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Tityustoxin-Kα, a Structurally Novel and Highly Potent K^+ Channel Peptide Toxin, Interacts with the α-Dendrotoxin Binding Site on the Cloned Kv1.2 K^+ Channel

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SUMMARY

The interaction between two nonhomologous K^+ channel toxins, *Tityus serrulatus* (scorpion) toxin tityustoxin-Kα (TsTX-Kα) and *Dendroaspis angusticeps* (snake) toxin dendrotoxin (α-DTX), was investigated on K^+ currents in B82 fibroblast cells transformed to express the Kv1.2 K^+ channel. As demonstrated previously, α-DTX was a potent blocker of the K^+ current (K_d, 2.8 nM). Recombinant TsTX-Kα produced a similar block of the current but was 1 order of magnitude more potent (K_d, 0.21 nM). TsTX-Kα did not affect the kinetic properties of the current or its voltage dependence of activation. Experiments with excised and cell-attached patch recordings demonstrated that TsTX-Kα blocks the K^+ channel by binding to an extracellular site. In the presence of TsTX-Kα the blocking potency of α-DTX was reduced, whereas the potency of 4-aminopyridine, which also blocks the channel, was unaffected. α-DTX caused a rightward shift in the scaled concentration-response curve for TsTX-Kα, the magnitude of which was reasonably well predicted by a model in which there is a competitive interaction between the two peptide toxins. We conclude that TsTX-Kα and α-DTX block the Kv1.2 K^+ channel by binding to the same or closely related sites.

A variety of peptide toxins that are potent blockers of voltage-gated or Ca^{2+}-activated K^+ channels have been isolated from the venoms of invertebrates and lower vertebrates (1, 2). However, studies of the K^+ channel-blocking activity of the peptides in native cells are hampered by the presence of multiple K^+ channel types. The combination of molecular cloning techniques with modern expression methods has made it possible to obtain cells bearing a single population of molecularly defined K^+ channels, thus allowing more detailed studies of drug-channel interactions than are possible in native cells (3–5). Fibroblast cells stably transfected with Kv1.2 K^+ channel cDNA constitute such a system. In a previous study we used this cell line to investigate the interactions of the peptide toxins α-DTX, CTX, and mast cell-degranulating peptide with the Kv1.2 K^+ channel (6).

Recently, several novel K^+ channel toxins were purified from the venom of the scorpion *Tityus serrulatus* (7, 8). One of these toxins, TsTX-Kα, is a 37-amino acid polypeptide that shares substantial sequence homology with CTX (8).[1] Using radioligand binding and measurements of ^{86}Rb efflux, TsTX-Kα was found to be a potent and selective blocker of delayed rectifier-like voltage-gated K^+ channels in rat brain synaptosomes (9). A-type (inactivating) voltage-gated K^+ channels were not blocked by TsTX-Kα, but the toxin was able to abolish the potent blocking action exerted by α-DTX on these channels. Moreover, TsTX-Kα has also been shown to be a selective blocker of noninactivating K^+ currents in cultured central neurons (10). In the present study we sought to investigate the actions of TsTX-Kα further by using patch-clamp recording techniques in fibroblast cells bearing the Kv1.2 K^+ channel. This channel was highly sensitive to block by TsTX-Kα, and our results indicate that the toxin binds to the same site, or a closely associated site, as does the structurally dissimilar snake toxin α-DTX.

Materials and Methods

Cell culture and electrophysiology. Fibroblast cells stably transfected with Kv1.2 cDNA were used in these studies (CL1023 cell line).

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[1] R. S. Rogowski, J. H. Collins, B. K. Krueger, and M. P. Blaustein, manuscript in preparation.

ABBREVIATIONS: α-DTX, α-dendrotoxin; CTX, charybdotoxin; TsTX-Kα, tityustoxin-Kα; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; 4-AP, 4-aminopyridine; TFA, trifluoroacetic acid.

The construction of the expression plasmid and the transfection method have been described previously (6). The transformed fibroblast cells were cultured in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, NY) supplemented with 5% fetal calf serum and 200 μg/ml geneticin (Sigma Chemical Co., St. Louis, MO), at 37° in a humidified atmosphere containing 10% CO_2. Every 3 or 4 days the culture medium was changed and every 7 days the cells were split with 0.025% trypsin (Sigma). The day after splitting the cells were grown in culture medium without geneticin, and 4–8 days after splitting they were used for electrophysiology. The culture medium was replaced with bathing solution containing (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5.6 D-glucose, and 5 HEPES. The pH was adjusted to 7.4 with NaOH and sucrose was added to adjust the osmolality to 330–335 mOsm. Recordings were performed on the stage of an inverted phase-contrast microscope at room temperature (21°). Patch electrodes (2–4 MΩ for whole-cell recordings and 5–10 MΩ for excised patch and cell-attached recordings) were prepared from filament-containing thin-wall glass capillary tubes (1.5-mm outer diameter; World Precision Instruments, New Haven, CT) with a two-stage vertical pipette puller (model L/M-3P-A; Adams & List Associates, Westbury, NY). The electrodes used for whole-cell and outside-out patch recordings were filled with intracellular solution containing (in mM) 150 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, and 5 HEPES. The pH was adjusted to 7.2 with KOH and sucrose was added to adjust the osmolality to 320–325 mOsm. Patch electrodes used for inside-out and cell-attached patch recordings were filled with bathing solution. The tips of all electrodes were heat-polished. Electrodes were coated with Sigmacote (Sigma) and bath levels were maintained at the lowest level possible to minimize stray capacitance.

