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Mallotoxin is a Novel Human *Ether-a-go-go*-Related Gene (hERG) Potassium Channel Activator

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d) Abbreviations: hERG, human ether-a-go-go-related gene; MTX, Mallotoxin; CHO, Chinese hamster ovary; I-V, current-voltage relationship; BK, large conductance voltage and Ca^{2+}-activated K^{+}; PKC, protein kinase C

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Abstract

Human *ether-a-go-go*-related gene (hERG) encodes a rapidly activating delayed rectifier potassium channel that plays important roles in cardiac action potential repolarization. Although many drugs and compounds block hERG channels, activators of the channel have only recently been described. Three structurally diverse synthetic compounds have been reported to activate hERG channels by altering deactivation, inactivation, or by unidentified mechanisms. Here, we describe a novel, naturally occurring hERG channel activator, Mallotoxin (MTX). The effects of MTX on hERG channels were investigated using the patch-clamp technique. MTX increased both step and tail hERG current with an EC_{50} of 0.34 and 0.52 μM, respectively. MTX leftward shifted the voltage-dependence of hERG channel activation to less depolarized voltages (~24 mV at 2.5 μM). In addition, MTX increased hERG deactivation time constants. MTX did not change the half-maximal inactivation voltage of the hERG channel, but it reduced the slope of the voltage-dependent inactivation curve. All of these factors contribute to the enhanced activity of hERG channels. During a voltage-clamp protocol using pre-recorded cardiac action potential, 2.5 μM MTX increased the total potassium ions passed through hERG channels by ~5-fold. In conclusion, MTX activates hERG channels through distinct mechanisms and with significantly higher potency than previously reported hERG channel activators.

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Introduction

The human *ether-a-go-go*-related gene (hERG) (Warmke and Ganetzky, 1994) encodes rapidly activating delayed rectifier potassium channels that conduct the cardiac I_{Kr} (Sanguinetti et al., 1995; Trudeau et al., 1995). Although existing in many different cell types (Farrelly et al., 2003; Sarzani et al., 2006; Warmke and Ganetzky, 1994), hERG channels are highly expressed in cardiac myocytes where they function to restore resting membrane potential following action potential generation (see (Sanguinetti and Tristani-Firouzi, 2006) for a recent review).

Many drugs with varying structures and therapeutic targets have been found to block hERG channels, which, in turn, prolong the QT interval and cause long QT syndrome (De Ponti et al., 2002; De Ponti et al., 2000). However, hERG channel openers have been described only recently. RPR260243 (Kang et al., 2005), PD-118057 (Zhou et al., 2005), and NS1643 (Hansen et al., 2006; Casis et al., 2006) are distinct chemical entities that activate hERG channels through a variety mechanisms, i.e. by slowing channel deactivation, reducing channel inactivation, or by unidentified mechanisms. To date, no naturally occurring hERG channel activators have been identified.

We report for the first time that Mallotoxin (MTX), a natural occurring substance, is a potent and unique hERG channel activator. MTX, extracted from a tree named *Mallotus phillippinensis*, was originally described as an inhibitor for protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II and III, and elongation factor-2 kinase (Gschwendt et al., 1994b; Gschwendt et al., 1994a). Recently, MTX has been shown to

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potently leftward shift the conductance-voltage relationship of the large conductance voltage and Ca^{2+}-activated K^+ channel (Zakharov et al., 2005). When we tested MTX on hERG channels, we found that MTX increased both step and tail hERG current by leftward shifting the voltage-dependence of hERG activation and slowing channel deactivation. These actions distinguish MTX as a novel naturally occurring hERG channel activator.

Methods

Cell culture

Chinese hamster ovary (CHO) cells were stably transfected to express hERG channels (Biocat ID: 97761, GlaxoSmithKline) and used for patch-clamp recording. The reason we chose CHO cells to stably express hERG channel is that CHO cells have little endogenous, voltage-dependent potassium channels and therefore are widely used to express voltage-gated potassium channels (Zeng et al., 2005). CHO cells were maintained at 37 ˚C with 5% CO_2 in six-well culture dishes with Iscoves DMEM F-12 nutrient mixture, supplemented with 10% FBS, 100 U/ml penicillin and streptomycin, and 0.5 mg/ml geneticin (Invitrogen, Carlsbad, CA).

