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Tityustoxin-K(alpha) blockade of the voltage-gated potassium channel Kv1.3

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1 We investigated the action of TsTX-Kα on cloned Kv1.3 channels of the *Shaker* subfamily of voltage-gated potassium channels, using the voltage–clamp technique. Highly purified TsTX-Kα was obtained from the venom of the Brazilian scorpion *Tityus serrulatus* using a new purification protocol. Our results show that TsTX-Kα blocks Kv1.3 with high affinity in two expression systems.

2 TsTX-Kα blockade of Kv1.3 channels expressed in *Xenopus* oocytes was found to be completely reversible and to exhibit a pH dependence. The K_D was 3.9 nM at pH 7.5, 9.5 nM at pH 7.0 and 94.5 nM at pH 6.5.

3 The blocking properties of TsTX-Kα in a mammalian cell line (L929), stably transfected to express Kv1.3, were studied using the patch–clamp technique. In this preparation, the toxin had a K_D of 19.8 nM at pH 7.4.

4 TsTX-Kα was found to affect neither the voltage-dependence of activation, nor the activation and deactivation time constants. The block appeared to be independent of the transmembrane voltage and the toxin did not interfere with the C-type inactivation process.

5 Taken as a whole, our findings indicate that TsTX-Kα acts as a simple blocker of Kv1.3 channels. It is concluded that this toxin is a useful tool for probing not only the physiological roles of Kv1.2, but also those mediated by Kv1.3 channels.
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Abbreviations: cRNA, complementary ribonucleic acid; $I_{Control}$, current in the absence of toxin; I_{Toxin}, current in the presence of toxin; PAGE, polyacrylamide gel electrophoresis; TEA, tetraethylammonium; TsTX-Kα, Tityustoxin-K(alpha)

Introduction

The voltage-gated family of potassium channels is formed by members that play crucial roles in the generation of electrical signals and excitability control in many cell types (Hille, 2001). Since they are essential for cell function, alteration of K^+ channel conduction or gating properties is a potent action of toxins present in venoms of different species. The venom of the Brazilian scorpion *Tityus serrulatus*, for instance, contains a collection of peptides that affects cell function by interacting with various ion channels (Kirsch *et al*., 1989; Legros *et al*., 1996).

TsTX-Kα, a constituent of this venom, was initially described as a peptide able to block the ^{86}Rb efflux through noninactivating, delayed-rectifier-type K^+ channels in synaptosomes, without changing the efflux through inactivating (also called A-type) K^+ channels (Blaustein *et al*., 1991; Rogowski *et al*., 1994). Its selective action on a voltage-gated noninactivating K^+ current was also described in hippocampal and cerebellar neurons (Eccles *et al*., 1994). In contrast, in neurons of the dorsal root ganglion (DRG), TsTX-Kα has been found to selectively block a low voltage-activated, partially inactivating K^+ current (IKf). These results indicate that the channels responsible for generating IKf in DRG neurons represent a different subtype, neither being the channels responsible for the inactivating K^+ current seen in rat synaptosomes, nor those inactivating channels found in hippocampal and cerebellar neurons, all of which are insensitive to this toxin (Matteson & Blaustein, 1997).

TsTX-Kα has also been tested on cloned α subunits that form functional homomeric K^+ channels. In fibroblast cells transformed to express Kv1.2, TsTX-Kα blocked the K^+ current with very high affinity (Werkman *et al*., 1993). The sensitivity of Kv1.2, but not of Kv1.1 and 1.4 homomers, to TsTX-Kα was also demonstrated in the *Xenopus* oocyte expression system (Hopkins, 1998). Coexpression of the TsTX-Kα-sensitive subunit (Kv1.2) with either α subunit

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Kv1.1 or Kv1.4 showed that the blocking affinity of the heteromers depends not only on the sensitive but also on the insensitive subunit. This observation showed that a more complete understanding of the subunit specificity and of the effect of subunit composition upon the toxin blocking affinity is necessary for the toxin to be useful as a tool to deduce the molecular identity of native channels.

