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The LRRC26 Protein Selectively Alters the Efficacy of BK Channel Activators

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The LRRC26 Protein and BK Channel Activators

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Abbreviations: BK, large conductance Ca-activated K channel; MTX, mallotoxin; parSlo, native parotid Slo gene; H-A model, Horrigan-Aldrich BK channel allosteric gating model.

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Abstract

Large Conductance, Ca^{2+} -activated K channel proteins are involved in a wide range of physiological activities so there is considerable interest in the pharmacology of BK channels. One potent activator of BK channels is maitotoxin (MTX) which produces a very large hyperpolarizing shift of the voltage-gating of heterologously expressed BK channels and causes a dramatic increase in the activity of BK channels in human smooth muscle cells. We found, however, that MTX shifted the steady-state activation of BK channels in native parotid acinar cells by only 6 mV. This was not because the parotid BK isoform (parSlo) is inherently insensitive to MTX since MTX shifted the activation of heterologously expressed parSlo channels by 70 mV. Even though MTX had a minimal effect on steady-state activation of parotid BK channels, it produced an approximate two-fold speeding of the channel gating kinetics. The BK channels in parotid acinar cells have a much more hyperpolarized voltage activation range than BK channels in most other cell types. We found that this is likely due to an accessory protein, LRRC26 that is expressed in parotid glands: expressed parSlo + LRRC26 channels were resistant to the actions of MTX. Another class of BK activators is the benzimidazolones which includes NS-1619. While the LRRC26 accessory protein strongly inhibited the ability of MTX to activate BK channels, we found that it had only a small effect on the action of NS-1619 on BK channels. Thus, the LRRC26 BK channel accessory protein selectively alters the pharmacology of BK channels.

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Introduction

K-selective ion channel proteins form the largest and most diverse class of ion channels. Most of these channels are composed of four identical subunits assembled to provide a narrow K-selective pore and the crystal structures of several of these have been solved (Doyle et al., 1998; Jiang et al., 2002; Long et al., 2005). BK (or maxi-K) channels are unique members of this very large class in that they are activated by both membrane voltage and by a ligand: Ca^{2+} . At negative potentials and at low Ca^{2+} levels these channels are closed. Depolarization, sensed by positively charged amino acid residues in the 4th membrane spanning (S4) domain of each channel subunit, increases the channel open probability (e.g. Diaz et al., 1998). Ca^{2+} binds to a region in the very large C-terminus of each subunit and produces a hyperpolarizing shift in channel activation. The co-operative interplay between membrane potential and Ca^{2+} has been the subject of extensive study for many years (Horrigan and Aldrich, 1999; Horrigan et al., 1999; Magleby, 2003; Rothberg and Magleby, 1999; Rothberg and Magleby, 2000) and see review by Magleby (2003). These efforts have culminated in a concise and quantitative description of the allosteric gating properties of BK channels: the Horrigan-Aldrich (H-A) model (Horrigan and Aldrich, 2002).

In addition to their unique ability to be activated by both voltage and Ca^{2+} , BK channels are modulated by a variety of endogenous signaling molecules. Likely as a result of these special properties, BK channels are widely distributed in the body and play a variety of important physiological roles. They are involved in fluid and electrolyte secretion (e.g. Romanenko et al., 2006), control of smooth muscle contraction (Brayden and Nelson, 1992), and control of electrical activity in many regions of the brain (reviewed in Salkoff *et al.*, 2006). Because of

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their prominent physiological roles BK channels have been actively targeted for the development of therapeutic compounds (e.g. Garcia et al., 1995; Garcia et al., 2007; Gribkoff et al., 2001; Jensen et al., 2003; Marx and Zakharov, 2009; Nardi and Olesen, 2008). One class of potentially therapeutic drugs increases the activation of BK- even at relatively negative membrane voltages. Many such compounds have been identified and most of these act by producing a hyperpolarizing shift of channel activation (reviewed by Gribkoff *et al.*, 1997). One class of such activators is the benzimidazolones exemplified by NS-1619 and its structural analogs (Strobaek et al., 1996). A very powerful addition to the list of BK modulating compounds has recently been identified: mallotoxin (rottlerin), a lipophilic product isolated from a powder covering the capsules of the *Mallotus philippinensis* tree. Concentrations of mallotoxin (MTX) in the μM range shift BK channel activation by more than 100 mV (Zakharov *et al.*, 2005). These large effects of MTX occur on native BK channels in human smooth muscle cells as well as heterologously expressed channels. Both the benzimidazolones and MTX have been proposed for therapeutic uses (Marx and Zakharov, 2009; Teuber et al., 2003).

As noted above, BK channels are involved in fluid and electrolyte secretion in such tissues as salivary glands. Defects in fluid secretion underlie several pathological conditions including oral pain, increased dental caries, and infections by opportunistic microorganisms such as *Candida albicans*. It is possible that activation of K channels in these cells would promote fluid secretion and so BK-activators could have therapeutic value in salivary gland pathologies. We tested MTX on BK channels in mouse parotid acinar cells and, contrary to the findings described above, found a negligible effect of this compound on the voltage dependence of steady-state activation. We investigated the reasons for the lack of effectiveness of this activator on parotid

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BK channels and found that a recently identified accessory protein, LRRC26 (Yan and Aldrich, 2010) caused BK channels to lose their sensitivity to the gating shift of MTX. We tested the specificity of the LRRC26 accessory protein by examining the action of another BK channel activator, NS1619, on BK channels with and without the protein. We found a minimal effect of the accessory protein on the effectiveness of NS1619. Thus, the LRRC26 accessory protein can selectively control the sensitivity of BK channels to channel modulators.

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Materials and Methods

Parotid acinar cell preparation

Single acinar cells were enzymatically isolated from CD-1 mouse parotid glands with techniques that have previously been described in detail (Thompson and Begenisich, 2006). All animals used in this study were housed in a pathogen-free area at the University of Rochester and all procedures for animal handling, maintenance and surgery were approved by the University of Rochester Committee on Animal Resources.

Cell culture and heterologous expression

The mouse parotid $K_{Ca1.1}$ variant (parSlo; see (Nehrke *et al.*, 2003) was cotransfected (~0.8 μ g) into CHO-K1 cells using a Nucleofector machine (Amaxa Biosystems, MD) according to the manufacturer's instructions along with 0.1 μ g of the fluorescent vector EYFPpdc315 for transfected cell identification and with or without 0.5 μ g of human LRRC26 in pCMV6-XL5 (Origene, MD). Cells were used 24 h after transfection. CHO-K1 cells were obtained from ATCC and grown in Ham's F-12K medium + L-glutamine (ATTC, VA) with 10% FBS and maintained in a CO₂ incubator with 5% CO₂.

Solutions

Two external (bath) solutions were used. One solution (5-K) was designed to mimic physiological cation conditions and contained (in mM): 135 Na-glutamate, 5 K-glutamate, 2 CaCl₂, 2 Mg Cl₂, 10 HEPES, pH 7.2. The use of glutamate rather than Cl allowed the measurement of K currents from the parotid acinar cells without significant contribution from the native Cl channels in these cells. A second external solution (Hi-K) contained (in mM) 135 K-

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glutamate, 2 CaCl₂, 2 MgCl₂, 10 HEPES, pH 7.2. The nominally “0” Ca²⁺ internal solution contained (in mM): 135 K-glutamate buffered to pH 7.2 with 10 HEPES and 10 BAPTA with no added Ca²⁺. In some experiments we used an internal solution designed to contain 10 μM free Ca²⁺: 135 mM K-glutamate, 5 mM HEDTA, 3.6 mM CaCl₂, 10 mM HEPES, pH 7.2.

Parotid acinar cells express two types of Ca²⁺-activated K channels: BK and IK1 (Nehrke et al., 2003). IK1 channels are not activated at low Ca²⁺ levels (Ishii et al., 1997; Romanenko et al., 2010) and our experiments with parotid cells were done with the zero Ca²⁺ solution. Thus, IK1 channels will not interfere with our measurements of BK channel currents from the parotid cells. The lack of IK1 currents in our measurements can be seen by the absence of a significant time independent component in the raw currents records of Fig. 1- a hallmark of IK1 channels (e.g. Ishii *et al.*, 1997). Mallotoxin (rothlerin) and NS1619 were purchased from Sigma, stored at -20 C° in aliquots in DMSO and diluted to produce the final working concentrations.

Electrophysiological recordings

Whole cell currents were acquired at room temperature using Axopatch 200B amplifier and Digidata 1320A digitizer (Axon Instruments, Foster City, CA) at 50 kHz sampling rate and filtered online at 5 kHz using a lowpass Bessel filter. Patch pipettes were made from quartz (Garner Glass Co., Claremont CA) or borosilicate glass (Warner Instrument Corp.) with typical resistance values between 3 and 6 MΩ. A minimum value of 90% series resistance compensation was used with most values near 95%. Voltage clamp steps were 100 or 150 msec in duration from a -60 mV or -70 mV holding potential except in experiments with expressed parSlo in the absence of Ca²⁺ where a holding potential of +30 mV was used to allow

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depolarizations to beyond 200 mV. With this high-voltage, whole-cell protocol it was difficult to maintain a tight seal so in most cases the data in the absence and presence of the activators were obtained from separate cells. Single channel activity was measured with the Hi-K solution in whole cell mode.

Data analysis

The voltage dependence of channel activation was estimated from steady-state conductance as: $I_{SS}/(V_m - V_K)$ where V_K is the K^+ equilibrium potential. These conductance-voltage relations were fit by the Boltzmann equation:

$$G(V_m) = G_{max}/(1 + \exp[-z(V_m - V_h)F/RT])$$

where z is the effective valence that controls the voltage sensitivity (steepness) of activation, V_h is the voltage at half activation, and F , R and T have their usual thermodynamic meanings. Relative activation was computed by normalizing the conductance values by the maximum level obtained from the fit. Important constraints for analysis of BK channel properties with the Horrigan-Aldrich (H-A) model (see text) require channel activation data at very low open probabilities in nominally Ca^{2+} -free internal solutions. Thus, the data for the H-A analysis (Fig. 7) was done with the Hi-K solution. BK channel activation at positive potentials was estimated as relative conductance from whole-cell steady-state currents. At negative potentials we measured single channel activity in whole-cell mode and determined the channel fractional open time. The measured single channel conductance and the maximum whole cell conductance for that cell were used to compute the open probability.

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Single exponential time functions were fit to the time course of current activation in order to obtain channel gating time constants. These fits were done using Clampfit 9.2 or software of our own design using the Simplex Algorithm. The voltage dependence of these time constants was fit by the expectation of a single open-closed transition with voltage dependent, forward and backward rate constants a and b respectively:

$$\text{Time Constant} = 1/(a + b)$$

$$\text{where } a = a_0 \cdot \exp(z_a V_m F/RT) \text{ and } b = b_0 \cdot \exp(z_b V_m F/RT) \quad (1)$$

Fits of Boltzmann's equation and Eq. 1 were made in Origin 6.1. Mean values are reported with standard error of the mean (SEM) values.

