A Gastropod Toxin Selectively Slows Early Transitions in the Shaker K Channel’s Activation Pathway

Jon T. Sack,1,2 Richard W. Aldrich,2 and William F. Gilly1

1Hopkins Marine Station of Stanford University, Department of Biological Sciences, Pacific Grove, CA 93950
2Department of Molecular and Cellular Physiology, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305

ABSTRACT A toxin from a marine gastropod’s defensive mucus, a disulfide-linked dimer of 6-bromo-2-mercaptotryptamine (BrMT), was found to inhibit voltage-gated potassium channels by a novel mechanism. Voltage-clamp experiments with Shaker K channels reveal that externally applied BrMT slows channel opening but not closing. BrMT slows K channel activation in a graded fashion: channels activate progressively slower as the concentration of BrMT is increased. Analysis of single-channel activity indicates that once a channel opens, the unitary conductance and bursting behavior are essentially normal in BrMT. Paralleling its effects against channel opening, BrMT greatly slows the kinetics of ON, but not OFF, gating currents. BrMT was found to slow early activation transitions but not the final opening transition of the Shaker ILT mutant, and can be used to pharmacologically distinguish early from late gating steps. This novel toxin thus inhibits activation of Shaker K channels by specifically slowing early movement of their voltage sensors, thereby hindering channel opening. A model of BrMT action is developed that suggests BrMT rapidly binds to and stabilizes resting channel conformations.

KEY WORDS: Shaker • Kv • gating • potassium channel • neurotoxin

INTRODUCTION

The activation path of Shaker (ShBΔ) potassium channels consists of many voltage-dependent transitions between closed states, culminating in a single open state (Hoshi et al., 1994; Schoppa and Sigworth, 1998a). Transitions early in the activation pathway determine the characteristic delay and sigmoidal time course with which K current (Ig) rises after a positive voltage step (Zagotta et al., 1994a; Schoppa and Sigworth, 1998c; Ledwell and Aldrich, 1999). Early voltage-dependent transitions are thought to occur independently in each of the channel’s four α-subunits (Zagotta et al., 1994a; Schoppa and Sigworth, 1998c). Early transitions also determine the time course of ON gating current (Ig,ON) (Bezanilla et al., 1994; Zagotta et al., 1994a; Schoppa and Sigworth, 1998c), which reflects movements of the channel’s voltage sensors.

After these early transitions, the four subunits appear to undergo one or more highly cooperative steps, resulting in the channel opening to potassium permeation. These late steps involve only 10–20% of the total gating charge (Schoppa and Sigworth, 1998c; Ledwell and Aldrich, 1999) and normally occur so quickly that they have only minor effects on the time course of Ig,ON or Ig activation. When channels deactivate, these late steps are first in the reverse pathway. When the channels deactivate, these late steps are rate limiting for both Ig,OFF and Ig “tail” currents as the channels close (Zagotta et al., 1994a; Schoppa and Sigworth, 1998c).

Later activation steps are kinetically adjacent to the open state, and much has been learned about them by studying the behavior of macroscopic and single-channel Ig. This experimental advantage has aided identification of specific mutations that separate the final cooperative opening step from earlier gating transitions (Baker et al., 1998; Schoppa and Sigworth, 1998b; Smith-Maxwell et al., 1998b; Ledwell and Aldrich, 1999; Mannuzzu and Isacoff, 2000). The dynamics of states later in the activation pathway have also been probed with a variety of compounds that preferentially block open channels (Armstrong, 1966, 1971; Choi et al., 1993; Holmgren et al., 1997; Brock et al., 2001; Melishchuk and Armstrong, 2001). One gating modifier, 4-aminopyrididine, has been reported to inhibit only the final transition to the open state, revealing the energetics of that final opening step (Armstrong and Loboda, 2001; Loboda and Armstrong, 2001).

In contrast, early gating transitions have been far more difficult to study. Although gating currents pro-
vide an informative signal about these transitions, details about individual early steps have been difficult to extract. Pharmacological approaches using ligands that specifically alter early gating steps would be of great value in studying early transitions, but few agents of this sort are presently available.

Recently, we identified the disulfide-linked dimer of 6-bromo-2-mercaptoptryptamine (BrMT) as a novel toxin that slows K-channel activation gating (Kelley et al., 2003).

BrMT was isolated from a marine snail (Calliostoma canaliculatum) and is a component of the gastropod’s defensive mucus. This toxin has a unique pharmacology, as it slows activation of Kv1 (Shaker-type), Kv4, and EAG channels, but not Kv2 or Kv3 channels (Kelley et al., 2003).