In the present study, we investigate the interaction of TsTX-Kα with mouse Kv1.3 (*m*Kv1.3) channels in two heterologous expression systems: *Xenopus* oocytes and naive mammalian cells (L929). Two-electrode voltage-clamp experiments in oocytes provided an excellent screen of the inhibitory activity of TsTX-Kα on distinct *Shaker* alpha subunits. Patch-clamp recordings were performed in L929 cells stably expressing Kv1.3 channels, to add more physiological significance to the results of toxin–channel interaction by providing an environment close to that of the cells from which Kv1.3 is derived. This second approach also overcame some limitations of the oocyte expression system, such as the inaccurate temporal resolution of the voltage-clamp response, which contaminates fast kinetics (Stühmer, 1998). We have found that TsTX-Kα blocks homomeric Kv1.3 channels with high affinity. The toxin acts as a simple blocker of this channel, occluding the pore but without changing the kinetic properties of the channel.

Methods

Purification procedure

T. serrulatus venom was extracted and chromatographed as previously described (Arantes *et al*., 1989). Reverse-phase liquid chromatography of lyophilized fraction XI was performed in a Shimadzu HPLC system, using a 4.6 mm × 25 cm column (Shimadzu Corp., Tokyo, Japan) previously equilibrated with 0.1% (v/v) trifluoroacetic acid. Elution was performed with a 0–60% (v/v) acetonitrile linear gradient in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1.0 ml min^{-1}. Absorbance was monitored at 214 nm. PAGE for basic proteins was run as described by Arantes *et al*. (1989). Stock solutions of TsTX-Kα (10^{-6} M) were prepared in water and 0.1% BSA, 100 mM NaCl, 10 mM Tris (pH 7.4) and stored at −20°C.

Cell culture and electrophysiology

Oocyte isolation, handling and measurements were performed as described previously (Stühmer, 1998). Two-electrode voltage–clamp recordings were made 1–10 days after cRNA injection. The oocytes were bathed in a solution containing (in mM): 90.0 sodium gluconate, 2.5 potassium gluconate, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10.0 HEPES, pH: 7.5. The vitelline membrane of the oocytes was left intact. Voltage and current electrodes were filled with 2.0 M KCl solution and exhibited a typical initial resistance between 0.5–1.0 MΩ. Currents were recorded using a Turbo TEC-10CD amplifier (NPI Electronics, Tamm, Germany). The data were collected and analyzed using the Pulse/Pulse-fit software (Heka, Lambrecht, Germany). Linear capacitive and leak currents were subtracted from the macroscopic currents, using a modified P/n protocol implemented in Pulse.

The L929 cells stably transfected with mouse Kv1.3 (Grissmer *et al*., 1994) were kindly provided by Dr George Chandy from the Department of Physiology and Biophysics (University of California, Irvine, U.S.A.). The cells were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 1 mM Na-pyruvate, 100 U ml^{-1} penicillin-G sodium and 100 μg ml^{-1} streptomycin sulfate in a humidified, 5% CO_2 incubator. Cells expressing Kv1.3 channels and also a neomycin resistance gene were selected by adding 250 μg ml^{-1} of geneticin to the culture medium. The media, sera and reagents were purchased from Life Technologies (Invitrogen, Carlsbad, CA, U.S.A.). The cells were detached using a trypsin/EDTA solution (Biochrom AG, Berlin, Germany), seeded on glass coverslips and subjected to patch-clamp analysis within 6 h.

During recordings, cells were bathed in a solution that contained (mM): 155.0 NaCl, 4.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 5.0 HEPES, adjusted to pH 7.4 with NaOH (300–310 mosm kg^{-1} H_2O). The internal (pipette) solution contained (mM): 140.0 KF, 2.0 $MgCl_2$, 1.0 $CaCl_2$, 10.0 EGTA, 10.0 HEPES, adjusted to pH 7.2 with KOH (290–300 mosm kg^{-1} H_2O). Patch-clamp recordings were made in the whole-cell configuration (Hamill *et al*., 1981). All membrane currents were recorded using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.) interfaced to a computer *via* a Digidata 1200 A/D converter running pClamp6 (Axon Instruments). Electrodes were pulled from borosilicate glass capillaries (B150-86-15, Sutter Instrument, Novato, CA, U.S.A.) to have a resistance of around 2.0 MΩ when measured in the bath. Series-resistances, when in the whole-cell mode, ranged from 4.0 to 7.0 MΩ, and were electronically compensated up to 80%. The liquid junction potential between the internal (pipette) and external (bath) solution was offset at the beginning of the experiment and no further corrections were applied. The sampling frequency and filter settings varied between experiments and will be presented with the description of the particular result. All electrophysiological experiments were performed at 22–24°C. A gravity-driven perfusion system (1.0 ml min^{-1}) was used to exchange the bath solution (0.4 ml). Chemicals used for electrophysiological studies were of analytical grade and from Sigma (St Louis, MO, U.S.A.).