Previous studies have revealed significant patch to patch variability in the half-activation voltage of heterologously expressed Slo channels (Horrigan et al., 1999; Stefani et al., 1997) possibly due to variable redox states (DiChiara and Reinhart, 1997). Computing mean voltage-activation relations with such variability distorts the shape of the relation such that it no longer reflects the true voltage sensitivity. In order to deal with this issue some authors (e.g. Horrigan and Aldrich, 2002; Orio and Latorre, 2005) have chosen to shift the activation curves from multiple cells so that the half-activation voltage from each cell coincides with the overall mean value and, through an interpolation procedure, compute a mean voltage-activation relation. With the exception of the H-A analysis of expressed parSlo in Fig. 7 we chose to simply report the mean V_h and z values from Fits of Boltzmann's equation. This issue is exacerbated by the inevitable variability

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of the shift produced by MTX so for the H-A analysis of MTX-treated parSlo BK channels we chose to use activation data at positive potentials from a single cell that had V_h and z values very close to the mean values. Part of the H-A analysis employs single channel open probability measurements at very negative potentials where the distorting effect is less problematic. Therefore, for that analysis we used mean activation values from several cells. The H-A analysis was done in Origin 6.1. Under normal conditions in zero Ca^{2+} , the H-A V_J parameter (see text) is quite similar to the voltage at which the activation time constant achieves its maximum value (Horrigan and Aldrich, 2002) so, for our H-A analysis of expressed parSlo BK channels we constrained V_J to be within 20 mV of this value (Fig. 2B). The z parameters were constrained to be between 0 and 2. Parameter seed values for the fitting algorithm were those from our previous similar analysis (Romanenko *et al.*, 2010). For the analysis of the MTX-treated channels (Fig. 7B), the z parameters were fixed at the values from the fit to the control data and the V_J value was constrained to be within 20 mV of the voltage at which the activation time constant achieved its maximum value (100 mV, see Fig. 2B).

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Results

As described in Introduction, the compound mallotoxin (MTX) exerts a powerful effect on BK channel activation by producing a very large hyperpolarizing shift in the voltage dependence of channel activation (Zakharov *et al.*, 2005). Thus, various pathologies, including those associated with salivary gland hypo-function, could be improved by such a compound. As a first step in evaluating MTX as a possible therapeutic treatment for hypo-salivation, we tested its action on BK channels in mouse parotid acinar cells. Since any such therapeutic agent would need to act on intact cells under physiological conditions we used whole cell recordings with physiological K concentrations (see Methods): 5 and 135 mM for extracellular and intracellular K, respectively. BK channels are activated by both voltage and by intracellular Ca^{2+} but in order to simplify analysis, we initially used intracellular solutions with Ca^{2+} chelated to near zero levels (see Methods).

MTX action on BK channels in parotid acinar cells

As illustrated in Fig. 1A, 5 μM MTX had only a very small effect on the voltage dependence of steady-state activation of native BK channels in parotid acinar cells under these physiological conditions. The figure illustrates the voltage dependence of parotid BK channel activation, computed as conductance from whole cell currents in the absence (○) and presence of 5 μM MTX (■). The solid lines are fits of the Boltzmann equation from which the activation midpoint voltage, V_h , and effective valence (or steepness), z , were obtained (see Methods). MTX treatment had a minimal effect on the voltage dependence of BK. The V_h value for the control cell was about 38 mV and that for the MTX-treated cell was about 40 mV. There also appeared

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to be a small decrease in the voltage sensitivity of activation as judged by a slight decrease in the z parameter. The minimal effect on the half-activation voltage, V_h , and the small decrease in voltage sensitivity, z , were consistent findings: the mean V_h value for 16 control cells was 42 ± 1.4 mV and was 36 ± 2.8 mV for 8 cells treated with 5 μ M MTX. The mean z parameter decreased with MTX treatment from 1.7 ± 0.04 to 1.4 ± 0.05 . The small hyperpolarizing shift in V_h and small decrease in z were both significant at the $p = 0.05$ level. Thus, MTX treatment produced only a small, 6 mV, hyperpolarizing shift of parotid BK channel activation and a modest decrease in the voltage sensitivity of channel activation. These findings are summarized in Table 1.

The data in Fig. 1B shows that the inability of MTX to affect the voltage dependence of native parotid BK channels was not the result of an inability of MTX to interact with the channel. The insets contain raw current traces from a cell in the absence (Control) and presence of MTX as indicated. It is clear that MTX increased the speed of activation of these native BK channels. The main figure (Fig. 1B) contains pooled time constant data obtained in the absence (○) and presence (■) of 5 μ M MTX. Treatment with MTX produced an approximate two-fold decrease in the activation time constant with little or no shift of its voltage dependence.

The very small effect of MTX on the voltage dependence of parotid BK channel activation was quite surprising since a 10-fold lower concentration (0.5 μ M) has been reported to produce a 100 mV shift of heterologously expressed BK channels and a dramatic increase in activity of BK channels in human smooth muscle cells (Zakharov *et al.*, 2005). This discrepancy could potentially be the result of differences between the parotid BK channel isoform (Nehrke *et al.*,

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2003) and the mSlo1 clone used in the heterologous expression experiments of Zakharov *et al.*, (2005). However, as noted above, Zakharov *et al.*, (2005) also found strong effects of MTX on the BK channels in human smooth muscle and the parotid isoform is rather similar to hSlo in that it differs at only 5 individual and the last 8 conceptually translated amino acids (see alignment in Nehrke *et al.*, 2003). Nevertheless, to more rigorously test for a possible isoform-specific action, we assessed the effect of MTX on heterologously expressed parotid BK (parSlo) channels and an example of the results is illustrated in Fig. 2.

MTX on heterologously expressed parSlo BK channels

The insets of Fig. 2A contains examples of whole-cell BK currents recorded from a control cell and from a cell treated with 5 μ M MTX as indicated. The control currents (lower inset) were recorded at potentials from 120 to 220 mV in 20 mV increments and from 60 to 160 mV for the MTX data (upper inset). As indicated by the arrows, a depolarization to near 120 mV was necessary to elicit detectable BK current in the absence of MTX but only 60 mV was needed in the presence of MTX.

The open circles in the main plot of Fig. 2A illustrate the voltage dependence of BK channel activation as determined from the relative conductance-voltage relation (see Methods). As is typical of heterologously expressed BK channels in low Ca^{2+} in the absence of auxiliary subunits or other modulation factors, activation occurs only at very large depolarizations (Butler *et al.*, 1993). The solid line is a fit of the Boltzmann relation to the data from which it can be determined that a depolarization to 189 mV was required to activate half of the channels. Treatment with 5 μ M MTX allowed the channels to activate at much smaller depolarizations

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with a half-activation potential (V_h) of 116 mV (filled squares): a 73 mV hyperpolarizing shift. The mean V_h value from 5 control cells was 188 ± 2.0 mV and 118 ± 4.3 mV from 8 MTX-treated cells. The steepness of the activation curve (as indicated by the Boltzmann z parameter) was unchanged: a mean value of 1.2 ± 0.04 for both control and MTX-treated cells. Thus, 5 μ M MTX produced a very large, 70 mV hyperpolarizing shift of the voltage dependence of expressed parSlo BK channel activation. In addition the data in Fig. 2B show that MTX appeared to produce a similar (~ 80 mV) hyperpolarizing shift in the voltage dependence of the activation time constant with little or no change in the peak time constant value. These very large effects of MTX on the expressed parotid BK channel are consistent with the findings of Zakharov *et al.*, (2005) and show that the expressed parSlo isoform was certainly sensitive to MTX treatment. Consequently, there must be some special property of BK channels in native parotid acinar cells that alters their response to MTX.

ParSlo + LRRC26 BK channels

A comparison of the control data in Figs. 1A and 2A shows that native parotid BK channels activate at potentials about 150 mV more hyperpolarized than the same channels in the heterologous expression system. In many types of cells, BK channels (at low intracellular Ca^{2+} levels) need very large depolarizations for activation see review by Latorre *et al.*, (1989)- just as required for the heterologously expressed parSlo channels illustrated in Fig. 2. In contrast, BK channels in parotid (see Fig. 1A) and submandibular (Romanenko *et al.*, 2007) salivary glands can be activated at relatively negative voltages. A few other cell types share this property including mammalian inner hair cells (Thurm *et al.*, 2005) and prostate cancer cells (Gessner *et al.*, 2005). It was recently shown that a BK channel auxiliary protein (LRRC26) is responsible

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for the hyperpolarized activation of the BK channels in these prostate cancer cells (Yan and Aldrich, 2010). In addition to the prostate LRRC26 is also highly expressed in normal salivary glands and at low levels in colon, pancreas, and intestine (Egland *et al.*, 2006). In addition, the LRRC26 protein was found in human parotid exosomes along with TMEM16A and NKCC1, proteins which are primarily, if not exclusively, expressed in acinar cells (Gonzalez-Begne *et al.*, 2009). These findings lead us to postulate that the hyperpolarized activation range of parotid acinar cell BK channels was due to their association with the LRRC26 protein. We tested this possibility by co-expressing parotid BK channels and the human LRRC26 protein and an example of the results are illustrated in Fig. 3.

The expressed parSlo data in the absence of MTX from Fig. 2 are replotted in Fig. 3 (○) along with data (■) from a cell coexpressing parSlo and the LRRC26 protein. As noted above, very large depolarizations were required to activate channels formed only of parSlo subunits. The results in Fig. 3A show that parSlo channels associated with the LRRC26 channel were activated at potentials about 140 mV more negative than channels formed by only the parSlo subunits. There was also an increase in the steepness of the voltage dependence of steady-state activation as indicated by an increase in the *z* parameter. The effects of LRRC26 expression on parSlo steady-state activation are summarized in Table 1. LRRC26 expression resulted in a similar hyperpolarizing shift of the voltage dependence of channel gating kinetics and an almost two-fold slowing of the maximum time constant value as illustrated in Fig. 3B. Representative raw currents are illustrated in the insets and the slower kinetics of the channels with the LRRC26 protein are evident. These findings are quite similar to those recently reported for the actions of LRRC26 on mSlo channel gating (Yan and Aldrich, 2010).

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MTX action on ParSlo + LRRC26 BK channels

The results illustrated in Fig. 3 show that, as predicted, co-expression of LRRC26 resulted in BK channels with gating properties much more like the channels in native parotid cells than channels consisting of only the parSlo subunit. We next tested our hypothesis that the very different effects of MTX on native parotid and expressed parSlo BK channels could be accounted for by the LRRC26 protein. Thus, we examined the actions of MTX on parSlo + LRRC26 channels and the results are illustrated in Fig. 4.