Patch-clamp

Whole-cell and inside-out patch currents were recorded from CHO cells stably expressing hERG channels with an Axopatch 200B amplifier and Digidata 1322A digitizer (Molecular Devices, Union City, CA). Glass electrodes (2-4 MΩ resistance) were pulled from thin wall glass (WPI, Sarasota, FL) using a P-97 horizontal puller (Sutter, Novato, CA) and fire polished with MF-830 micro forge (Narishige, Long Island, NY). In whole-cell recording, bath solution contained (in mM) 140 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Glucose, and 10 HEPES, pH=7.4 with NaOH. Pipette solution contained (in mM) 119 K-gluconate, 15 KCl, 5 EGTA, 5 K_2ATP, 3.2 $MgCl_2$, 0.01 PIP_2, 5 HEPES, pH=7.2 with KOH. In inside-out patch recording, both bath and pipette solution contained (in mM) 140 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 HEPES, pH=7.2 with KOH. Currents were elicited by different voltage protocols (described in text and figure legends) and

acquired with pCLAMP 8 software (Molecular Devices, Union City, CA) at room temperature.

Chemicals

MTX (Sigma, St. Louis, MO), also called rottlerin (see insert of Fig. 1B for structure), and dofetilide (APIN chemicals LTD, Abingdon, UK) were made as 10 mM dimethyl sulfoxide stock solutions and diluted in external buffer solutions to desired concentrations. The highest dimethyl sulfoxide concentration in solution was 0.1%. The effects of MTX on hERG channels were measured at the steady-state. Each cell was exposed to a single concentration of MTX; in addition, two cells were tested at both 0.1 and 10 μM.

Data analysis

All data were analyzed and illustrated using pCLAMP 8, Origin 7, and GraphPad Prism 4, and presented as mean ± S.E. (n). Step current amplitude was measured as the mean during the last 100-ms of the 2-s voltage step and tail current was measured at the peak when membrane potential returned to -50 mV. To generate normalized current-voltage relation (I-V), step current amplitudes at various voltages were normalized to the maximal step current amplitude of the cell prior to MTX treatment. Peak tail currents were normalized to the maximal peak tail current and fitted with a single Boltzmann function to determine the voltage-dependence of channel activation. Activation or deactivation time constants were obtained by fitting the rising phase of the step current traces or decay of tails with a single- or double-exponential function,

respectively. The hERG current induced by a pre-recorded cardiac action potential in the presence or absence of MTX were normalized to the maximal current amplitude of the cell prior to MTX application.

Statistic significance ($P < 0.05$) was determined using the paired student's *t*-test.

Results

hERG current in CHO cells was induced by a 2-s depolarizing step to +20 mV from a holding potential of -80 mV, followed by a 2-s repolarizing step to -50 mV to measure tail current and repeated at 10-s interval (insert of Fig. 1A). The effects of 2.5 μM MTX on hERG current and its time course are illustrated in Fig. 1. MTX increased the amplitude of both step and tail current (Fig. 1A). The effects of MTX developed gradually and reached steady-state in 6 to 7-min (Fig. 1B). The MTX activated current was completely blocked by 100 nM dofetilide, a potent hERG channel blocker (Kamiya et al., 2006; Snyders and Chaudhary, 1996). In inside-out patch setting, similar results were observed when MTX was applied to the cytoplasmic side of the channel, and the effect was washable (Fig. 1C). Peak tail current amplitude increased by 140 ± 40 % (n=4).

We then used different voltage-clamp protocols to study the effects of MTX on hERG channel in more detail. hERG currents were elicited by 2-s depolarizing steps to different voltages (from -70 to +60 mV in 10-mV increments) from a holding potential of -80 mV, followed by a 2-s hyperpolarizing step to -50 mV to measure tail currents (insert of Fig. 2A). MTX increased step and tail current amplitudes in a broad range of membrane potentials (Fig. 2B), and dofetilide (100 nM) blocked hERG currents activated by MTX (Fig. 2C).

The normalized step current-voltage relationship (I-V) curve has a characteristic bell shape due to very fast inactivation of hERG channels (Spector et al., 1996) and the effect of MTX was concentration-dependent (Fig. 3A). The activating threshold of hERG channels was lowered by MTX and the voltage I-V peaked was also shifted from +30 mV (control) to +10 mV (10 μM MTX). The voltage-dependent activation of hERG channels measured from the normalized peak tail currents revealed that MTX shifts the voltage-dependence of activation to less depolarized voltages (Fig. 3B). The half maximal activation voltage ($V_{1/2}$) was reduced from +12.2 ± 0.3 mV (28) to +7.1 ± 0.6 mV (8), -2.7 ± 0.8 mV (8), -11.4 ± 0.6 mV (11), and -14.4 ± 0.7 mV (7) by 0.1, 0.5, 2.5 and 10 μM MTX respectively. The $V_{1/2}$ value at each concentration was significantly different from the control ($P< 0.05$), whereas the slope factor was not (Fig. 3B).