Results

TsTX-Kα purification

In testing all fractions obtained from the initial purification of *T. serrulatus* venom (Arantes *et al*., 1989), we observed that fraction XI was the most active against potassium channels (results not shown). This fraction accounted for 2.5–3.5% of the total soluble protein present in the sample. Figure 1 shows the result from reverse-phase HPLC of fraction XI, illustrating that it is composed of at least six different subfractions (Peaks XI-1 to XI-6). Mass spectrometry analysis of subfraction XI-4, a toxin representing 12–13% of the fraction XI, revealed a molar mass of 3942 Da. The purity of this subfraction was confirmed by PAGE (Figure 1, inset), where XI-4 appeared as a single band. Complete sequence analysis (results not shown) confirmed that fraction XI-4 was the toxin TsTX-Kα.

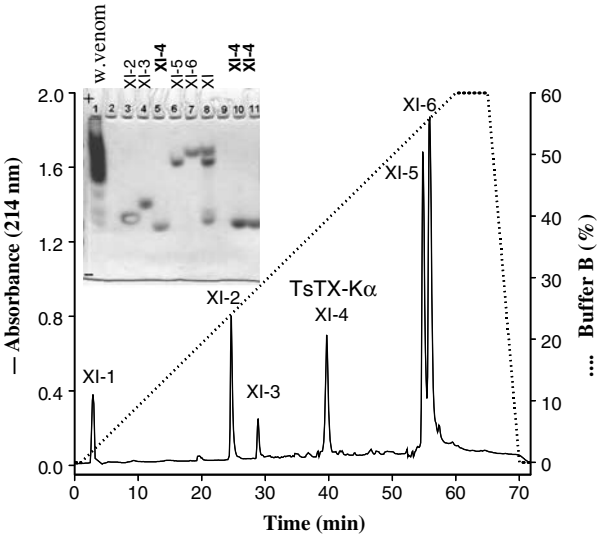

Figure 1 Reverse-phase HPLC of fraction XI (0.3 mg). Adsorbed proteins were eluted with a linear acetonitrile gradient (0–60%) in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1.0 ml min^{-1}. Absorbance was monitored at 214 nm. Buffer B was 60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The peak XI-4 corresponds to TsTX-Kα. Inset, PAGE on a 10% (w/v) acrylamide gel at pH 4.5. Lane 1: *T. serrulatus* whole venom; lane 3: fraction XI-2; lane 4: fraction XI-3; lanes 5, 10 and 11: fraction XI-4; lane 6: fraction XI-5; lane 7: fraction XI-6; lane 8: fraction XI.