Fig. 4A shows an example of the voltage dependence of steady-state activation of parSlo + LRRC26 BK channels from the same cell before (○) and during treatment with 5 μM MTX (■). Unlike the very large, 70 mV hyperpolarizing shift that MTX produced in cells without the LRRC26 protein (e.g. Fig. 2A), its presence limited, in this case, the shift to about 19 mV. The mean V_h value for all 6 similarly treated cells was 35 ± 3.6 mV and the mean shift produced by MTX was 17 mV. Fig. 4B illustrates activation time constants obtained in the absence (○) and presence (■) of 5 μM MTX. A comparison of these data with those in Fig. 2B reveals that MTX had rather different effects on the activation kinetics of BK channels with the LRRC26 protein than those without this accessory protein. Instead of a large, parallel shift of the voltage dependence of the time constants of parSlo-only channels (Fig. 2B), MTX produced a much smaller shift along with a decrease in the peak time constant of parSlo + LRRC26 BK channels. In summary, co-expression of LRRC26 with parSlo produced BK channels with intrinsic gating properties and sensitivity to MTX that reflected the properties of the channels in native parotid cells.

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MTX action on heterologously expressed parSlo BK channels with high internal Ca^{2+}

As shown in Figs. 1 and 4, MTX had a much smaller effect on steady-state activation of native and expressed parSlo + LRRC26 BK channels than on channels formed only of parSlo subunits (Fig. 2). The native and parSlo + LRRC26 channels have a much more hyperpolarized activation range than parSlo-only channels. Thus, it is possible that the inability of MTX to produce a hyperpolarizing shift of the activation of these channels was due to their already rather negative activation range. We tested this idea by examining MTX action on expressed parSlo BK channels whose activation was shifted to more hyperpolarized potentials by inclusion of Ca^{2+} in the intracellular solution (Fig. 5).

Fig. 5A shows that 10 μM internal Ca^{2+} caused expressed parSlo BK channels to activate at potentials even more negative than native BK channels or channels co-expressed with LRRC26. Nevertheless, MTX still produced a strong shift of channel activation to even more negative potentials. We found a mean V_h value of 35 ± 2 mV ($N = 10$) in the absence of MTX and mean value of -3 ± 3 mV in the presence of 5 μM MTX. Thus, MTX was able to produce a 38 mV hyperpolarizing shift even of BK channels with an already hyperpolarized activation range- not as large as the 70 mV shift observed in zero Ca^{2+} but considerably larger than the shift of native BK channels or expressed parSlo + LRRC26 channels.

It should be noted that this finding complements the results from an analogous set of experiments by (Zakharov *et al.*, 2005). Those authors measured the ability of intracellular Ca^{2+} to shift BK channel activation in the presence of MTX whereas we tested the efficacy of MTX in the presence of elevated Ca^{2+} . Their study was with cell-free, inside out patches whereas our results

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were obtained from intact cells. Nevertheless, we came to the same conclusion that the shifts produced by intracellular Ca^{2+} and MTX were independent of one another.

Fig. 5B illustrates the voltage dependence of activation time constants in the absence (○) and presence (■) of 5 μM MTX in the presence of 10 μM Ca^{2+} . MTX appears to have produced mostly a simple, parallel hyperpolarizing shift of the voltage dependence of the activation time constant, reminiscent of its action on channels consisting of only the parSlo α subunit. This conclusion is somewhat weakened by our lack of activation time constants in the presence of MTX at sufficiently negative potentials to visualize the maximum time constant value. However, the solid line, which provides a reasonable description of the MTX data, is the result of fitting Eq. 1 to the data but with the constraints that the effective valance values (z_a and z_b) maintain their control values; that is, a good fit was obtained by allowing only a simple shift of the voltage dependence.

The LRRC26 accessory protein and other BK channel activators

As noted in Introduction, there are several classes of BK channel activator compounds and a relevant question for this study was if the LRRC26 interfered with BK activators other than MTX. Benzimidazolones, like NS1619, are structurally very different from MTX but both produce large hyperpolarizing shifts in BK channel activation. Gessner *et al.*, (2005) found that NS1619 caused a substantial shift of activation of BK channels in the LNCap prostate cell line. These channels contain the LRRC26 protein suggesting that its ability to interfere with BK activators may not be universal. To specifically examine the role of LRRC26 on BK channel activation by NS1619 we tested the effectiveness of NS1619 on heterologously expressed ParSlo

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channels and on ParSlo channels expressed with the LRRC26 accessory protein and examples of the results are illustrated in Fig. 6.

Fig. 6A contains voltage activation data measured in the nominal absence of Ca^{2+} from a cell expressing BK channels composed of only parSlo subunits in the absence (○) and presence (■) of 50 μM NS1619. The V_h value for the activation in the absence of NS1619 was 170 mV and 108 mV in its presence: a 62 mV hyperpolarizing shift. In a total of 6 similar experiments the mean V_h value without NS1619 was 181 ± 4.5 mV, similar to values described above and the mean hyperpolarizing shift produced by 50 μM NS1619 was 61 ± 4.8 mV. Part B of Fig. 6 shows that a somewhat smaller but still large shift is produced in channels with the LRRC26 protein. In the example shown the V_h value for activation was 54 mV and 4 mV in the absence and presence of NS1619, respectively. In a total of 6 similar experiments the mean V_h value without NS1619 was 51 ± 2.9 mV, similar to values described above and the mean hyperpolarizing shift produced by 50 μM NS1619 was 47 ± 3.2 mV. That is, the NS1619-induced shift of BK channel activation was reduced by only 23% in the presence of the LRRC26 channel. In contrast, under similar conditions, the LRRC26 protein inhibited the MTX-induced gating shift by 76%.

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Discussion

Several new findings have resulted from this study. In contrast to previous findings on smooth muscle BK channels, mallotoxin had very little effect on the voltage dependence of activation of BK channels in native parotid acinar cells. However, MTX was not entirely without effect as it produced a two-fold increase in the speed of parotid BK channel activation. We showed that the limited action of MTX on the voltage dependence of BK channel activation in parotid cells was likely due to the existence of a BK channel accessory protein, LRRC26, which is expressed in salivary glands including parotid (Egland *et al.*, 2006; Gonzalez-Begne *et al.*, 2009). Co-expression of the parotid BK isoform (parSlo) with LRRC26 produced channels that mimicked many of the properties of those in the native cells including: (1) a hyperpolarized activation range; (2) resistance to MTX modification of steady-state activation; and (3) a speeding of gating kinetics by MTX. We suggest that the relatively small differences between native parotid and parSlo + LRRC26 BK channels may be due to an incomplete stoichiometry in the heterologous expression system. Finally, we showed that while the LRRC26 protein inhibited the gating shift produced by MTX, it had a minimal effect on the gating shift produced by NS1619. This result shows that the LRRC26 protein has a selective effect on BK channel pharmacology.

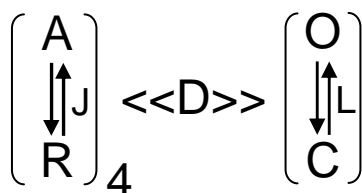
One possible mechanism for the inability of MTX to shift the voltage dependence of BK channels formed with the LRRC26 protein is that the accessory protein prevents binding of MTX to the channel complex. This does not seem to be the case since even though MTX did not produce a significant activation shift of BK + LRRC26 channels it did produce a substantial increase in the speed of activation of this BK channel complex (Figs. 1 and 4). The fact that MTX increased the kinetics of BK + LRRC26 channels but had no effect on the kinetics of BK

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channels without LRRC26 (Fig. 2) suggests another mechanism in which MTX displaces the LRRC26 protein reversing the slowing effect by the accessory protein and replacing the shift produced by LRRC26 by its own gating shift. The data in Figs 1 and 3 show that this idea is quantitatively reasonable. The peak time constants of parotid and parSlo + LRRC26 channels were quite similar and MTX caused the time constant of parSlo + LRRC26 channels to approach that of parSlo-only channels. Thus, the simple idea that MTX displaces the LRRC26 protein is quantitatively consistent with both the increase in activation kinetics and the minimal effect on the voltage dependence of activation of BK channels with the LRRC26 protein.

The functional basis for MTX and LRRC26 action

More insight into the mechanism by which MTX and the LRRC26 protein compete could come from an understanding of the mechanisms by which they produce their large hyperpolarizing shifts of the BK activation process. The Horrigan-Aldrich allosteric model for BK channel activation provides a framework for analyzing the functional basis for the actions of LRRC26 and MTX. The full H-A model describes how voltage and Ca^{2+} act synergistically to control BK channel activation but the actions of MTX and the LRRC26 protein were investigated in the nominal absence of Ca^{2+} . Under these conditions, the H-A model can be schematically represented as follows:



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In this scheme the voltage sensors in each of the four BK channel subunits have a resting (R) and an activated (A) conformation controlled by a voltage dependent equilibrium constant, J . The channel pore has a closed (C) and an open, conducting (O) conformation governed by the voltage dependent equilibrium constant L . There is a cooperative interaction between the voltage sensors and the pore represented by the D parameter.

The overall channel open probability for this scheme is given by:

$$P_o = \frac{L(1+JD)^4}{L(1+JD)^4 + (1+J)^4} \quad (2)$$

where

$$L = L_0 \exp\left(\frac{z_L V_m F}{RT}\right)$$

$$J = \exp\left(\frac{z_J F(V_m - V_J)}{RT}\right)$$

This, Ca^{2+} -free version of the H-A model has five independent parameters: L_0 , z_L , z_J , V_J , and D and the goal then is to define the set of parameters that best describes measured activation data. At very negative potentials where the open probability is very small, P_o is simply equal to L and so obtaining these data greatly improves the process of defining L_0 and z_L . These data can be obtained from measurements of single channel currents at negative potentials which required the use of an external solution with an elevated level of K^+ . Yan and Aldrich (2010) did this type of analysis on data from inside/out patches of cells expressing hSlo channels with and without the LRRC26 accessory protein. They found that their data from the BK-only channels could be

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well-described by the H-A model with a set of parameters similar to those that have been reported previously. They went on to show that the data from channels with the LRRC26 protein could be accounted for if the allosteric coupling parameter D was greatly increased. We did a similar analysis of data from heterologously expressed parSlo channels in the absence and presence of MTX and the results are illustrated in Fig. 7.

The open circles in Fig. 7 represent the voltage dependence of expressed parSlo channels and the associated black lines are from our H-A model analysis with the indicated parameters (Basic Set). These values are within the range of those previously published for heterologously expressed mSlo, hSlo, and parSlo channels (Bao and Cox, 2005; Horrigan and Aldrich, 2002; Orio and Latorre, 2005; Romanenko et al., 2010; Wang et al., 2006; Yan and Aldrich, 2010).

Also shown in Fig. 7 are activation values for expressed parSlo channels treated with MTX (■) including values obtained from single channel measurements (see Methods) at very negative potentials. While the data used for the analysis of the control data were pooled from many cells the activation data in MTX at positive potentials is from a single, representative cell (the data at negative potentials are from several cells). For reasons described in Methods we feel that this approach allowed the most accurate estimation of the BK channel activation in the presence of MTX.

