Inactivation of BK Channels Mediated by the NH$_2$ Terminus of the β3b Auxiliary Subunit Involves a Two-Step Mechanism: Possible Separation of Binding and Blockade

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ABSTRACT A family of auxiliary β subunits coassemble with Slo α subunit to form Ca$^{2+}$-regulated, voltage-activated BK-type K$^+$ channels. The β subunits play an important role in regulating the functional properties of the resulting channel protein, including apparent Ca$^{2+}$ dependence and inactivation. The β3b auxiliary subunit, when coexpressed with the Slo α subunit, results in a particularly rapid (~1 ms), but incomplete inactivation, mediated by the cytosolic NH$_2$ terminus of the β3b subunit (Xia et al., 2000). Here, we evaluate whether a simple block of the open channel by the NH$_2$-terminal domain accounts for the inactivation mechanism. Analysis of the onset of block, recovery from block, time-dependent changes in the shape of instantaneous current-voltage curves, and properties of deactivation tails suggest that a simple, one step blocking reaction is insufficient to explain the observed currents. Rather, blockade can be largely accounted for by a two-step blocking mechanism (C$_*$ ⇒ O$_*$ ⇒ O$^*$ ⇒ I$^*$) in which preblocked open states (O$^*_n$) precede blocked states (I$^*_n$). The transitions between O$^*$ and I are exceedingly rapid accounting for an almost instantaneous block or unblock of open channels observed with changes in potential. However, the macroscopic current relaxations are determined primarily by slower transitions between O and O$^*$. We propose that the O to O$^*$ transition corresponds to binding of the NH$_2$-terminal inactivation domain to a receptor site. Blockade of current subsequently reflects either additional movement of the NH$_2$-terminal domain into a position that hinders ion permeation or a gating transition to a closed state induced by binding of the NH$_2$ terminus.

KEY WORDS: channel block • K$^+$ channels • gating mechanisms • Ca$^{2+}$- and voltage-gated K$^+$ channels • mSlo channels •

INTRODUCTION

Following perturbations that favor activation of an ion channel, a number of ion channels inactivate: specifically, ion channel open probability diminishes despite the continued imposition of conditions that would normally maintain the channels in open states. For many voltage-dependent K$^+$ channels including the ShakerB channel, an important type of inactivation, often termed ball-and-chain inactivation (Bezanilla and Armstrong, 1977; Hoshi et al., 1990), is thought to result from the movement of an NH$_2$-terminal peptide domain into a position that physically occludes the mouth of the ion permeation pathway. For inactivation of the ShakerB K$^+$ channel, several lines of evidence support the view that the association of the NH$_2$-terminal domain with its binding site directly results in blockade of the ion channel. First, the kinetic features of block can be described by a simple, one-step blocking reaction in which binding of the inactivation domain and block are identical. Second, cytosolic channel blockers can impede ShakerB K$^+$ channel inactivation, suggesting the native inactivation domain and cytosolic blockers share portions of a common binding site at the mouth of the ion permeation pathway (Choi et al., 1991; Demo and Yellen, 1991). Third, blockade by untethered NH$_2$-terminal peptides of the ShakerB K$^+$ channel exhibits features consistent with a simple first-order blocking reaction in which association of the peptide directly results in inhibition of ion permeation (Murrell-Lagnado and Aldrich, 1993a,b).

Ca$^{2+}$-regulated, voltage-activated BK-type K$^+$ channels can also exhibit a somewhat similar type of inactivation (Solaro and Lingle, 1992; Solaro et al., 1997; Ding et al., 1998), mediated by the NH$_2$-terminal domains of either of two auxiliary β subunits, the β2 (Wallner et al., 1999; Xia et al., 1999) or the β3b (Uebel et al., 2000; Xia et al., 2000). Although the inactivation of large conductance Ca$^{2+}$-regulated, voltage-
activated K⁺ channels (BK). Like Shaker K⁺ channel inactivation, is mediated by NH₂-terminal domains, several aspects of the inactivation of BK channels in chromaffin cells and of channels containing the cloned β2 subunit differ from the simple picture of ball-and-chain inactivation proposed for voltage-dependent K⁺ channels. In particular, cytosolic blockers of BK channels fail to slow the onset of inactivation, suggesting that such blockers do not impede the movement of the inactivation domain to its blocking site (Solaro et al., 1997; Xia et al., 1999). Furthermore, channel reopening does not occur during the recovery process, which is indicative that the conformational change associated with channel closing can occur independent of the recovery from inactivation (Solaro et al., 1997). Thus, inactivation mediated by the β2 subunit may involve a site that is not homologous to that involved in Shaker B K⁺ channel inactivation, and perhaps may involve a mechanism of inactivation somewhat different from that described for Shaker K⁺ channels.

To provide further insight into the mechanisms of BK channel inactivation, here we have examined the properties of inactivation mediated by the β3b subunit in more detail. In contrast to inactivation mediated by the β2 subunit (Wallner et al., 1999; Xia et al., 1999), inactivation produced by the β3b subunit exhibits a much faster, although incomplete, inactivation (Uebele et al., 2000; Xia et al., 2000). The β3b subunit is one of at least four splice variants of the β3 subunit (Uebele et al., 2000). Like the β2 subunit, inactivation mediated by the β3b subunit is not inhibited by occupancy of the BK channel by cytosolic channel blockers (Xia et al., 2000), leaving open the possibility that despite the kinetic differences, the underlying target of the inactivation domain for each subunit might be similar. Here we ask, to what extent does a simple pore-blocking model account for inactivation mediated by the β3b subunit? Taking advantage of the unique kinetic characteristics of inactivation conferred by the β3b subunit, we show that inactivation requires a minimum of two kinetic steps, one of which must be a conducting, but preinactivated state. One physical conception of the two-step mechanism is that the binding step for the association of the NH₂-terminal inactivation domain may be separated from the blocking step itself.

Expression Constructs

The preparation of all constructs used in this work has been described previously (Xia et al., 2000). The Xenopus oocyte expression vector pBF was used to subclone all the DNA constructs (Xia et al., 1998). Constructs in which the COOH and NH₂ termini were removed, previously termed β3b-D3 (COOH terminus-deleted) and β3b-D4 (NH₂ terminus-deleted), are here called β3b-3C and β3b-DN, respectively.

Expression in Xenopus Oocytes

Methods of expression in Xenopus oocytes were as described previously (Xia et al., 1999). SP6 RNA polymerase was used to synthesize cRNA for oocyte injection after DNA was linearized with MluI (Xia et al., 1998). 10–50 nl of cRNA (10–20 ng/µl) was injected into stage IV Xenopus oocytes harvested 1 d before. To ensure a molar excess of β subunits to αβ subunits, we usually injected α/β at 1:1 or 1:2 ratios by weight, although, in some cases, ratios up to 1:5 were also examined.

After injection, oocytes were maintained at 17°C in ND96 (96 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, and 3.0 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate, 100 µM penicillin, 100 µg/ml streptomycin, and 50 mg/ml gentamicin. Oocytes were used 1–7 d after injection of cRNA.

Electrophysiology

All currents were recorded in inside-out patches (Hamill et al., 1981). Currents were typically digitized at 10–50 kHz, although, in some cases, 100-kHz sampling frequencies were used. Currents were filtered at 5–50 kHz (Bessel low-pass filter; –3 dB) during digitization.

During seal formation, oocytes were bathed in ND-96. After excision, patches were quickly moved into a flowing 0 Ca²⁺ solution (contaminant Ca²⁺ plus 5mM EGTA). For inside-out recordings, the pipet extracellular solution was 140 mM potassium methanesulfonate, 20 mM KOH, 10 mM HEPES, and 2 mM MgCl₂, pH 7.0. Test solutions bathing the cytoplasmic face of the patch membrane contained 140 mM potassium methanesulfonate (MES), 20 mM KOH, 10 mM HEPES, and 5 mM HEDTA, pH 7.0, and one of the following: 5 mM EGTA (for nominally β-Ca²⁺, 0.5-µM, and 1-µM Ca²⁺ solutions), 5 mM HEDTA (for 4- and 10-µM Ca²⁺ solutions), or no added Ca²⁺ buffer (for 60 µM, 100 µM, 300 µM, and 5 mM Ca²⁺ solutions). The procedures for calibration of Ca²⁺ solutions have been described previously (Xia et al., 1999, 2000). Briefly, for a given Ca²⁺-sensitive electrode, a commercial set of Ca²⁺ standards (WPI) was used to define a calibration curve. Ca²⁺ concentrations of the methanesulfonate-based solutions were determined based on this calibration curve. The values obtained from the commercial set of Ca²⁺ standards were identical to a set of chloride-based Ca²⁺ solutions prepared in this lab in which free Ca²⁺ was defined by a computer program (EGTAETC, obtained from E. McCleskey, Vollum Institute, Portland, OR) using published stability constants. Local perfusion of membrane patches was as described previously (Solaro and Lingle, 1992; Solaro et al., 1997).

Voltage commands and acquisition of currents was accomplished with pClamp7.0 or pClamp8.0 for Windows (Axon Instruments). Current values were measured using ClampFit (Axon Instruments), converted to conductances, and then fit with a custom nonlinear least squares fitting program. Conductances were determined in three ways: from tail currents, from the peak current at a given activation potential, and from steady-state current at a given activation potential. Single conductance-voltage (G-V) curves for activation were fit with a Boltzmann equation with the form:

\[
G(V) = G_{\text{max}} / \left(1 + \exp \left( - \frac{V - V_{0.5}}{k} \right) \right)
\]

where \(V_{0.5}\) is the voltage of half-maximal activation of conductance, and \(k\) is the voltage dependence of the activation process (mV). G-V curves for a single patch over a range of Ca²⁺ concentrations were also fit with a set of equations based on a Monod-
Two Models for Inactivation

This paper has two primary aims. The first aim is to provide a detailed description of the behavior of currents arising from coexpression of the β3b subunit with Slo α subunits. The second aim is to present a kinetic model that appears to account for many of the novel aspects of the observed currents. To help guide the presentation and evaluation of the results, it is useful to introduce here the two primary blocking models that will be considered.

Typically, inactivation of a variety of ion channels, including the Shaker type voltage-dependent K⁺ channels (Hoshi et al., 1990; Choi et al., 1991; Demo and Yellen, 1991), can be modeled by a simple open channel block scheme given as follows.

\[
C_n \xrightarrow{k_o} O_n \xrightarrow{k_p} I_n
\]

(Scheme 1)

The hallmark of this model is that binding of an inactivation domain to its binding site directly produces functional blockade. Molecules that block the binding of the inactivation domain to its blocking site compete with the inactivation mechanism, resulting in the slowing of inactivation. Furthermore, occupancy of the blocking site by the inactivation domain prevents the direct return of inactivated channels to closed states, therefore, resulting in channel unblocking and reopening during recovery from inactivation. For the case in which \( n = 5 \), Scheme 1 corresponds to the voltage-dependent MWC model considered by Cox et al. (1997), with the addition of the blocking reaction from the open states.