Whole-cell recordings were made with an Axoclamp 2A amplifier (Axon Instruments, Burlingame, CA) in discontinuous single-electrode voltage-clamp mode (sampling rates, 15–23 kHz). Excised and cell-attached patch recordings were made with an Axopatch 200A amplifier (Axon).

Voltage command pulses were generated and membrane currents were acquired under the control of a microcomputer by using an Axolab-1 interface with the CLAMPEX program of the pCLAMP software package (Axon). Ensembles of patch currents were generated by applying 10 consecutive 400-msec depolarizing voltage steps at 15-sec intervals. Currents were filtered at 1 kHz with the single-pole low-pass filter of the Axoclamp 2A or the four-pole Bessel filter of the Axopatch 200A. Digitized currents were stored on magnetic media for later analysis.

Drug application. α-DTX and TsTX-Kα stock solutions were made in distilled water. These stock solutions were diluted to the required final concentration immediately before the start of the experiments. 4-AP (Sigma) solutions were prepared freshly each day. Solutions were applied to the cells with a nine-barrel, gravity-fed, perfusion system based on the design of Tang *et al.* (11) [see also the report by Werkman *et al.* (6)]. Only one solution was applied at a time (flow rate, 0.5 ml/min) and the level of the solution in the dish was maintained at a constant level with an aspirator. In the case of inside-out patch recordings, intracellular solution was applied to the cell upon establishment of the high resistance seal. Inside-out patches were then formed by pulling the electrode away from the cell.

In the concentration-response experiments, we examined two toxin concentrations per cell. Five consecutive 1-sec depolarizing steps from −60 to +30 mV were applied at 20-sec intervals. Two to 3 min after the onset of perfusion with the lower toxin concentration, an additional five steps were applied. After a 5-min wash-out period the same protocol was repeated using the higher toxin concentration. The averages of the steady state currents for each set of five pulses were used to calculate the percentage of block (see below). In experiments examining the interaction between drugs, one of the blocking agents was present throughout the entire experiment (except during the wash-out periods).

Data analysis. In the whole-cell voltage-clamp experiments, the CLAMPFIT program of the pCLAMP software package was used to measure the steady state current amplitudes (taken as the leak-subtracted current level at the end of the voltage command pulse). For the experiments with excised and cell-attached patch recordings, the CLAMPFIT program was used to average the current traces; most of the capacitive transient and leak current was eliminated by subtracting currents obtained with hyperpolarizing voltage command steps equal in magnitude to the depolarizing steps. The NFIT program (Island Products, Galveston, TX) was used for curve fitting. Data are expressed as mean ± standard error (unless otherwise indicated).

Percentage block induced by the toxins was calculated according to the formula $(1 - I_{toxin}/I_{control}) \times 100$, where $I_{control}$ is the steady state current amplitude before toxin application and I_{toxin} is the steady state current amplitude obtained after toxin application. Conductance values were calculated according to the relationship $G = I/(V - V_R)$, where I is the steady state current, V is the step potential, and V_R is the K^+ reversal potential (previously determined to be −73 mV in the CL1023 fibroblast cells) (6). The conductance values at each potential were fit to the Boltzman equation

$$G = \frac{G_{max}}{1 + \exp[(V_{½} - V)/A]} \quad (1)$$

where G_{max} is the maximal conductance, $V_{½}$ is the voltage for half-maximal conductance, V is the step potential, and A is a slope factor. Concentration-effect data were fit to the logistic equation for a 1:1 binding reaction

$$B = \frac{100}{1 + K_d/[\text{toxin}]} \quad (2)$$

where [toxin] is the toxin concentration and K_d is the concentration of toxin resulting in 50% block.

Toxins. TsTX-Kα was purified from the venom of the Brazilian scorpion, *T. serrulatus* (7); α-DTX was from the Eastern green mamba snake, *Dendroaspis angusticeps* (12).