In the context of the H-A model there are three general ways to produce a hyperpolarizing shift of activation: (1) a hyperpolarizing shift in the voltage sensor equilibrium (i.e. a more negative V_J value); (2) a hyperpolarizing shift in the open-closed equilibrium (i.e. a larger L_0 value); or

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(3) an increase in the coupling between voltage sensor activation and channel opening (i.e. an increase in D). The simulations illustrated in Fig. 7A demonstrate that no single one of these parameter changes could account for the action of MTX. A shift of V_J from 165 to 100 mV reproduced the data at positive potentials but was not within an order of magnitude value of the data at the most negative voltages. A larger V_J shift would produce a better match to the data at negative potentials but would then not match the rest of the data. The increase in L_0 shown provides a good description of the negative voltage data but not of the data at depolarized potentials. As can be seen in the figure, no value of D was able to describe any part of the MTX data.

Thus, the MTX data could not be described by a change in any single aspect of BK channel gating. Indeed we found that the only way to accurately describe the action of MTX was to allow all three parameters to change. Fig. 7B shows that a modest increase in L_0 coupled with a 70 mV hyperpolarizing shift of V_J and a reduction in the coupling parameter D provided an accurate description of the MTX data. A reduction in D , on its own, would produce a depolarizing shift so the changes in L_0 and especially V_J overcome this effect. Thus, it appears that MTX and the LRRC26 protein exert their actions on BK channel activation by quite different mechanisms. Our analysis indicates that MTX produces its shift primarily through a hyperpolarizing shift of the voltage sensor equilibrium voltage, V_J , while Yan and Aldrich, (2010) concluded that LRRC26 produces its hyperpolarizing activation shift by increasing the allosteric coupling factor D .

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Summary

BK channels are valuable targets for therapeutic interventions and many compounds have been identified that modify BK channel gating. One such compound is mallotoxin (MTX) which produces a large increase in the activation of smooth muscle BK channels. In contrast, we showed that MTX had only a minor effect on steady-state activation of BK channels in parotid acinar cells. We hypothesized that this was due to the presence in parotid cells of LRRC26, a protein known to produce a hyperpolarizing shift of BK channel activation and one that is expressed in these cells. We confirmed this hypothesis by showing that MTX was only weakly effective on steady-state activation of parotid BK channel isoform co-expressed with the LRRC26. We showed that the action of the LRRC26 protein was selective: it strongly interfered with the hyperpolarizing shift produced by MTX but had a minimal effect on the hyperpolarizing activation shift produced by the benzimidazole BK activator NS1619. We suggest that the ineffectiveness of the MTX on BK channels with the LRRC26 protein was the result of a competition between MTX and the LRRC26 protein for their action on the BK channel gating machinery. Presumably, the physical chemical properties of NS1619 allow it to act independently of the LRRC26 protein. Indeed, Zakharov *et al.*, (2005) showed that MTX shifts BK channel activation even in the presence of NS1619. Our results highlight the fact that the screening of BK channel drugs needs to include consideration for BK accessory proteins like LRRC26 and that it may be possible to take advantage of this behavior to design tissue-specific BK channel modifying drugs.

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Authorship Contributions

Participated in research design: Begenisich, Almassy.

Conducted experiments: Almassy

Performed data analysis: Begenisich, Almassy

Wrote or contributed to the writing of the manuscript: Begenisich, Almassy

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Footnotes

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Legends for Figures

Figure 1. MTX action on BK channels in native parotid cells. (A) Voltage dependence of relative BK conductance in a representative mouse parotid acinar cell in the absence (○) and presence (■) of 5 μ M MTX. Lines: fits of the Boltzmann equation (see Methods) with the indicated parameters. (B) Voltage dependence of activation time constant. Pooled data in the absence (○, N = 8 – 15) and presence (■) of 5 μ M MTX (N = 6 -7 except at -30 mV with N = 2). Solid lines are fits of Eq. 1 (see Methods) to the data. Insets: raw current data from a cell in the absence and presence of MTX as indicated. Voltages of 0, 20, 40, and 60 mV. Calibration: 1 nA, 20 msec.

Figure 2. MTX shifts activation of heterologously-expressed parotid BK channels. (A) Voltage dependence of relative BK conductance in the absence (○) and presence (■) of 5 μ M MTX. Solid lines: fits of the Boltzmann equation (see Methods) with the indicated parameters. Insets: raw BK currents in the absence (voltages: 60, 80, 100, 120, 140, 160 mV) and presence (voltages: 120, 140, 160, 180, 200 mV) of 5 μ M MTX as indicated. For each condition the smallest detectable current and the associated voltage are indicated. Calibration: 2.5 nA, 20 msec (Control); 1 nA, 20 msec (MTX). (B) Voltage dependence of activation time constant. Pooled data in the absence (○, N = 5 - 19) and presence (■) of 5 μ M MTX (N = 4 – 10). Solid lines are fits of Eq. 1 (see Methods) to the data.

Figure 3. Actions of LRRC26 on BK channel gating. (A) Voltage dependence of expressed parSlo channel conductance without (○, from Fig. 2A) and with (■) association with the

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LRRC26 protein. Solid lines: fits of the Boltzmann equation with the indicated parameters. (B) Voltage dependence of the channel activation time constant without (○, N = 5 – 19, from Fig. 2B) and with (■) the associated LRRC26 protein (N = 5). Solid lines are fits of Eq. 1 to the data. Insets: raw currents from parSlo only and parSlo + LRRC26 channels as indicated. Currents from parSlo channels elicited by depolarizations from 140 to 220 mV in 20 mV increments; calib: 1 nA/25 msec. Currents from parSlo + LRRC26 channels elicited by depolarizations from 20 to 100 mV in 20 mV increments; calib: 2 nA/25 msec.

Figure 4. MTX action on ParSlo + LRRC26 BK channels. (A) Voltage dependence of relative BK conductance in the absence (○) and presence (■) of 5 μM MTX. Solid lines: fits of the Boltzmann equation (see Methods) with the indicated parameters. (B) Voltage dependence of activation time constant in the absence (○, N = 5) and presence (■) of 5 μM MTX (N = 4 except N = 3 at 0 mV). Solid lines are fits of Eq. 1 (see Methods) to the data.

Figure 5. MTX shifts expressed parSlo activation in high Ca²⁺. (A) Voltage dependence of relative BK channel conductance from a cell in the absence (○) and presence (■) of 5 μM MTX. Solid lines: fits of the Boltzmann equation (see Methods) with the indicated parameters. (B) Voltage dependence of activation time constant in the absence (○, N = 5 - 7) and presence (■) of 5 μM MTX (N = 4 - 5). Solid lines are fits of Eq. 1 (see Methods) to the data. All measurements were made with 10 μM intracellular Ca²⁺.

Figure 6. NS1619 on parSlo and parSlo + LRRC26 BK channels. (A) Voltage dependence of heterologously expressed parSlo BK channel conductance in the absence (○) and presence

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(■) of 50 μ M NS1619 (B) Voltage dependence of parSlo + LRRC26 BK channel conductance in the absence (○) and presence (■) of 50 μ M NS1619. Solid lines: fits of the Boltzmann equation (see Methods) with the indicated parameters.