TsTX-Kα blockade of Kv1.3 channels in Xenopus *oocytes*

As previously described for the *Xenopus* oocyte expression system, mRNA transcribed from the cloned Kv1.3 potassium channel cDNA induces a delayed-rectifier-like current that exhibits fast activation and slow inactivation during depolarizing pulses (Stühmer *et al*., 1989; Douglass *et al*., 1990; Grissmer *et al*., 1990). Figure 2a shows representative current traces in response to depolarizing voltage steps for *m*Kv1.3 channels expressed in *Xenopus* oocytes before and after the addition of 4.0 nM TsTX-Kα to the extracellular solution at pH 7.5. In an oocyte bathed by a solution that contained 20.0 nM TsTX-Kα and had a pH 6.5, the blockade of Kv1.3 currents was considerably smaller (Figure 2b). Figure 2c illustrates the concentration-dependence and pH-sensitivity of the blockade induced by TsTX-Kα on Kv1.3 currents elicited by depolarization to +40 mV from a holding potential of −80 mV. Acidifying the bathing solution shifted the concentration–response curve to the right, revealing a decrease in the affinity of the toxin. The K_D was 3.9 nM at pH 7.5, 9.5 nM at pH 7.0 and 94.5 nM at pH 6.5. The K_D changed 10-fold in the pH range between 6.5 and 7.0, whereas it changed only two-fold between pH 7.0 and 7.5, suggesting the involvement of a protonatable histidine residue (pK_a near 7.0) in the interaction. It has been suggested that there are significant through-space electrostatic interactions between charges on the toxin and charges on the channel (Stocker & Miller, 1994). Thus, it seems likely that the protonation of histidine residues at low pH would destabilize binding of toxin to the receptor site of the channel due to electrostatic interactions of positively charged histidine imidazole group(s) with basic residue(s) on the toxin (Thompson & Begenisich, 2000). The TsTX-Kα-induced block was reversible as shown in the Figure 2d.

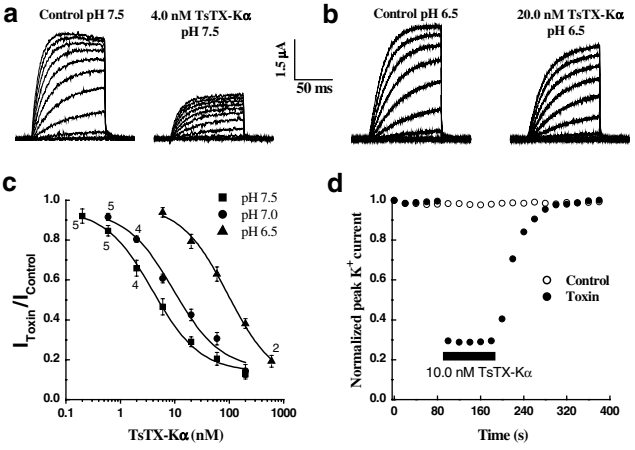

Figure 2 Basic properties of TsTX-Kα blockade of currents through cloned *m*Kv1.3 channels expressed in *Xenopus* oocytes. The currents were elicited by pulses ranging from −50 to +50 mV in 10 mV steps from a holding potential of −80 mV, in the absence (left) and presence (right) of the toxin in the bath solution, which had the pH adjusted to either 7.5 (a) or 6.5 (b). The interpulse interval was 30 s. Lowpass filtering was at 2 and 1 kHz and the sampling rate 10 and 5 kHz in (a) and (b), respectively. (c) Dose–response curves determined at different pH. The fraction of unblocked current ($I_{Toxin}/I_{Control}$) was calculated by measuring the peaks of currents elicited by voltage steps from −80 to +40 mV. Bars indicate s.e.m., and the number of repetitions averaged, if not exactly three, are given next to the data points. The solid curves represent fit of the equation: $I_{Toxin}/I_{Control} = K_D/(K_D + [T_x])$ to the experimental points, where $[T_x]$ indicates the toxin concentration and K_D is the dissociation constant. The best fit predicts a K_D of 3.9, 9.5 and 94.5 nM for pH 7.5, 7.0 and 6.5, respectively. (d) Reversibility of the block of Kv1.3 channels by TsTX-Kα (pH 7.5). Peak currents elicited by pulses from −80 to +40 mV, applied every 20 s, were normalized to the maximum value and plotted against time in control and in the presence of toxin.

