ACTIVATION OF THE BK (SLO1) POTASSIUM CHANNEL BY MALLOTOXIN
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Pharmacologic approaches to activate K+ channels represent an emerging strategy to regulate membrane excitability. Here we report the identification and characterization of a lipid soluble toxin, mallotoxin (rottlerin), which potently activates the large conductance voltage and Ca2+ activated K+ channel (BK) expressed in a heterologous expression system and human vascular smooth muscle cells, shifting the conductance/voltage relationship by >100 mV. Probing the mechanism of action, we discover that the BK channel can be activated in the absence of divalent cations (Ca2+, Mg2+), suggesting that mallotoxin’s mechanism of action involves the voltage-dependent gating of the channel. Mallotoxin-activated channels remain incrementally sensitive to Ca2+ and β subunits. In comparison to other small hydrophobic poisons, anesthetic agents and protein toxins that inhibit ion channel activity, mallotoxin potently activates channel activity. In certain respects, mallotoxin acts as a BK channel β1 subunit-mimetic, preserving BK channel’s Ca2+ sensitivity, yet adjusting the set-point for BK channel activation to a more hyperpolarized membrane potential.

Introduction
Large conductance voltage and Ca2+-activated potassium channels (BK channels), formed by α subunit tetrameric association, are encoded by a single gene SLO (1-3). BK channels mediate a feedback regulation countering an increase in intracellular Ca2+ [Ca2+]i, and membrane depolarization. Electrophysiological experiments have shown that BK channels are particularly abundant in smooth muscle, where they modulate contractile activity. Although BK channels are expressed to a lesser extent in neurons, they play important roles in the regulation of transmitter release and spike shaping (4-6).

The BK channel α subunit has seven transmembrane domains (S0 to S6), with a GYG sequence (K+ selectivity) containing pore-forming region (7). The channel has a unique C-terminus with four hydrophobic regions (S7-S10), but can be alternatively spliced, leading to significant variability in functional properties such as single channel conductance, gating characteristics, Ca2+ sensitivity and pharmacology (8,9). Ca2+ sensitivity is mediated by the Ca2+ bowl motif in the C-terminus region (10) and the RCK domain (regulator of the conductance of K+) (11). The S4-S5 region and RCK domain are also responsible for Mg2+ regulation (12,13). The channels are modulated by multiple regulatory pathways including Mg2+, phosphorylation, oxidation, heme, and auxiliary β subunits (12,14-16). Functional diversity is increased by channel association with a β subunit, which alters voltage and Ca2+ responses and some pharmacologic characteristics.

Pharmacologic approaches to activate BK channels represent a potential strategy to control membrane excitability. BK channel openers could be effective for diseases mediated through muscular and neuronal hyperexcitability such as asthma, urinary incontinence, hypertension, coronary spasm, convulsion or anxiety (17). Recent work has also suggested a role for K+ channel activators for post-stroke neuroprotection (18). Despite the identification of several BK channel openers (17), relatively little is known about the interaction sites/mechanism of action. Moreover, many of the compounds are either
relatively weak, require either intracellular application or the presence of a β subunit and/or Ca\(^{2+}\), or demonstrate inhibitory effects at high concentrations. Mallotoxin (MTX; otherwise known as rottlerin), a natural product isolated from Mallotus phillippinensis (19), is often used as a putative PKCδ inhibitor (20), although recent work would suggest that it is neither specific nor effective at concentrations utilized (up to 20 µM) (21). During ongoing investigations into the regulation of BK channels, we observed that MTX potently activated the BK channel in a Ca\(^{2+}\) and phosphorylation-independent manner, shifting the conductance-voltage (G-V) relationship by >100 mV in a hyperpolarized direction. Moreover, the BK channels remain sensitive to MTX in the presence of the BK β1 or β2 subunits. Our findings suggest that MTX may modulate the voltage sensitivity of the channel, leading to a hyperpolarizing shift in the G-V relationship.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

MTX (rottlerin) was obtained from three sources, Sigma, Calbiochem and Biomol. Different batches/sources produced similar results. Stock solutions (10 mM) of MTX dissolved in DMSO were stored at –20°C for up to 3 months and were thawed immediately before use. The final concentration of DMSO was not higher than 0.1% by volume. TEA/iberiotoxin and most salts were obtained from Sigma.