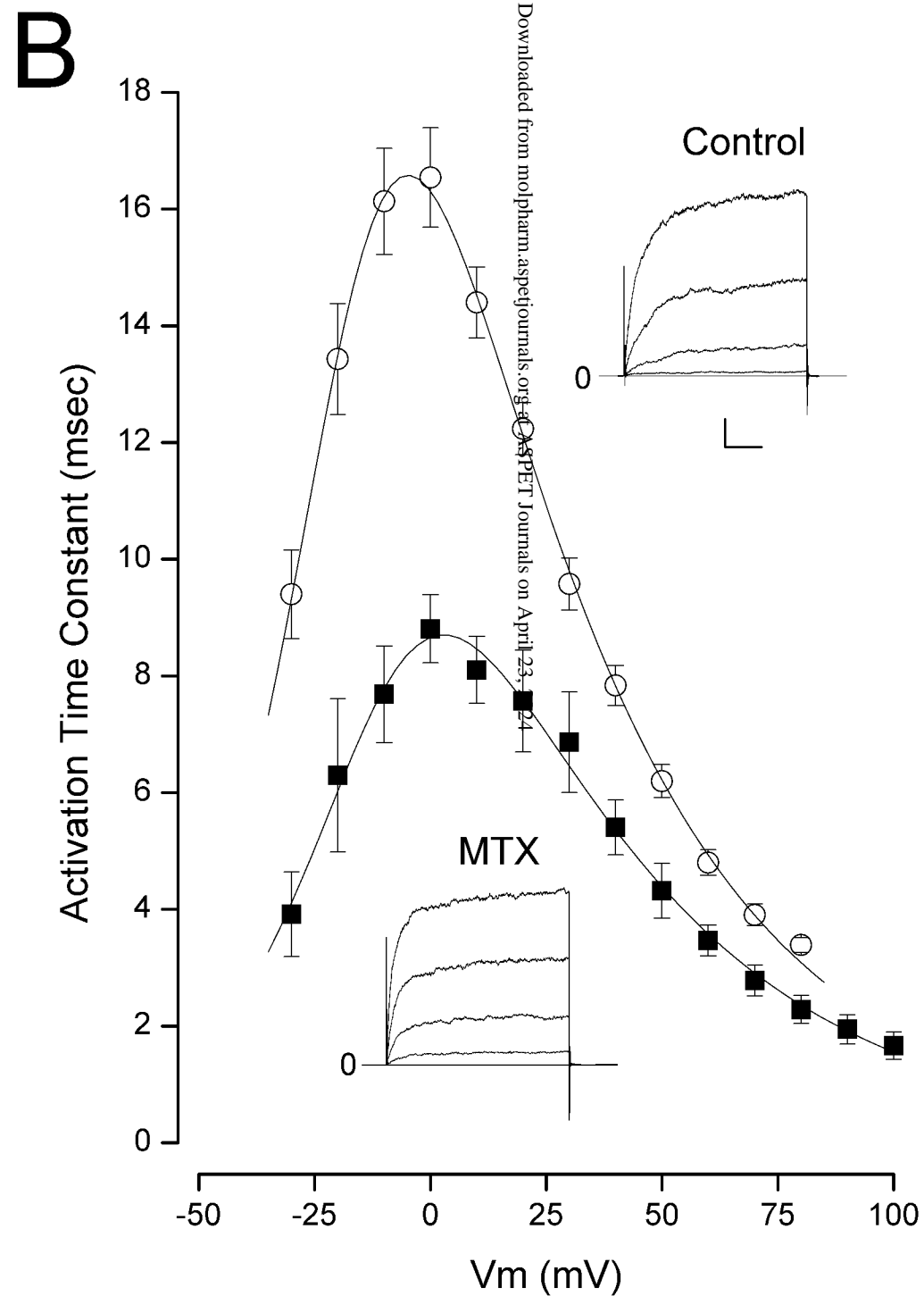
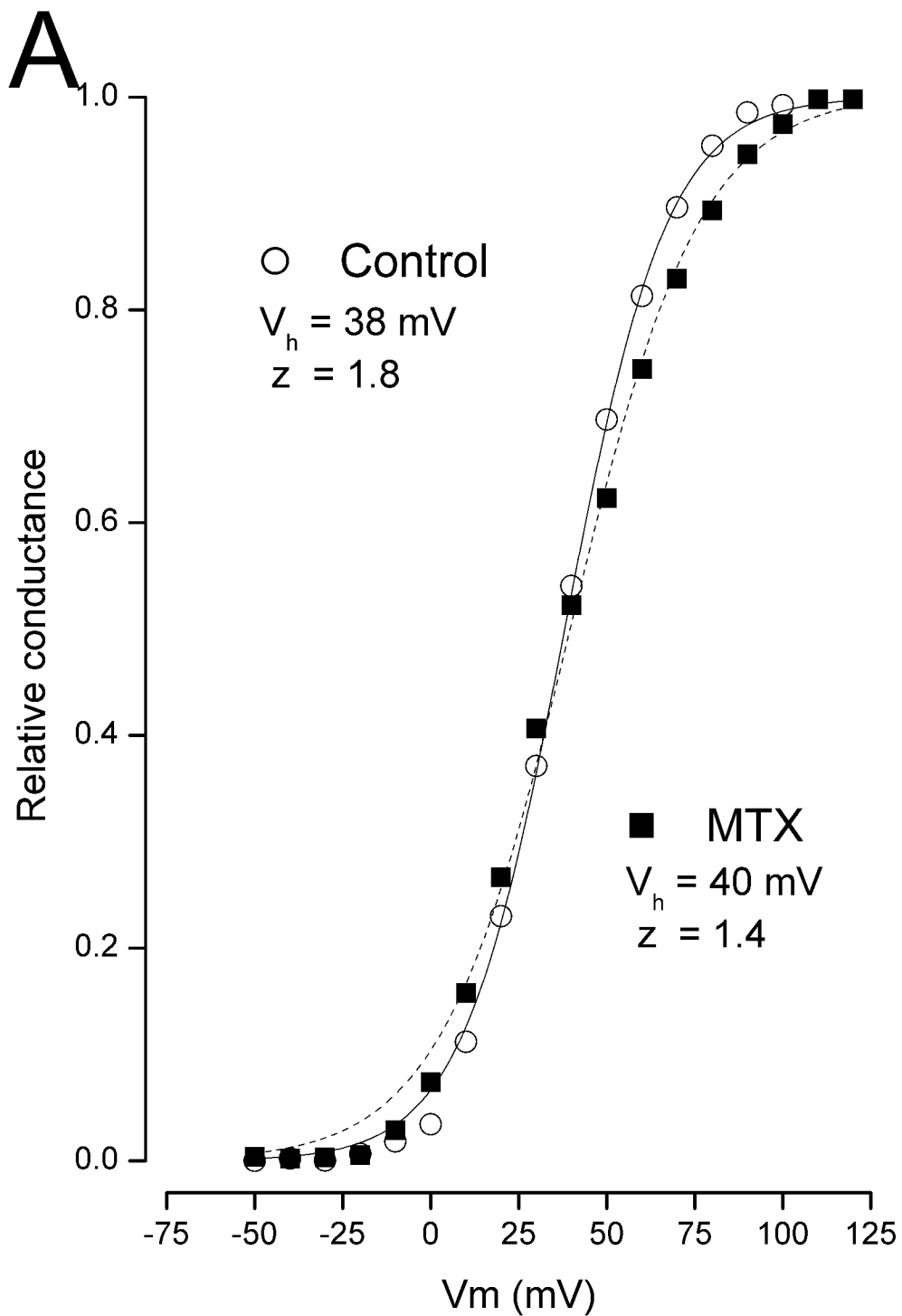
Figure 7. H-A model simulations of the activation of expressed parSlo channels in the absence and presence of MTX. Voltage dependence of relative activation of heterologously expressed parSlo BK channels in the absence (○) and presence (■) of 5 μ M MTX. Solid lines: Simulation of the H-A model with the indicated parameters (see text for additional details). Control data are pooled from several cells: N = 4 for $V_m \geq 140$ mV, N = 3 for V_m from 100 to 140 mV; data at +50 mV are single channel measurements from three individual cells. MTX data at large depolarizations are from a single cell with activation properties representative of the mean values of 5 cells. MTX data at negative voltages are mean values from 6 cells except for the value at -120 mV which is the mean from 3 cells.

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Tables

Table 1. Summary of BK channel activation parameters.

	Parotid			parSlo			parSlo + LRRC26		
	Control	MTX	shift	Control	MTX	shift	Control	MTX	shift
V _h (mV)	42 ± 1.4	36 ± 2.8	6	188 ± 2.0	118 ± 4.3	70	52 ± 2.0	35 ± 3.6	17
z	1.7 ± 0.04	1.4 ± 0.05		1.2 ± 0.04	1.2 ± 0.04		2.1 ± 0.05	1.5 ± 0.11	
N	16	8		5	8		7	6	