We also consider a second type of blocking scheme, which postulates a set of preinactivated, open states (O*ₜ) that precede entry into inactivated states (Iₜ):

\[
C_n \xrightarrow{k_o} O_n \xrightarrow{k_f} O^* \xrightarrow{k_b} I_n
\]

(Scheme 2)

One physical picture of this model would be that binding of an inactivation domain to a site on the channel precedes a subsequent transition to the inactivated state. The primary mechanistic distinction between Schemes 1 and 2 is that, in the latter, binding of the inactivation domain to its binding site is distinct from the transition that produces inactivation. However, alternative conceptions of the two steps in Scheme 2 are also possible.

How do these two categories of models differ in terms of the characteristics of the currents they predict? From macroscopic currents, Scheme 2 might be expected to differ from Scheme 1 in three primary
ways. First, dependent on the relative rates of various transitions, channels behaving in accordance with Scheme 2 might show some unusual features in the tail currents after repolarization from inactivating potentials. This might be apparent either as a rising phase in the tail current itself or as a slowing in the time constant of deactivation. Second, Scheme 2 predicts additional complexity in the recovery from inactivation than would be predicted by Scheme 1. Third, Scheme 2 predicts that, at different times during a tail current, channels should be occupying different types of open states. Experiments below test these various possibilities and show that Scheme 2 provides a better explanation for α + β3b currents than Scheme 1.

### Rapidly Inactivating Currents Mediated by the β3b Subunit

Message for the β3b subunit was coexpressed with Slo α subunits in *Xenopus* oocytes. Currents obtained from inside-out patches expressing α + β3b subunits were both voltage- and Ca2+ dependent with activation shifted to more negative potentials as cytosolic Ca2+ was increased. The general properties of the currents arising from coexpression of β3b with α subunits have been presented elsewhere (Xia et al., 2000). Briefly, at strong depolarizations and higher Ca2+, α + β3b currents exhibit a very rapid, although incomplete, inactivation (Fig. 1). At 300 μM Ca2+, the time constant of the inactivating portion of current is ~0.5–1 ms at potentials positive to +40 mV. Above +50 mV, current inactivates to a steady-state level that is ~10–50% of the peak value, dependent on voltage, but largely independent of Ca2+. The substantial level of steady-state sustained current indicates that both the onset and recovery of the blocking reaction are rapid. Inactivation requires the NH2 terminus of the β3b subunit (Xia et al., 2000). Typical conductance-voltage curves measured from the peak current level, the tail current, and from the steady-state outward current are plotted in Fig. 1.

#### Deactivation of α + β3b Currents after Brief Activation Steps and Longer Inactivating Steps

An unusual feature of α + β3b currents is the exceedingly rapid recovery from inactivation upon repolarization (Xia et al., 2000). A repolarizing voltage step at the peak of outward current results in a tail current of amplitude essentially identical to that after a repolarization during a steady-state level of inactivation (Fig. 2). This result demands that a large number of channels that are blocked at the activation potential reopen before the peak of the tail current. In Fig. 2 A, tail currents are compared at a repolarization potential of −80 mV after either a 40-ms or a 1-ms depolarizing step to +160 mV. Peak outward current during the depolarization to +160 mV occurs somewhat before 1 ms, whereas, at 40 ms, the outward current is at the steady-state–inactivated level. Despite the differences in amount of current at +160 mV for the two traces, the current after the repolarization to −80 mV is actually somewhat greater when channels are mostly inactivated at +160 mV. When a 2-ms inactivation step is compared with the 40-ms inactivation step, qualitatively similar results are obtained (Fig. 2 B). Similar results were obtained with tail currents at −160 mV (not shown).

The fact that the tail current after the 1-ms activation step is actually smaller than that after either a 2 or 40 ms activation step presumably occurs because the peak of
In the activated states to open states at potentials from only further emphasizes the point that recovery from inactivation through different open states than when they have not inactivated.

To evaluate this possibility, we now address several questions related to the tail current behavior of $\alpha + \beta 3b$. First, is the slowing of the tail current decay rate with duration of the activation step related to the inactivation process? Second, is the inward current increase during the tail current associated with the recovery from inactivation and how fast is the recovery from inactivation? Third, during repolarization to any potential, is the fast recovery from inactivation complete?

The Prolongation of Deactivation Observed for $\alpha + \beta 3b$ Currents Does Not Appear To Be Related to the Inactivation Mechanism

The deactivation of currents arising from the Slo $\alpha$ subunit alone was examined at various potentials for both 10 and 300 $\mu$M for command steps ranging in duration from 0.2 to 10 ms (Fig. 3, A1 and A2 for $-80$ mV and 10 $\mu$M $Ca^{2+}$). Over this range of command steps, we observed a small, but distinct, increase in deactivation $\tau_d$ at both 10 $\mu$M $Ca^{2+}$ and 300 $\mu$M $Ca^{2+}$, with some increase occurring even after the peak of outward current activation (Fig. 4). In all cases, current deactivation was reasonably described with a single exponential time course (Fig. 3 B). This prolongation was observed whether leak subtraction was used or not. With activation steps of 0.5–10 ms, the magnitude of this prolongation was on the order of 1.5–2-fold (Fig. 4, A and B).

Coexpression of $\alpha$ subunits with a $\beta 3$ subunit lacking the inactivating NH$_2$ terminus (construct $\beta 3b$-$\Delta N$; Xia et al., 2000) resulted in exponentially decaying tail currents that decayed more slowly at both 10 (Fig. 3 B) and 300 $\mu$M than those arising from the $\alpha$ subunit
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Like the α subunit alone, α + β3b-ΔN currents exhibited a slight slowing in τd as a function of activation step duration (Fig. 4). When deactivation was examined for either wild-type α + β3b currents (Fig. 3 C) or for α + β3b-ΔC (Fig. 3 D; a construct lacking the β3b-ΔC), there was also a 1.5–2-fold slowing of τd as command step duration was increased. However, in contrast to the nonactivating currents resulting from α alone or α + β3b-ΔN, both α + β3b and α + β3b-ΔC currents exhibited an unblocking component in their tail currents (Fig. 3 C and D). The magnitude of this unblocking component increased as the duration of the activation step was increased.

These results indicate that a 1.5–2.0-fold slowing of τd occurs with increases in activation step duration for all four types of currents. Thus, the slowing of deactivation is unrelated to the inactivation mechanism itself. Here, we make no attempt to provide an explanation for this slowing, but note that a simple voltage-dependent MWC model (Cox et al., 1997) does not predict an activation-dependent slowing of deactivation (Fig. 4, C and D). In our experiments, the occurrence of activation-dependent slowing of deactivation was not dependent on current amplitude among different patches, and also occurred with 300 μM Ca²⁺ in which 50 μM of the crown ether Ba²⁺ chelator, 18C6TA, was included (not shown).
With an Intact Inactivation Domain, Tail Currents Exhibit a Fast Unblocking Process

The results in Fig. 2 show that recovery from inactivation for $\alpha + \beta 3b$ currents must occur very rapidly, since the tail current conductance at the earliest discernible times greatly exceeds the steady-state conductance at positive potentials. However, as noted above, for both $\alpha + \beta 3b$ and $\alpha + \beta 3b-\Delta C$ currents, at more positive deactivation potentials following the initial essentially instantaneous recovery in current amplitude, there is a secondary shoulder or slight increase in inward tail current (Figs. 2 and 3). This is evaluated more explicitly in Fig. 5. After repolarization to either $-40$ or $-80$ mV, there is a nonohmic step to an initial tail current amplitude that shows a secondary increase reaching a peak in $\langle 1 \text{ ms}\rangle$. At more negative potentials ($-120$ and $-160$ mV), after the initial decay of uncompensated patch capacitance, there is only a shoulder that precedes the onset of deactivation. Tail currents recorded after brief activation steps (Fig. 5, A and B) show a brief voltage-dependent shoulder before the onset of deactivation, although only at $-40$ mV is there any indication of an actual unblocking process before the peak tail current. Comparison of tail currents resulting from constructs containing the $\beta 3b$ inactivation domain ($\alpha + \beta 3b$ and $\alpha + \beta 3b-\Delta C$) to those lacking the inactivation domain ($\alpha$ alone and $\alpha + \beta 3b-\Delta N$) indicate that this unblocking process is unique to channels exhibiting inactivation (Fig. 3).

For better comparison, tail currents were normalized to their peak amplitudes for either the shorter activation steps (Fig. 5 B) or longer activation steps (Fig. 5 C). With 10-μs sampling and 10 kHz filtering, the solid lines (Fig. 5, B and C) show the best fit of a two exponential function to each of the tail currents. Over the range of $-40$ mV to $-160$ mV, for the examples shown, the fitted values for the initial, rapid unblocking time constant ($\tau_u$) ranged from $\sim 250 \mu s$ at $-40$ mV to $\sim 120 \mu s$ at $-160$ mV. This 100–250-μs unblocking relaxation is too slow to account for the “instantaneous” unblocking of current that produces the nonohmic discrepancy between the steady-state conductance and the initial conductance of the tail currents.

These results indicate that, although the slowing of $\tau_d$ with command step duration appears unrelated to the inactivation process, there are two components of the inactivation process that are discernible in the tail currents: a nonohmic, almost instantaneous unblocking of channels, followed by a rapid unblocking relaxation ($\tau_u$) or shoulder in the tail current. The shoulder in the tail currents is not observed in the absence of the NH$_2$-terminal inactivation domain.

Fig. 4 shows that $\tau_d$ is similar for both $\alpha + \beta 3b$ and $\alpha + \beta 3b-\Delta N$ currents. This indicates that the time course of current deactivation is not markedly influenced by any molecular transitions requiring the NH$_2$-terminal inactivation domain. This result indicates that the rate limiting steps in current deactivation are not coupled to transitions involved in recovery from inactivation. This assertion is also supported by experiments below in which the NH$_2$-terminus is enzymatically removed with trypsin.

