Purification and Characterization of Three Inhibitors of Voltage-Dependent K^+ Channels from *Leiurus quinquestriatus* var. *hebraeus* Venom†

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Received January 11, 1994; Revised Manuscript Received April 1, 1994®

ABSTRACT: Three new toxins from the venom of the scorpion *Leiurus quinquestriatus* var. *hebraeus* have been identified on the basis of their ability to block the *Shaker* K^+ channel. These toxins have been purified using HPLC techniques and characterized as 38 amino acid peptides by mass spectroscopy, amino acid analysis, and sequence determination. Their chemical identity was confirmed by producing fully functional synthetic toxins using recombinant methods. These peptides are potent inhibitors of the *Shaker* K^+ channel (K_d < 1 nM) as well as the mammalian homologues of *Shaker*. They are related to other previously described K^+ channel toxins, but form a new subclass within the larger family of K^+ channel inhibitors derived from scorpion venom. We have named these toxins agitoxin 1, 2, and 3, respectively.

The discovery of selective peptide inhibitors has played an instrumental role in the development of our current understanding of K^+ channels. As structural probes, they have led to the identification of the pore-forming region, and as well they have made it possible to infer gross structural features of the outer vestibule of K^+ channels (MacKinnon & Miller, 1989; MacKinnon et al., 1990; Stampe et al., 1994). In addition, they have allowed biochemical studies of ion channel proteins (Garcia-Calvo et al., 1994), and they have also provided a way to define the physiological role of K^+ channels in tissues of interest (Leonard et al., 1992).

The scorpion toxin K^+ channel inhibitors fall into a family of related peptides that includes charybdotoxin (ChTX),[1] iberiotoxin (IbTX), and others. Several studies have addressed the mechanism by which these toxins inhibit K^+ channels: they bind to a receptor site in the extracellular vestibule and prevent ion conduction by occluding the pore (MacKinnon & Miller, 1988; Giangiacomo et al., 1991; Miller, 1988). A pore-blocking mechanism has now been established both in the case of high-conductance Ca^{2+}-activated K^+ (maxi-K) channels and in the case of voltage-activated K^+ channels (Goldstein & Miller, 1993). This finding is not surprising since the maxi-K and voltage-activated K^+ channels have similar amino acid sequences, especially near their pore-forming region where the toxins are known to bind. The idea that many K^+ channels share a common toxin receptor site has been reinforced by the finding that single amino acid modifications in the toxin binding region can either increase or decrease the affinity of a given toxin for a specific channel (Oliva et al., 1991b). For example, the *Shaker* K^+ channel is highly sensitive to ChTX if the mutation Phe425 → Gly425 is induced into the toxin binding region, but the native channel displays 1000 times lower affinity (Oliva et al., 1991c; Goldstein & Miller, 1993).

It was previously reported that ChTX is a high-affinity inhibitor of the native *Shaker* K^+ channel (MacKinnon et al., 1988), but more recent experiments using synthetic ChTX showed only low-affinity inhibition (Oliva et al., 1991a). Therefore, different toxin(s) in the *Leiurus* venom must account for high-affinity inhibition of the native *Shaker* K^+ channel. In this study, we set out to identify and characterize the toxin(s) directed against *Shaker*.

Three new 38 amino acid peptides are described, and their sequences are confirmed by synthesis using recombinant techniques. These toxins, agitoxins (AgTX), are related to other known K^+ channel toxins. In particular, they are most closely related to kaliotoxin (KTX) (Crest et al., 1992; Romi et al., 1993). Although these new toxins maintain a number of expected features including the position of six cysteine residues and several other residues known to be important for the activity of ChTX, they are unique in the following respects. (1) They begin with the sequence Gly-Val-Pro. (2) They have a high Pro content. (3) They lack residues Trp and Tyr, and as a consequence they are nearly invisible at 280 nm. (4) They appear to be specific for the *Shaker* K^+ channel and many of the mammalian homologues of *Shaker*. We show that these toxins and not ChTX account for the previously described activity against the *Shaker* K^+ channel.