**Molecular biology and cell culture**

Constructs encoding BK channel (mSlo1) mutants were obtained using site-directed mutagenesis (QuikChange XL kit; Stratagene). The Ca\(^{2+}\) bowl mutation was D900N and D901N in mslo sequence. All clones were sequenced on both strands prior to use. Transfections into human embryonic kidney (HEK293) were performed using LipofectAmine 2000 (Invitrogen). In most electrophysiologic experiments, HEK293 cells stably expressing BK channels were utilized. Human aortic smooth muscle cells were obtained from Cambrex.

**Electrophysiology**

Petri dishes with plated HEK293 cells were mounted on the stage of an inverted microscope and served as a perfusion chamber. Control solution was perfused through the chamber by gravity at a rate of ~0.5 ml min\(^{-1}\). Experimental solutions were applied by local perfusion, which allowed relatively rapid (< 10 sec) changes of experimental solutions near the cell. For excised patches, a fast perfusion system was utilized, which permitted complete replacement of the perfusion solution near the patch within 1 sec. All recordings in whole cell, cell attached and excised patch (inside-out and outside-out) configurations were performed at room temperature. Data was acquired on an Axopatch 200B amplifier (Axon Instruments) at 5 kHz using a Digidata (Axon Instruments) and pClamp 9. In some cases, data were imported into Origin 7.5 for curve fitting. Pipette resistance was between 2-5 MΩ. For a majority of whole-cell and excised patch experiments, intracellular solution contained (mM): 158 KCl, 5 TES, 5 EGTA, pH=7.0. The bath solution contained (mM): 158 KCl, 5 TES, 1 MgCl\(_2\), pH=7.0. In some experiments, low Cl– micropipettes and bath solutions containing (mM): 5 KCl, 140 KOH, 1 MgCl\(_2\), 2 EGTA, 10 HEPES, pH adjusted to 7.2 with methanesulfonic acid. HEK-293 cells used in experiments had average capacitance C=15.3+0.8 pF (n=52), series resistance was R\(_s\)=8.7+0.5 MΩ (n=52). Activation was measured as conductance since symmetrical K+ gives a consistent unitary conductance across the voltage range. Curves were generated with a standard Boltzmann relationship in which \(G/G_{\text{max}} = 1/(1+\exp [(V-V_{1/2})/k])\). Conductance-voltage (G-V) curves were generated from tail currents, except for Fig. 1B (maximum current). For each patch, G-V curves were fit to a Boltzmann function to provide estimates of \(V_{50}\) (the voltage of half-activation). BK channels were identified by their characteristic voltage and Ca\(^{2+}\) dependence. Single channel analysis was performing utilizing Clampfit9. Single channel amplitudes were measured by using an all-points histogram of current records. Open probability was determined over at least 10 sec of recording. In all cases, the 10 sec recording window was characteristic of > 3 min recording for each test point. All error bars represent S.E.M. Paired t-test was utilized where appropriate. For Ca\(^{2+}\) sensitivity experiments, patches were exposed to MTX through pipette and bath. Ca\(^{2+}\) concentrations were determined using http://www.stanford.edu/~cpatton/webmaxc/webm
MTX induced a non-significant increase in leak current in whole cell configuration. The maximum net current was –9.8 pA/pF at –200 mV and 13 pA/pF at +200 mV, which were not blocked by standard K⁺ and Cl⁻ inhibitors.

RESULTS

MTX activates BK channels

MTX (C₃₀H₂₈O₈; MW 516.55) is a hydrophobic, lipid soluble natural product isolated from a powder (Kamala) covering the capsules of the Mallotus phillippinensis tree(19). Kamala has long been used in India as a therapy for tape worms, potentially based upon its paralyzing effects on motor nerves and muscle(22). On the basis of a study reporting MTX’s relative specificity for PKCδ inhibition(20), a role for PKCδ in a variety of biological events including apoptosis and cell differentiation has been described. However, more recent data suggest that MTX (20 µM) is ineffective in blocking PKCδ activity in vitro(21,23), but may uncouple mitochondria in intact cells (10 µM) (24).