In this paper we determine that BrMT inhibits activation of Shaker K channels by a novel mechanism. We find that BrMT specifically slows early voltage-dependent activation transitions, and a model is proposed for the mechanism by which BrMT slows these activation steps. This work identifies BrMT as a novel reagent that selectively stabilizes Shaker’s voltage sensors in their resting conformation.

MATERIALS AND METHODS

BrMT Solutions

BrMT was purified from hypobranchial glands of Calliostoma canaliculatum as described previously (Kelley et al., 2003). All concentrations of BrMT cited refer to the active dimeric form. BrMT was diluted from an aqueous stock solution containing residual acetonitrile and trifluoroacetic acid from the purification process. These solvents had no significant effect on channel properties at the dilutions used to study BrMT. BrMT-containing solutions were frozen for long-term storage at −80°C.

BrMT appeared to be light sensitive. After several days of exposure to fluorescent laboratory lighting, degradation was apparent in the UV/visible absorbance spectrum of BrMT solutions. The intensity of the UV/visible absorbance spectrum of BrMT in physiological salt solutions decreased after contact with many different surfaces, suggesting that BrMT was being retained. Materials that appeared to retain BrMT included: polyethylene, polypropylene, polycarbonate, glass, and quartz. The amount of BrMT that a piece of plastic or glassware could retain appeared saturable. To prevent loss of BrMT from solutions, polytetrafluoroethylene (PTFE; Teflon®) was used whenever practical.

Channel Expression

Oocytes. Xenopus laevis oocytes were surgically removed, defolliculated with collagenase, and stored at 17°C in ND96 solution (in mM): 96 NaCl, 2 KCl, 1.8 CaCl2, 2 MgCl2, 5 HEPES (pH 7.6) plus 10 μg/ml gentamycin. The Drosophila ShakerBΔ-46 (ShBΔ) constructs had NH2-terminal residues 6–46 deleted to eliminate fast, N-type inactivation (Hoshi et al., 1990). Unless noted otherwise, a ShBΔ construct with C-type inactivation minimized by the T449V mutation (Lopez-Barneo et al., 1993) was used to study the effects of BrMT on activation with minimal interference from channel inactivation. The ILT construct (ShBΔ V369F/I372L; S376T) (Smith-Maxwell et al., 1998b) retained a threonine at position 449. The full coding regions of all constructs were verified by nucleotide sequencing. K channel RNA was transcribed with the mMessage Machine® 17 kit (Ambion) following the manufacturer’s protocols, and oocytes were injected with RNA 2–7 d before recording.

Mammalian cells. The ShBΔ channel expressed in CHO-K1 cells (American Type Culture Collection) contained the additional mutations C301S, C308S and T449V (Holmgren et al., 1996) and was a gift of G. Yellen, Harvard University. The effects of BrMT on these channels in CHO cells were similar to ShBΔ expressed in oocytes. Cells were plated onto untreated glass coverslips and transfected using calcium phosphate as described elsewhere (Brock et al., 2001). Recordings were performed 1–4 d posttransfection.

Electrophysiology

Macroscopic currents. Excised oocyte patch recordings were made at 22°C in the outside-out or inside-out configuration (Hamill et al., 1981) using an Axopatch 200A or Axopatch 1-B amplifier (Axon Instruments, Inc.). Data were acquired with a ITC-16 interface (Instrutech) on a Macintosh computer (Apple) running Pulse acquisition software (HEKA Electronik). The stimulus pulse was sometimes filtered at 20 kHz to minimize fast capacitance transients. Records were filtered at 10 kHz and digitized at 50 kHz. Most traces shown were digitally smoothed with a 2 kHz Gaussian filter. P/~n leak subtraction was used. Holding potential was −80 mV unless otherwise mentioned.

The external solution for I~K recordings contained (in mM): 115 NaCl, 10 KCl, 2 MgCl2, 2 CaCl2, 20 HEPES (pH 7.2 with HCl). The internal solution contained (in mM): 50 KF, 60 KCl, 10 EGTA, 20 HEPES (pH 7.2 with HCl). Pipette tip resistances with these solutions were <3 MΩ. In some outside-out patches, effects similar to internal application of BrMT would slowly develop after minutes of exposure to BrMT. To prevent this apparent accumulation of dimeric BrMT in the patch pipette, 2 mM tris-carboxyethylphosphine (TCEP) was added to the internal solution (see Fig. 1). Solution pH was then returned to 7.2 with n-methyl-d-glucamine (NMG). Aliquots of this internal solution were frozen at −20°C until use.

Solutions were applied to patches in a continuous stream using a delivery manifold with a 100 μm diameter port (DAD-12; ALA Scientific Instruments) and a back pressure of 150–300 mm Hg. Solution accessibility was verified for each patch by tetraethy lammonium block of I~K.