**Figure 5.** Inactivation is associated with the appearance of a rapid unblocking component in the tail current. In A, a protocol similar to that used in Fig. 2 was used to examine tail currents at repolarization potentials of $-40$, $-80$, $-120$, and $-160$ mV with 10 μM Ca$^{2+}$. In the right-hand traces of A, points show every twentieth digitized value for currents resulting from a 40-ms activation step. In B, normalized tail currents after the 2-ms activation step (from A) are shown for the indicated repolarization potentials. Only every fifth digitized value (50 μs) is displayed. At the most positive repolarization potential (−40 mV), there is a pronounced lag before the tail current begins to decay in an exponential fashion. At more negative potentials, after a brief capacitative current, the current begins an exponential decay fairly rapidly. Lines correspond to a fitted two exponential function, $A_1 \cdot \exp(-t/\tau_u) + A_2 \cdot \exp(-t/\tau_d) + \text{offset}$, where $\tau_u$ corresponds to an unblocking relaxation, and $\tau_u$ corresponds to the deactivation time course. At $-160$ mV, $\tau_u = 0.087$ ms and $\tau_d = 0.44$ ms; at $-120$ mV, $\tau_u = 0.113$ ms and $\tau_d = 0.98$ ms; at $-80$ mV, $\tau_u = 0.172$ and $\tau_d = 1.50$ ms; and at $-40$ mV, $\tau_u = 0.134$ and $\tau_d = 2.16$ ms. In C, normalized tail currents activated after a 40-ms activation step are shown over the same range of repolarization potentials. At both $-40$ and $-80$ mV, there is a distinct increase in tail current after the capacitative transient. At both $-120$ and $-160$ mV, the shoulder of current before the onset of exponential decay is more pronounced than in B. Open symbols again show every fifth digitized data value, whereas solid lines correspond to a two exponential fit to the decay time course. Fitted values were as follows: at $-160$ mV, $\tau_u = 0.128$ ms and $\tau_d = 0.57$ ms; at $-120$ mV, $\tau_u = 0.173$ ms and $\tau_d = 1.42$ ms; at $-80$ mV, $\tau_u = 0.292$ ms and $\tau_d = 2.415$ ms; and at $-80$ mV, $\tau_u = 0.183$ and $\tau_d = 5.64$ ms.

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**Figure 5.** Inactivation is associated with the appearance of a rapid unblocking component in the tail current. In A, a protocol similar to that used in Fig. 2 was used to examine tail currents at repolarization potentials of $-40$, $-80$, $-120$, and $-160$ mV with 10 μM Ca$^{2+}$. In the right-hand traces of A, points show every twentieth digitized value for currents resulting from a 40-ms activation step. In B, normalized tail currents after the 2-ms activation step (from A) are shown for the indicated repolarization potentials. Only every fifth digitized value (50 μs) is displayed. At the most positive repolarization potential (−40 mV), there is a pronounced lag before the tail current begins to decay in an exponential fashion. At more negative potentials, after a brief capacitative current, the current begins an exponential decay fairly rapidly. Lines correspond to a fitted two exponential function, $A_1 \cdot \exp(-t/\tau_u) + A_2 \cdot \exp(-t/\tau_d) + \text{offset}$, where $\tau_u$ corresponds to an unblocking relaxation, and $\tau_d$ corresponds to the deactivation time course. At $-160$ mV, $\tau_u = 0.087$ ms and $\tau_d = 0.44$ ms; at $-120$ mV, $\tau_u = 0.113$ ms and $\tau_d = 0.98$ ms; at $-80$ mV, $\tau_u = 0.172$ and $\tau_d = 1.50$ ms; and at $-40$ mV, $\tau_u = 0.134$ and $\tau_d = 2.16$ ms. In C, normalized tail currents activated after a 40-ms activation step are shown over the same range of repolarization potentials. At both $-40$ and $-80$ mV, there is a distinct increase in tail current after the capacitative transient. At both $-120$ and $-160$ mV, the shoulder of current before the onset of exponential decay is more pronounced than in B. Open symbols again show every fifth digitized data value, whereas solid lines correspond to a two exponential fit to the decay time course. Fitted values were as follows: at $-160$ mV, $\tau_u = 0.128$ ms and $\tau_d = 0.57$ ms; at $-120$ mV, $\tau_u = 0.173$ ms and $\tau_d = 1.42$ ms; at $-80$ mV, $\tau_u = 0.292$ ms and $\tau_d = 2.415$ ms; and at $-80$ mV, $\tau_u = 0.183$ and $\tau_d = 5.64$ ms.

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Trypsin-mediated Removal of Inactivation Removes the Unblocking Relaxation in the Tail Current, but Does Not Alter the Tail Current Decay Rate

The similarity in τu (once the unblocking relaxation is complete) for α + β3b, α + β3bΔC, and α + β3bΔN currents suggests that similar molecular transitions govern the deactivation time course in all cases. This requires either that transitions into and out of blocked states do not occur during the decay phase of the tail currents or that such transitions do not impede channel closure. We next compared tail currents resulting from activation of α + β3b channels with 10 μM Ca2+ before (Fig. 6, A and B) and after removal of inactivation by trypsin (Fig. 6, C and D). After trypsin, outward current at +160 mV appears to activate more slowly and is markedly larger. In contrast, tail current amplitudes exhibit a more modest increase after trypsin. The calibration bar in A also applies to C, while B and D share a calibration bar.

Tail current amplitude at more positive repolarization potentials exhibits a larger increase following trypsin than those at more negative potentials. After trypsin application, the tail current decay follows a relatively simple exponential time course, whereas, before trypsin, there is a brief rising phase. In E, time constants of deactivation (τd) are plotted for α + β3b currents before (○) and after (■) trypsin and compared with τd for currents arising from α alone (□). Tail currents for intact α + β3b were fit with a two exponential function to approximate the fast unblocking shoulder of current and then the deactivation of current. Points show means and standard deviations for at least four patches.

Trypsin removes the unblocking relaxation observed in α + β3b tail currents. In A, panels from top to bottom compare tail currents at the four indicated repolarization potentials, before and after (line with points) trypsin application. Despite the extensive unblocking that occurs during repolarization, the ability of trypsin to increase the tail current amplitude suggests that even at negative potentials some voltage-dependent block of channels persists. At −120 mV, tail current amplitudes before trypsin are ~70% of those after trypsin application. For the traces after trypsin application, the points correspond to every fifth digitized value. The horizontal bars overlaid on the rising phase of the tail currents indicate the calculated level of tail current expected at the repolarization potential assuming an instantaneous ohmic step from the residual level of current at +160 mV. A small segment of the steady-state–inactivated current at +160 mV is shown for the traces before trypsin application. Filter, 10-kHz bandwidth; sampling period, 10 μs. In B, each pair of traces was normalized to the same peak amplitude. For the trace obtained before trypsin application, the deactivation time course was fit with the following two exponential function: A1 · exp(−t/τu) + A2 · exp(−t/τd) + offset. At −120, −100, −80, and −60 mV, the unblocking relaxations were 0.18, 0.13, 0.20, and 0.24 ms, respectively, whereas the subsequent deactivation time constants were 0.40, 0.57, 0.71, and 0.98 ms, respectively. After trypsin, a fit of A1 · exp(−t/τu) + C yielded time constants of 0.42, 0.52, 0.74, and 1.06 ms.

Figure 6. Effects of trypsin on α + β3b currents. In A, currents in an inside-out patch expressing α + β3b channels were activated by the indicated voltage protocol with 10 μM Ca2+. In B, the tail currents at potentials from 0 mV to −180 mV are displayed at higher magnification. In C, trypsin was briefly applied to the same patch shown in A resulting in removal of inactivation, whereas D shows the tail currents after trypsin at higher magnification. After trypsin, peak outward current at +160 mV appears to activate more slowly and is markedly larger. In contrast, tail current amplitudes exhibit a more modest increase after trypsin. The calibration bar in A also applies to C, while B and D share a calibration bar.

Tail current amplitude at more positive repolarization potentials exhibits a larger increase following trypsin than those at more negative potentials. After trypsin application, the tail current decay follows a relatively simple exponential time course, whereas, before trypsin, there is a brief rising phase. In E, time constants of deactivation (τd) are plotted for α + β3b currents before (○) and after (■) trypsin and compared with τd for currents arising from α alone (□). Tail currents for intact α + β3b were fit with a two exponential function to approximate the fast unblocking shoulder of current and then the deactivation of current. Points show means and standard deviations for at least four patches.

Trypsin generates a relaxation that follows an instantaneous ohmic step from the residual level of current at +160 mV. A small segment of the steady-state–inactivated current at +160 mV is shown for the traces before trypsin application. Filter, 10-kHz bandwidth; sampling period, 10 μs. In B, each pair of traces was normalized to the same peak amplitude. For the trace obtained before trypsin application, the deactivation time course was fit with the following two exponential function: A1 · exp(−t/τu) + A2 · exp(−t/τd) + offset. At −120, −100, −80, and −60 mV, the unblocking relaxations were 0.18, 0.13, 0.20, and 0.24 ms, respectively, whereas the subsequent deactivation time constants were 0.40, 0.57, 0.71, and 0.98 ms, respectively. After trypsin, a fit of A1 · exp(−t/τu) + C yielded time constants of 0.42, 0.52, 0.74, and 1.06 ms.

Figure 7. Trypsin removes the unblocking relaxation observed in α + β3b tail currents. In A, panels from top to bottom compare tail currents at the four indicated repolarization potentials, before and after (line with points) trypsin application. Despite the extensive unblocking that occurs during repolarization, the ability of trypsin to increase the tail current amplitude suggests that even at negative potentials some voltage-dependent block of channels persists. At −120 mV, tail current amplitudes before trypsin are ~70% of those after trypsin application. For the traces after trypsin application, the points correspond to every fifth digitized value. The horizontal bars overlaid on the rising phase of the tail currents indicate the calculated level of tail current expected at the repolarization potential assuming an instantaneous ohmic step from the residual level of current at +160 mV. A small segment of the steady-state–inactivated current at +160 mV is shown for the traces before trypsin application. Filter, 10-kHz bandwidth; sampling period, 10 μs. In B, each pair of traces was normalized to the same peak amplitude. For the trace obtained before trypsin application, the deactivation time course was fit with the following two exponential function: A1 · exp(−t/τu) + A2 · exp(−t/τd) + offset. At −120, −100, −80, and −60 mV, the unblocking relaxations were 0.18, 0.13, 0.20, and 0.24 ms, respectively, whereas the subsequent deactivation time constants were 0.40, 0.57, 0.71, and 0.98 ms, respectively. After trypsin, a fit of A1 · exp(−t/τu) + C yielded time constants of 0.42, 0.52, 0.74, and 1.06 ms.
tion time course and peak outward current is increased over 10-fold. Tail currents after trypsin exhibit a much smaller increase in amplitude, and no longer show the unblocking relaxation characteristic of the intact α + β3b currents. This indicates that the unblocking relaxation in the tail currents requires an intact inactivation mechanism. However, the trypsin-induced increase in tail current amplitude suggests that, even though unblock after repolarization is rapid, it is not complete, even at the most negative repolarization potentials.