We observed that exposure of stably BK channel (mSlo1) expressing HEK cells to MTX potently activated channel activity. Bath application of MTX (0.5 µM) significantly shifted the V₅₀ for BK channel activation by 118 ± 7.3 mV (n=21; Fig. 1A), recorded during a series of voltage clamp steps in whole cell configuration ([Ca²⁺], ~ 20 nM). Reversible activation of BK channels occurs in a concentration dependent manner over a range of MTX concentrations (100 nM – 20 µM; EC₅₀ <0.5 µM; Supplemental Fig. 1). The EC₅₀ for MTX’s effect on BK channel activation is markedly lower than the concentration utilized as a protein kinase inhibitor (PKCδ IC₅₀ 3-6 µM)(20). MTX (1 µM) had no effect on endogenous HEK cell K⁺ and/or Cl⁻ currents (Fig. 1B). Bath application of MTX activated other BK channel isoforms (rat and Drosophila α subunits), but failed to modulate heterologously expressed Kv1.1 and Ca v1.2 channels (data not shown), suggesting at least partial specificity. TEA (5 mM), a pore blocker of BK channels significantly and reversibly inhibited the MTX-induced BK current (Fig. 1C). Apparent from the current traces (Fig. 1A; arrow), MTX had a pronounced effect upon channel deactivation, increasing the time constant at -80 mV from 0.6 ± 0.2 to 1.7 ± 0.1 ms (n=8; Supplemental Fig. 2).

We compared MTX to one of the originally described, prototypic BK channel activators, NS-1619(25); whereas NS-1619 (10 µM) shifted V₅₀ for activation by 19.6 ± 3 mV (n=6), the addition of MTX (0.5 µM) markedly and incrementally shifted V₅₀ by an additional 100 ± 26 mV (n=3) in the absence of Ca²⁺ (Fig. 1D).

In whole cell configuration, BK channel activation, in response to bath application of MTX developed progressively, over a period of several minutes (Fig. 2A), with the activating effect outlasting its application duration, partially and slowly reversing with washing. In contrast, no significant BK channel activation was observed after application of MTX (20 µM) through the pipette, despite the presence of a ~200 fold higher concentration than the minimum effective concentration tested (100 nM) and >5 fold increased recording time (25 min) (Fig. 2B). To rule out potential run-down of the channel activity during the prolonged recording, subsequent application of the compound to the bath reproduced the activating effects on peak current and deactivation kinetics (Fig. 2B-insets), as well as the hyperpolarizing shift in G-V curve (Fig. 2B; right panel). These results suggest that limited membrane access through intra-pipette delivery, potentially due to cytoarchitecture and/or the physical properties of the compound might preclude adequate accumulation required for the activating effects. Taken together, MTX potently activates heterologously expressed and native BK channel, at a concentration that would be very unlikely to affect other cellular processes reported to be modulated by MTX (23,24,26-28).

BK β subunits do not affect MTX activity

BK α subunits associate, in a tissue-dependent fashion, with β subunits. In smooth muscle, the channel is composed of the pore forming α subunit and β1 subunit, which increases the apparent Ca²⁺ and voltage sensitivity (29,30). In contrast, the β2 subunit primarily affects channel inactivation (31). We examined the effect of MTX on the mouse BK α, co-expressed with either the auxiliary β1 or β2 subunits. We found that co-
expression of the β1 subunit did not reduce the MTX-induced shift in the G-V relationship (Fig. 3A). Similarly, MTX did not perturb the β2 subunit induced channel inactivation (Fig. 3B).

MTX activates BK channels in a cell-free system
In the setting of a detached patch, a second messenger mediated process is very unlikely due to the lack of substrates/organelles and a disruption of normal intracellular biochemical/biophysical pathways(32). In outside-out patches pulled from HEK cells stably expressing BK channels and cultured hVSMC, significant activation (increase in single channel NPo (Fig. 4B, F), without significant change in unitary conductance (Fig. 4D)) was observed after bath application of 0.5 µM MTX, which reversed upon wash-out (Fig. 4C). The increase in Po was marked by an increase in open time, consistent with the prolongation of tail current (deactivation) kinetics observed in whole cell patch configuration (Fig. 1A). In inside-out patch configuration, bath application of MTX (0.5 µM) also caused significant activation (Fig. 4G-H), marked by an increase in NPo and open dwell time, without significant change in unitary conductance (Fig. 4I). The reversible activation of heterologous and native BK channels by MTX in a cell-free configuration in the absence of Ca2+, Mg2+ and ATP implies that a phosphorylation event and/or cytosolic factor (cytochrome c, oxidation) could not explain the MTX induced activation, suggesting a potentially novel mechanism leading to BK channel activation.