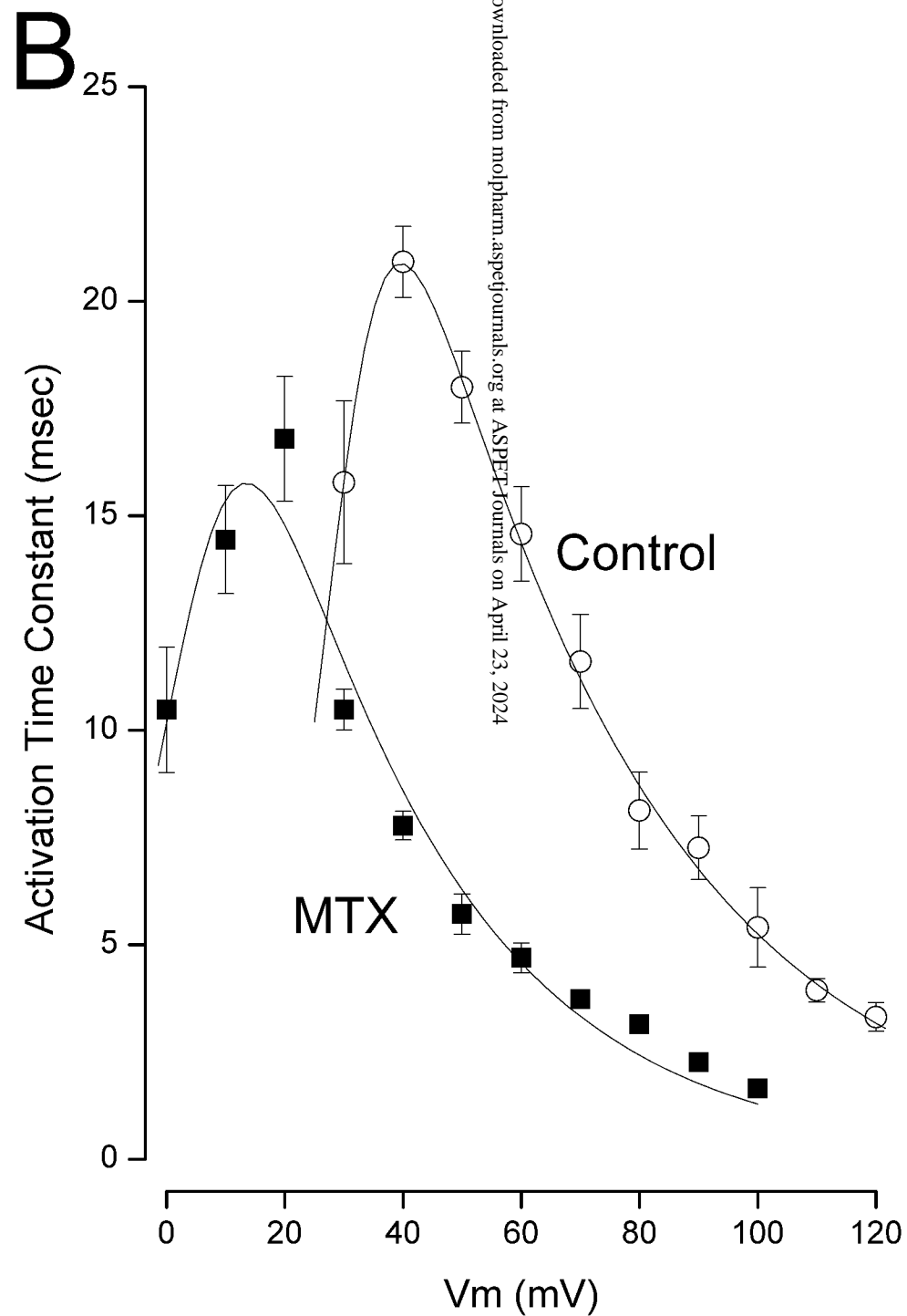
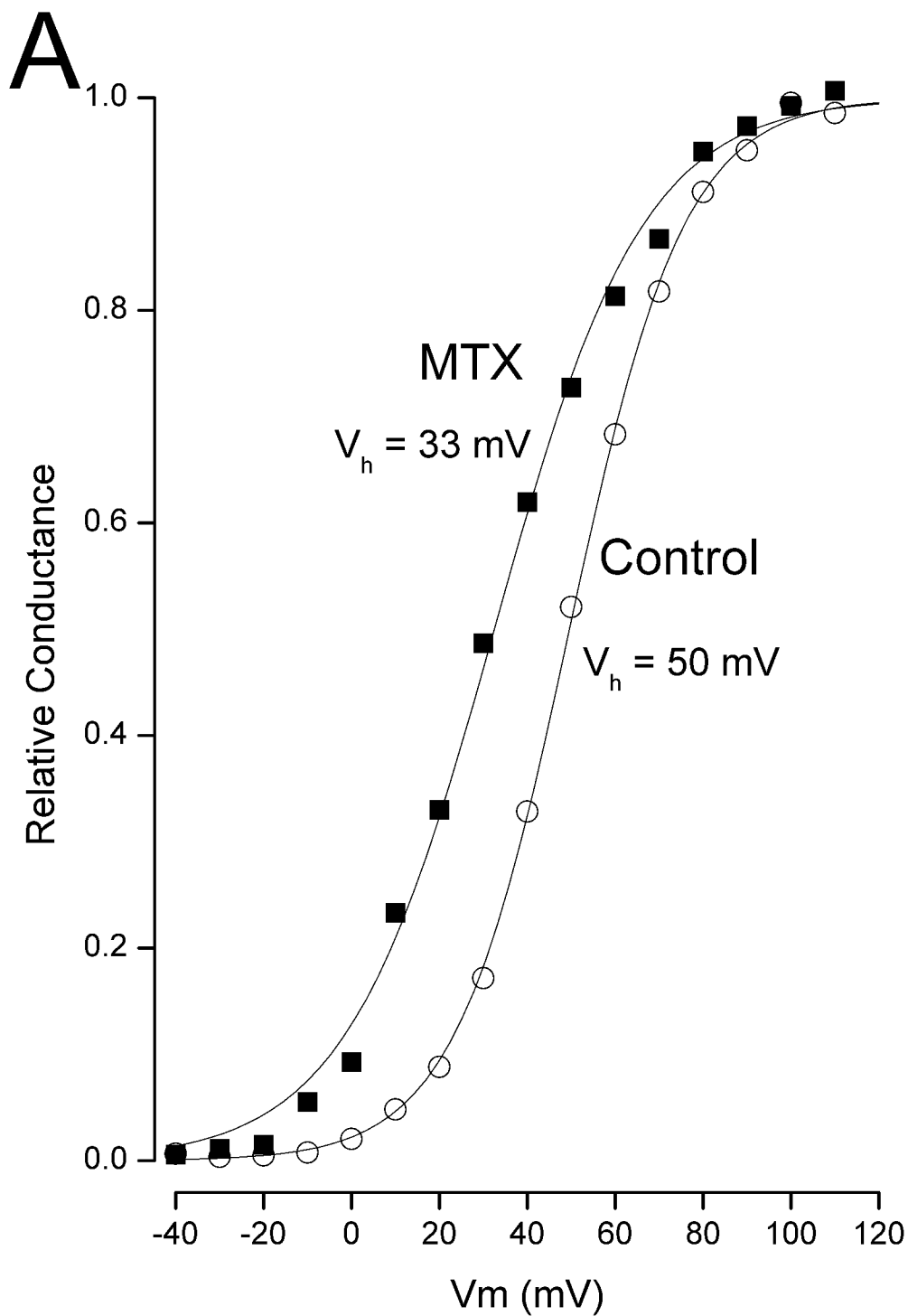
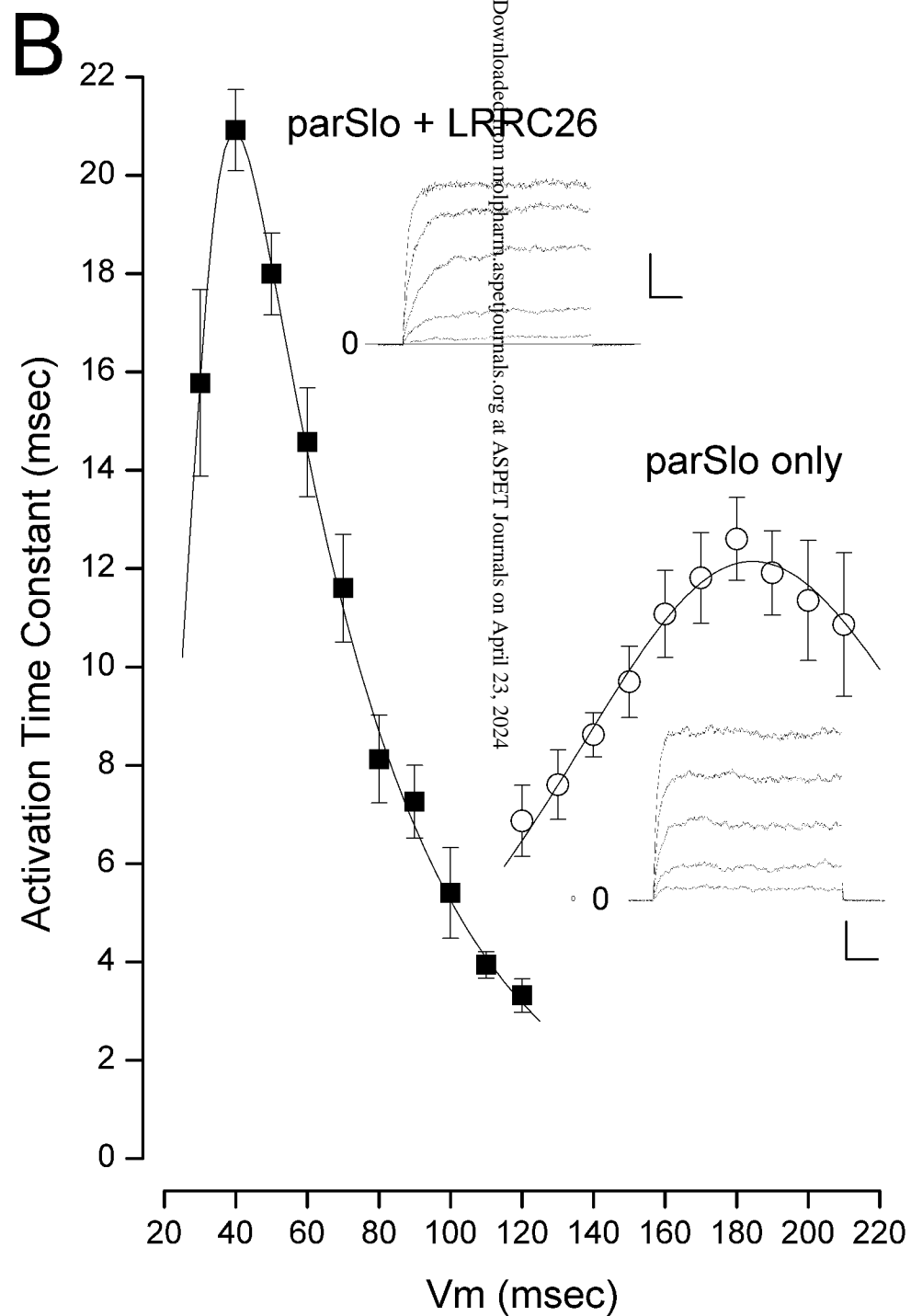