For simplicity, we used the step and peak tail currents following the 0 mV depolarization step to determine the concentration-response curves of MTX. EC_{50}s were 0.34 μM for the step and 0.52 μM for the tail currents (Fig. 3C).

We then studied whether the voltage-dependent inactivation of hERG channels was affected by MTX using a voltage-clamp protocol described by Zou and his colleagues (Zou *et al.*, 1998). Currents were elicited by a 1-s depolarizing step pulse to +20 mV from a holding potential of -80 mV, followed by a 10-ms hyperpolarizing step to different voltages (from -150 mV to 20 mV in 10-mV increments), then by a 1-s depolarizing step back to +20 mV (insert of Fig. 4). The current amplitude at +20 mV following the 10-ms hyperpolarizing steps to different voltages was normalized by the maximal amplitude

following -150 mV step to obtain the voltage-dependent inactivation curve. The half maximal inactivation voltage ($V_{1/2}$) in control was -57.0 ± 0.4 mV (8) and the slope factor was 20.1 ± 0.4 mV (8). In the presence of 2.5 μM MTX, the $V_{1/2}$ and the slope factor were -55.5 ± 0.4 mV (8) and 25.4 ± 0.3 mV (8), respectively. The slope factors were significantly different ($P < 0.05$), whereas the $V_{1/2}$ values were not.

We also analyzed the activation and deactivation kinetics of hERG channels in the presence and absence of 2.5 μM MTX. The activation phase of step currents was well fitted by a single-exponential function. Due to small amplitudes, traces below 0 mV were not used. The activation time constants of hERG channels were decreased significantly ($P< 0.05$) by 2.5 μM MTX (Fig. 5). Deactivation time constants of hERG channels were derived by fitting the decay phase of tail currents with a double-exponential function (Kang et al., 2005; Wang et al., 2003). In the presence of 2.5 μM MTX, both slow time constants (τ_s) and fast time constants (τ_f) were increased significantly compared with the controls ($P < 0.05$) (Fig. 6). The fraction of the slow component measured by $A_s/(A_s+A_f)$ was also increased significantly by MTX (Fig. 6).

Finally, we used a pre-recorded action potential (Fig. 7A) from a rabbit ventricular myocytes as a voltage-clamp stimulus to examine the overall hERG channel responses caused by MTX during an action potential. Current traces before and after MTX application were normalized to the maximal current amplitude of the cell prior to MTX treatment (Fig. 7B). We integrated the area under positive current traces to calculate the relative change of the total potassium ions conducted by hERG channels. The

results demonstrate that 2.5 μM MTX increased the total potassium ions passed through hERG channels by 4.8 ± 0.4 (5) fold during the action potential stimulus. The inward tail current was also significantly increased (Fig. 7B).

Discussion

We found that MTX, a naturally occurring substance, potently activates hERG channels. It increased both step and tail hERG currents at sub-micromolar concentrations. It leftward shifted the voltage-dependence of hERG channel activation to less depolarized voltages. In addition, MTX increased hERG deactivation time constants. Therefore, MTX is a novel and potent hERG channel activator with a unique mechanism of action.

MTX is the most potent hERG channel activator reported to date. When compared to the three previously reported hERG channel activators, MTX is at least ten times more potent than PD-118057 (Zhou et al., 2005), NS1643 (Casis et al., 2006; Hansen et al., 2006), and RPR260243 (Kang et al., 2005). MTX activates hERG channels by mechanisms distinct from those of the other hERG activators. RPR260243 modifies hERG channel mainly by slowing the deactivation kinetic (Kang et al., 2005), NS1643 reduces hERG channel inactivation (Casis et al., 2006), and PD-118057 changes neither the kinetics nor voltage-dependence of hERG channel activation (Zhou et al., 2005). In contrast, MTX activates hERG channels by shifting the voltage-dependence of activation to less depolarized voltages. In addition, it also alters the kinetics to make the hERG channel deactivate slower. These actions of MTX on hERG channels resemble the effects of a benzodiazepine L3 on I_{ks} (KCNQ1+KCNE1) channels (Salata et al., 1998). The voltage-dependent inactivation of hERG channels is much less sensitive to MTX than its activation, consistent with the idea that hERG channel has distinct voltage sensors for activation and inactivation (Johnson, Jr. et al., 1999). The half-maximal

inactivation voltage $V_{1/2}$ was unaffected by MTX. However, MTX reduced the slope of voltage-dependent inactivation curve resulting in reduced hERG inactivation at membrane potentials corresponding to cardiac action potentials. All of these actions contribute to MTX activation of the hERG channel. However, it is unclear whether similar or disparate molecular mechanisms underlie these actions.