MTX activation of BK channels is not use dependent
MTX activation of BK channel is associated with a time dependent process, although we could not exclude the possibility that repetitive interrogation through voltage pulse protocols contributed to the activation in a use dependent manner. To address this, we utilized a protocol in which we exposed HEK cells stably expressing BK channels to bath application of MTX (0.5 µM), while hyperpolarizing the membrane to −80 mV without intervening voltage protocols. During the exposure to MTX, we observed the progressive development of an inward BK current, which was not due to depolarization-induced movement of the voltage sensors (Fig. 5). Moreover, the first voltage protocol interrogation of the BK channels after this period using a ramp protocol indicated a hyperpolarized shift in the I/V curve. Therefore, in contrast to β-scorpion toxin activation of Na+ channels that requires depolarization and outward movement/trap of the voltage sensors(33), MTX-induced BK channel activation is not use-dependent.

Allosteric Ca2+ sensor mechanism is not required for MTX induced activation
The molecular mechanisms mediating the allosteric regulation of BK channels by Ca2+ have recently been identified by structural correlation to the M. thermautothrophicum K+ channel(11) and site-directed mutagenesis(34). The C-terminus, containing the Ca2+ bowl(10) and the regulator of K+ conductance (RCK) domain (11,34) is hypothesized to inhibit the channel, with the inhibitory activity removed by binding Ca2+ (Fig. 6A) As previously reported (10,34), mutation of residues within the Ca2+ bowl (D900N, D901N) and RCK1 domain, (D367A) significantly blunted the Ca2+ sensitivity of the channel (Fig. 6B). However, MTX sensitivity was preserved in BK channels harboring these Ca2+ sensitivity-modifying mutations (Fig. 6C). This finding supports the conclusion that BK channel activation upon cellular exposure to MTX is not due to an increase in intracellular Ca2+ concentration, since even with the disabling of the Ca2+ sensors, MTX potently shifts the G-V relationship.

We then took advantage that BK channels are modulated allosterically by voltage and Ca2+, and that the channel’s gates can open without voltage sensor movement(35). In the presence of MTX, Ca2+ sensitivity (shift in V50 of G-V curve) is additive and unchanged compared to control conditions (Fig. 6D), implying that allosteric opening of the channel gates by Ca2+ does not perturb the effects of MTX. This finding uniquely complements recent work demonstrating the relative independence of the BK channel allosteric Ca2+ and voltage sensors(34,35).
DISCUSSION
Pharmacologic approaches to activate BK channels represent an emerging strategy to control membrane excitability in neurons and vascular smooth muscle. Despite the increasing number of natural and synthetic BK channel openers, relatively little is known about the interaction sites and mechanism of action. As shown in Fig. 1D, one of the prototypic BK channel agonist, NS-1619 had a markedly smaller effect on the G-V relationship as compared to the dramatic shift in G-V relationship observed with MTX. Moreover, many of these compounds are ineffective with extracellular exposure (due to lack of membrane permeability) or require a β subunit or other modulator (such as Ca\(^{2+}\)). Our data clearly demonstrate that MTX requires no other factor. This is important from a clinical perspective, as certain diseases/conditions (i.e. hypertension, aging) are associated with altered (decreased) β subunit expression (36,37). MTX does not activate the L type calcium channel (Ca\(_{v1.2}\)) or K\(_{v1.1}\), suggesting at least partial specificity. Thus, based upon potency, chemical structure and mode of action, MTX represents a unique compound.

MTX activates the channel, in a Ca\(^{2+}\) and phosphorylation independent manner. Our data also demonstrate that the BK channel remains sensitive to intracellular Ca\(^{2+}\) and to β subunit modulation in the presence of MTX. The minimum effective concentration tested (0.1 µM) is markedly lower than the reported IC\(_{50}\) for MTX induced inhibition of protein kinases (including PKC\(\delta\)). Thus, some of the previously reported cellular effects of MTX (rotterlein) may be attributable to activation of BK channels. The activation was not dependent upon cellular structures/signaling pathways, since it reversibly occurred in a relatively cell free configuration (inside-out and out-outside patch), albeit with a slightly decreased efficacy in inside-out compared to outside-out configuration). Although detached patches can contain cytoskeleton proteins and/or signaling molecules, the reproducible nature of our results and their reversibility, strongly suggest that the MTX mediated effects were due to a direct effect upon the channel protein. In addition, MTX does not affect the allosteric Ca\(^{2+}\) sensor mechanism based upon the findings that modification of the Ca\(^{2+}\) sensors through site-directed mutagenesis did not perturb MTX channel activation, and Ca\(^{2+}\) sensitivity was completely intact and incremental after MTX exposure.