Construction of synthetic TsTX-Kα gene. To construct a gene that encodes the 37-amino acid TsTX-Kα molecule (8), two oligonucleotides were prepared. The sequences of these oligonucleotides were as follows (shown 5′ to 3′): oligonucleotide A (sense strand), AGGTTTTCATCAACGCTAAATGCCGCGGTTCTCCGGAATGCC-TGCCGAAATGCAAAGAA; and oligonucleotide B (antisense strand), GCGCTCTAGACTACGGGTAGCATTTGCATTTACCGT-TCATGCATTTACCAGCAGCTTTACCGATAGCTTCTTTGCATT-TCGGCAGGCA. These oligonucleotides were designed such that they contained a 21-base pair overlap at their 3′ ends. To construct the synthetic gene, the two oligonucleotides were annealed by the mixing of equimolar amounts of each, heating to 70° for 5 min, and slow cooling to 37°. At that point all four deoxynucleoside triphosphates were added to a concentration of 0.25 mM and 10 units of modified T7 DNA polymerase (Sequenase; United States Biochemicals, Cleveland, OH) were added. After 30 min at 37°, the annealed extended product (126 base pairs) was purified by agarose gel electrophoresis using the glass bead method of purification (Qiagen, Chatsworth, CA).

The purified product was first ligated into the pFLAG expression vector (Kodak/International Biotechnologies Inc., New Haven, CT) as follows. The vector was digested with *Hind*III, followed by brief treatment with mung bean nuclease to produce blunt-ended DNA. After inactivation of the nuclease, the vector was digested with *Xba*I and the DNA was gel purified as outlined above. The synthetic DNA was also digested with *Xba*I and introduced into the pFLAG vector. Clones that contained inserts were analyzed by DNA sequencing. The pFLAG 1 vector was found to give no measurable expression of the fusion protein. Therefore, oligonucleotide C was designed to allow introduction of the toxin sequence into a second plasmid, pBluescript KS+. The sequence of oligonucleotide C was as follows (5′ to 3′): sense strand, GGGCGTCGACTACAAGGACGACGAT; this contains a *Sal*I restriction site as well as sequences corresponding to nucleotides 164–181 of the pFLAG vector. This oligonucleotide was used to amplify (by polymerase chain reaction) the toxin sequence from the pFLAG vector with

oligonucleotide B. The polymerase chain reaction product was digested with *SalI*, ligated with the *SalI*/*XbaI* fragment from the pBluescript polylinker, cut with *XbaI*, and finally ligated into *XbaI*-digested pBluescript. This product was sequenced on both strands and found to be correct. At that point, the *SalI*/*HindIII* fragment containing the toxin sequences was transferred to the pSR9 expression plasmid (13), which had been digested with *SalI* and *HindIII*. Thus, the final clone, termed pSR9.TsTX-Kα, was designed to produce a fusion protein containing T7 gene 9 sequences, the pFLAG epitope, and an enterokinase cleavage site linked to the toxin sequences. This expression vector therefore allows 1) efficient induction of expression by T7 RNA polymerase, 2) increased fusion protein stability and solubility due to the gene 9 fusion sequences, 3) facile identification of the fusion protein during expression and purification by using the anti-Flag M2 antibody (Kodak/IBI), and 4) efficient cleavage of the toxin from the fusion product with enterokinase, resulting in an intact toxin amino terminus.

Expression and purification of recombinant TsTX-Kα fusion protein. Initial expression studies showed inducible expression of a protein of the correct molecular weight that was clearly identifiable both with Coomassie blue staining and with Western blots using the anti-Flag M2 antibody (data not shown). Large-scale expression and purification of the toxin fusion protein were performed essentially as described (14). Briefly, pSR9.TsTX-Kα was introduced into *Escherichia coli* strain BL21(DE3) and grown in LB medium with 100 μg/ml ampicillin. Fusion protein expression was induced by the addition of isopropyl β-D-thiogalactoside to 0.5 mM when the cultures reached an A_{600} of 0.8–1.0. Cells were harvested 2–3 hr later and the TsTX-Kα fusion protein was purified as described (14).

The fusion protein was cleaved with enterokinase (EK-3, 300 units/mg of fusion protein; Biozyme Laboratories, San Diego). The enterokinase was incubated with fusion protein at 37° for 24 hr and filtered through an Acrodisc 13 (0.2-μm syringe filter; Gelman Sciences, Ann Arbor, MI). The filtered product was applied to an Aquapore RP-300 (Pierce, Rockford, IL) reverse phase column (220 × 4.6 mm) equilibrated with 30 ml of 0.075% TFA, with a flow rate of 1 ml/min. The cleavage components bound to the column were washed for 5 min; a linear gradient from 0.075% TFA in water to 0.075% TFA in acetonitrile over 60 min was then used to elute the toxin. Fractions were collected every 1 min and dried in a SpeedVac (Beckman Instruments, Palo Alto, CA). The retention time of the recombinant TsTX-Kα was identical to that of the native toxin.

Results

Whole-cell voltage-clamp recordings were made from 59 transformed CL1023 fibroblast cells. The mean ± standard deviation of the membrane potential was −30.3 ± 5.0 mV, and the input resistance (as determined by applying 20-mV hyperpolarizing steps under voltage clamp) was 860 ± 208 MΩ.

Recombinant TsTX-Kα block of the K^+ current. Recombinant TsTX-Kα produced a potent, concentration-dependent block of the K^+ current activated by depolarization from a holding potential of −60 mV. As illustrated in Fig. 1A, 0.3 nM TsTX-Kα reduced the current by >50%. From the concentration-response experiments, a K_d value of 0.21 nM was obtained (see below). The TsTX-Kα block was largely reversible at concentrations of <1 nM (~5 min of wash); at higher concentrations, however, relatively longer wash-out periods (~20 min) were required and full recovery was usually not obtained.