Blocking properties of mKv1.3 currents by TsTX-Kα in L929 cells

Figure 3a shows representative recordings of Kv1.3 currents in L929 cells stably expressing the channel before and after the addition of 20.0 nM TsTX-Kα to the bath. This experiment shows that at 20 nM TsTX-Kα blocks about 50% of the current. The current–voltage relationships in the presence and absence of TsTX-Kα are plotted on the right-hand side. In order to exclude the possibility that the reduction in peak current was induced by a shift in the activation curve, conductance–voltage relationships before and during the perfusion were constructed (Figure 3b, left). Conductance (g) was calculated according to Ohm's law: $g = I/(V - V_R)$, where I is the peak current, V the applied potential and V_R the reversal potential. The reversal potentials determined in the absence and presence of the toxin were from the peak tail currents and found to be around −76 mV (Figure 3b, middle and right), close to the potassium equilibrium potential calculated using the Nernst equation (−80.9 mV), assuming an activity coefficient of 0.75 for 140 mM KF. The experimental data points were fitted by a Boltzmann function of the form:

$$g = g_{min} + \frac{g_{max} - g_{min}}{1 + e^{-(V_m - V_0)/K}}$$

where g is the peak K^+ conductance at a given potential, g_{min} and g_{max} are the minimum and maximum cell conductances, V_0 is the voltage at which half-activation occurs and K a slope

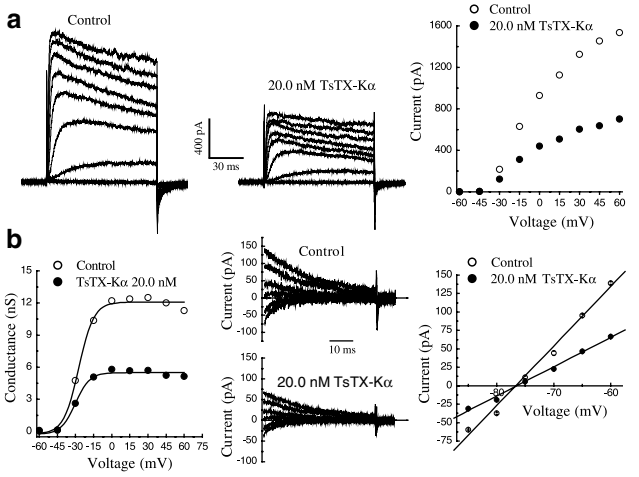

Figure 3 Effects of TsTX-Kα on Kv1.3 currents in L929 cells. (a) Whole-cell currents elicited by voltage pulses ranging from −60 to +60 mV, in 15 mV steps, from a holding potential of −80 mV, in the absence (left) and presence (middle) of the toxin. The interpulse-interval was 30 s. The resultant current–voltage relationships, taken from the peak currents, are shown on the right-hand side. (b) The plot on the left-hand side shows the conductance–voltage relation for the K^+ currents presented in (a). Fit lines are from a Boltzmann function, as described in the text. Values for V_0 and K are −30.0 and 5.9 mV in the absence and −27.3 and 6.2 mV in the presence of TsTX-Kα, respectively. The center panel shows tail currents recorded from the same cell at voltages between −85 and −60 mV, in 5 mV steps, after applying a 10 ms pulse to +40 mV from a holding potential of −90 mV. On the right-hand side, the peak tail currents in both conditions are plotted against voltage. The reversal potential, determined by a linear fit, was −76.7±6.7 mV for control and −76.5±5.7 mV in the presence of toxin ($n=3$). Lowpass filtering was at 2 and 5 kHz and the sampling rate 10 and 20 kHz in (a) and (b), respectively.

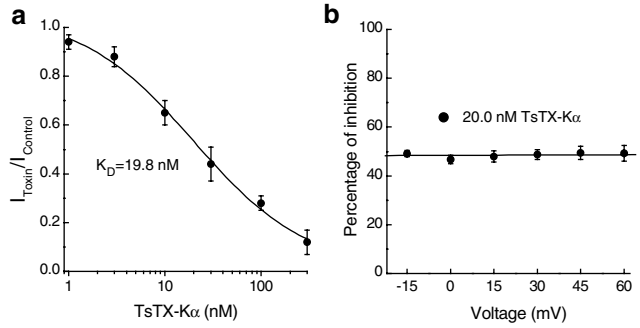

Figure 4 Concentration-dependent inhibition of Kv1.3 currents in L929 cells (a). The fraction of unblocked current was calculated as $I_{Toxin}/I_{Control}$ measured at the peak of depolarizing steps from −80 to +40 mV. Bars indicate s.e.m. ($n=4$). The solid curves represent the fits obtained as described in Figure 2 giving a K_D of 19.8 nM. (b) Block of Kv1.3 channels by TsTX-Kα is voltage-independent. The percentage of inhibition was calculated according to the formula $(1-I_{Toxin}/I_{Control})\times 100$, where the peak currents in the presence and absence of the toxin were measured by depolarization to the indicated voltages from a holding potential of −80 mV ($n=5$ L929 cells).