Taken together, these data suggest that MTX might alter the voltage sensitivity of the channel, potentially by directly interacting with the voltage sensor or indirectly through modification of the allosteric voltage input. Consistent with this hypothesis, we have found a voltage dependence of MTX effects, with increased efficacy upon membrane hyperpolarization (Zakharov and Marx, unpublished results), suggesting that the position of the voltage sensors, potentially due to the unmasking of a favorable MTX binding site and/or increasing the likelihood of interaction with MTX, may be important for MTX-induced activation of the BK channel. In contrast to voltage, opening of the channel gates by Ca\(^{2+}\) (Fig. 6D) does not reduce MTX induced activation of the channel.

In animal models and humans, alterations of BK channel activity, in part through either a β1 subunit mutation or decreased β1 subunit expression has been associated with either low or increased prevalence of hypertension respectively(38). Recent work has also suggested an important role for BK channels in C. elegans, with gain of function BK channel mutants displaying depression of locomotion and egg-laying behaviors(39). MTX is purified from kamala, which has been used in India for centuries as a remedy for tape-worm(40). In 1910, the anti-helminthic effects of kamala were hypothesized to be due to a paralyzing effect on the worm’s motor nerves and muscle(22). Our findings suggest that the apparent molecular basis for this remedy is the MTX-induced activation of BK channels.

Activation of BK channels by pharmacologic means may be clinically relevant as a cytoprotective agent after trauma/hypoxic episodes and/or treatment of disorders with increased smooth muscle tone. In certain respects, MTX acts as a BK channel β1 subunit-mimetic, preserving BK channel’s Ca\(^{2+}\) sensitivity, yet shifting the G-V curve to a physiologic Ca\(^{2+}\) concentration and adjusting the set-point for BK channel activation to a more hyperpolarized membrane potential. The findings suggest the
possibility of utilizing MTX as a scaffold to design novel modifiers of ion channel voltage sensitivity, leading to enhancement of channel activity.
References:
Acknowledgements
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Figure 1 MTX activates BK channels. (A) Exemplar current traces from whole cell voltage clamp recordings ([Ca^{2+}]_i \sim 20 \text{nM}) from HEK cells stably expressing BK channels (mSlo1) under control conditions and after bath application of MTX (0.5 \mu M). G-V curves (right) were generated from tail current analysis for control conditions (square) and after MTX exposure (circles) utilizing Boltzmann function (n=21, p< 0.0001; paired t-test). Error bars represent S.E.M. Control solutions contained 0.1% DMSO. (B) Exemplar current traces from whole cell voltage clamp recordings from non-BK expressing HEK cells under control conditions and after MTX exposure (1 \mu M). (C) Representative G-V curves from whole cell current recordings ([Ca^{2+}]_i \sim 20 \text{nM}) demonstrating extracellular TEA (5 mM; diamonds) inhibition of MTX-induced (triangles) activation of BK channels. MTX and MTX +TEA curves were generated from same protocol, thus demonstrating an \sim 90\% relative inhibition of current at 0 \text{mV}. (D) Time course-current (left) and G-V (right) plots demonstrating effects of sequential extracellular application of NS-1619 (10\mu M; diamonds) and MTX (0.5 \mu M; triangles).
Figure 2 BK channel is preferentially activated by bath application of MTX (A) Representative time course of recorded current at +70 mV (left) and G-V curves (right) of recombinant BK (mSlo1) current before (black), during extracellular application (orange) and wash-out (black) of MTX (0.5 µM) (n=3). (B) Representative time course of recorded current at +70 mV (left) and G-V curves (right) of recombinant BK (mSlo1) currents recorded during intracellular application of MTX (20 µM) in whole cell patch configuration. After 25 min, the cell was exposed to bath application (orange) of MTX (0.5 µM). Insets demonstrate series of current traces for voltage steps from a holding potential of 0 mV, with steps from –20 to +200 mV for 100 ms, followed by return to +45 mV for 1 min and 25 min; for superfusion of MTX, steps were from –100 to +200 mV.
Figure 3 Co-expression of either β1 or β2 subunits with BK α subunit does not alter MTX activity. (A) Exemplar current traces from whole cell voltage clamp recordings of HEK cells transfected with BK α + β1 subunits under control (Cont) conditions (open symbols) and after bath (filled symbols) application of MTX (0.5 μM). G-V curves (right) were generated from tail current analysis. Pipette solution contained either ~0 free Ca²⁺ (square) or 3 μM free Ca²⁺ (circle). Traces are representative of at least 3 separate experiments for each condition. (B) Exemplar current traces from whole cell voltage clamp recordings of HEK cells transfected with BK α + β2 subunits, under control (cont) conditions and after bath application of MTX (0.5 μM). G-V curves (right) were generated from tail current analysis for control conditions (square) and after MTX exposure (circle). Pipette solution contained ~0 free Ca²⁺. All data shown are representative of at least 3 similar experiments.
**Figure 4** MTX activates BK channels in cell-free system. (A-C) Representative outside-out patch single channel traces recorded at +30 mV of recombinant BK (mSlo1) from control conditions and after bath application of MTX (0.5 µM). Activation was found in 100% of experiments (n=18) Open dwell time (To) and NPo are indicated for each condition. (D) I-V curve demonstrating no MTX-induced change in unitary conductance. Error bars represent SEM. (E,F) Representative outside-out patch single channel current traces recorded at +60 mV in ~20nM Ca²⁺ and amplitude histograms of BK channel from hVSMC before and after bath application of MTX (0.5 µM). (G,H) Inside-out patch single channel current traces and amplitude histograms of recombinant murine BK stably expressed in HEK293 cells recorded at +30 mV before and after bath application of MTX (0.5 µM). MTX activation was found in 88% and no effect in 12% (n=8). (I) I-V curve demonstrating no MTX-induced change in unitary conductance. Error bars represent SEM.
Figure 5 MTX activation is not use-dependent. Representative I/V curves generated from ramp protocols (500 ms) obtained in whole cell configuration immediately before and 4 min after MTX application (0.5 μM). During a 5 min period in which the membrane potential was held at –80 mV, current was monitored for 3 sec every 13 sec.
**Figure 6** Allosteric Ca\(^{2+}\) sensor mechanism is not responsible for MTX induced activation. 