This study expands the list of scorpion toxin K^+ channel inhibitors by identifying a new subclass with potent activity against *Shaker* and its mammalian counterparts. It is interesting to see that the K^+ channel inhibitors are displaying diversity that mirrors the diversity of K^+ channels.

MATERIALS AND METHODS

Materials. *Leiurus quinquestriatus* var. *hebraeus* venom was obtained from Alomone Labs, Jerusalem, Israel. ChTX was purchased from Peninsula Laboratories. Bovine blood "Restriction Protease Factor Xa" and endoproteinase Lys-C were obtained from Boehringer Mannheim. T4 DNA polymerase and T4 DNA ligase were from Bio-Rad (Richmond, CA), Taq DNA polymerase was from Perkin-Elmer Cetus

† This work was supported in part by NIH Grant GM43949 and by funds from the W. M. Keck Foundation to R.M.

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® Abstract published in *Advance ACS Abstracts*, May 15, 1994.

[1] Abbreviations: HPLC, high-performance liquid chromatography; maxi-K, high-conductance Ca^{2+}-activated K^+ channel; ChTX, charybdotoxin; IbTX, iberiotoxin; AgTX, agitoxin; FPLC, fast performance liquid chromatography; r-AgTX, recombinant AgTX; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NxTX, noxiustoxin; MgTX, margatoxin; KTX, kaliotoxin.

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(Norwalk, CT), T7 RNA polymerase was from Promega (Madison, WI), nucleotide triphosphates were from Pharmacia (Piscataway, NJ), and restriction enzymes were from New England Biolabs (Beverly, MA).

Purification of Toxins. Lyophilized venom of the scorpion *L. quinquestriatus* var. *hebraeus* was suspended in 20 mM sodium borate, pH 9.0, at a concentration of 5 mg/mL. After being vortexed, the suspension was clarified by centrifugation at 27000*g* for 15 min. The supernatant was removed, filtered through a Millex-GV filter (0.20-μm pore size, Millipore), and loaded onto a Mono S FPLC column (HR10/10, Pharmacia LKB Biotechnology Inc.) equilibrated with 20 mM sodium borate, pH 9.0. After the absorbance of the eluate had returned to base line, bound material was eluted with a linear gradient of NaCl (0.37 M/h) at a flow rate of 2 mL/min. Fractions were separated and assayed for their ability to inhibit *Shaker* K^+ currents expressed in *Xenopus* oocytes. Fractions displaying inhibitory activity were loaded onto a 300-Å pore size C_{18} reversed-phase HPLC column (0.46 × 25 cm) that had been equilibrated with 10 mM trifluoroacetic acid. The column was eluted with a linear gradient of isopropyl alcohol/acetonitrile (2:1; 0–20%, 30 min) at a flow rate of 0.5 mL/min, and monitoring was done at two different wavelengths, 280 and 215 nm. Fractions were collected, lyophilized, assayed for activity, and further purified using either a C_{18} or a C_8 reversed-phase HPLC column but eluting with a shallower gradient (5–13.5%, 30 min). Individual peaks were collected, lyophilized, and stored at –80 °C until use.

Amino Acid Sequence Determination. Purified toxins were reduced, alkylated with iodoacetic acid, and repurified by reversed-phase chromatography as described previously for ChTX (Gimenez-Gallego et al., 1988). Modified toxin was digested with endoproteinase Lys-C at 37 °C for 24 h in 75 mM sodium phosphate, pH 7.6, at a ratio of 30 μg of toxin per enzyme unit. Fragments from the digestion were purified by reversed-phase chromatography. Approximately 0.2 nmol of either reduced–alkylated toxin or endoproteinase Lys-C fragments was loaded onto a Porton peptide support filter, and Edman degradation was performed using a Porton 2090 microsequencer. Phenylthiohydantoin derivatives were analyzed using an on-line detection system. Typical repetitive yields were 94%

Amino Acid Analysis and Determination of Extinction Coefficient. Samples containing ca. 1 nmol of purified toxins were subjected to acid hydrolysis and derivatized, and the phenylthiocarbamyl amino acid derivatives were identified by reversed-phase chromatography using a Pico-Tag (Waters) column. The absorbance spectrum of purified toxins was digitized in a Beckman DU 7400 UV/VIS spectrophotometer. Protein extinction coefficients were calculated by determining the amino acid composition of an aliquot of toxin and then correlating protein content with the recorded absorbance at 280, 235, and 215 nm, respectively.