Single-channel currents. Single-channel recordings were made from outside-out oocyte patches. Unless mentioned otherwise, methods were as described for macroscopic currents. An Axopatch 200A amplifier was used in capacitive feedback mode, and data were initially filtered at 5 kHz. Pipette tip resistances were 3–10 MΩ. All analysis was performed on steps from −100 to +40 mV in the presence or absence of 5 μM external BrMT. Traces were leak subtracted using a partial average of blank sweeps compiled with the Patch Machine program (available at http://
open-time analysis, idealization of a sweep was terminated upon the occurrence of substate conductance in the record (Hoshi et al., 1994; Zheng et al., 2001). Mean open times were determined from the square root of logarithmically binned open durations (Sigworth and Sine, 1987). The sweep interval for single-channel experiments was ~200 ms and was partially determined by the processing speed of the acquisition computer. This rapid pulsing rate was not always sufficient for channels to recover from inactivation, and resulted in multiple sequential blank sweeps.

**Gating currents.** Many unsuccessful attempts were made to measure effects of BrMT on ShBΔ \( I_K \) from outside-out oocyte patches, the preparation used for the rest of the experiments in this paper. During application of BrMT, current leak from patches routinely increased, especially at the extreme voltages necessary to perform proper leak subtraction pulses. These leak artifacts induced by BrMT were found to be less severe in mammalian cells patch-clamped in the whole-cell configuration. Effects of BrMT on \( I_K \) were therefore recorded from whole CHO-K1 cells transiently expressing ShBΔ.

Unless mentioned otherwise, methods were as described for measuring macroscopic ionic currents. Data were acquired with an Axopatch 200A amplifier, filtered at 10 kHz, and sampled at 50 kHz.

Solutions were chosen to prevent ShBΔ channels from becoming defunct in the absence of permeant ions (Melishchuk et al., 1998). The external (bath) solution contained (in mM): 150 tetraethylammonium chloride, 2 MgCl2, 2 CaCl2, 20 HEPES (pH 7.2). The internal (pipette) solution contained (in mM): 5 CsCl, 86 NMG-Cl, 50 NMG-F, 10 EGTA, 20 HEPES (pH 7.2).

A low-volume (~100 μl) chamber was used for whole-cell recordings. Addition of BrMT was accomplished by flowing a volume >0.5 ml through the chamber. This volume was determined to produce >95% solution exchange as calibrated using tetraethylammonium block of \( I_K \) from ShBΔ channels.

**Analysis and Graphing**

Analysis and graphing were performed with IgorPro software (WaveMetrics), which performs nonlinear least-squares fits using a Levenberg-Marquardt algorithm. All statistics noted are mean ± SE.

Conductance-voltage relations were determined from \( I_K \) amplitude at −100 mV after an activating pulse of sufficient duration to maximize \( I_K \). Conductance data were fit using the fourth power of a Boltzmann distribution function:

\[
g_K = A \left( \frac{1}{1 + e^{\frac{V}{zF}} \frac{1 - V_{1/2}}{V}} \right)^4, \tag{1}\]

Here \( A \) is maximal conductance, \( z \) is the number of elementary charges responsive to \( V \), the applied voltage, and \( V_{1/2} \) is the voltage at which a single Boltzmann distribution reaches half its maximal value. After fitting with Eq. 1, conductance data \( f \) were normalized such that \( A = 1 \).

**RESULTS**

**Sidedness of BrMT Activity**

When applied to the extracellular side of an outside-out patch, 5 μM BrMT greatly slows macroscopic potassium current (\( I_K \)) through ShBΔ channels and reduces its peak amplitude (Fig. 1 A). Deactivation kinetics after the activating pulse are unaffected by BrMT.
Fundamentally different effects are seen with application of BrMT to the intracellular side of an inside-out patch (Fig. 1 B). In this case, activation kinetics are not slowed, and $I_K$ declines during the activating pulse. In addition, deactivation is obviously slowed. These effects are characteristic of classic “open-channel block” (Armstrong, 1966) of the type produced by long-chain tetraethylammonium derivatives and mildly hydrophobic amines (see Brock et al., 2001). It appears likely that intracellular BrMT, which contains two amine groups, blocks open K channels in a similar manner. These clear differences between intracellular and extracellular application strongly suggest that BrMT does not rapidly cross the plasma membrane, otherwise both effects should be apparent with bath application to patches of either configuration. BrMT thus appears to have distinct internal and external sites of interaction with ShΔB channels, and different mechanisms are responsible for modifying $I_K$ in the two cases.

When BrMT’s disulfide bond is reduced, external application of the monomeric compound does not slow ShΔB activation (Kelley et al., 2003). Similarly, monomeric BrMT does not block ShΔB channels from the internal side (Fig. 1 C).

Throughout the rest of this paper, only effects of externally applied BrMT will be discussed.