TsTX-Kα did not produce a consistent change in the time course of the K^+ current (Fig. 1B). However, during the prolonged recordings required to acquire data on the toxin block, a slight change in the kinetic properties of the current (a modest speeding of the rising phase and a small increase in the degree of inactivation at more depolarized potentials) was often observed. This could not be attributed to an effect of the toxin, because similar changes occurred in prolonged (≥15-min) control recordings. The amount of block obtained for the first pulse 2 min after onset of toxin perfusion was in general not different from that obtained with later pulses, indicating that TsTX-Kα block does not occur through a slow use-dependent mechanism. (Occasionally, the current amplitude declined slightly during the five control depolarizing steps and in these experiments no greater decline was observed in the presence of TsTX-Kα.)

Conductance-voltage relationships before and during toxin perfusion were constructed from experiments like the one depicted in Fig. 1A. The conductance at each potential was normalized to the maximum conductance under control conditions (G_{max}) and the mean normalized conductance values were fit to eq. 1 (Fig. 1C). In experiments with three cells, there was no significant shift in either the $V_{\frac{1}{2}}$ or slope values of the voltage-conductance curves, indicating that TsTX-Kα does not affect the voltage dependence of the current.

Comparison of native and recombinant TsTX-Kα. The blocking potencies of recombinant TsTX-Kα and natural TsTX-Kα (purified from the *T. serrulatus* venom) (7) were compared in experiments with four transformed fibroblast cells. The synthetic and natural toxins produced essentially identical levels of block (at 0.3 nM, 60.9 ± 2.2% and 60.2 ± 2.3%, respectively). Because recombinant TsTX-Kα was more readily available, it was used in the remainder of the experiments.

Evidence that TsTX-Kα acts by binding to an extracellular site. To investigate the site of action of TsTX-Kα, we carried out experiments with excised outside-out and inside-out patches. Because of the high level of expression of the Kv1.2 K^+ channel protein in CL1023 cells, patch currents consisted of multiple channel openings. TsTX-Kα (1 nM) was able to block the patch current only when applied to outside-out patches (Fig. 2), indicating that it acts by binding to an extracellular site on the channel and is not able to cross the membrane. This latter conclusion is supported by experiments in the cell-attached configuration, where the toxin was inactive (Fig. 2, *right*). (In this recording configuration, access of the toxin to the external portion of the channel is occluded by the patch electrode.) In similar experiments, comparable results were obtained with α-DTX, indicating that it also blocks only from the outside.[2] The block produced by 1 nM TsTX-Kα in the outside-out patches was largely although not completely reversible, as was the case in the whole-cell voltage-clamp experiments (see above). In the cell-attached recordings, a more pronounced inactivation of the patch current was observed, compared with that in the outside-out and inside-out recordings (Fig. 2). The reason for this difference is not known but could relate to the absence of intracellular factors in the excised patch recordings or uncertainty as to the true membrane potential in the cell-attached recordings.

Apparent competition between TsTX-Kα and α-DTX. To investigate a possible interaction between TsTX-Kα and α-DTX, we quantified the block produced when the peptides were applied together. α-DTX at a concentration of 2.5 nM blocked approximately 50% of the K^+ current. However, in the presence of 1 nM TsTX-Kα (which by itself reduced the current by about 90%), 2.5 nM α-DTX blocked the residual current by only

[2] T. R. Werkman and M. A. Rogawski, unpublished observations.