Bacterial Strains and Plasmids. Escherichia coli DH5a was used for plasmid propagation, and strain BL21(DE3) was used for expression of the fusion protein (Studier & Moffatt, 1986). Plasmid pCSP105 (Park et al., 1991) was a gift from C. Miller (Brandeis University).

Construction of AgTX Plasmids. An $AgTX_1$ gene was made from two synthetic oligonucleotide duplexes and inserted into the pCSP105 vector as was previously described for ChTX (Park et al., 1991). From this construction, a toxin–T7 gene9 fusion protein was produced, and toxin was cleaved prior to final purification. Two versions of the toxin gene were made, one encoding a factor Xa site (Park et al., 1991) and another encoding an enteropeptidase site (Escobar et al., 1993) in the linker between T7 gene9 and the toxin. Most of the toxin was prepared as described below using the factor Xa construction. An $AgTX_2$ gene was prepared by mutating an $AgTX_1$ gene using PCR mutagenesis. DNA sequences were confirmed by dideoxy sequencing across the toxin-encoding DNA.

Expression and Purification of r-AgTXs. E. coli BL21-(DE3) harboring the AgTX plasmids were cultured and induced with isopropyl 1-β-D-thiogalactopyranoside. Purification of the fusion protein was done essentially as described (Park et al., 1991). Fractions containing the fusion protein were identified by Coomassie staining of SDS–PAGE gels, combined, and dialyzed overnight against 20 mM Tris-HCl, 100 mM NaCl, and 0.5 mM β-mercaptoethanol, pH 8.3. $CaCl_2$ was then added to a final concentration of 3 mM, and 1 μg of factor Xa was added per 200 μg of fusion protein. The recombinant AgTXs were purified from the cleavage mixture using Mono S FPLC and C_{18} reversed-phase HPLC columns as described above for native toxins. The composition of the purified material was verified by Edman degradation, amino acid hydrolysis, and mass spectroscopy.

Potassium Channel Mutagenesis and Expression. The *Shaker* H4 (Kamb et al., 1988), Kv1.3, Kv1.6 (Swanson et al., 1990), and Kv2.1 (Frech et al., 1989) K^+ channels were in a Bluescript vector (Stratagene, Lajolla, CA) to allow amplification, mutagenesis, and RNA transcription for study of channels expressed in *Xenopus* oocytes. The Kv1.1 K^+ channel (Baumann et al., 1988) was present in a pGEM-3Z vector (Promega) modified to contain untranslated sequences of the *Xenopus* β-globin gene to enhance expression in oocytes (Liman et al., 1992). The Kv1.1 channel contained a single amino acid difference (Ser357Ala) when compared to the published sequence of the native channel. RNA transcription using T7 polymerase and *Xenopus* oocyte injections were carried out using previously described protocols (MacKinnon et al., 1988). Mutagenesis of the *Shaker* K^+ channel was carried out as previously described (MacKinnon & Miller, 1989). The *Shaker* H4 K^+ channel was modified by deleting amino acids 6–46 to remove fast inactivation (Hoshi et al., 1990) as previously described (Heginbotham & MacKinnon, 1992). (The amino acid numbering corresponds to the unmodified *Shaker* K^+ channel.)