**Voltage Dependence of BrMT Effects on $I_K$ Activation**

The most striking effect of externally applied BrMT is the degree to which it slows $K$ channel activation. BrMT greatly slowed activation of $I_K$ from ShΔB channels at every voltage tested (Fig. 2 A). In contrast, deactivation kinetics were unaffected by BrMT (Fig. 2 B). As might be expected of a ligand that slows activation transitions, BrMT displaced the midpoint of the $g_K-V$ relation to more positive voltages (Fig. 2 C). In addition, the $g_K-V$ curve appears to saturate at a lower conductance level in BrMT. Thus, $K$ channels are not only slow to open in the presence of BrMT, but a fraction of $g_K$ is eliminated and cannot be recovered by increasing the stimulus potential. After channels were opened in BrMT, the K conductance retained an ohmic instantaneous $I_K-V$ relation similar to control (Fig. 2 D), indicating that reduction of peak $I_K$ in BrMT is not due to a rapid, voltage-dependent block of the ShΔB conduction path.

To compare the degree of slowing induced by BrMT at different voltages, the final 50% of $I_K$ rise was fit with a single exponential to determine a time constant ($\tau$).
The time constant of deactivation after an activating pulse was determined by fitting the entire decay phase of \( I_K \) with a single exponential. Activation and deactivation time constants in the absence (\( \tau_{\text{control}} \)) or presence of BrMT (\( \tau_{\text{BrMT}} \)) are compared in Fig. 1 E over a range of voltages. An index of slowing in BrMT is given by \( \tau_{\text{BrMT}}/\tau_{\text{control}} \). Deactivation maintains a \( \tau_{\text{BrMT}}/\tau_{\text{control}} \) ratio of \( \sim 1 \) over all voltages tested, while activation is always slower in BrMT. Activation is uniformly slowed \( \sim 10 \)-fold with little voltage-dependence evident between \(-10 \) and \(+100 \) mV. This is the same voltage range where \( g_K \) is largely saturated in BrMT (Fig. 2 C), and hence rates of deactivation transitions are expected to be too slow to affect \( I_K \) rise. The similar degree of slowing over this wide voltage range suggests that the intrinsic voltage-sensing of the K channel is responsible for the voltage dependence of ShBD activation kinetics in BrMT. A different voltage dependence would be expected if \( I_K \) rise in BrMT was a manifestation of voltage-dependent unblock (MacKinnon and Miller, 1988; Goldstein and Miller, 1993).

Concentration Dependence of BrMT

ShBD activation is progressively slowed by increasing the concentration of BrMT (Fig. 3 A). At no concentration which induces significant slowing is there any indication of rapidly activating channels that are unaffected by BrMT. Activation slowing (Fig. 3 B) and reduction of \( I_K \) amplitude (Fig. 3 C) become notable at a concentration of \( \sim 2 \) \( \mu \)M, and both effects increase smoothly up to \( 20 \) \( \mu \)M. As the concentration of BrMT is increased, the midpoint of ShBD’s \( g_K \)-V relation is shifted to increasingly positive voltages (Fig. 3 D). These graded effects on ShBD \( I_K \) suggest that increasing the concentration of BrMT may slow individual channels in a graded fashion.

Effects of External BrMT on Single K Channels

To better understand the mechanisms of \( I_K \) reduction and slowing by BrMT, \( I_K \) from single ShBD K channels was examined. In 5 \( \mu \)M BrMT, although activation is slowed, single channel \( I_K \) appears otherwise similar to the control condition (Fig. 4, A and B). Ensemble averages of sweeps where channels open (Fig. 4 C) demonstrate that BrMT slowing of macroscopic \( I_K \) is recapitulated at the single-channel level. In the presence of 5 \( \mu \)M BrMT, time to peak \( I_K \) in ensemble averages was \( \sim 40 \) ms (Fig. 4 C), but the cumulative first-latency plot (Fig. 4 D) demonstrates that channels continue to open until somewhat later. This discrepancy is not large, but it suggests that slow inactivation partially obscures the time course of K channel activation in BrMT, even though the ShBD channels used for single-channel recordings have inactivation minimized.

BrMT slows latency to first opening (Fig. 4, D and H), but after this initial opening ShBD behaves similar to control. The unitary conductance in 5 \( \mu \)M BrMT does not change (Fig. 4, E and J) and cannot account for the decrease in peak \( I_K \) seen in macroscopic \( I_K \) (\( P < 0.0001, \) Student’s \( t \) test; see Fig. 4 J, legend). The distri-
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Distribution of open times was also little altered by BrMT (Fig. 4, F, G, and K). Likewise, the probability that a channel was open, provided it had opened previously during a voltage step, was unaffected by BrMT (Fig. 4 L). The similar conductance level, mean open time and conditional open probability suggest that BrMT does not affect transitions near the open state.