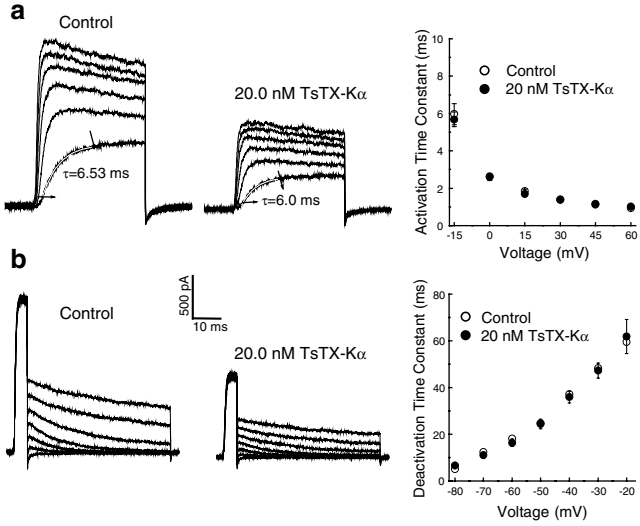

Figure 5 TsTX-Kα does not affect the opening (a) and closing (b) kinetics of Kv1.3-mediated currents in L929 cells. Activation and deactivation time constants were calculated by fitting single-exponential functions to the current records in the presence and absence of the toxin. (a) Cells were depolarized to voltages between −15 and +60 mV, from a holding potential of −90 mV, at intervals of 20 s. Fits were from 10 to 90% of the current amplitude, as shown in the record to −15 mV. The plot on the right-hand side shows mean±s.e.m. of the activation time constants under control conditions and in the presence of TsTX-Kα ($n=5$). (b) Tail currents were elicited by voltage steps ranging from −80 to −20 mV, after a short activating pulse (10 ms) to +40 mV. On the right, mean± s.e.m. of the decay time constants of the tail currents, with and without toxin, are presented ($n=5$). All records were filtered at 5 kHz and sampled at 20 kHz.

factor. In the four cells studied, the values of V_0 and K did not change significantly in the presence of the toxin; −29.1±1.3 and 6.1±1.4 mV in the control and −27.7±1.2 and 6.3±1.1 mV after the addition of TsTX-Kα.

The concentration–response curve for Kv1.3 inhibition by TsTX-Kα in L929 cells is presented in Figure 4a. The K_D in this case was 19.8 nM. We evaluated the voltage-dependence of the interaction of TsTX-Kα with Kv1.3 by determining the percentage of block of the whole-cell current induced by 20.0 nM of TsTX-Kα at different membrane potentials (Figure 4b). The results show that the percentage of block induced by a given toxin concentration is independent of the applied test potential.

The effect of TsTX-Kα on the opening kinetics of Kv1.3 was also evaluated. The activation time constants were calculated by fitting single-exponential functions to the records (Figure 5a, left). In T lymphocytes, it has been shown that the activation time constants of Kv1.3 decrease as the amplitude of depolarizing pulses increases. Their values are below 10 ms for voltages more positive than −15 mV (Cahalan *et al*., 1985). In L929 cells, we observed similar values with time constants that are not affected by the presence of 20.0 nM TsTX-Kα (Figure 5a, right). Deactivation rates (channel closing upon repolarization) were determined by fitting single-exponential functions to the K^+ tail currents during repolarization to potentials ranging from −80 to −20 mV after a short (10 ms) activating pulse to +40 mV (Figure 5b, left). We did not observe any significant difference between the deactivation kinetics of Kv1.3 channels in control conditions and in the presence of 20.0 nM of TsTX-Kα as shown in Figure 5b on the right-hand side.