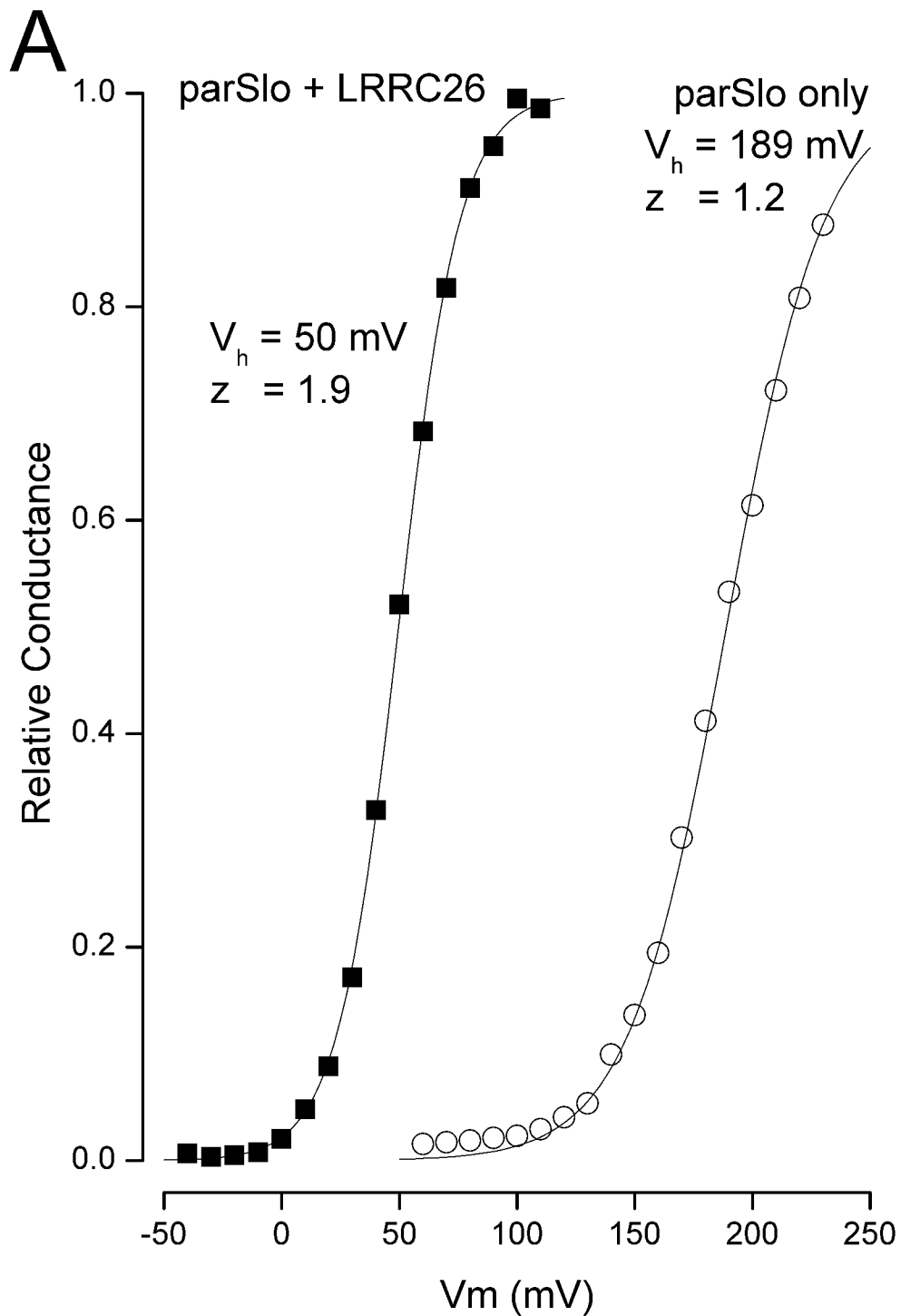
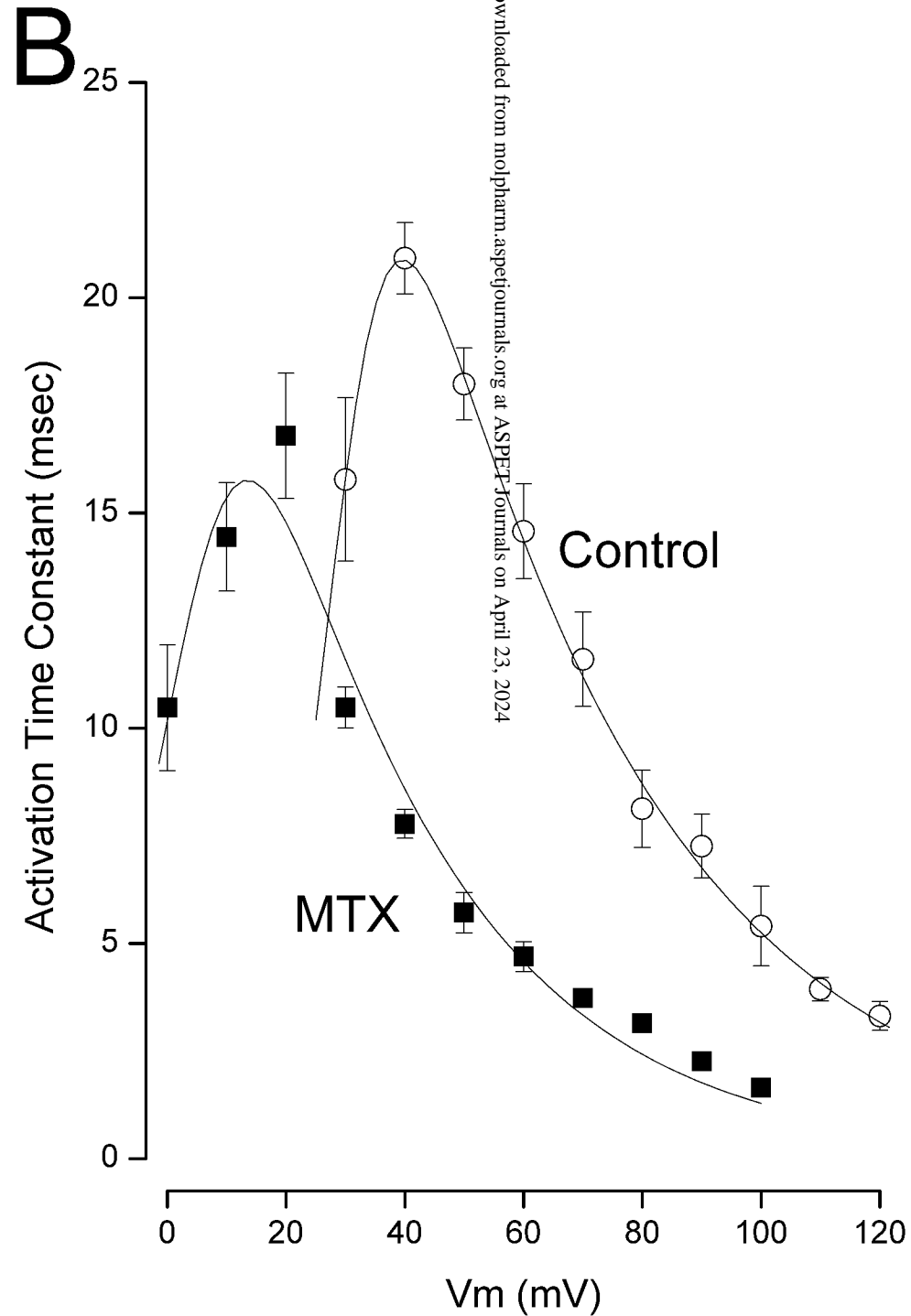
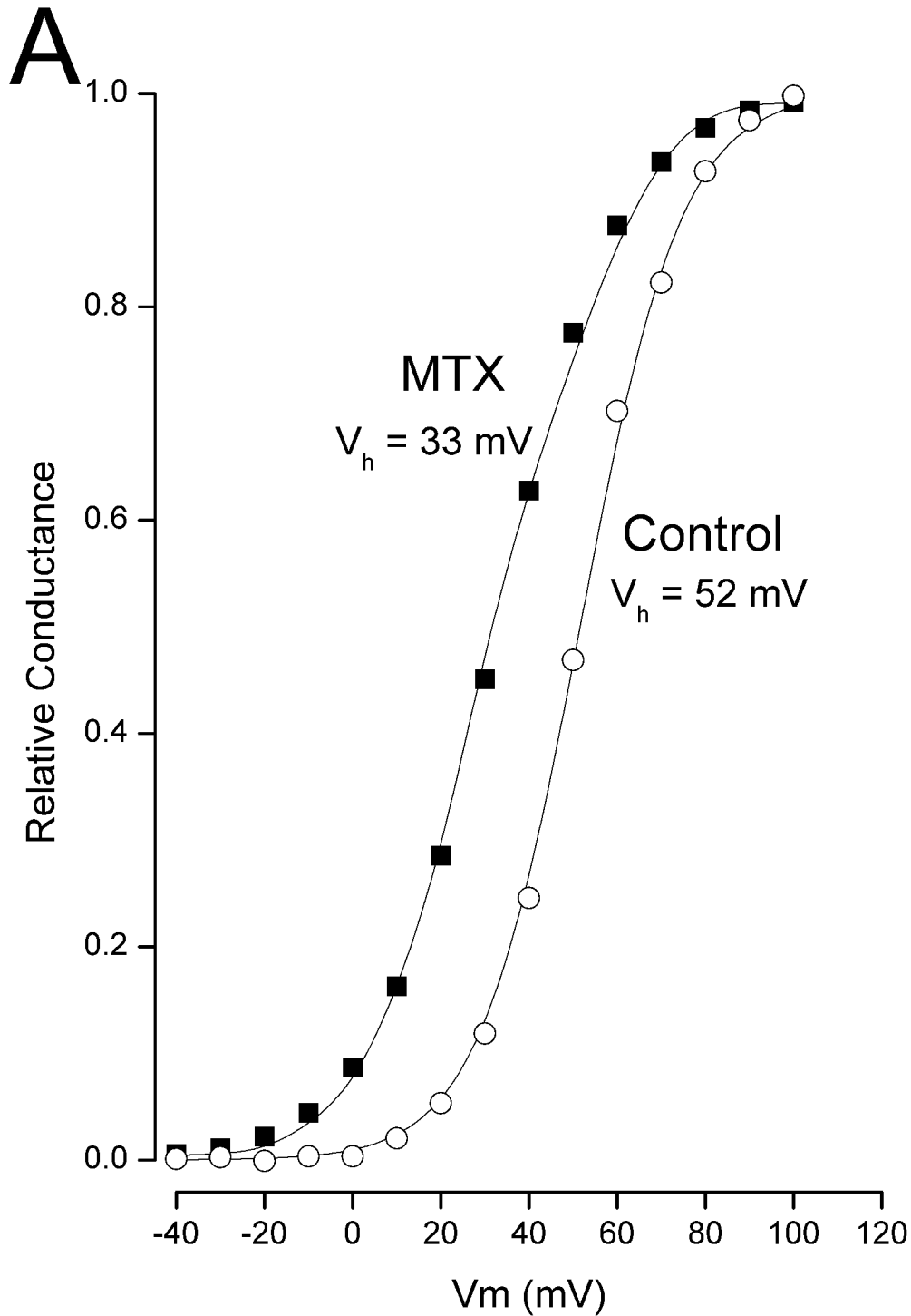