Electrophysiological Recording. Channels were expressed to a level where 0.5 –2 μA of current was recorded during a depolarizing step from a holding potential of –70 mV to potentials between 0 and –20 mV. The oocyte membrane potential was controlled and current recorded using a commercially available two-microelectrode voltage clamp amplifier (Warner Instruments) operating under computer control. Data were filtered at 1 kHz using an 8-pole Bessel filter (Frequency Devices), sampled every 98 or 196 μs, and stored on a computer disk for subsequent analysis. Control records were taken prior to the addition of toxin, and then toxin was added to the bathing medium during continuous perfusion of the bath. Bovine serum albumin was added at a concentration of 50 μg/mL to all toxin solutions to minimize nonspecific sticking of toxin to the recording chamber and perfusion tubes. In most experiments, toxin was removed from the perfusate after equilibrium blockade was reached and current was recorded episodically to demonstrate full recovery. Due to the slow dissociation rate in the very high-affinity interactions (i.e., $AgTX_2$ and Kv1.3), it was not always possible to achieve full recovery. In all experiments, the recording solution contained (in millimolar) 96 NaCl, 2 KCl, 1 $MgCl_2$, 0.3 $CaCl_2$, and 5 HEPES, pH 7.6 (NaOH). All experiments were carried out at room temperature, 21–23 °C.

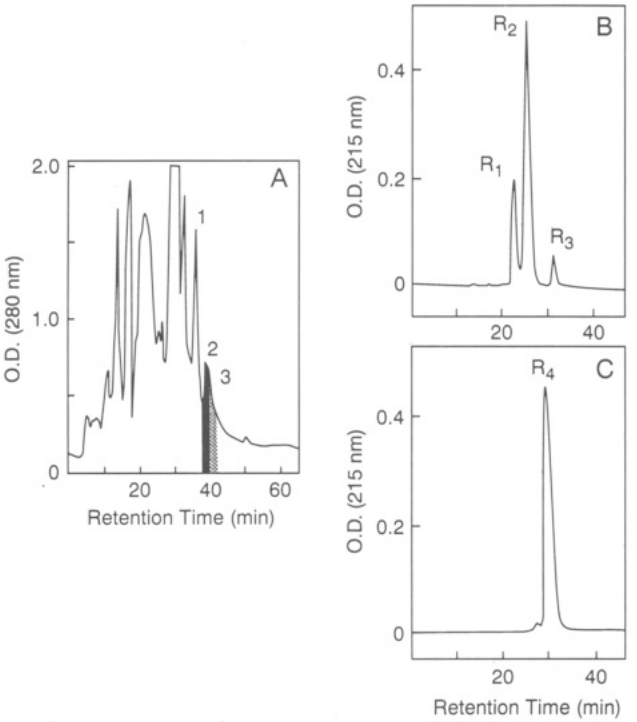

FIGURE 1: Purification of agitoxins. (A) *Ion*-exchange chromatography. Lyophilized venom from *L. quinquestriatus* var. *hebraeus* was extracted as indicated under Experimental Procedures and loaded onto a Mono S HR10/10 column (Pharmacia-LKB) equilibrated with 20 mM sodium borate, pH 9.0, at a flow rate of 2 mL/min. Buffer B was 500 mM NaCl in 20 mM sodium borate, pH 9.0, and the gradient was applied at a rate of 0.37 M/h. Individual fractions were collected, and those displaying inhibitory activity against the *Shaker* K^+ channel are indicated (fractions 2 and 3). (B and C) Reversed-phase HPLC of active fractions from the Mono S column. Individual fractions from the Mono S column (2, panel B; 3, panel C) were loaded onto a C_8 (B) or C_{18} (C) reversed-phase column equilibrated with 10 mM trifluoroacetic acid. The column was eluted with a linear gradient of isopropyl alcohol/acetonitrile (2:1) in 4 mM trifluoroacetic acid as indicated under Experimental Procedures. Absorbance was monitored at 215 nm.