The single-channel currents indicate that BrMT decreases macroscopic $I_K$ by increasing the probability that a channel will fail to open during a voltage pulse. Under control conditions, 26% of repetitive 100-ms sweeps contained no openings with the rapid pulsing protocol used, whereas in BrMT 51% were blank.

These blank sweeps were not included in analyses of single-channel properties. The blanks sweeps tended to occur in long sequential clusters, and are likely due to incomplete recovery from inactivation. This result suggests that BrMT may reduce peak $I_K$ by stabilizing an inactivated state.

Effects of External BrMT on K-channel Gating Current

Actions of BrMT described above point to an inhibitory interaction with the voltage-dependent gating processes underlying activation of ShBΔ channels. These voltage-dependent processes give rise to a gating current ($I_g$) that is measurable in the absence of conduct-
ing ions. If BrMT slows voltage-dependent activation transitions then it might also be expected to alter $I_k$.

$ShB\Delta$ gating current during activation ($I_{ON}$) was markedly slowed when exposed to 5 μM BrMT (Fig. 5 A). This effect parallels BrMT’s slowing of $I_k$ activation, and demonstrates that BrMT inhibits voltage-dependent processes that underlie $I_k$ activation.

In BrMT, a component of $I_{ON}$ appears to decay very slowly. The integral of this current ($Q_{ON}$) slowly rises after 10 ms of activating stimulus (Fig. 5 B), but this slow charge movement was difficult to quantitate accurately, as slight displacements from zero greatly affect integration over long time periods. It is likely that some $ON$ charge moves too slowly to be clearly resolved.

$I_{OFF}$ during deactivation was little affected by BrMT (Fig. 5 C). In conducting solutions, BrMT reduces the peak level of $I_k$ during an activating pulse (Figs. 1–3). This loss of $I_k$ appears to be due to some channels failing to open during the activating pulse. The conservation of total OFF gating charge ($Q_{OFF}$ in 5 μM BrMT vs. control = 1.02 ± 0.02, $n = 4$ cells, Fig. 5 D) indicates that $ShB\Delta$’s gating machinery continues to function in BrMT, even in channels that fail to open. This suggests that the reduction in peak $I_k$ by BrMT is the product of a subpopulation of Shaker channels with functional voltage sensors that are for some reason unavailable for opening during the activating pulse. When $ShB\Delta$ is C-type inactivated by the W434F mutation, the channels have apparently normal gating currents, yet fail to open during activating pulses (Yang et al., 1997). Analogously, the reduction of $I_k$ in BrMT may be the result of a stabilized inactivated state from which $ShB\Delta$’s gating machinery continues to operate.

$ShB\Delta$ ILT Identifies Gating Steps Affected by BrMT

The activation pathway of $ShB\Delta$ involves many transitions before channel opening. The slowing of both $I_k$ and $I_k$ indicates that BrMT slows some of these transitions, but these experiments do not clearly define which transitions are affected. The exact number and nature of existing activation transitions are not known. It is clear that a minimum of five activating transitions must occur to account for the sigmoidicity of $ShB\Delta$ activation (Zagotta et al., 1994b). The most thorough models of $ShB\Delta$ activation require each subunit of the tetrameric channel to undergo at least three transitions before channel opening (Schoppa and Sigworth, 1998c; Ledwell and Aldrich, 1999). It is difficult to identify which of these many transitions are affected by BrMT, because $ShB\Delta$ activation transitions occur at overlapping voltage ranges, and a stimulus to any activating voltage will trigger multiple activating transitions.

The $ShB\Delta$ gating mutant, V369I;I372L:S376T (ILT) cleanly separates gating steps into distinct voltage ranges (Smith-Maxwell et al., 1998b; Ledwell and Aldrich, 1999). A simple activation scheme depicting differences between “wild-type” $ShB\Delta$ and ILT is shown in Fig. 6 A. The ILT mutations shift channel opening to very positive voltages, whereas the majority of activation steps and gating charge movements still occur at negative voltages. When activated from −140 to 0 mV, $ShB\Delta$ will traverse all the transitions along its activation path. When activated by the same voltage step, the ILT channel will move most of its gating charge, yet not open. A greater activating potential, e.g., +140 mV, is required to open ILT channels. This separation of activation transitions into distinct voltage ranges allows a test of which transitions are affected by BrMT: early transitions that occur at negative voltages, or the later transitions at positive voltages that allow channel opening.

When ILT channels are stepped from −140 to +140 mV, the full activation pathway is traversed before channel opening. When ILT channels are stepped from 0 to +140 mV, only the later part of the activation path is
BrMT’s dramatic slowing of \( I_k \) activation and \( I_{gON} \) in “wild-type” ShBΔ.