Although the rate of C-type inactivation of Kv1.3 channels is reduced by TEA (Grissmer & Cahalan, 1989), a classical

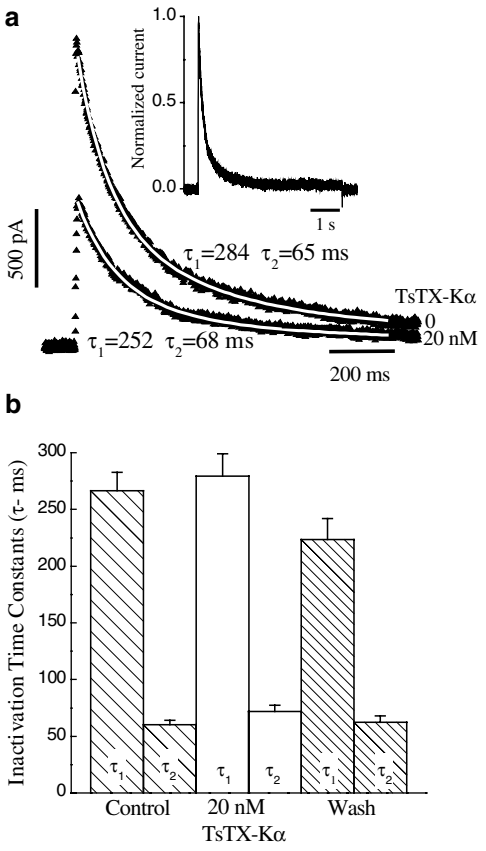

Figure 6 The inactivation time constants of Kv1.3 currents in L929 cells are not affected by TsTX-Kα. (a) Currents recorded with 5 s pulses to +40 mV from a holding potential of −80 mV in the absence and presence of 20 nM TsTX-Kα. The first 1600 ms are shown in order to best visualize the double exponential fits to the decay phase of the current. The fit begins at 15 ms and ends at 1500 ms after the start of the pulse. The inset shows normalized full traces (control and toxin) almost exactly superimposed. (b) Inactivation time constants (mean ± s.e.m., $n = 7$) obtained as in (a). τ_1 is the slow component, while τ_2 is the fast component.

blocker of this channel, 20.0 nM TsTX-Kα, merely scaled down the current without altering its inactivation kinetics. This is illustrated in Figure 6a, where the time course of current decay is fitted by two exponentials. The time constants obtained under control conditions and in the presence of the toxin were not significantly different (Figure 6b).

Discussion

In this study, we report additional pharmacological properties of TsTX-Kα, a toxin from the venom of the Brazilian scorpion *T. serrulatus*, and an alternative protocol for its purification. We show that at low nanomolar concentrations, TsTX-Kα blocks homomeric Kv1.3 channels. This result confirms TsTX-Kα as a blocker of some voltage-gated potassium channels members of the Kv1 (*Shaker*) subfamily.

The highly purified TsTX-Kα used in the present work was obtained by a fast and reliable purification scheme, involving only two chromatographic steps and two lyophilizations. This procedure differs from the previously described method for purifying TsK4, the name first given to TsTX-Kα (Blaustein *et al*., 1991). In our case, only two lyophilizations were required because the solutions used (NH_4HCO_3 for the first and trifluoroacetic acid in acetonitrile for the second chromatographic run) are easily removed. This procedure yields approximately 0.3–0.4 mg of purified toxin from 100 mg of soluble venom. The toxin exhibited high activity, indicating that the purification did not alter its three-dimensional structure.