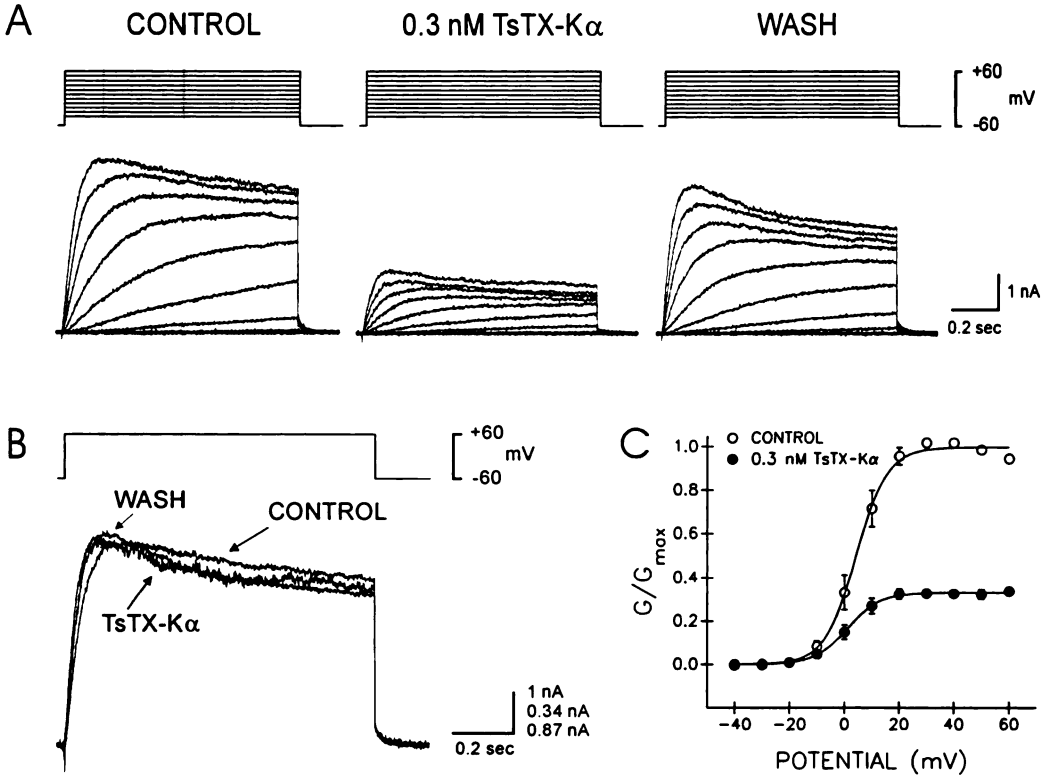

Fig. 1. TsTX-Kα blocks the K+ current in fibroblast cells transfected with Kv1.2 cDNA. A, Currents in a cell before (*left*), during (*middle*), and after (*right*) perfusion with 0.3 nM TsTX-Kα. Currents were evoked with families of 1-sec depolarizing voltage steps from a holding potential of −60 mV to potentials ranging from −40 to +60 mV. B, Scaled current *traces* from the experiment in A, demonstrating that TsTX-Kα does not alter the kinetic properties of the current. C, Conductance-voltage relationships before and during TsTX-Kα perfusion. The *smooth curves* represent the best fits to the mean normalized conductance values, using eq. 1. The $V_{½}$ values for activation before and during TsTX-Kα perfusion were 4.4 ± 2.2 and 1.5 ± 2.3 mV, respectively; the slope factors of the voltage-conductance curves before and after TsTX-Kα perfusion were 5.4 ± 0.2 and 5.8 ± 0.4, respectively. The *points* represent the means ± standard errors of experiments with three cells.