RESULTS

Purification and Characterization of Shaker K^+ Channel Toxins. Venom from the scorpion *Leiurus quinquestriatus* var. *hebraeus* was fractionated beginning with ion-exchange chromatography using a Mono S FPLC column (Figure 1A). The Mono S column gives a better separation of peaks than the SP-Sephadex column used in a previous method (Anderson et al., 1988). Peak 1 is known to contain the bulk of ChTX activity (Gimenez-Gallego et al., 1988). The nearby peaks labeled 2 and 3 were collected and further fractionated by reversed-phase HPLC on either a C_{18} or C_8 column. A second HPLC run derived from peaks 2 and 3 in Figure 1A is shown in Figure 1B,C. At each step, the individual fractions were tested for their ability to inhibit the *Shaker* K^+ channel expressed in *Xenopus* oocytes. Four major peaks of activity were found. Three active components (Figure 1B) originated from peak 2 in Figure 1A, and a fourth was from peak 3 (Figure 1C). These peaks appeared to be chromatographically pure and were therefore subjected to chemical analysis.

Individual peaks were reduced, carboxymethylated, and repurified by reversed-phase HPLC. Each of the four repurified peptides was subjected to Edman degradation, and also they were digested with endoproteinase Lys-C for sequencing of peptide fragments. Two of the peaks, R_1 and R_4, were not blocked. The sequence of R_1 is shown in Figure 2A together with the sequence from the individual internal fragments. Because of previous experience with sequencing other peptides of known composition, we suspected that the last residue was lost from the filter and would have to be determined by other methods. Amino acid analysis and mass spectroscopy (M_r = 4014.8) were consistent with there being an additional Lys residue not apparent in the amino acid sequence (Table 1).

FIGURE 2: Amino acid sequence determination of agitoxins. (A) Complete amino acid sequence of $AgTX_1$. A sample of purified $AgTX_1$ was reacted with iodoacetic acid and the alkylated toxin purified by reversed-phase HPLC. The toxin was digested with endoproteinase Lys-C, and the fragments were purified by reversed-phase HPLC. Amino-terminal Edman degradation was carried out on Porton peptide support filters using a Porton microsequenator. The last residue of $AgTX_1$, Lys, is highlighted to indicate that its identification was done by a different approach. (B) Complete amino acid sequence of $AgTX_2$ and $AgTX_3$. The same sequence strategy as outlined above was employed to determine the amino acid sequence of $AgTX_2$ and $AgTX_3$.

Table 1: Amino Acid Analysis of $AgTX_1$, $AgTX_2$, and $AgTX_3$[a]

	residues/mol of toxin					
	$AgTX_1$		$AgTX_2$		$AgTX_3$	
	obs	int	obs	int	obs	int
aspartic acid, asparagine	3.01	3	3.27	3	3.0	3
glutamic acid, glutamine	1.06	1	1.37	1	1.24	1
serine	1.05	1	2.05	2	1.07	1
glycine	4.95	5	4.10	4	3.86	4
histidine	0.97	1	0.92	1	0.87	1
arginine	1.04	1	1.92	2	1.78	2
threonine	1.92	2	1.90	2	1.92	2
alanine	1.00	1	1.05	1	1.00	1
proline	3.95	4	3.89	4	4.81	5
tyrosine	ND[b]		ND		ND	
valine	1.92	2	2.04	2	1.91	2
methionine	0.90	1	2.20	2	1.97	2
cysteine	5.97	6	5.85	6	5.80	6
isoleucine	1.95	2	2.04	2	1.92	2
leucine	1.0	1	ND		ND	
phenylalanine	1.0	1	1.03	1	0.97	1
lysine	5.61	6	4.92	5	4.70	5

[a] Samples of purified AgTXs were analyzed for their amino acid composition as described under Experimental Procedures. The observed (obs) and integer (int) values of residues per mole of toxin are presented. The absorbance spectrum of purified toxins was digitized and protein extinction coefficients were calculated as described under Experimental Procedures. These values were 26.2, 6.75, and 0.59 mM^{-1} cm^{-1} at 215, 235, and 280 nm, respectively, for $AgTX_1$ and 49.6, 7.48, and 0.56 mM^{-1} cm^{-1} at 215, 235, and 280 nm, respectively, for $AgTX_2$. [b] ND, not detected.