The effect of MTX on activation kinetics may be considered secondary to the leftward shift of voltage-dependence of hERG channels, since the activation time constants are not significantly decreased if we adjust for the effect of negative shift in the channel's voltage dependence of activation caused by MTX (e.g., compare the activation time constant at 10 mV with MTX to the one at 30 mV without MTX).

Since MTX is a PKC inhibitor (Gschwendt et al., 1994b) and hERG channels are regulated by PKC (Barros et al., 1998; Thomas et al., 2003), a question arises as to whether MTX activates hERG channels via PKC inhibition. Our results demonstrated that MTX could activate hERG channels from cytoplasmic side in inside-out patch configuration. It is unlikely that secondary messenger systems exist in such a setting. Furthermore, 20 μM MTX has little inhibitory effect on PKC (Davies et al., 2000), while 0.5 μM MTX exerts a profound effect on hERG channels. Therefore, it is likely that MTX alters voltage-dependent activation and kinetics of hERG channels by a direct, yet unknown molecular interaction, but not through kinase inhibition. MTX has been suggested to alter the voltage sensitivity of large conductance voltage and Ca^{2+}-activated K^+ (BK) channel by directly interacting with the voltage sensor or indirectly

through modification of the allosteric voltage input (Zakharov et al., 2005). Sphingomyelinase D from spider venom activates Kv2.1 channel by enzymaticly modifying phospholipid (Ramu et al., 2006), which may indicate a direction to further explore the molecular mechanism of MTX activation of hERG channels.

Since MTX affects many aspects of the voltage-dependence and kinetics of hERG channels, we used a pre-recorded action potential as a voltage stimulus to evaluate the overall increase of potassium ions through hERG channels by MTX. In the presence of 2.5 μM MTX, the increase is about 5-fold at room temperature. However, as hERG is very sensitive to temperature (Vandenberg et al., 2006), the effect of MTX on I_{Kr} during cardiac action potentials could be quite different. Further studies using cardiac myocytes at 37 ˚C and site-directed mutagenesis on hERG channels are required to explore the physiological effects of MTX on cardiac action potentials and the molecular basis of the mechanism(s) of MTX. As a commercially available, potent hERG channel activator, MTX represents a useful tool for the investigation of the physiology of hERG channel physiology and hERG “channelopathies” related to long QT syndrome, short QT syndrome and cardiac arrhythmias.

Acknowledgments

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Legends for figures

Figure 1. MTX increased both step and tail hERG current. (A) Whole-cell currents are elicited by a voltage-clamp protocol (insert) before, and after application of 2.5 μM MTX, and 2.5 μM MTX+100 nM dofetilide. (B) Time course of the step (filled square) and peak tail current amplitudes (open circle). The application of MTX (insert) and dofetilide is indicated by arrows. (C) Inside-out macro-patch currents are elicited by a voltage-clamp protocol (insert) before, after application of 2.5 μM MTX, and after wash.

Figure 2. MTX activated hERG channels at various membrane potentials. Whole-cell currents were induced by a voltage-clamp protocol (insert) before (A), and after the application of 2.5 μM MTX (B), and 2.5 μM MTX +100 nM dofetilide (C).

Figure 3. MTX activated hERG channels in a concentration-dependent manner. Normalized step (A) and peak tail current-voltage relationships (B) were generated as described in the text in the absence and presence of 0.1, 0.5, 2.5 and 10 μM MTX. Normalized peak tail current-voltage relationships were fitted with the Boltzmann equation to obtain half maximal activation voltage ($V_{1/2}$) and slope factor (k): $I/I_{max}=1/(1+\exp[(V_{1/2}-V_t)/k])$. Dose response of MTX (C) was measured at 0 mV step (filled squares) and the following peak tail currents (open circles). Current amplitudes were normalized to the control prior to MTX application and plotted against MTX concentrations. The resulting curves were fitted with the logistic equation to obtain EC_{50} values.

Figure 4. Effect of MTX on voltage-dependent hERG channel inactivation. The voltage-clamp protocol is shown as an insert. Peak tail currents were normalized to the maximal peak tail current and plotted against voltages with (open circles) or without 2.5 μM MTX (filled squares). The curves were fitted with the Boltzmann function: $I/I_{max}=1/(1+\exp[(V_t-V_{1/2})/k])$.

Figure 5. MTX decreased hERG channel activation time constants. In the voltage range from 0 to +50 mV, step currents were fitted with a single-exponential function to obtain the activation time constants with (open circles) or without 2.5 μM MTX (filled squares). * Indicates $P< 0.05$.