The effects of trypsin on tail currents are shown in more detail in Fig. 7. Comparison of the peak tail current amplitudes before and after trypsin (Fig. 7 A) shows that, when inactivation is intact, although repolarization produces an extremely rapid unblocking of current, the peak tail current amplitude is still reduced over twofold increase in the peak tail current amplitudes before and after trypsin (Fig. 7 A) shows that, when inactivation is intact, although repolarization produces an extremely rapid unblocking of current, the peak tail current amplitude is still reduced.

Moreover, the trypsin-induced increase in tail current amplitude suggests that, even though unblock after repolarization is rapid, it is not complete, even at the most negative repolarization potentials.

One cautionary comment is that there are unusual nonohmic aspects of instantaneous currents arising from the α + β3b subunits that are observed after removal of the NH2 terminus. As will be seen below, the underlying intrinsic instantaneous current-voltage (I-V) properties conferred by the β3b subunit exhibit marked outward rectification, in marked distinction to the inward rectification produced by rapid block by the NH2 terminus. Thus, if we assume that channels with the intact NH2 terminus behave similarly, the discrepancy between the predicted level of current after the repolarizing voltage step and the observed instantaneous current after unblocking would be even greater. Irrespective of the impact of the outward rectification, it is clear that the tail currents upon repolarization during steady-state inactivation exhibit both an instantaneous component of unblocking, and then a slower time-dependent unblocking.

Do any observations presented to this point place any constraints on the type of model that might account for the results? The existence of two distinct unblocking components in the tail currents argues that at least two steps are involved in the inactivation process, which is consistent with Scheme 2, but not Scheme 1. On the other hand, the absence of any marked effect of the NH2-terminal-mediated blocking mechanism on τd is at variance with both linear blocking schemes.

Properties of Instantaneous Current-Voltage (I-V) Curves after Recovery from Steady-state Inactivation

As another approach to evaluation of the rapid recovery process, we examined the amplitude of tail currents over potentials from -180 mV through +180 mV after

![Figure 8](image-url)

Figure 8. Properties of instantaneous current-voltage (I-V) curves following repolarization from a steady-state-inactivated condition. In A, currents were activated by the indicated voltage protocol at various Ca2+ (0, 1, 4, and 10 μM Ca2+). For all Ca2+, there is a large, immediate nonohmic increase in tail current indicative of rapid unblocking from inactivation. In B, the current was measured 100 μs after the nominal time of the repolarizing voltage step and normalized to the current measured with the step to -100 mV. The shape of the I-V curve is identical for all Ca2+. In C, the mean and standard deviation for the instantaneous I-V curve from five patches at 10 μM Ca2+ are plotted, along with the best fit of Eq. 5. For the fit with solid circles, Gmax was constrained to 0.305 ± 24.0; K2(0) = 51.7 ± 49.4 and Q2 = 0.154 ± 0.020e. For the fit with diamonds, Gmax was constrained to 2.0 with K2(0) = 2.27 ± 0.045 and Q2 = 0.209e ± 0.005e. For the open circles, Gmax was constrained to 1.0 with K2(0) = 0.504 ± 0.07 and Q2 = 0.341e ± 0.03e.
inactivation at +160 mV (Fig. 8 A). To avoid assumptions about the time course of the decay process, the current amplitude at a time point 100 μs after the nominal time of the repolarizing voltage-step was measured. Current amplitudes were normalized to the current measured at −100 mV. These instantaneous I-V curves were essentially identical at all Ca2+ concentrations (Fig. 8 B), and exhibited strong inward rectification.

Results so far suggest that the initial tail current amplitude reflects something about the fractional recovery from inactivation. The fact that trypsin results in an increase in tail current amplitude shows that the unblocking process is not complete. Thus, the instantaneous recovery to a new partially blocked level would imply that the initial tail current amplitude reflects a new voltage-dependent equilibrium between open and inactivated states. By assuming for the moment that any curvature in the instantaneous I-V arises from rapid development of a new voltage-dependent equilibrium between O*\textsubscript{n} and I\textsubscript{n}, we can extract some estimates of the properties of that proposed equilibrium and ask whether the shape of the I-V curve is consistent with other aspects of our results.

As a first approximation, we assume that, at +160 mV, channels are in a rapid equilibrium between states O*\textsubscript{n} and I\textsubscript{n} with occupancy of states (O\textsubscript{n}) assumed to be negligible. After repolarization, the new conductance at any potential would be given solely by the new equilibrium between O*\textsubscript{n} and I\textsubscript{n}. Therefore, an estimate of the relative current (I) at any potential (V) is given by:

\[ I(V) = V \cdot G_{\text{max}} \cdot k_b(V)/k_b(V) + k_b(V). \]

Since the ratio I/O* is given by K\textsubscript{2}(V) = k_b(V)/k_b(V),

\[ I(V) = V \cdot G_{\text{max}}/\left(1 + K_2(V)\right). \]

With K\textsubscript{2}(V) = K\textsubscript{2}(0) \cdot \exp(Q_2FV/RT).

\[ I(V) = V \cdot G_{\text{max}}/\left(1 + K_2(0) \cdot \exp((Q_2/FV)/RT)\right). \quad (3) \]

Although the three parameters provided by Eq. 3 (G\textsubscript{max}, K\textsubscript{2}(0), and Q_2) are not well constrained by the shape of the curve in Fig. 8 C, evaluation of the data in terms of Eq. 3 can place some limits on this postulated equilibrium. Points were fit over the range of −90 mV through +160 mV. Values at more negative potentials are likely to be underestimated because of more rapid deactivation at those potentials. Fig. 8 C shows the result of three cases: (1) fitting with all parameters unconstrained; (2) fitting with G\textsubscript{max} constrained to be maximal at −100 mV; and (3) fitting with G\textsubscript{max} constrained to twice the value observed at −100 mV. Case 2 corresponds to the assumption that all channels return to open states at −100 mV. Case 3 is in accordance with the observation that the tail current amplitude approximately doubles at −100 mV after trypsin application. In this case, K\textsubscript{2}(0) = 2.27 ± 0.045 and Q_2 = 0.21 ± 0.005 (Fig. 9 A).

Since the ratio of I/O* at a given potential is given by k_b(V)/k_b(V) or K\textsubscript{2}(V), the relative occupancy of O* and I at different voltages can be calculated (Fig. 9 B). This analysis suggests that occupancy of blocked states is still appreciable at potentials negative to 0 mV, consistent with the effects of trypsin on the tail current amplitude at negative potentials (Fig. 6 C). At each voltage, the amplitude of the tail current before and after trypsin application, therefore, was determined (Fig. 9 C) along with the values for K\textsubscript{2}(V) obtained from the fit shown in A, fractional occupancy in O*\textsubscript{n} (○) and I\textsubscript{n} (●) was calculated and plotted as a function of the potential at which the tail current was measured. In C, tail current amplitudes (normalized to −100 mV) were measured for three patches before (●) and after (○) removal of inactivation with trypsin. In D, the ratio (○) of tail current amplitude at 100 μs before trypsin application to that after removal of inactivation is plotted as a function of repolarization potential. Note the correspondence of these values to those for O*\textsubscript{n} in panel B. The solid line is a fit of the following equation:

\[ f(V) = \frac{f_{\text{max}}}{1 + K(0) \cdot \exp(-V/\tau)}, \]

where f\textsubscript{max} = 0.613, K(0) = 3.32 and \( \tau = -0.503. \)
With the ratio of tail current before and after trypsin (Fig. 9 D). This fractional unblock revealed by trypsin (Fig. 9 D) correlates well with the proposed fractional occupancy of states (\(O_n^*\)) plotted in Fig. 9 B, which was calculated from the curvature of the instantaneous I-V curve. It should also be noted that, after trypsin, the instantaneous I-V curve exhibits an unusual outward curvature. This is an intrinsic property of channels arising from \(\alpha + \beta 3b\) coexpression and is unrelated to any secondary effect of trypsin (Zeng et al., 2001).

Paired Pulse Recovery Protocols Also Reveal Multiple Components of Recovery from Inactivation

A standard approach to defining kinetic properties of an inactivation process is to examine recovery from inactivation using a paired pulse protocol. This sort of protocol uses a variable recovery interval at a repolarizing potential to define the fractional recovery from inactivation. For Scheme 1, if upon repolarization most channels rapidly return to states \(O_n\), a subsequent depolarizing voltage-step at the peak of the tail current would be expected to result solely in an ohmic step of current corresponding to the number of channels still open during the tail current. The ohmic step would then be followed by a subsequent time-dependent inactivation of open channels governed by the \(O_n\) to \(I_n\) transitions. With longer recovery intervals, the ohmic current would be expected to decrease, as more channels have returned to closed states (\(C_n\)). In contrast, the predictions from Scheme 2 are more complex.

Such an experiment is shown in Fig. 10. After development of steady-state inactivation at +160 mV, the effect of recovery steps of different duration on current activation during a subsequent step to +160 mV was examined. Recovery was examined with 10 \(\mu M\) \(Ca^{2+}\) at either −80 mV (Fig. 10 A) or −120 mV (Fig. 10 B). Quite remarkably, with short recovery intervals (e.g., 50–250
The recovery process is seen more clearly with the faster time base traces in Fig. 10, A and B. For the shortest recovery interval (50 μs), a subsequent depolarization to +160 mV results in a return to almost the same steady-state–blocked level of current (indicated by a triangle) once the brief capacitative transient is complete. With longer recovery periods, the level of current after completion of the capacitative transient slowly increases with increases in repolarization duration. In addition, there begins to be a time-dependent activation of outward current, and then the slower inactivation of current. With even longer recovery periods, the step change in current diminishes as the amount of current that exhibits time-dependent activation and inactivation increases. Two aspects of the current levels marked by the triangles should be noted. First, given the amount of current present in the tail currents at either −80 or −120 mV, the level of current during the subsequent step to +160 mV is markedly nonohmic, indicating that channels that are open during the tail current have re-blocked instantaneously during the depolarization. Second, although there is some correspondence between the unblocking observed in the tail current and the slow increase in the instantaneous current level (triangles) after the depolarization, the instantaneous currents actually reach a peak value that lags the peak of the tail current. Thus, the instantaneous current seen at +160 mV does not simply reflect the total number of channels open during the tail current, but must reflect something about the particular open states occupied at different times during the tail current.