The specificity of BrMT for early transitions in ILT also demonstrates that late activation transitions are pharmacologically distinguishable from early charge movements. Thus, early and late charge movements must represent distinct classes of conformational transitions.

**DISCUSSION**

**Primary Features of BrMT Action Against ShBΔ Channels**

BrMT slows early, but not late activation steps in the ShBΔ activation pathway.

This activation scheme (Scheme I) is a simplified depiction of more complex published models (Schoppa and Sigworth, 1998c; Ledwell and Aldrich, 1999). The proposition that BrMT slows voltage-dependent steps early in ShBΔ’s activation pathway takes into account the following points:

1. **BrMT slows \( I_k \) activation and does not affect deactivation.** Activation kinetics are profoundly slowed by BrMT, and time constants from the slowed \( I_k \) rise have the same voltage dependence as control \( I_k \) (Fig. 2). This suggests that BrMT slows the early gating steps that are rate limiting for \( I_k \) rise under control conditions (Zagotta et al., 1994a; Schoppa and Sigworth, 1998c; Smith-Maxwell et al., 1998b; Ledwell and Aldrich, 1999). The lack of any BrMT effect on \( I_k \) deactivation clearly indicates that BrMT does not affect the rate-limiting deactivation transitions near the open state.

2. **BrMT slows opening, but once open, single channels behave normally.** BrMT lengthens latency to first opening, but does not alter unitary conductance amplitude, mean open time, or steady-state open probability (Fig. 4). This suggests that BrMT stabilizes the channel’s closed states, but has little effect on the open state or transitions late in the activation pathway. The rapid, flickery closing transitions near the open state (Hoshi et al., 1994; Schoppa and Sigworth, 1998c; Smith-Maxwell et al., 1998b; Ledwell and Aldrich, 1999). The lack of any BrMT effect on \( I_k \) deactivation clearly indicates that BrMT does not affect the rate-limiting deactivation transitions near the open state.

3. **BrMT selectively slows \( I_{gON} \) gating charge movement.** A large, fast component of \( I_{gON} \) is slowed by BrMT (Fig. 5). This \( I_{gON} \) component represents voltage-sensor movements early in the activation pathway (Zagotta et

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**SCHEM I**

**Figure 6.** BrMT slows early but not late activating transitions of ShBΔ ILT. \( I_k \) from ShBΔ V369I;S372L;S376T (ILT) in outside-out patches. HP = −60. Pipette solution did not contain TCEP. (A) Scheme depicting gating of ILT channels. (B) \( I_k \) rise from ILT channels at +140 mV after 60-ms prepulses to either −140 or 0 mV. Note the slight delay apparent from −140 mV. (C) ILT activation during a step from −140 mV. \( I_k \) in 5 μM BrMT is scaled to match peak control \( I_k \). Open circle: mean time to half maximal \( I_k \). (D) ILT activation during a step from 0 mV. \( I_k \) in 5 μM BrMT is scaled to match peak control \( I_k \). Open circle: mean time to half maximal \( I_k \).
al., 1994a; Schoppa and Sigworth, 1998c), and BrMT clearly impedes these steps. After channels have been fully activated, $I_{OFF}$ is little affected by BrMT. Failure of BrMT to alter this deactivation-related signal again indicates that BrMT does not greatly affect deactivation transitions.

4. BrMT does not affect the final opening step. BrMT only slows early activation steps of the ShβΔ ILT mutant. When ILT channels open after traversing only late activation steps (Fig. 6), BrMT does not slow $I_K$ activation.

These findings clearly demonstrate that BrMT modifies ShβΔ gating by selectively slowing voltage-sensitive transitions early in the activation pathway. How does BrMT binding slow activation in such a fashion? To address this question, a key feature of BrMT’s effect was examined further: the graded slowing of $I_K$ as BrMT concentration is increased.

**Rapid Binding of BrMT to Closed Channels Can Account for Graded Activation Slowing**

To understand the mechanism by which BrMT slows activation of ShβΔ K channels, two models of activation slowing are tested here. One assumes that BrMT binds and unbinds channels more slowly than they activate, while the other assumes that BrMT is always at a rapid binding equilibrium with K channels.

**Slow BrMT Binding**

If there is little ligand exchange during activation, binding sites occupied by BrMT would remain occupied during channel activation, and vacant sites remain vacant. A simple model where one BrMT molecule binds a channel and slows its activating transition is depicted in Scheme II.

![Scheme II](image)

In this scheme, two populations of channels exist: those with and those without BrMT. Channels without BrMT activate normally, while those bound to BrMT activate more slowly. The equilibrium constant ($K_{eq}$) determining the ratio of bound versus unbound channels is simply:

$$K_{eq} = \frac{[BrMT]}{K_D}$$  \hspace{1cm} (2)

The Shaker channel contains four identical subunits. If we assume BrMT binds independently to each subunit, then the proportion of channels with 0, 1, 2, 3, or 4 BrMT bound at any given time would follow a binomial distribution as depicted in Scheme III.