Figure 6. MTX increased hERG deactivation time constants. In the voltage range from +20 to +50 mV, tail currents were fitted with a double-exponential function to obtain the slow time constants τ_s (A), fast time constant τ_f (B), and fraction of the slow component (C) with (open circles) or without 2.5 μM MTX (filled squares). * Indicates $P < 0.05$.

Figure 7. MTX increased total potassium ions conducted by hERG channels during an action potential stimulus. Whole-cell currents were excited with a test protocol using a pre-recorded action potential trace (A). B: hERG current traces before and after MTX application were normalized to the maximal current amplitude of the cell prior to MTX treatment. Dotted line indicates zero current.

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Figure 1

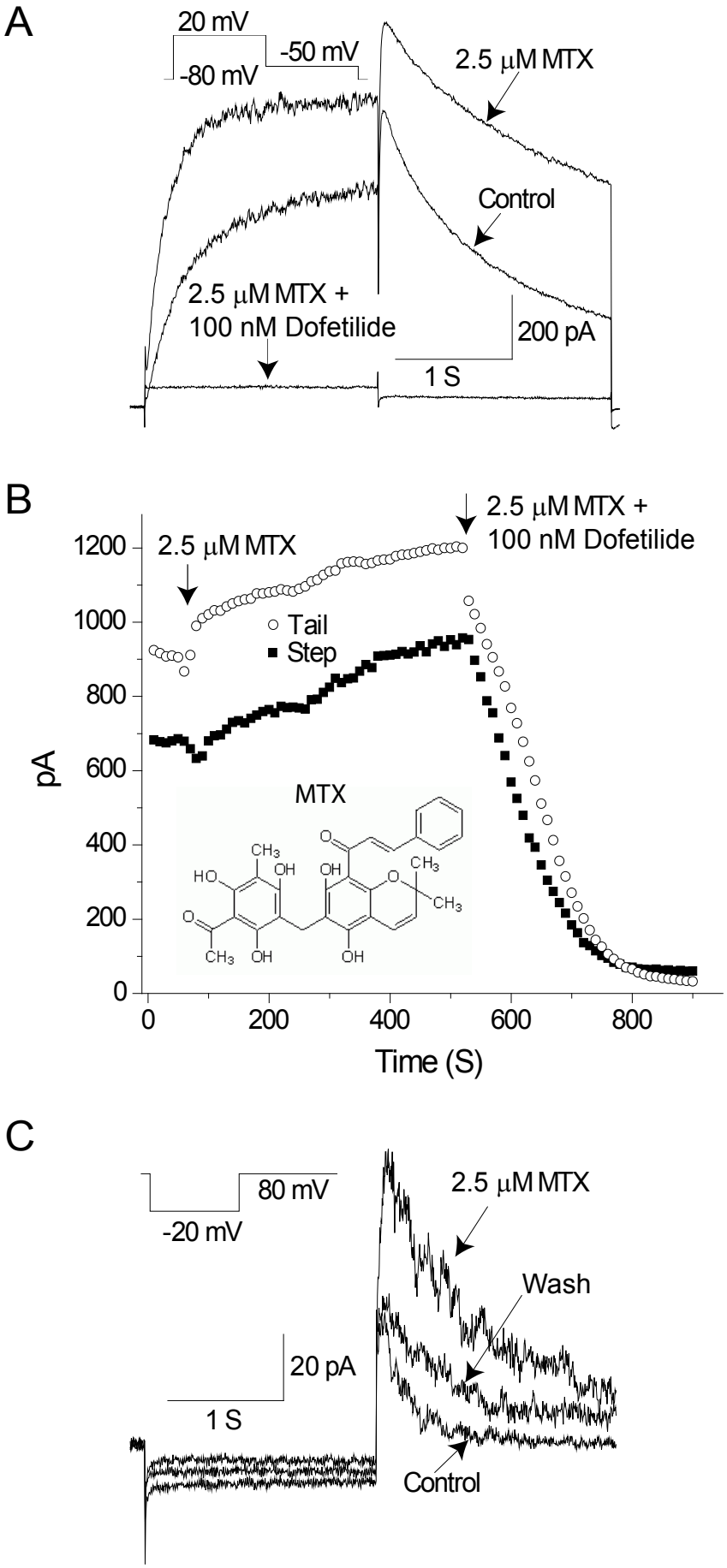

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Figure 2

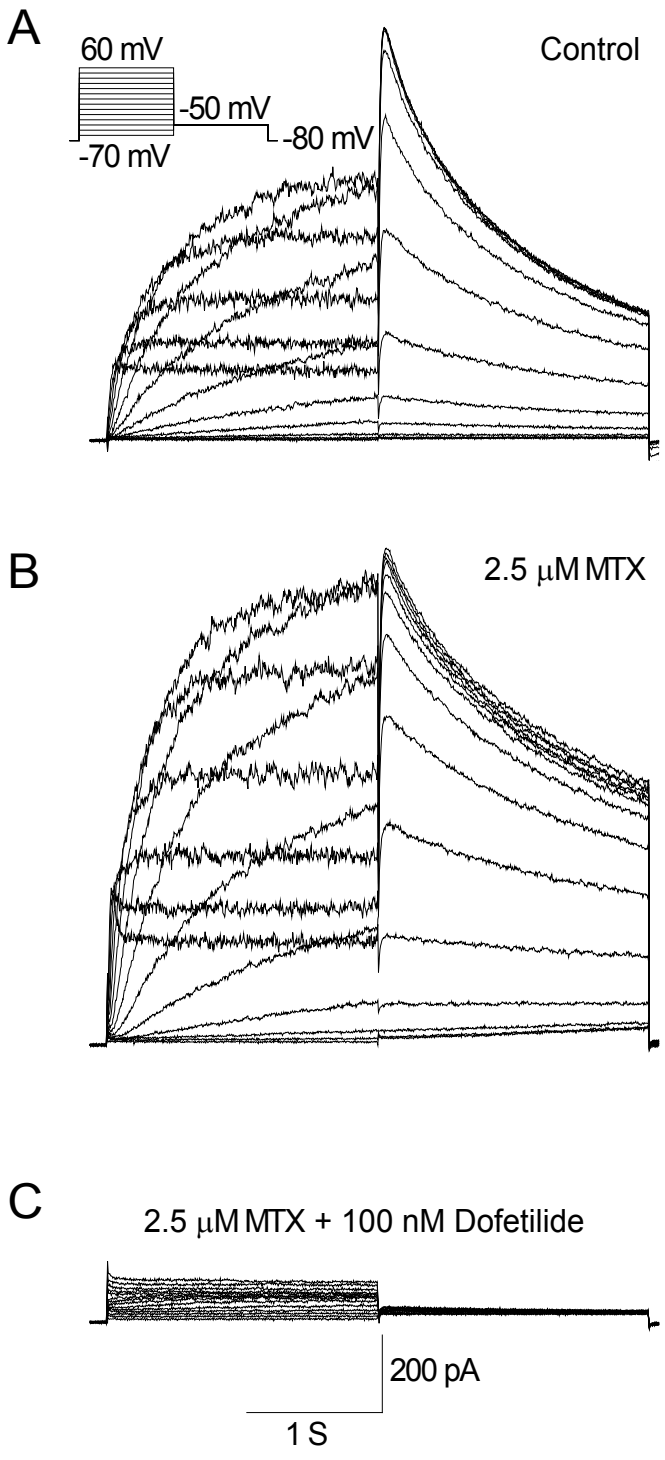

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Figure 3

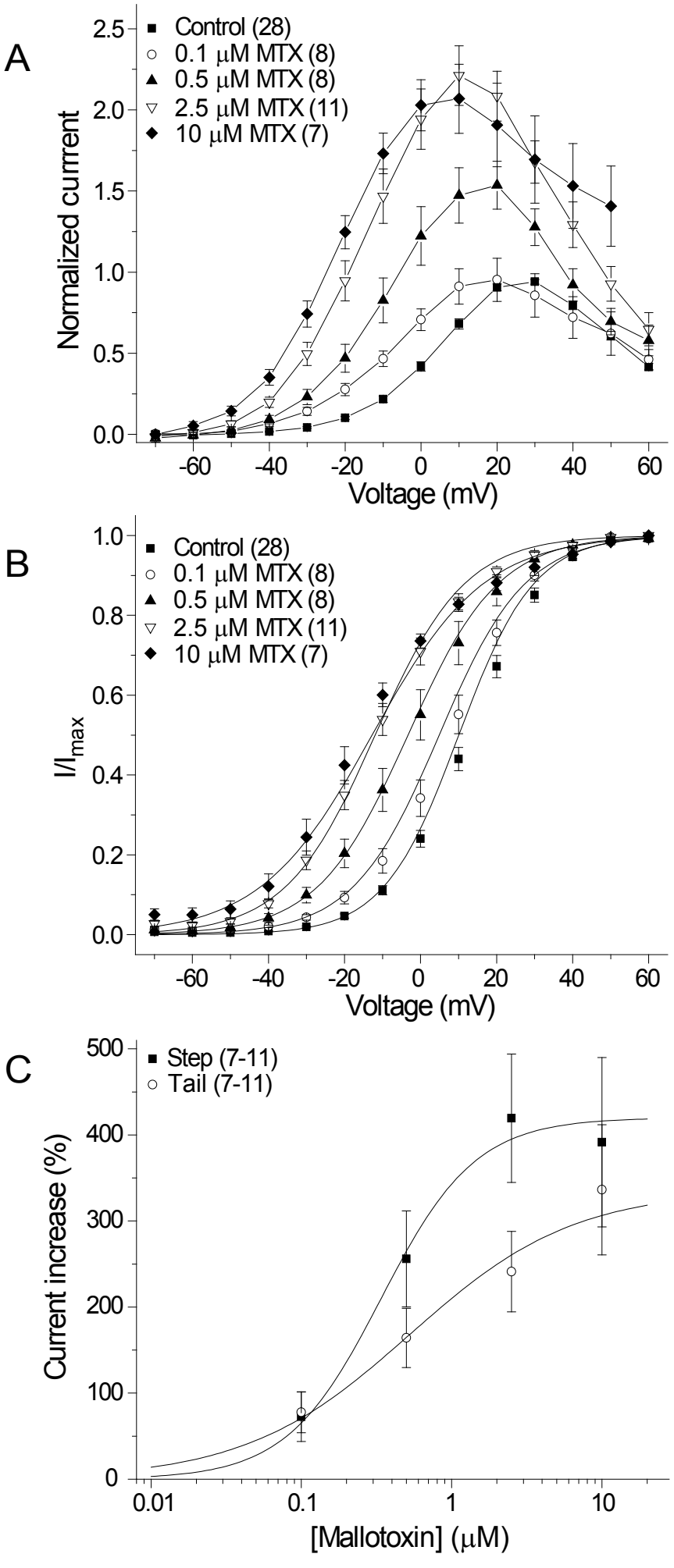