These observations can be explained in terms of Scheme 2 as follows. During the depolarizing step, channels are in rapid equilibrium between the conducting, preinactivated states (O*n), and the nonconducting, inactivated states (I*n). Upon repolarization, channels rapidly reach a new equilibrium between O*n and I*n, with greater occupancy of O*n. However, when channels are in O*n, a subsequent depolarization causes an almost instantaneous return to the original voltage-dependent equilibrium between O*n and I*n, because of the rapid rates of the transitions between O*n and I*n. Thus, the original steady-state current level at +160 mV is reached immediately. With longer recovery times, channels from O*n will return to normal open states, O. This will result in a small, time-dependent increase in the total number of channels in open states, resulting in the unblocking and shoulder in the tail current.
currents. For those channels in $O_n$, a subsequent depolarization would be expected to result in an ohmic step of current, before those channels subsequently enter $O^*_n$, and then inactivate. Only as the fraction of channels reentering $C_n$ increases will the slower time-dependent activation and inactivation become more pronounced. In contrast, Scheme 1 predicts that, after unblocking associated with even the shortest repolarizations, the subsequent depolarization should result in an ohmic step in outward current, followed by a 1-ms blocking relaxation. This is clearly not observed.

Kinetically, the number of blocking and unblocking components observed in our data requires a minimum of four distinct sets of states. Is it possible that a model containing two nonconducting inactivated states ($C \rightleftharpoons O \rightleftharpoons I \rightleftharpoons I$) would account equally well for the results? The simple answer is no. The characteristics of the unblocking process require that the blocking scheme contain at least two distinct types of open states. One type of open state is indicated by a component of current that undergoes an $\sim$1-ms relaxation to the inactivated condition. A second type of open state is indicated by the fact that there exists a component of current that exhibits an essentially instantaneous block and unblock. This is shown explicitly in the next section.

**Instantaneous Macroscopic I-V Curves at Different Times during the Tail Currents Also Indicate that $\alpha + \beta 3b$ Channels Occupy Unique Open States**

We have just proposed that, at different times in the tail current, the relative occupancy of states $O_n$ and $O^*_n$ varies. Because of the rapid block of channels in states $O^*_n$, instantaneous I-V curves arising from channels in $O^*_n$ would be expected to differ from instantaneous I-V curves arising from channels in state $O_n$. The relative curvature of the instantaneous I-V curve, therefore, should differ at early and later times in the tail current, depending on the relative occupancy of each proposed open state. If a conventional type of blocking model involving a single open state were correct, the shape of the instantaneous I-V should be unchanged.

To test this idea, depolarizations to potentials between $-120$ and $+160$ mV with $300 \mu$M Ca$^{2+}$ were applied at either 100 or $500 \mu$s after a repolarization to $-160$ mV (Fig. 11). Current levels at $100 \mu$s after the nominal application of the depolarizing voltage-step were measured and plotted for $\alpha$ alone and $\alpha + \beta 3b$. Currents from the $\alpha$ subunit alone exhibited a largely linear instantaneous I-V after either 100- or $500-\mu$s repolarizing steps (Fig. 11 A). In contrast, $\alpha + \beta 3b$ currents exhibited the expected inward rectification, but the rectification was less pronounced after the $500-\mu$s step to $-160$ mV (Fig. 11 B). This supports the idea that the relative occupancy of channels in different open states has changed during the tail current. Specifically, the fraction of channels in states $O_n$ has increased relative to the fraction in states $O^*_n$. A similar experiment was done for currents arising from $\alpha + \beta 3b-\Delta N$ (Fig. 11 C). The instantaneous I-V curve exhibited a nonlinear outwardly rectifying behavior (Zeng et al., 2001), but the shape of the I-V curve was identical after either 100- or $500-\mu$s repolarizing steps. Similar outward rectification in the instantaneous I-V was also observed after trypsin-mediated removal of inactivation of $\alpha + \beta 3b$ channels (Fig. 9 C).

The novel change in instantaneous I-V curves as a function of time during the $\alpha + \beta 3b$ deactivation tails supports the view that channels must occupy different sets of open states during the deactivation process. This change in occupancy of open states would also be expected to occur during current activation as channels sequentially progress from $O$ to $O^*$. To confirm this expectation, instantaneous I-V curves were generated at

![Figure 12. Instantaneous I-V curves for $\alpha + \beta 3b$ currents change as a function of duration of the activation step. In A, $\alpha + \beta 3b$ currents were activated with $10 \mu$M Ca$^{2+}$ with the voltage protocol shown on the top, except the command step duration was varied as indicated on the figure. Traces on the right are faster time base records of the traces on the left focusing on the properties of currents after repolarization. The vertical bars show the time points at 0 and $80 \mu$s relative to the nominal time of the voltage-step. For the $200-\mu$s activation step, note that currents after repolarization to voltages negative to zero are more closely spaced than those positive to zero, indicative of the intrinsic outward rectification. Sampling period, $10 \mu$s; filter, $10 \text{kHz}$. In B, current amplitudes were measured at the $80-\mu$s time point after repolarization for each voltage, normalized to the trace at $-100$ mV, and plotted as a function of repolarization potential.](image)
Kinetic Properties of Inactivation of $\alpha + \beta$3b Currents

Here, we provide an empirical description of some of the kinetic properties of $\alpha + \beta$3b currents and consider how these processes may relate to Scheme 2.

The Time Constant of Macroscopic Inactivation ($\tau_i$). Upon depolarization from negative holding potentials with Ca\textsuperscript{2+} of 10 µM and higher, $\alpha + \beta$3 currents rapidly activate and inactivate with $\tau_i$ of $\sim$0.5–1.5 ms. Fig. 13 A plots $\tau_i$ as a function of voltage with 300 µM Ca\textsuperscript{2+}. $\tau_i$ becomes largely Ca\textsuperscript{2+} independent above 10 µM Ca\textsuperscript{2+}, and exhibits only a weak voltage dependence (Xia et al., 2000). From Scheme 2, transitions from both $O_n$ to $O^*_n$ and $O^*_n$ to $I_n$ might be expected to contribute to the observed relaxation. However, based on the rapidity of instantaneous unblocking during repolarization and reblocking during a subsequent depolarization, this implies that both $k_o$ and $k_u$ are very fast relative to $k_i$ and $k_r$. This argument is, in fact, the basis for the analysis of the curvature in the instantaneous I-V curve (Figs. 8 and 9). Because of the rapidity of $k_o$ and $k_u$, a channel that enters $O^*_n$ may enter and exit states ($I_n$) many times before returning to $O_n$. Therefore, this would suggest that the macroscopic inactivation time constant, $\tau_i(V)$, would involve an apparent unblocking rate, $k'_i(V)$, defined by:

$$\tau_i(V) = 1/(k_i(V) + k'_i(V)),$$

where

$$k'_i = k_i(k_o/(k_o + k_u)) = k_i \cdot 1/(1 + K_i(V)).$$

Recovery from Inactivation. A number of macroscopic relaxations are observed with the recovery protocol in Fig. 10. Specifically, over some potentials, an unblocking relaxation ($\tau_d$) was observable in the tail current, whereas relaxations could also be measured in the time constant of recovery ($\tau_u$) of current activated at +160 mV using a paired pulse protocol. $\tau_u$ was difficult to measure reliably, but exhibited a slight voltage dependence with values in the range of 100–250 µs, being faster at more negative potentials (Fig. 13 B). No substantial difference in $\tau_u$ was observed between tail currents recorded with 10 or 300 µM Ca\textsuperscript{2+}, although the scatter in the estimates makes this point uncertain. Because multiple relaxations are observed in association with the recovery protocols, at negative potentials, it is tenuous to associate particular time constants with specific postulated molecular steps. However, $\tau_u$, the unblocking relaxation, is about an order of magnitude more rapid than either the paired pulse recovery relaxation or the tail current deactivation time constant. Thus, in accordance with Scheme 2, we propose that the unblocking relaxation would primarily correspond to movement of channels from $O^*_n$ to $O_n$, presumably also reflecting $1/(k_i(V) + k'_i(V))$. However, the unblocking relaxation might also reflect other kinetic processes if, for example, substantial reopening of channels may occur at particular [Ca\textsuperscript{2+}] or in the case that channels in preinactivated states ($O^*_n$) may directly deactivate. $\tau_u$ was measured from the paired-pulse protocol for 10 and 300 µM at three different potentials (Fig. 13 C).
These values, in the range of 0.75–1.5 ms, are of the same order as \( \tau_d \) measured at more positive potentials, but are also essentially identical to the time constants of deactivation (\( \tau_d \)) measured from the tail current. What is the significance of \( \tau_r \)? There is some suggestion that the underlying recovery process is actually better described by a two exponential time course (Fig. 10, E and F), but the two components are difficult to discern with the methods used here. However, the general correspondence of the \( \tau_r \) to \( \tau_d \) would argue that return of channels to states \( C_n \) is the primary determinant of the recovery in current amplitude.

**Steady-state Properties of Currents Predicted by Scheme 2**

The results of three separate types of protocols (Figs. 4 and 5, Fig. 10, and Figs. 11 and 12) exhibit features qualitatively consistent with Scheme 2, but inconsistent with Scheme 1. Here, we examine predictions for macroscopic G-V curves expected for either scheme. Critical to the evaluation of an inactivation model is the selection of an appropriate model for current activation. We are certainly aware of the complexities of the activation behavior of BK channel gating particularly under conditions of low or high Ca\(^{2+}\) (McManus and Magleby, 1988; Cox et al., 1997; Cui et al., 1997; Horrigan and Aldrich, 1999; Rothberg and Magleby, 1999). However, over a wide range of Ca\(^{2+}\) concentrations, the equilibria between closed and open states follows a simple behavior approximated by the voltage-dependent MWC gating model (Cox et al., 1997). Thus, here we use a 10-state MWC activation model in conjunction with the generalized blocking models of Schemes 1 and 2. The general 10-state MWC activation model (Cox et al., 1997) when expanded in accordance with Scheme 2 is given by:

\[
\begin{align*}
C_1 & \rightleftharpoons C_2 \rightleftharpoons C_3 \rightleftharpoons C_4 \rightleftharpoons C_5 \\
O_1 & \rightleftharpoons O_2 \rightleftharpoons O_3 \rightleftharpoons O_4 \rightleftharpoons O_5 \\
O_1^* & \rightleftharpoons O_2^* \rightleftharpoons O_3^* \rightleftharpoons O_4^* \rightleftharpoons O_5^* \\
I_1 & \rightleftharpoons I_2 \rightleftharpoons I_3 \rightleftharpoons I_4 \rightleftharpoons I_5
\end{align*}
\]

(SCHMIE 2a)

where each horizontal row corresponds to Ca\(^{2+}\) association steps. If we assume that rates of entry and exit into O\(_n^*\) and I\(_n\) are identical for O\(_1\)–O\(_5\), the following relationships are defined, with \( Q_1 \) and \( Q_2 \) representing net charge movement associated with particular reaction steps, and \( K_1 \) and \( K_2 \) representing equilibrium constants:

\[
\begin{align*}
O^*/O &= K_1(V) = k_e(V)/k_d(V) \\
I/O^* &= K_2(V) = k_b(V)/k_u(V)
\end{align*}
\]

Assuming that, at any command voltage, steady-state current arises from occupancy in states O\(_n\) and O\(_n^*\), the fractional conductance is given by:

\[
G_n(V) = (O_n + O_n^*)/(C_n + O_n + O_n^* + I_n).
\]

With O/C = \( K_0(V) \),

\[
\begin{align*}
G_n(V) &= K_0(V) + K_0(V) \cdot K_1(V)/(1 + K_0(V) + K_0(V) \cdot K_1(V) + K_0(V) \cdot K_1(V) \cdot K_2(V)) \\
&\quad \text{or} \\
G_n(V) &= (1 + K_1(V))/(1 + 1/K_0(V) + K_1(V) \cdot K_2(V)).
\end{align*}
\]

We further assume that Ca\(^{2+}\) binding among O\(_n^*\) and among I\(_n\) is identical to the Ca\(^{2+}\) association and dissociation steps among O\(_n\). From this, the steady-state conductance arising from Scheme 2 following from Cox et al. (1997) is given by:

\[
G_n(V) = (1 + K_1(V))/(1 + B \cdot L(0) \cdot \exp(-QFV/RT) + K_1(V) + K_1(V) \cdot K_2(V)),
\]

where B, L(0), and Q are as defined for Eq. 2 in Materials and Methods.