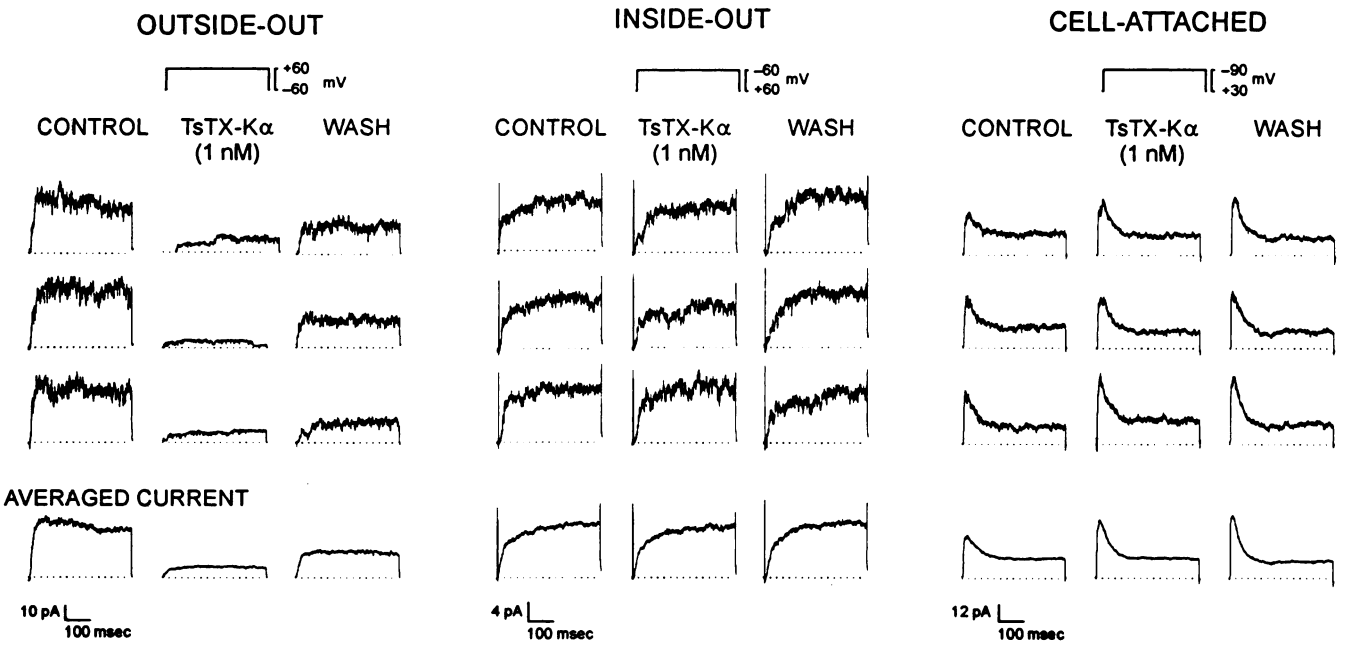

Fig. 2. TsTX-Kα (1 nM) blocks K+ currents in excised outside-out patches (*left*) but does not affect currents in excised inside-out (*middle*) and cell-attached (*right*) patch recordings. Currents were evoked by 400-msec depolarizing voltage steps applied at 15-sec intervals. In the excised patch recordings, the steps were from −60 to +60 mV, whereas in the cell-attached patch recordings the steps were from the pipette potential of +30 to −60 mV. [Assuming a resting membrane potential of −30 mV (see Results), the transmembrane potential would step from −60 to +60 mV.] As in the example shown, a slight increase in peak current often occurred during the course of the cell-attached patch recording, whether or not toxin was present. The ensemble currents were generated by averaging all 10 of the currents acquired per epoch; only three representative *traces* are shown.

~20% (Fig. 3A). In contrast, in the presence of 1 mM 4-AP [which is a potent antagonist of the expressed Kv1.2 channel (15) and at this concentration produced 80–90% block], 2.5 nM α-DTX still evoked a 50% block of the residual current (Fig. 3B). In the reverse experiment, 0.3 mM 4-AP produced the same fractional block (~55%) in the presence or absence of 1 nM TsTX-Kα (data not shown). These results indicate that α-DTX and TsTX-Kα do not act independently to block the K+ current, whereas the blocking actions of 4-AP and the peptides occur at noninteracting sites. To characterize the interaction between the peptide toxins further, we determined the concentration-response relationships for TsTX-Kα in the absence and