Figure 3





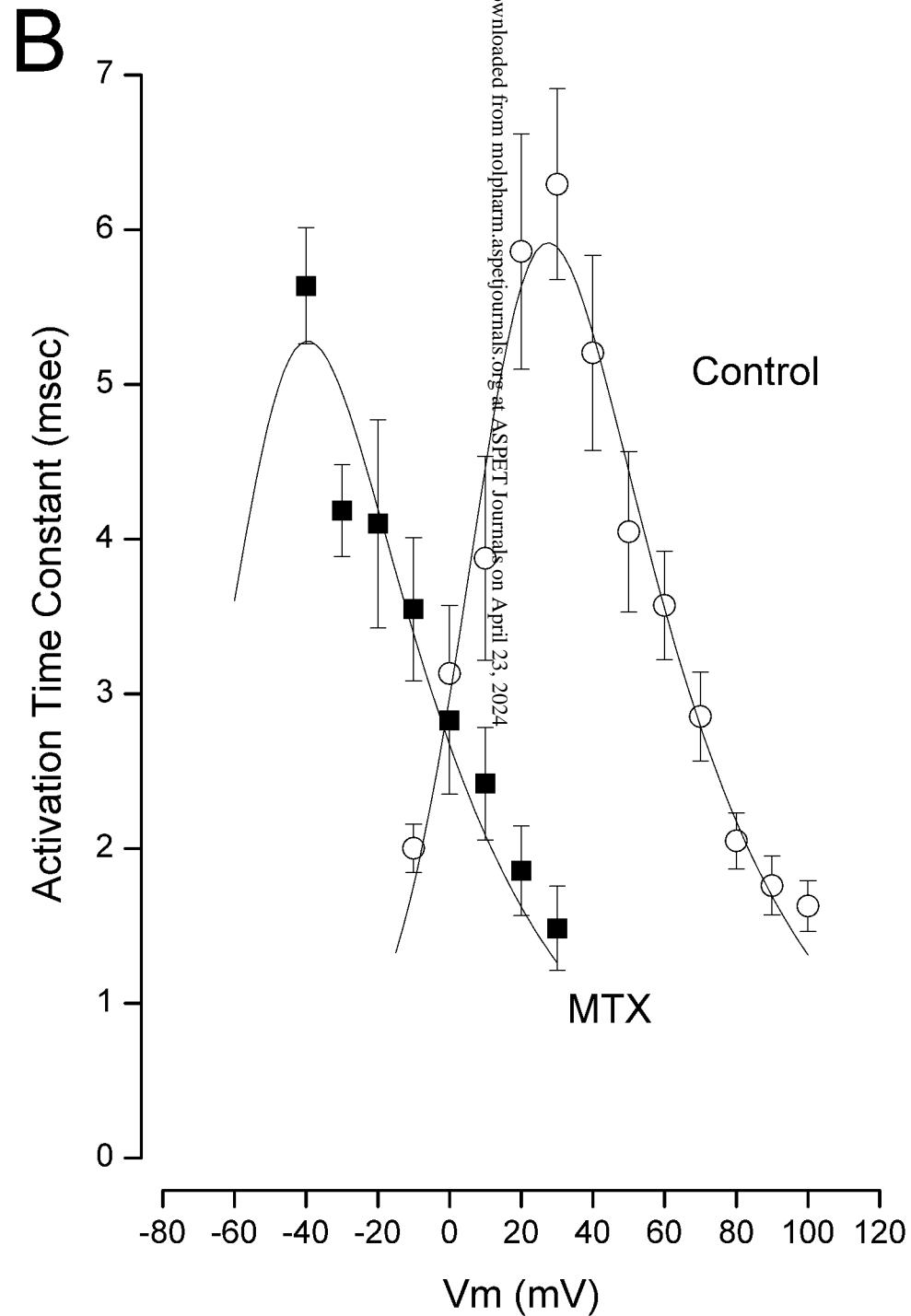
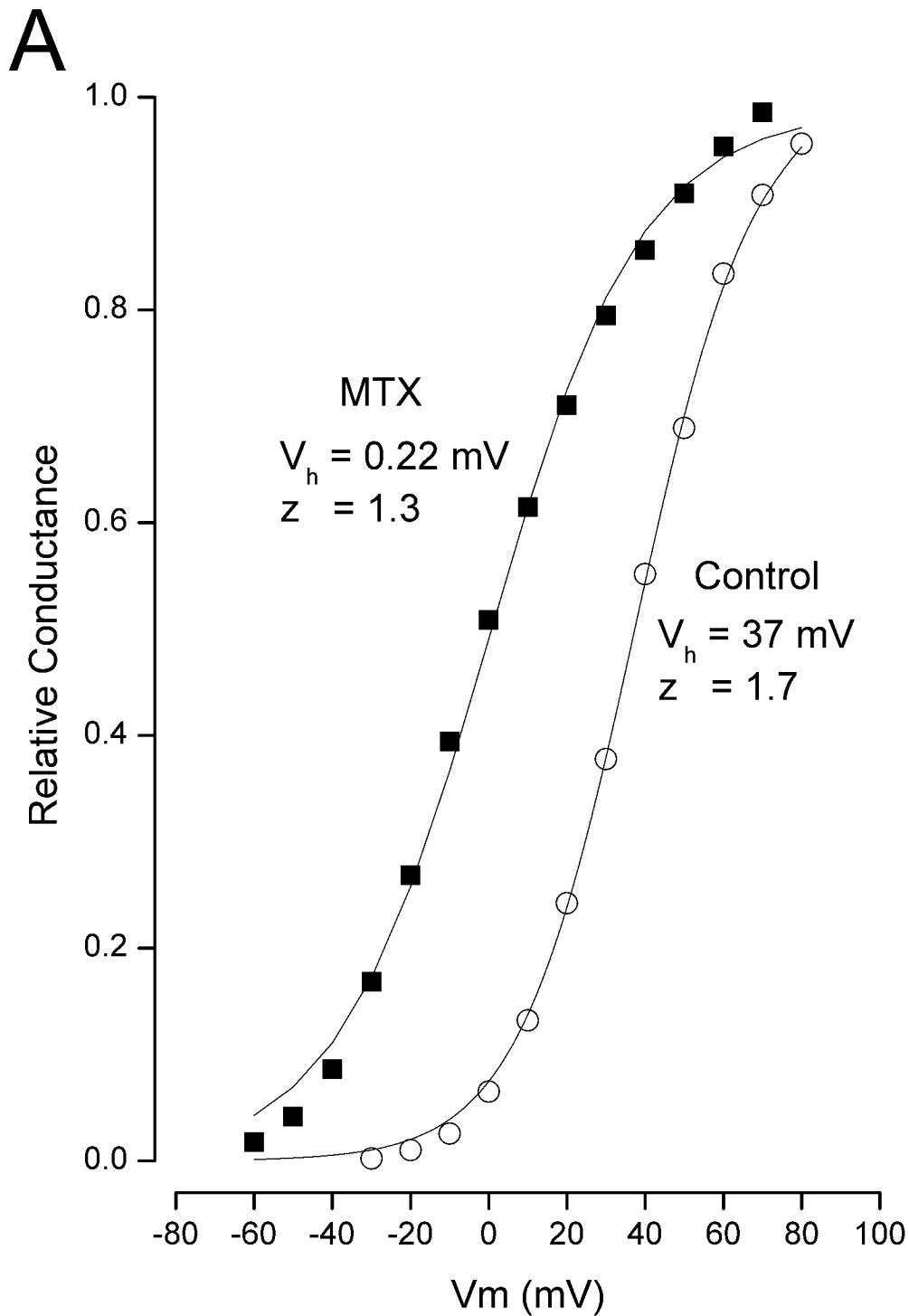
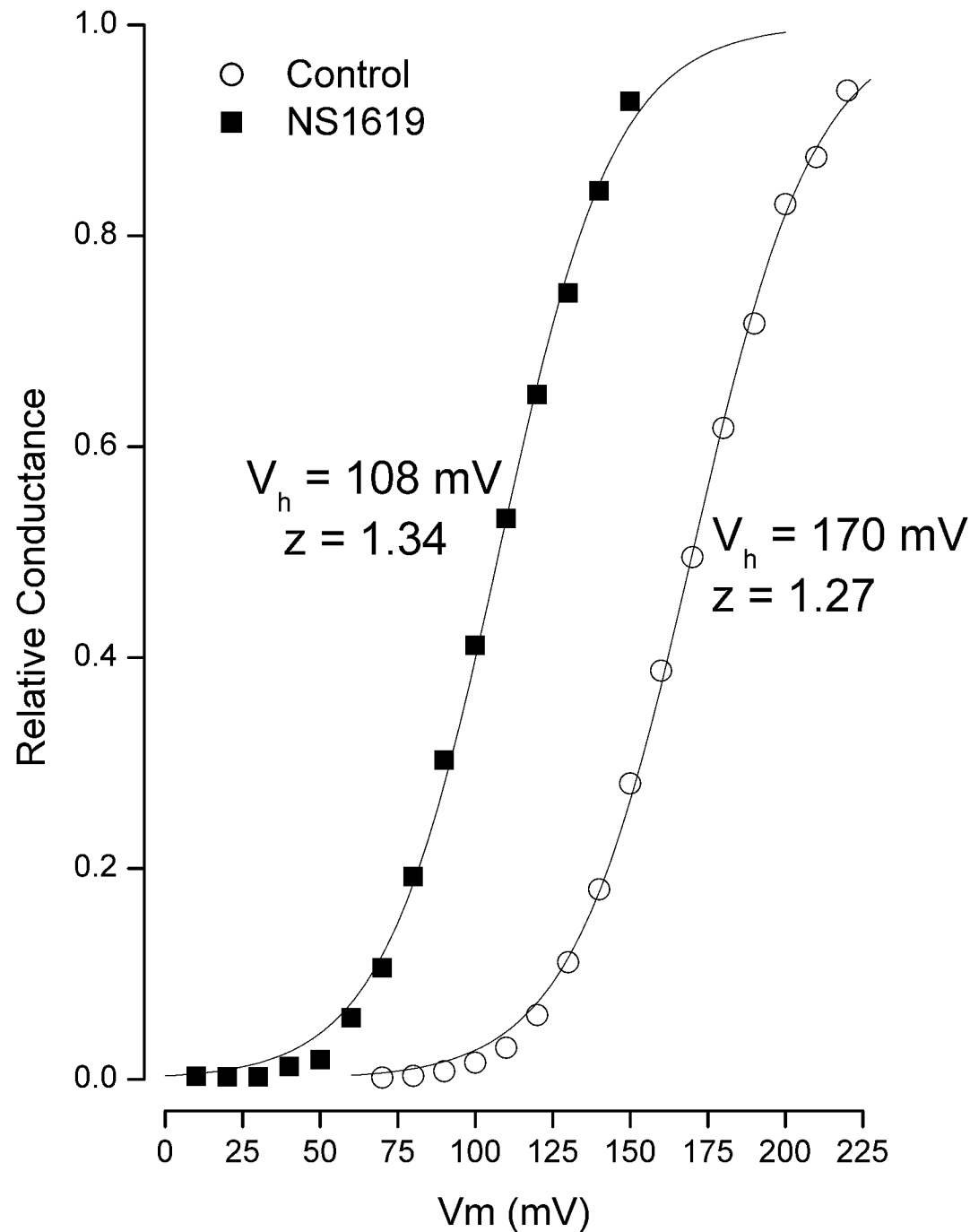


Figure 6

A

parSlo



B

parSlo + LRRC26

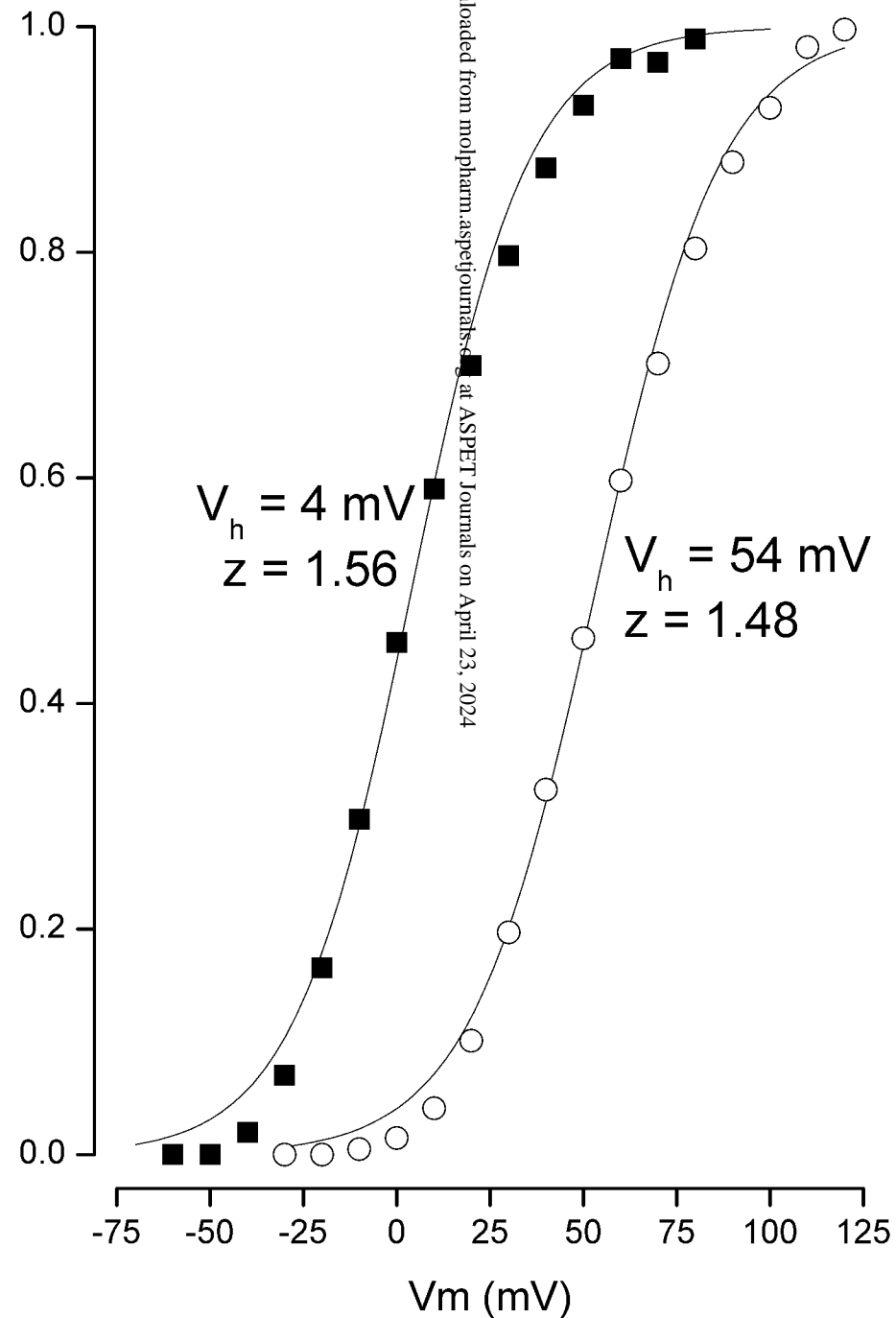


Figure 7

Heterologously Expressed ParSlo (Only)