For tail currents, we assume that, because of rapid unblocking during repolarization, all channels in I\(_n\) immediately return to O\(^*\). As shown experimentally, this does not appear to be the case, i.e., repolarization results in a new voltage-dependent equilibrium between O\(^*\) and I\(_n\). However, at very negative repolarization potentials this simplifying assumption is not unreasonable. The peak tail currents will therefore reflect occupancy in states O, O\(^*\), and I immediately before the repolarizing voltage-step. Thus,

\[
G_t(V) = (O_n + O_n^* + I_n)/(C_n + O_n + O_n^* + I_n).
\]
\[ G_t(V) = (K_0(V) + K_0(V) \cdot K_1(V) + \\
K_0(V) \cdot K_1(V) \cdot K_2(V)) / (1 + K_0(V) + \\
K_0(V) \cdot K_1(V) + K_0(V) \cdot K_1(V) \cdot K_2(V)) \]

or

\[ G_t(V) = (1 + K_1(V) + K_1(V) \cdot K_2(V)) / (1 + B \cdot L(0) \cdot \\
\exp(-QFV/RT) + K_1(V) + K_1(V) \cdot K_2(V)) \quad (6) \]

Similar equations, except lacking the term for \( K_2(V) \), can be generated to describe the behavior of currents arising from an expanded version of Scheme 1 (15 states). Given the empirical requirement that unblocking from inactivated states is exceedingly rapid, here we consider only the case where channels blocked in accordance with Scheme 1 reopen very rapidly after repolarization, such that channels in both O and I contribute to the tail currents. More detailed evaluation of the discriminatory capability of Scheme 1 and Scheme 2 is presented in the Online Supplemental Materials (available at http://www.jgp.org/cgi/content/full/117/6/583/DC1). In short, this evaluation shows that families of G-V curves are unlikely to prove of much use in discerning between the two types of blocking models. However, there are two interesting facets of G-V curves predicted by either Scheme. Because of rapid unblocking and the contribution of inactivated channels to the tail currents, the \( V_{0.5} \) for G-V curves measured from tail currents exhibits a pronounced leftward shift at lower \( \text{Ca}^{2+} \) relative to channels activated in the absence of inactivation. This shift in the \( V_{0.5} \) is also accompanied by a steeper voltage dependence of the G-V curve.

Both the B1 and B2 subunits produce profound shifts in the curves for activation of conductance as a function of voltage at a given \( \text{Ca}^{2+} \) (McManus et al., 1995; Wallner et al., 1995, 1999; Xia et al., 1999). For non-inactivating currents, such curves are typically determined from tail current measurements, whereas for inactivating currents, determination of the G-V curves for activation can be more complicated. We have previously reported that the \( \beta_3 b \) subunit produces shifts in the \( V_{0.5} \) for activation at lower \( [\text{Ca}^{2+}] \) than is typically observed for other \( \beta \) subunits (Xia et al., 2000). Our analysis here indicates that, irrespective of whether blockade occurs by either Scheme 1 or Scheme 2, a rapid blocking and unblocking process can result in an apparent shift in the \( V_{0.5} \) for activation at low \( \text{Ca}^{2+} \). Thus, the observed shift in \( V_{0.5} \) seen with the \( \beta_3 b \) subunit at low \( \text{Ca}^{2+} \) (Xia et al., 2000) is consistent with a fast unblocking mechanism, but does not by itself distinguish between Scheme 1 or Scheme 2.

To define parameters for Scheme 2 that might be consistent with our results, families of G-V curves obtained from \( \alpha + \beta_3 b \) currents were fit to either Scheme 1 or Scheme 2. Fig. 14 displays normalized tail current and steady-state current G-V curves for \( \alpha + \beta_3 b \) currents from one patch measured with 0, 0.5, 1, 4, 10, and 300 \( \mu\text{M} \text{Ca}^{2+} \). In A1 and A2, conductance values were fit with Eqs. 5 and 6 derived from Scheme 2, whereas, in B1 and B2, values were fit with similar equations derived from the expanded 15-state version of Scheme 1. Not unexpectedly, neither model clearly fits the data set better than the other. In both cases, the approximate spacing of the G-V curves is faithfully described. For either scheme, the shape of the steady-state G-V curves is reasonably well-described from 4 \( \mu\text{M} \text{Ca}^{2+} \) and higher. However, in both cases, the experimentally measured steady-state conductance at the most positive voltages, particularly at 0 \( \text{Ca}^{2+} \), underestimates that expected for these models (see Fig. 1). This is observed, even though the maximal tail current conductance is similar at either 0 \( \text{Ca}^{2+} \) or 300 \( \mu\text{M} \text{Ca}^{2+} \). This observation was consistently observed in all patches.

The greater reduction in steady-state current at 0 \( \text{Ca}^{2+} \) than at higher \( \text{Ca}^{2+} \) is rather unusual and was

**Figure 14.** Families of G-V curves obtained from both steady-state and tail current measurements do not distinguish between Schemes 1 and 2. Tail current amplitudes and steady-state current amplitudes were measured in one patch at 0, 0.5, 1, 4, 10, and 300 \( \mu\text{M} \text{Ca}^{2+} \). The normalized conductance is plotted as a function of command potential with tail current measurements in the left column (A1 and B1) and steady-state current estimates (A2 and B2) on the right. In A, both panels were fit simultaneously with the expanded MWC version of Scheme 2 defined by Eqs. 5 and 6. \( K_C = 10.11 \pm 0.75; K_0 = 0.66 \pm 0.05; L(0) = 1775.74 \pm 1280; Q = 1.26e \pm 0.13; K_1(0) = 0.31 \pm 0.10; Q_1 = 0.44e \pm 0.14; K_2(0) = 2.17 \pm 3.6; Q_2 = 0.19e \pm 0.21. \) In B, both panels were fit simultaneously with similar equations defined by Scheme 1, assuming essentially instantaneous recovery from I to O at the tail current potential. \( K_C = 11.20 \pm 0.17; K_0 = 0.75 \pm 0.04; L(0) = 1996.3 \pm 243; Q = 1.41e \pm 0.045; K_1(0) = 0.71 \pm 0.14; Q_1 = 0.342e \pm 0.04. \)
noted previously (Xia et al., 2000). This observation would require that, at saturating activation, the equilibrium among channels in $O_n$, $O^*_n$, and $I_n$ would differ at low and high Ca$^{2+}$, with greater relative occupancy of $I_n$ at lower Ca$^{2+}$. This would require models in which inactivation from different states $O_n$ is not identical. One of the limitations of the voltage-dependent MWC gating model is that it fails to account for the multiple closed and open states observed for activation of BK channels at 0 Ca$^{2+}$ (Horrigan and Aldrich, 1999; Horrigan et al., 1999; Nimigean and Magleby, 2000; Talukder and Aldrich, 2000). In such cases, activation at 0 Ca$^{2+}$ is presumably driven exclusively by movement of voltage sensors in each subunit, whereas, at high Ca$^{2+}$, Ca$^{2+}$-binding steps become important. Thus, to account for both the voltage-dependent and Ca$^{2+}$-dependent properties of the channel, two-tiered activation models for BK channels have been proposed (Rothberg and Magleby, 1999; Cox and Aldrich, 2000). Future analysis will have to consider whether inactivation might proceed somewhat differently from open states occupied at low Ca$^{2+}$ relative to those at high Ca$^{2+}$. We also considered that the reduction in steady-state currents in 0 Ca$^{2+}$ might arise from a Ca$^{2+}$-dependent inhibition of the blocking process. However, such a scheme would require that Ca$^{2+}$-dependent inhibition be saturable by $\sim$1 $\mu$M Ca$^{2+}$ and could not result from simple mass-action competition between Ca$^{2+}$ and the inactivation mechanism.

**Scheme 2 Predicts the Unusual Behavior of the $\alpha + \beta$3b Currents**

To test whether Scheme 2 can reproduce the key features of the $\alpha + \beta$3b currents, we have used estimates for activation rates for the MWC model taken from Table I of Cox et al. (1997). From Scheme 2, the following parameters for the kinetic steps involved in block were defined:

- $k_f(0)$ and $z_1$, giving $k_f(V) = k_f(0) \cdot \exp(z_1 F V/RT)$
- $k_r(0)$ and $z_2$, giving $k_r(V) = k_r(0) \cdot \exp(z_2 F V/RT)$
- $k_b(0)$ and $z_3$, giving $k_b(V) = k_b(0) \cdot \exp(z_3 F V/RT)$
- $k_u(0)$ and $z_4$, giving $k_u(V) = k_u(0) \cdot \exp(z_4 F V/RT)$

Our goal was to identify a set of rates that generally reproduces the essential features of the observed currents.

**The Role of $k_b$ and $k_u$**

Let us consider first the properties of the rapid equilibrium between $O^*$ and $I$. To account for the instantaneous block and unblock, $k_b$ and $k_u$ must be sufficiently fast that there is no detectable relaxation, at least under normal recording conditions. Thus, the actual rates of $k_b$ and $k_u$ are arbitrary based on the information available to us, and were chosen so that, except at infinite bandwidth, relaxations between $O^*$ and $I$ would not be observable. The relative voltage dependence of the two rates and the ratio of the rates at positive potentials are very critical in terms of defining the steady-state levels of current at the positive potentials. Empirically, it is observed that outward current levels at different voltages tend to remain constant over a range of positive voltages (as seen in the actual data records of Fig. 1). This required values for the net charge associated with the $O^*$ to $I$ equilibrium of $\sim$0.2e–0.25e. This agrees with the value of 0.21e obtained from fitting the instantaneous I-V curve in Fig. 8.