![Scheme III](image)

The most extensive models of ShβΔ activation suggest that early transitions occur independently in each subunit (Zagotta et al., 1994a; Schoppa and Sigworth, 1998c; Ledwell and Aldrich, 1999; Mannuzzu and Isacoff, 2000). When subunits activate independently, they are not affected by the activation state of other subunits. Fig. 7 B demonstrates a model in which BrMT slows activation of each subunit when bound. With such a model, biphasic $I_K$ rise is seen at subsaturating concentrations of BrMT, rather than the graded slowing of activation seen experimentally. This model clearly does not recapitulate the experimentally observed slowing of $I_K$. Many other attempts were made with related slow-binding models to mimic the effects of BrMT, but no plausible scheme was ever found to produce an appropriately graded slowing of activation.

**Rapid BrMT Binding**

To construct a model in which BrMT produces a graded slowing of activation, BrMT was assumed to rapidly bind resting channel conformations. If BrMT rapidly binds and unbinds a channel on a time scale faster than activation, then the graded slowing with increasing BrMT concentration can be simply accounted for. Scheme IV depicts BrMT binding only to subunits with voltage sensors in a resting conformation, such that they cannot activate when BrMT is bound.

![Scheme IV](image)

A double-headed arrow connecting BrMT to a white subunit indicates that BrMT can rapidly bind a resting subunit and prevent its activation. Grayed subunits have undergone their activating transition, and no longer bind BrMT. The hollowed channel depicts the open state. If BrMT binding is at a rapid equilibrium relative to the rate of voltage-sensor activation, the de-
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gree of activation slowing of each subunit in Scheme IV is determined by:

$$\frac{\tau_{BrMT}}{\tau_{control}} = 1 + \frac{[BrMT]}{K_D} \quad (3)$$

This mechanism of slowing is similar to how rapid open channel blockers, such as internal tetraethylammonium, slow I_K deactivation (Armstrong, 1966, 1971; Choi et al., 1993). In this scheme, activation slowing is proportional to the probability that BrMT is bound. As the concentration of BrMT is increased above its K_D, activation of each subunit becomes slower because it is bound to BrMT a greater proportion of the time. If each subunit of a homotetrameric channel activates independently, then this activation model is analytically described by the fourth power of an exponential rise in which the underlying time constant of activation is determined by Eq. 3:

$$I_K = A \left(1 - e^{-(1+([BrMT]/K_D)^2)^4}\right) \quad (4)$$

I_K predicted by Eq. 4 produces a graded slowing of activation and increases the delay before I_K rise (Fig. 7 C). The striking similarity between this model and I_K activation in BrMT (Fig. 7 A) suggests that BrMT slows activation by rapidly binding and stabilizing resting voltage sensor conformations. The degree of slowing predicted by Eqs. 3 and 4 fits the slowing seen experimentally (Fig. 7 D), further implicating this fast-binding mechanism in BrMT’s slowing of ShBΔ I_K.

One potential inconsistency with this mechanistic proposal is the slow wash-out of BrMT seen experimentally. While ShBΔ channels activate on a millisecond time scale, the effects of BrMT require many seconds to completely wash-in or wash-out (unpublished data). Although this might indicate that BrMT binds and unbinds on a time scale much slower than channel activation, we suggest the wash-out kinetics of BrMT are unrelated to the microscopic rate of BrMT unbinding from the channel. The kinetics of BrMT wash-in and wash-out were highly variable, and wash-out from outside-out patches was often incomplete after minutes in BrMT-free solutions. Wash-in kinetics of the BrMT effect on ShBΔ I_K in mammalian cells or whole oocytes were even more variable and complete wash-out was rarely obtained in these preparations. The variable kinetics of wash-in and wash-out are likely due to a complex process dependent on solution flow and membrane topology rather than the microscopic binding rate of BrMT to K channels. This may reflect an accumulation of BrMT in or around the plasma membrane. The greasy aromatic rings of BrMT may aggregate with lipids and other hydrophobic molecules. BrMT has a propensity to stick to plasticware (see MATERIALS AND METHODS) and is strongly retained by hydrophobic col-