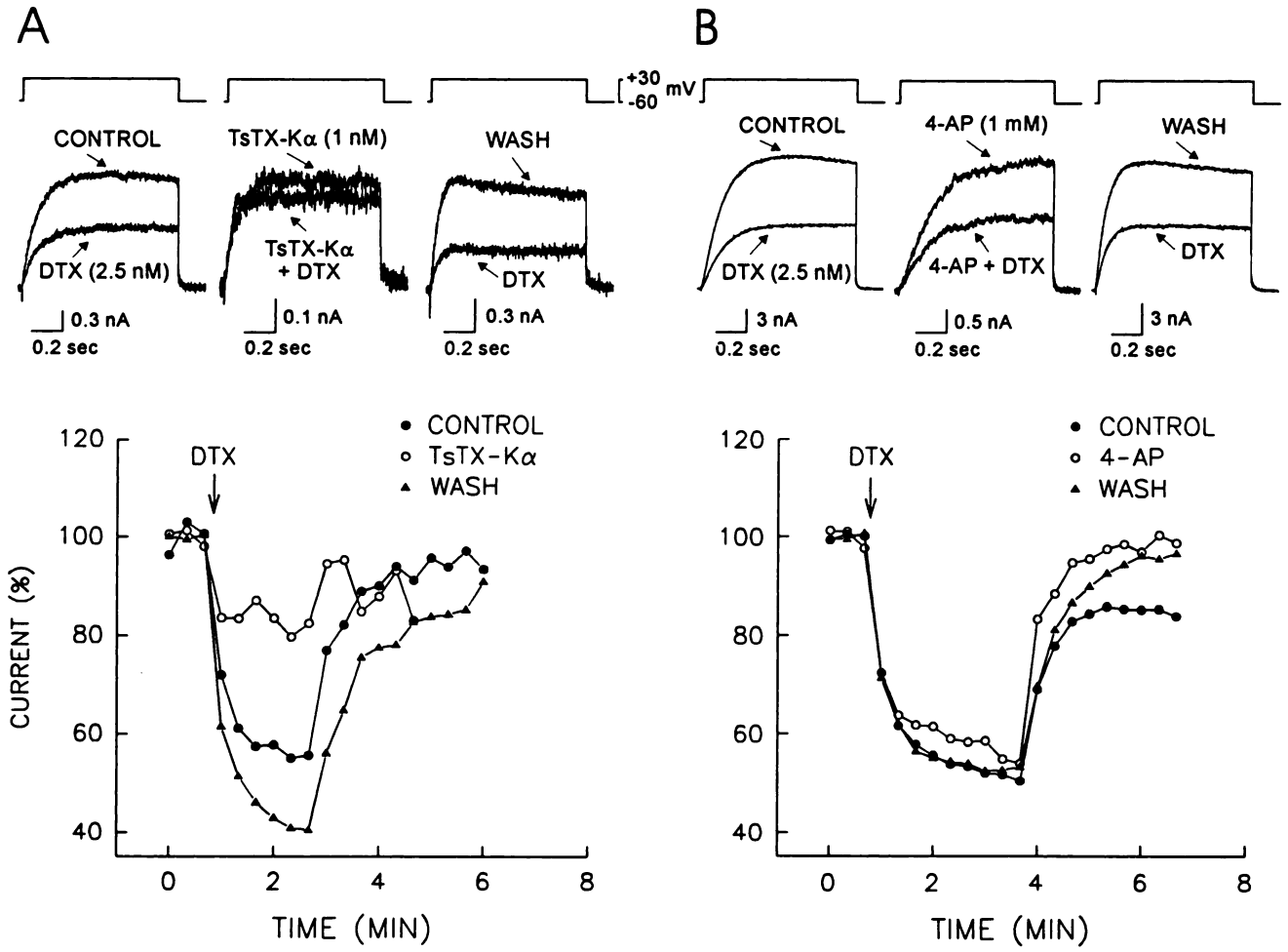

Fig. 3. The presence of 1 nM TsTX-Kα (A) but not 1 mM 4-AP (B) diminishes the ability of 2.5 nM α-DTX (*DTX*) to block the residual K^+ current. Currents were evoked by 1-sec voltage steps from −60 to +30 mV, applied at 20-sec intervals. *Bottom*, the blocking effect of α-DTX is shown before, during, and after application of TsTX-Kα or 4-AP; percentages were determined with respect to the mean amplitudes of three control steps preceding the α-DTX applications. *Top*, representative current *traces* obtained before and during each α-DTX application. The much smaller currents in the presence of TsTX-Kα and 4-AP have been enlarged to roughly match the control currents (note *scale bars*).

presence of 2.5 nM α-DTX (Fig. 4); the apparent TsTX-Kα K_d values were 0.21 and 0.69 nM, respectively. Assuming a competitive interaction between the toxins (i.e., binding to the same or a closely related binding site), the expected shift of the concentration-response curve can be determined from the relationship

$$K^*_{d(\text{TsTX-K}\alpha)} = K_{d(\text{TsTX-K}\alpha)}\left(1 + \frac{[\text{DTX}]}{K_{d(\text{DTX})}}\right) \quad (3)$$

where $K^*_{d(\text{TsTX-K}\alpha)}$ is the apparent dissociation constant in the presence of α-DTX at concentration [DTX], $K_{d(\text{TsTX-K}\alpha)}$ is the dissociation constant in the absence of α-DTX, and $K_{d(\text{DTX})}$ is the dissociation constant of α-DTX. Experiments with 10 cells showed that at 2.5 nM the batch of α-DTX used in this study resulted in an average of block of 55.2 ± 2.6%. Using this value in eq. 2 allowed us to estimate $K_{d(\text{DTX})}$ as ~2 nM. Applying this value in eq. 3 yielded a $K^*_{d(\text{TsTX-K}\alpha)}$ value of 0.47 nM, which is slightly lower than the experimental value. The result from this experiment is consistent with a fully competitive interaction between TsTX-Kα and α-DTX [with the deviation being due to the inaccuracy in our measurement of $K_{d(\text{DTX})}$]; however, we cannot exclude the possibility that there is a more complex interaction between the two toxins.

Discussion

In this study we investigated the block of Kv1.2 voltage-dependent K^+ channels by native and recombinant TsTX-Kα and studied the interaction of TsTX-Kα with other known blockers of the channel. An unexpected finding was the exceptionally high blocking potency of TsTX-Kα. We previously showed that DTX, CTX, and mast cell-degranulating peptide block the Kv1.2 K^+ channel at low nanomolar concentrations (6). However, TsTX-Kα was effective at 1 order of magnitude lower concentrations (K_d, 0.2 nM), so that it is perhaps the most potent K^+ channel-blocking peptide described to date. The natural and recombinant peptides had essentially identical blocking potencies, and they appeared to be biologically indistinguishable. Our results further demonstrate that there is an interaction between TsTX-Kα and the structurally dissimilar snake toxin α-DTX, suggesting that they act at the same or adjacent sites. In contrast, TsTX-Kα did not appear to interact with 4-AP.