**The Role of $k_f$ and $k_r$**

Several factors dictate appropriate choices of values for $k_f$ and $k_r$. First, given the mild voltage dependence of $\tau_o$, the voltage dependence of $k_f$ must be relatively weak. Second, if $k_r$ is too slow, it re-

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**Table I**

| Activation parameter | Range defined in Cox et al. | Values used for current simulations |
|----------------------|-----------------------------|------------------------------------|
| $L(0)$               | 1.647–2.029                 | 2,000                              |
| $Q$                  | 1.35–1.40e                  |                                    |
| $K_C$                | 8.68–11.0                   |                                    |
| $K_O$                | 1.04–1.1                    |                                    |
| $C_0 \rightarrow O_0$| 1.8–2.39                    | 2                                  |
| $C_1 \rightarrow O_2$| 5.0–7.0                     | 6                                  |
| $C_2 \rightarrow O_2$| 29–40                       | 40                                 |
| $C_3 \rightarrow O_3$| 130–295                     | 250                                |
| $C_4 \rightarrow O_4$| 300                         | 500                                |
| $q_o$                | 0.71–0.73e                  | 0.72e                              |
| $O_0 \rightarrow C_0$| 3.612–5.936                 | 4,000                              |
| $O_1 \rightarrow C_1$| 1.076–1.338                 | 1,200                              |
| $O_2 \rightarrow C_2$| 659–974                     | 800                                |
| $O_3 \rightarrow C_3$| 486–490                     | 500                                |
| $O_4 \rightarrow C_4$| 92–126                      | 100                                |

| Blocking parameters | $k_x(V) = k_x(0) \cdot \exp(z_x F V/RT)$ | $k_x(0)$ | $z_x$ |
|---------------------|------------------------------------------|---------|------|
| $k_x(V)$ $O_n \rightarrow O^*_n$ | 900                                      | 0.072   |      |
| $k_x(V)$ $O^*_n \rightarrow O_n$ | 750                                      | 0.361   |      |
| $k_x(V)$ $O^*_n \rightarrow O^*_n$ | 90,000                                   | 0.084   |      |
| $k_x(V)$ $I_n \rightarrow O^*_n$ | 80,000                                   | 0.14    |      |
| Equilibrium blocking parameters | $K_x(0)$ | net Q |
| $K_x(V)$ $O^*_n$ | 1.2                                      | 0.433e  |      |
| $K_x(V)$ $O^*_n$ | 1.12                                     | 0.225e  |      |

Equilibrium blocking parameters are defined by $K_x(V) = k_x(V)/k_x(0) = k_x(0) \cdot \exp(-z_x F V/RT)$ and $K_x(V) = k_x(V)/k_x(0) = k_x(0) \cdot \exp(-z_x F V/RT)$.

*(Cox et al., 1997)*
sults in an excessive slowing of tail currents. However, if $k_r$ is too fast and has appreciable voltage dependence, it will contribute to a voltage dependence of $\tau_i$ that is inconsistent with the results. The precise value of $k_r$ and its voltage dependence seem to be parameters that are most critical in trying to account for several aspects of the data. From these considerations, transition rates between O and O* were adjusted to result in values for the macroscopic $\tau_i$ and $\tau_d$ that approximate those seen experimentally. In our adjustment of parameters, we also took into account estimates for equilibrium values for $K_1(0)$, $Q_1$, $K_2(0)$, and $Q_2$ made from fits of the macroscopic tail and steady-state G-V curves in Fig. 14 and for estimates of $K_2(0)$ and $Q_2$ from fits to the instantaneous I-V curves in Figs. 8 and 9.

Based on the above, a set of values were chosen that reasonably reproduced the kinetic properties of the blocking and unblocking relaxations observed in the raw currents we have examined. Table II provides a listing of the values for the parameters used in the simulations and also those obtained from the two fitting procedures. In general, there is reasonable agreement among all values except for $K_2(0)$. Of all equilibrium values, that for $Q_2$ is probably the most accurately constrained, since it is this value that primarily defines the slope of the steady-state G-V curves at positive voltages.

**Effects of Short and Longer Activation Steps on Tail Currents.** Currents were simulated with a stimulation protocol similar to that used to activate currents in Fig. 2. Assuming 10 $\mu$M Ca$^{2+}$, tail currents were elicited at either −80 (Fig. 15, A1) or −160 mV (Fig. 15, A2) after either a 1- or 10-ms step to +120 mV. For tails examined at −80 mV, the tail current amplitude was essentially identical for both 1- and 10-ms command steps, despite the fact that the outward current at 1 ms greatly exceeded the outward current at 10 ms. Furthermore, with the repolarization to −80 mV, the tail current exhibits the shoulder of unblocking observed in the $\alpha + \beta 3b$ currents. For tails examined at −160 mV, the tail current after the 10-ms step actually exceeds that observed after the 1-ms step, similar to Fig. 5. Despite the unblocking shoulder in the tail current at −80 mV after the 10-ms step, single exponential functions fit to the tail currents after either 1- or 10-ms activation steps yielded similar values (see Fig. 15 legend).

**Paired Pulse Recovery Properties Predicted by Scheme 2.** Currents were simulated with parameters given in Table I with a stimulation protocol similar to that used in Fig. 10. Assuming 10 $\mu$M Ca$^{2+}$, a pair of test steps to +160 mV were separated by recovery steps to −80 mV ranging from 50 $\mu$s to 8 ms duration (Fig. 15 B). Similar to the $\alpha + \beta 3b$ currents, despite the rapid recovery from inactivation seen in the tail current amplitude, there is a slower increase in peak current amplitude during the second test step as the duration of the recovery interval is increased. With the shorter recovery steps (e.g., 50 $\mu$s), a subsequent depolarization results in a nonohmic step in current which only slightly exceeds the previous steady-state level of current at +160 mV. With longer recovery steps, the second step to +160 mV evokes a more ohmic step in current. These features recapitulate those observed for the $\alpha + \beta 3b$ currents. For brief recovery steps, there is a spike of current at the onset of the second step to +160 mV. This represents the ohmic current through channels in states O* that become blocked within 20 $\mu$s during the step to +160 mV and would not be detected with typical recording conditions.

**Scheme 2 Predicts Changes in the Instantaneous I-V Curves.** Instantaneous I-V curves were simulated with a protocol similar to that used in Fig. 11. After activation of current at +160 mV with 10 $\mu$M Ca$^{2+}$, a repolarization to −120 mV of either 100- or 500-$\mu$s duration preceded a subsequent step to potentials between −120

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**Table II**

*Equilibrium Parameters for Activation and Inactivation*

| Activation parameter | Range defined in Cox et al.* | Values from fit to G-V curves |
|----------------------|-----------------------------|-----------------------------|
| $I_i(0)$             | 1.647–2.029                 | 1.775 ± 1.280               |
| $Q_1$                | 1.35–1.40e                  | 1.26 ± 0.13e                |
| $K_C$                | 8.68–11.0                   | 10.11 ± 0.75                |
| $K_O$                | 1.04–1.1                    | 0.66 ± 0.005                |

| Equilibrium inactivation parameters | Values used for simulating currents | Values obtained from fit to G-V curves | Values obtained from fit to instantaneous I-V§ |
|-------------------------------------|-------------------------------------|----------------------------------------|---------------------------------------------|
| $K_x(V) = K_x(0) \cdot \exp(Q_x F V / RT)$ | $K_x(0)$ | $Q_x$ | $K_x(0)$ | $Q_x$ | $K_x(0)$ | $Q_x$ |
| $K_C(V)$                           | 1.2 | 0.433e | 0.51 ± 0.1 | 0.44 ± 0.014 |
| $K_O(V)$                           | 1.12 | 0.225e | 2.17 ± 3.6 | 0.192 ± 0.2 |

| Blocking parameters: | | |
|----------------------|---|---|
| $K_x(V) = K_x(0)$    | $K_x(0)$ | $Q_x$ |
| $K_C(V)$             | 1.2 | 0.433e |
| $K_O(V)$             | 1.12 | 0.225e |

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*(Cox et al., 1997).*

†(Fig. 14).

§(Fig. 8).
DISCUSSION

Rapid inactivation of voltage-dependent K⁺ channels mediated by NH₂-terminal domains, either of the pore-forming α subunits (Hoshi et al., 1990; Ruppersberg et al., 1991; Tseng-Crank et al., 1993) or of auxiliary β subunits (Rettig et al., 1994; Morales et al., 1995; Rasmusson et al., 1997), has typically been analyzed with a simple scheme in which binding of the inactivation domain to a binding site directly results in cessation of ion permeation (Hoshi et al., 1990; Demo and Yellen, 1991; MacKinnon et al., 1993). Kinetic features of inactivation onset and recovery support the idea that a single molecular step governs the blocking process. While in its inactivating position, the inactivation domain is thought to rest in intimate association with the mouth of the ion permeation pathway. This assertion is based on multiple types of evidence. Cytosolic blockers impede the movement of the inactivation domain to its blocking site (Choi et al., 1991). Occupancy of the ion permeation pathway by permeant ions favors dissociation of the inactivation domain from its binding site (Gomez-Lagunas and Armstrong, 1994). After repolarization, occupancy of its binding site by the inactivation domain inhibits movement of the open pore to a closed conformation resulting in channel reopening during recovery from inactivation (Demo and Yellen, 1991; Ruppersberg et al., 1991). Furthermore, residues in the S4-S5 loop are thought to contribute to the inactivation domain binding site and also influence ion permeation (Isaoff et al., 1991).