**Figure 7.** Graded activation slowing can be modeled by rapid, reversible binding of BrMT to resting voltage sensors. (A) ShBΔ I_K scaled to match peak current amplitudes. Thin trace: voltage step from −100 to +40 mV under control conditions. Thick traces: progressive slowing of activation at +40 mV by 1, 2, 5, 10, and 20 μM BrMT. (B) Predicted I_K from an activation model with slow BrMT binding to each of four subunits that undergo a single activating transition. Subunits bound to BrMT activate 20-fold slower than those without. This value was chosen because the maximal slowing of ShBΔ activation seen was ~20-fold in BrMT versus control (see D). The proportion of channels with 0, 1, 2, 3, or 4 BrMT bound was binomial. Thin line: control condition. Dotted line: saturating BrMT. Thick lines: [BrMT] = 0.2, 0.5, 1, 2, and 5 × K_D. (C) Predicted I_K from Eq. 4. This equation represents an n^4 model of activation with rapid BrMT binding as in Scheme IV. Increasing the concentration of BrMT slows activation by increasing the proportion of time subunits are bound to BrMT and thus unable to activate. Thin line: control condition. Thick lines: [BrMT] = 1, 2, 5, 10, and 20 × K_D. (D) Filled circles: ratio of \(\tau_{BrMT}\) and \(\tau_{control}\) at +40 mV, n = 4–9. Line: fit of rapid binding model (Eq. 3) to data. K_D from fit = 0.8 μM.
umnns during reverse phase HPLC (Kelley et al., 2003), indicating that BrMT is not highly soluble in aqueous solution. Also, some form of accumulation of BrMT could explain the tendency for micromolar concentrations of BrMT to disrupt the patch clamp seal.

If BrMT accumulates in or around membranes, the local concentration of BrMT around K channels would be different from that in the external solution. Thus, the apparent $K_D$ of 0.8 $\mu$M (Fig. 7 D) refers to the concentration of BrMT in aqueous solution and not to the presumably higher local concentration surrounding the channels. ShB$\Delta$’s actual $K_D$ for BrMT is therefore difficult to determine.

**Similarity to Divalent Cations?**

Activation of Kv1 family channels, including squid delayed rectifier (Gilly and Armstrong, 1982), Shaker B (Spires and Begenisich, 1994), and hKv1.5 (Zhang et al., 2001) channels, are inhibited by zinc and other divalent transition metal ions in a way that resembles the action of BrMT. Specifically, activation kinetics are slowed, but deactivation kinetics are affected to a much lesser extent. A key feature of the action of both BrMT and zinc ions is the selective and smoothly graded slowing of $I_K$ activation kinetics with little evidence for a population of unmodified channels at intermediate ligand concentrations (compare Fig. 3 A with Fig. 2 A of Gilly and Armstrong, 1982). In both cases the dramatic $I_K$ slowing is essentially voltage independent and the $g_{K-V}$ relationship is only modestly shifted.

Extracellular magnesium and other divalent ions also slow activation of EAG K channels. Magnesium induces a change in EAG $I_K$ that is qualitatively different from the effects of BrMT or divalent transition metals on Kv channels. The relative amplitudes of fast and slow components of EAG $I_K$ vary with magnesium concentration. However, the effect of magnesium on EAG appears mechanistically similar to the effect of BrMT on ShB$\Delta$. Magnesium induces a graded slowing of early EAG activation steps, slows $I_{KON}$, has no apparent effect on later activation steps, and no effect on deactivation (Terlau et al., 1996; Tang et al., 2000). Terlau and coworkers propose a model of activation slowing whereby magnesium stabilizes resting voltage-sensor conformations from which EAG is slow to activate. As the concentration of magnesium increases, more voltage sensors occupy this stabilized resting state. The biggest difference between the Terlau et al. (1996) magnesium versus EAG model and our BrMT versus ShB$\Delta$ model is that BrMT stabilizes a state already occupied at rest in the absence of BrMT.

The coupling of divalent ion binding to the graded slowing of $I_K$ activation has never been fully understood. As the effects of divalent ions are very similar to those of BrMT, a reinterpretation of their inhibitory effects using a rapid-binding model may prove fruitful.

**Usefulness of BrMT**

Here we show BrMT to be a unique gating modifier that inhibits specific gating steps early in a K channel’s activation pathway. BrMT acts by slowing the early activation steps. Other activation steps, which influence the time course of $I_K$ activation under control conditions, are rendered insignificant or “silent” because they occur much more quickly than the BrMT-slowed steps. Thus, BrMT can slow $I_K$ activation until its time course is determined almost solely by these BrMT-slowed steps. This allows the BrMT-sensitive steps to be more carefully studied. The ILT mutations, which specifically slow the final opening of Shaker channels, have been used to determine the voltage dependence and cooperativity among subunits during channel opening (Smith-Maxwell et al., 1998a,b; Ledwell and Aldrich, 1999). BrMT retards early voltage-sensor movements, and it can be used in an analogous fashion to study early gating steps. Work is currently ongoing to define biophysical properties of the early, BrMT-sensitive steps in Shaker’s activation path.

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