Recently, experiments using the *Xenopus* oocyte expression

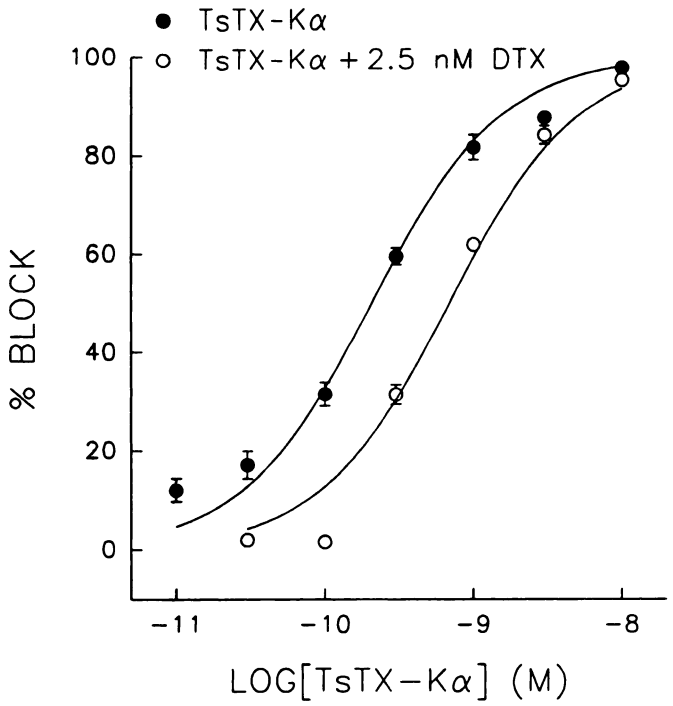

Fig. 4. Concentration-response curves for TsTX-Kα in the absence and presence of 2.5 nM α-DTX (*DTX*). The current was evoked with 1-sec depolarizing steps from −60 to +30 mV (with 20-sec pulse interval) and the percentage block was calculated according to the formula given in Materials and Methods. The percentage of block produced by TsTX-Kα in the presence of α-DTX was determined by assuming the current partly blocked by α-DTX in the absence of TsTX-Kα to be 100%. Each *point* represents the mean ± standard error of experiments with two or three cells. The *smooth curves* represent the best fits to the data according to eq. 2.

system have indicated that the level of expression may be a factor in determining the kinetic and pharmacological properties of the Kv1.2 K^+ channel (16). However, in fibroblast cells transfected with the Kv1.2 gene we did not observe any variation in the properties of the K^+ current, despite the fact that occasional cells displayed current intensities that were >1 order of magnitude larger or smaller than the norm.[3] Thus, the level of expression does not appear to be an important determinant of the fundamental channel properties in our mammalian cell expression system as it is in oocytes.

As with other K^+ channel-blocking toxins such as CTX, TsTX-Kα was active only when applied to the extracellular face of the membrane, indicating that, like CTX, TsTX-Kα has an external binding site. Mutational analysis has revealed that the CTX binding site is in the outer vestibule of the pore of the K^+ channel (4, 5, 17, 18). Other recent studies have indicated that the binding site for α-DTX is in the same region (19, 20), although it may not overlap completely with the CTX site (5). Taken together with our results demonstrating an interaction between TsTX-Kα and α-DTX, it can be concluded that TsTX-Kα may also act at a site in the external channel mouth. Despite its presumed location at this site, our data are consistent with the possibility that the drug blocks closed channels. Thus, we did not observe a change in the K^+ current time course or the slow use-dependent development of block. Nevertheless, we cannot entirely exclude an open channel-blocking mechanism because toxin binding could be fast, compared with channel activation, although this seems unlikely.

It is remarkable that TsTX-Kα and α-DTX, peptides with completely different amino acid sequences, appear to bind to similar domains of the Kv1.2 channel. α-DTX, a toxin present in the venom of the green mamba snake *D. angusticeps*, is composed of 59 amino acids (21). On the other hand, TsTX-Kα, which has structural similarities to two other scorpion toxins, CTX and noxiustoxin, consists of only 37 residues and shows no sequence homology to α-DTX (8), although TsTX-Kα and α-DTX both have three disulfide bridges (8, 12). The competitive interaction between TsTX-Kα and α-DTX suggests that the peptides share common or closely associated blocking sites. Alternatively, however, it is possible that α-DTX and TsTX-Kα do not share the same physical binding site but they sterically overlap one another when bound to their unique sites. Finally, the possibility that the toxins bind to distant sites but interact in an allosteric fashion cannot be excluded. In contrast to TsTX-Kα, 4-AP appears to block by binding to an independent site that does not interact with the α-DTX site. Consistent with our experimental results, work in a variety of systems including the Kv1.2 K^+ channel has suggested that the 4-AP binding site is located deep within the channel (22, 23).[4] The 4-AP binding domain is thus remote from the region of toxin binding, which, as noted above, is likely to be in the channel mouth (4, 5).

TsTX-Kα was previously observed to prevent the blocking effect of α-DTX on fast inactivating (A-type) voltage-dependent K^+ channels in brain synaptosomes, without itself blocking the channels (9). In contrast, the toxin is a potent blocker of noninactivating (delayed rectifier-like) voltage-dependent K^+ channels in synaptosomes and primary cultured rat brain neurons (9, 10). The nature of the unusual interaction between TsTX-Kα and α-DTX on A-type channels in synaptosomes is unknown. One possibility is that TsTX-Kα binds to the same site, or an adjacent site, on the A-channel as does α-DTX but unlike α-DTX does not occlude the mouth of the channel. Obviously this is not the case for the Kv1.2 channel, where TsTX-Kα is an even more potent blocker than α-DTX. Alternatively, TsTX-Kα could influence the binding of α-DTX in a more complex allosteric manner. Whatever the mechanism, it is apparent that there are important differences in the interactions between TsTX-Kα and α-DTX at diverse voltage-dependent K^+ channel types. Nevertheless, the present study supports the view that the structurally dissimilar peptide toxins bind to common or closely related sites on some voltage-dependent K^+ channels.

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