Inactivation mediated by NH₂ termini of BK β subunits (Wallner et al., 1999; Xia et al., 1999; Xia et al., 2000) shares with ShakerB inactivation (MacKinnon et al., 1993; Gomez-Lagunas and Armstrong, 1995) the

**Figure 15.** Scheme 2 predicts currents that exhibit properties similar to those observed for α + β₃b currents. In A, Scheme 2 (with parameters in Table I), assuming 10 μM Ca²⁺, was used to simulate currents with the indicated protocol (as in Fig. 2). Tail currents were compared at either −80 mV (A1) or −160 mV (A2), after either a short (1 ms) or longer (10 ms) activation step. Single exponential fits to the decay phases yielded time constants of 1.23 and 1.24 ms, for the shorter and longer activation steps, respectively. In A2, tail current was elicited at −160 mV. Time constants of deactivation were 0.227 ms (longer step) and 0.221 ms. In B, a paired-pulse protocol (as in Fig. 10) was used to model the recovery from inactivation with Scheme 2 (values from Table I, 10 μM Ca²⁺). Points show each simulated value (10 μs). The tail current exhibits the rapid nonohmic unblocking, followed by a slower unblocking. After the shorter recovery steps (500 μs), a subsequent depolarization to +160 mV results in rapid reblocking with only a small excess of current over the previous steady-state level of block. Longer recovery steps result in an increase in the initial amount of current observed during the step to +160 mV, even as the tail current at −80 mV decreases. For the transition rates between O* and I in Table I, at short recovery times the step to +160 mV also results in a 10–20-μs rapid spike of current associated with the rapid reblocking of channels from O* to I. Currents were simulated at 10 μs per point, and the spike of current lasted 1–2 points. In C, the indicated protocol (as in Fig. 11) was used to simulate currents assuming 10 μM Ca²⁺. In the middle panel, a 100-μs recovery step to −120 mV preceded a subsequent step to potentials between −120 and +180 mV. In the bottom panel, a 500-μs step to −120 mV was used to produce recovery from inactivation. In D, the instantaneous I-V from the traces in C were determined by measuring current values 30 μs after the nominal end of the recovery steps. Values were normalized to current amplitudes at −100 mV. At 30 μs, the spike of reblocking observed in the simulated currents is complete. The I-V curve becomes more linear after the 500-μs recovery step (●), which is indicative of greater occupancy of O*. Simulation frequency: 100 kHz.
fact that each of the up to four inactivation domains per channel appears to function independently to produce inactivation (Ding et al., 1998). However, unlike voltage-dependent K+ channel inactivation, cytosolic blockers do not impede the BK inactivation process (Solaro et al., 1997; Xia et al., 1999, 2000) and channels can recover from the inactivated state without passing through open states (Solaro et al., 1997). These results have suggested that the NH2 terminus of the β2 subunit may not directly occlude the mouth of the channel, although binding at a more superficial site might account for the blocking effect.

Inactivation mediated by the β3b subunit also exhibits features inconsistent with the simple occlusion model proposed for ball-and-chain inactivation mechanisms. First, inactivated channels exhibit two unblocking components after repolarization: an essentially nonohmic, instantaneous increase in tail current, followed by a small secondary time-dependent unblock. Second, after short repolarizations that drive inactivated channels back to open states, a subsequent depolarization will result in an essentially instantaneous reblock of those open channels, despite the slower inactivation kinetics observed when channels are activated from closed states. Third, instantaneous I-V curves generated at different times during deactivation indicate that channels occupy multiple kinds of open states.

To account for our observations, we proposed an extension to the simple, open channel block model, namely that a transition to a preinactivated open state precedes a subsequent blocking step. This model, given in Scheme 2a, reproduces most of the novel features of the α + β3b currents, including the instantaneous unblock and subsequent slower unblock in the tail currents, the characteristics of the paired pulse protocols, and the changes in the instantaneous I-V curves at different times during activation and deactivation. Although rates for Scheme 1 can be chosen to produce rapid unblocking upon repolarization, Scheme 1 is entirely unable to account for other aspects of the results. In contrast, using rates constrained by those suggested from experimental observations, it was possible to simulate currents from Scheme 2 that approximated the key kinetic and steady-state features of the α + β3 currents.

Although we have not systematically evaluated a variety of models, a number of factors suggest that Scheme 2 provides the simplest extension of Scheme 1 that can account for most of our results. As argued, at least two kinetic states must contribute to the blocking reaction. Furthermore, among other factors, the changes in the shape of the instantaneous I-V curve argue quite compellingly for the existence of a type of open state distinct from those that occur when the NH2 terminus is absent. This behavior of the instantaneous I-V curves, therefore, excludes models with a single category of open state but with multiple closed, inactivated states such as \( O \equiv I \equiv I_1 \). It might also be argued that the inactivated state represents an open state with less than a full-conductance, such that the blocking scheme would be \( O \equiv O_a \), where \( O_a \) has a conductance that is a small fraction of that of \( O \). Such a model could approximate our results if the single-channel conductance of \( O_a \) were markedly voltage-dependent. Such a model is, in essence, functionally equivalent to that given in Scheme 2, in that a voltage-dependent single-channel conductance approximates the rapid two-state transitions between \( O^* \) and \( I \). We prefer Scheme 2, however, since it provides an explicit kinetic step to account for the apparent voltage dependence of conductance.

The inward rectification resulting from the rapid block and unblock of open α + β3b channels is somewhat reminiscent of cytosolic blockade of BK channels produced by two peptides, bovine pancreatic trypsin inhibitor (BPTI) and dendrotoxin (DTX; Lucchesi and Moczydlowski, 1991; Moss and Moczydlowski, 1996; Favre and Moczydlowski, 1999). Blockade by these peptides is associated with the appearance of subconductance states that arise from rapid flickering between open and closed states (Moss and Moczydlowski, 1996). The subconductance states exhibit inwardly rectifying behavior, presumably because of the voltage dependence of the processes controlling opening and closing during the rapid flickering (Moss and Moczydlowski, 1996). These results have been interpreted in terms of a fluctuating barrier within the ion permeation pathway that is allosterically regulated by binding of BPTI or DTX to sites outside the channel pore. Analysis of blockade by Ba2+ in the presence and absence of BPTI has supported the idea that binding of Ba2+ to the channel is unaffected whether BPTI is bound or not (Lucchesi and Moczydlowski, 1991). Thus, BPTI and DTX appear to bind to sites that do not prevent access by Ba2+ (or TEA) to the cytosolic mouth of the channel. Similarly, TEA does not inhibit access of the α + β3b inactivation domain to the inactivation site. Thus, it is possible that both blockade by BPTI and inactivation may involve actions at sites peripheral to the ion permeation pathway, that then promote rapid flickery opening and closing of the channel. Future work will be required to assess whether block by BPTI is related the inactivation behavior described here.

**Aspects of α + β3b Currents Not Explained by the Two-Step Blocking Model**

**Outward Rectification in Instantaneous I-V Curves.** One characteristic of α + β3b currents not considered by the proposed model is the outward rectification observed in the instantaneous I-V curves, under conditions when inactivation is abolished or not yet developed. This is particularly apparent following removal of
inactivation by trypsin, and by molecular deletion of the β3b NH2 terminus (Zeng et al., 2001). The presence of the outward rectification following removal of NH2 and COOH termini indicates that the nonlinearity is unrelated to the inactivation mechanism we have studied and we expect that it will occur in parallel with any ongoing inactivation mechanism. Because of this nonlinearity in the instantaneous I-V curve, the shape of the steady-state and peak G-V curves arising from the α + β3b currents and also the instantaneous I-V curves with inactivation intact will be biased by this secondary effect. However, this phenomenon will have essentially no effect on any of the basic conclusions we have drawn that depend on the kinetic characteristics of the β3b inactivation mechanism.

Smaller Steady-state Currents at Lower Ca2+. Another unexplained aspect of α + β3b currents is that, at positive potentials with zero or very low Ca2+, the steady-state currents are smaller than would be expected based on the size of the tail currents (Fig. 1). The similarity of peak tail current after activation to very positive potentials at both 0 Ca2+ and 300 μM Ca2+ would suggest that, in accordance with any rapid blocking scheme, the same fraction of channels occupy activated states (e.g., O_n, O*_n, and I_n) at both low and high Ca2+. For the steady-state level of current to be less at lower Ca2+ would require that the distribution of channels among the activated states favor I_n at lower Ca2+. This condition cannot be achieved by any kind of model in which inactivation from all open states is identical. However, if the affinity of the inactivation domain to channels that are activated primarily as a consequence of voltage sensor movement was stronger than to channels activated primarily as a consequence of Ca2+-dependent transitions, it might be possible to account for this behavior. An extension of the 50-state two-tiered model (Horrigan and Aldrich, 1999; Rothberg and Magleby, 1999; Cox and Aldrich, 2000) to include inactivation might help address this possibility.

Lack of Contribution of Recovery from Inactivation to Tail Current Deactivation. Both the linear blocking models of Schemes 1 and 2 suggest that, during repolarization, when recovery from inactivation is not complete, the kinetics of blocking transitions should contribute to the tail current relaxation time course. However, two observations have indicated that the kinetics of the blocking reactions do not contribute to the final exponential decay of the deactivation time course. First, τ_d was similar for constructs either with or without the inactivating NH2 terminus. Second, τ_d was similar before and after removal of inactivation by trypsin. Both observations are obviously inconsistent with either Scheme 1 or Scheme 2. We envision two possible explanations for this lack of participation of blocking reactions in the deactivation time course. In one case, the rates of return of channels to states (O_n) may be sufficiently fast compared with the closing rates that deactivation time constants are unaffected by the blocking steps. This explanation seems unlikely, since there should then be little difference in peak tail current amplitude before and after trypsin application. On the other hand, the lack of influence of inactivation on deactivation kinetics could also arise, if there were pathways by which channels in inactivation-specific states (O*_n and I_n) could return to closed pathways without returning through O_n. A model of this kind is given in Scheme 3:

\[
\begin{array}{c}
C_n \rightleftharpoons O_n \\
\downarrow \quad \downarrow \\
C^*_n \rightleftharpoons O^*_n \\
\downarrow \\
I_n
\end{array}
\]

(Scheme 3)

In this model, occupancy of channels in states I_n and O*_n does not impede their return to closed states. This modification to Scheme 2, with appropriate rates, can still qualitatively reproduce the key features of the α + β3b currents, but does a better job of accounting for the properties of τ_d. In addition to explaining the lack of change in τ_d after the removal of the NH2 terminus, Scheme 3 may help account for the large increase in outward current after trypsin-mediated removal of inactivation. The enormous increase in the peak currents after trypsin application suggests that, with the inactivation mechanism intact, there may be more entry into inactivated states from closed states than would be predicted by linear blocking models. The moderate increase in tail current amplitudes after trypsin application indicates that a similar number of channels are activated in both cases. However, the discrepancy between peak current values before and after trypsin indicates that many channels that contribute to the tail current before trypsin are contributing less than expected to the outward current, raising the possibility of entry into inactivated states from closed states.

In sum, the results have indicated that Scheme 2 accounts for many unusual features of the α + β3b behaviors, although some aspects of the α + β3b currents remain unexplained. From considerations just given, a
matter to adjust the rates in Scheme 2 to obtain currents which qualitatively resemble those obtained with $\alpha + \beta_2$ coexpression. Future work in conjunction with mutational analysis of the $\beta_2$ and $\beta_3b$ NH$_2$ termini may allow molecular dissection of the elements necessary for the inactivation process and a separation of factors that may contribute to the binding and blocking steps.

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