(A) G-V curves, generated from tail current analysis/Boltzmann function (n=6) for inside-out patch recordings of HEK cells stably expressing BK channels (mSlo1). [Ca\(^{2+}\)]\(_i\): Square= ~20 nM; diamonds= 1 µM; triangles= 10 µM; circles 100 µM. Error bars represent SEM. (B) G-V curves, generated from tail current analysis/Boltzmann function (n=4) for inside-out patch recordings of HEK cells stably expressing BK channels (mSlo1) harboring Ca\(^{2+}\) bowl and D367A mutations. Error bars represent SEM. Symbols for [Ca\(^{2+}\)]\(_i\) are the same as in (a). (C) Representative G-V curves for control and MTX-induced activation of wild type BK channels (square) and BK channels harboring Ca\(^{2+}\) bowl and D367A mutations (circle). (D) Representative fitted (Boltzmann) G-V curves for control (black) inside-out patches and MTX (orange) treated inside-out patches at 0 (squares) and 1 µM (circles) Ca\(^{2+}\). Inset: Graph of Ca\(^{2+}\) sensitivity (change in V\(_{50}\) between the 2 Ca\(^{2+}\) concentrations denoted in x-axis) before (white) and after MTX exposure (orange).
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Supplemental Figure 1 Time course-current plot demonstrating dose response of MTX on BK channel activity. HEK cells stably expressing BK channels were subjected to whole cell voltage clamp with $[\text{Ca}^{2+}]_i \sim 20$ nM. Bath application of MTX (100 nM) caused the development of BK current at +70 mV over several min, which was ~80% reversed during a 10 min wash. Re-exposure of the cell to MTX (0.5 $\mu$M) caused the more rapid development of a larger BK current at +70 mV. BK currents were induced by a ramp protocol (500 ms) every 10 sec, from −50 mV to +150 mV, with a holding potential of −40 mV. The kinetics of reversibility were found to be dependent upon drug concentration and time of drug exposure.
Supplemental Figure 2 MTX slows deactivation kinetics. (A) Tail currents were evoked by 20 ms steps to indicated voltage (+200 mV, control; +50 mV, MTX) and then measured by stepping down to various voltages. Currents were fitted by single exponential. (B) Plot of deactivation time constants versus voltage. Error bars are SEM. Squares- control (n=10); circles- MTX (n=8).
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