Kv1.3 subunits are able to form heteromultimeric channels with other members of the *Shaker* subfamily (Wang *et al*., 1993; Shamotienko *et al*., 1997; Koschak *et al*., 1998; Coleman *et al*., 1999). The use of radiolabeled toxins in binding and immunoprecipitation studies have been very useful in evaluating subunit composition. The rat brain Hongotoxin-1 receptor, for example, always has a Kv1.2 subunit as an integral component (Koschak *et al*., 1998). Kv1.1 seems to be present in 75% of the toxin receptors, whereas Kv1.3 and Kv1.6 represent only minor components. Neither Kv1.1, Kv1.2 nor Kv1.3 appear to form homomultimers (Koschak *et al*., 1998). TsTX-Kα has been described as a potent blocker of expressed homomeric Kv1.2 channels (Werkman *et al*., 1993; Hopkins, 1998), without interacting with Kv1.1 or Kv1.4 channels (Hopkins, 1998). Taking into account the present results, one would expect that native homomeric and heteromeric K^+ channels containing Kv1.2 and/or Kv1.3 subunits will be sensitive to block by TsTX-Kα. The degree of block will presumably depend on the proportion of channels that contain either Kv1.3 and/or Kv1.2 subunits. The sensitivity may also depend on the other subunits present in a particular heteromer. Some subunits, Kv1.4 for instance, may decrease the blocking affinity of TsTX-Kα for a heteromultimeric channel that also contains sensitive subunits (Hopkins, 1998).

TsTX-Kα is commonly used as a pharmacological tool to verify the involvement of Kv1.2 subunits in the generation of the total K^+ current in native cells. Based on the sensitivity to the block of Kv1.3 channels by TsTX-Kα, reported in the present work ($K_D = 19.8$ nM in L929 cells), this channel might also be blocked by the concentrations of TsTX-Kα usually employed (100 nM) (Dodson *et al*., 2002). This observation indicates that the interpretation of experiments that use this toxin to pharmacologically dissect K^+ current components, especially on those cells where the level of coexpression of these subunits has not been evaluated by other approaches, is not simple.

The α subunit that forms the Kv1.3 channels has a histidine residue at position 404 and mutations at this position have been found to alter the Kv1.3 sensitivity to some toxins. This residue is located at the outer pore entrance of the channel and mutations at this site, for example to threonine (H404T), abolish the pH dependence of the block by charybdotoxin (Aiyar *et al*., 1995). Considering that TsTX-Kα has no histidine residue, the pH-dependence of the block observed in our results (Figure 2c) raises the possibility that protonation of Kv1.3 at H404 induces electrostatic repulsion of positively charged residues on the toxin. A similar effect has been observed for the interaction of TsTX-Kα with both a native and a cloned K^+ channel from squid (Ellis *et al*., 2001).

If the binding site of TsTX-Kα were deep within the transmembrane electric field, we should have observed some voltage-dependence of the blockade, as TsTX-Kα is a positively charged molecule. According to our results, where

we determined the remaining fractions ($I_{Toxin}/I_{Control}$) of the K^+ currents from the peak amplitudes at different test potentials, the blockade induced by TsTX-Kα on Kv1.3 channels is independent of the applied voltage (Figure 4b). However, the values obtained for the activation time constants of Kv1.3 channels (Figure 5a), suggest that the channel may open in a much shorter time than would be necessary to reach a new equilibrium of block at different membrane potentials. Therefore, our results indicate that there is no fast voltage-dependent block, but do not address the question of whether there is a slow voltage-dependent block. The conclusion that the block is voltage-independent implies that this peptide binds to the most external region of the Kv 1.3 channel (outer mouth of the channel pore), where the charged toxin molecule does not sense the transmembrane electric field.

Our results show that TsTX-Kα does not interact with the gating of Kv1.3 since it did not affect the voltage-dependence of channel activation (Figure 3b). Similar half-maximal activation in the absence and in the presence of the toxin suggests that TsTX-Kα simply blocks the channel, but does not shift the voltage-dependence of activation of the unblocked channel. According to Grissmer & Cahalan (1989) TEA^+ ions block Kv1.3 channels while also preventing inactivation. We observed that when Kv1.3 channels expressed in L929 cells are bathed in a physiological solution, TsTX-Kα induces blockade without altering the inactivation time constants of Kv1.3 currents.

Our data are consistent with the idea that TsTX-Kα blocks Kv1.3 channels by binding to them with high affinity, without changing their kinetic properties through effects on gating or on C-type inactivation. TsTX-Kα can be used to probe for *Shaker*-type K^+ channels that contain either or both Kv1.2 and Kv1.3 α subunits, therefore clarifying and extending the use of the toxin as a tool to evaluate the physiological roles of K^+ conductances in excitable and nonexcitable cells.

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