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Figure 4

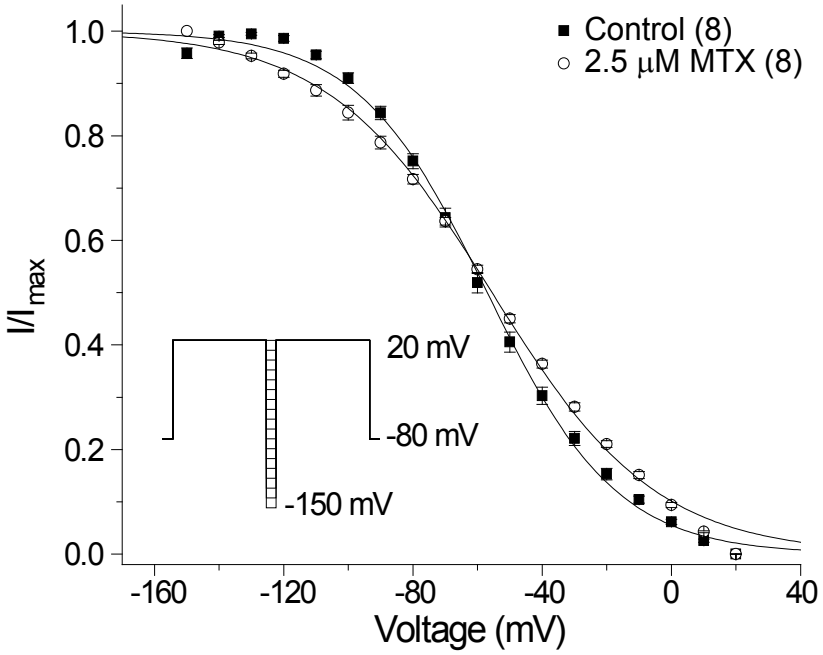

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Figure 5

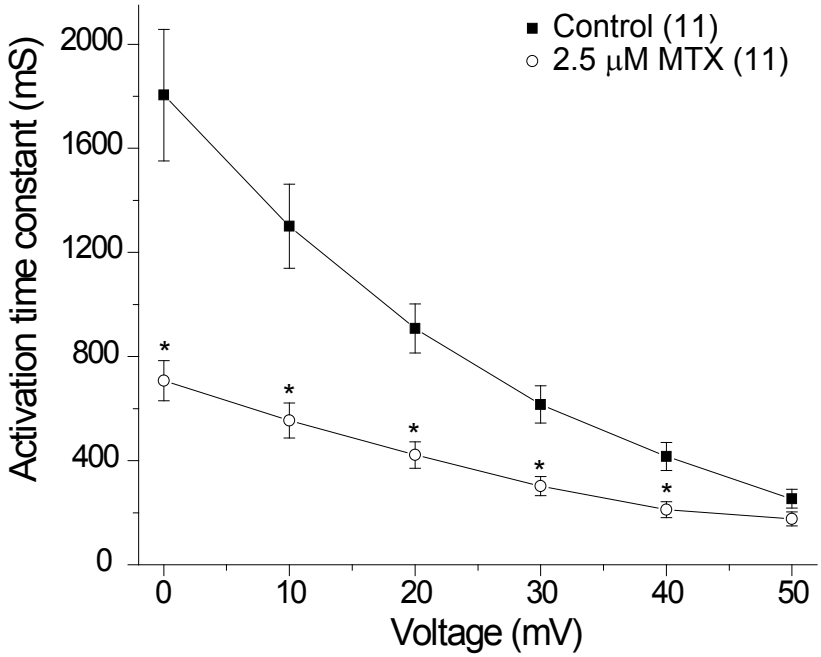

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Figure 6

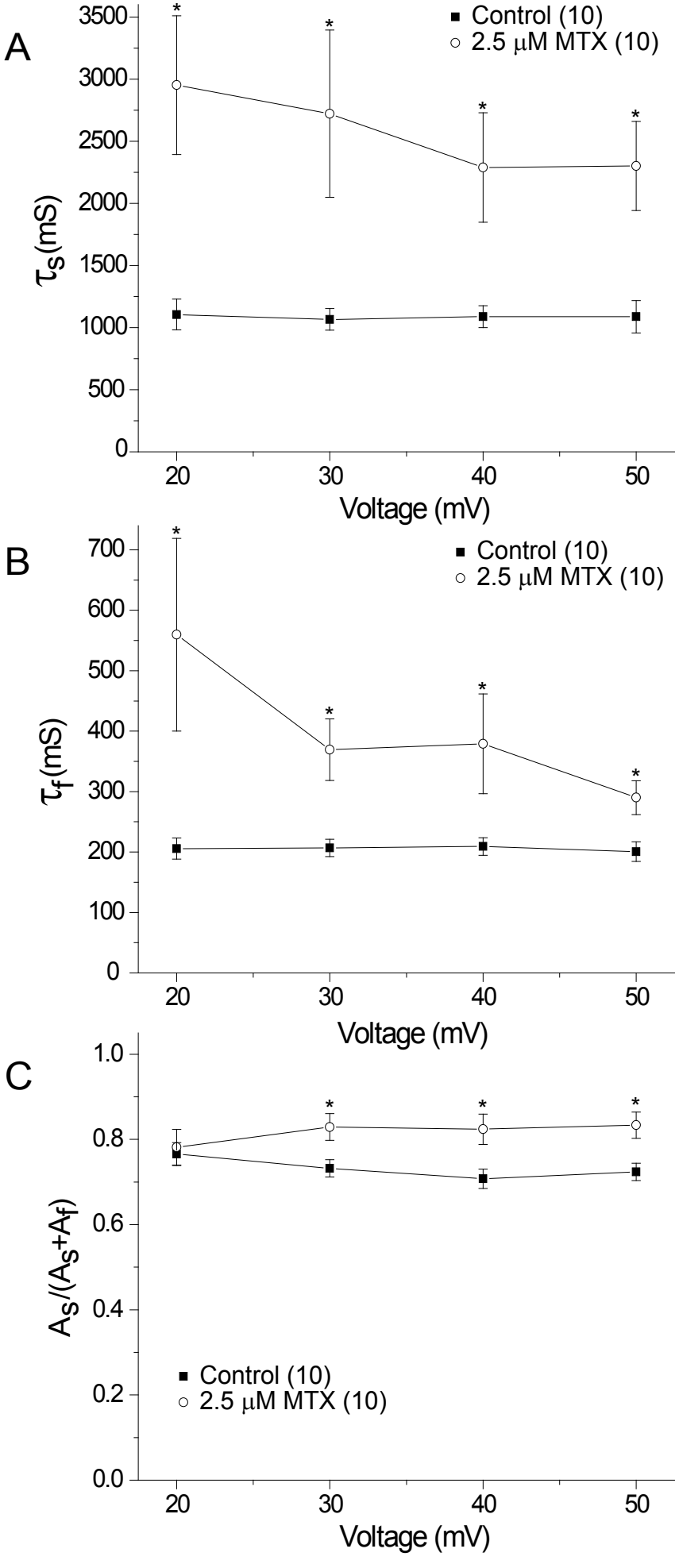

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Figure 7