We therefore conclude that the toxin consists of 38 amino acid residues and that the last residue is Lys. This sequence is identical to a peptide previously isolated from *L. quinquestriatus* venom (C. Dunnwiddie, E. Simpson, and J. Jacobs, unpublished observations), which had previously been termed leiurotoxin II (Vazquez et al., 1989). A similar sequencing strategy was used for peak R_4, and the sequence is shown in Figure 2B ($AgTX_2$). In addition, an active peak (not shown in Figure 1) was purified in parallel by a different method [SP Sephadex followed by reversed-phase HPLC as previously described (Anderson et al., 1988) except we monitored at 215 nm where a small peak was visible as a shoulder from the major peak]. The sequence is also shown in Figure 2B ($AgTX_3$). These three toxins all exhibited high-affinity blockade of *Shaker* K^+ channels expressed in oocytes. We

Table 2: Amino Acid Analysis of Recombinant $AgTX_1$ and $AgTX_2$[a]

	residues/mol of toxin			
	$AgTX_1$		$AgTX_2$	
	obs	int	obs	int
aspartic acid, asparagine	2.85	3	2.63	3
glutamic acid, glutamine	0.99	1	0.98	1
serine	0.99	1	1.77	2
glycine	4.77	5	4.06	4
histidine	0.95	1	0.95	1
arginine	1.03	1	2.08	2
threonine	1.86	2	2.06	2
alanine	0.98	1	1.06	1
proline	3.77	4	3.99	4
tyrosine	ND[b]		ND	
valine	1.90	2	1.96	2
methionine	1.05	1	1.73	2
cysteine	5.82	6		
isoleucine	1.85	2	1.73	2
leucine	0.99	1	ND	
phenylalanine	0.99	1	0.98	1
lysine	5.77	6	6.05	6

[a] Samples of purified r-AgTXs were analyzed for their amino acid composition as described under Experimental Procedures. The observed (obs) and integer (int) values of residues per mole of toxin are presented. [b] ND, not detected.

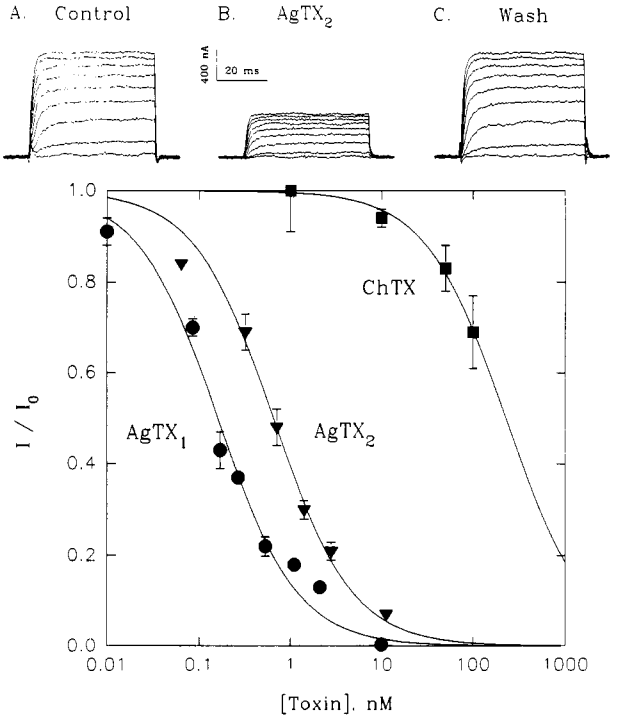

FIGURE 3: Inhibition of *Shaker* K^+ channels by AgTXs. (A, top panel) *Shaker* K^+ channels (containing a modification to remove N-type inactivation) expressed in *Xenopus* oocytes are shown in the absence and presence of 0.65 nM $AgTX_2$. Currents elicited by membrane depolarization are reduced at all voltages when toxin is added to the bath, and inhibition is fully reversed upon removal of toxin. The membrane potential was held at −80 mV and stepped between −40 and +40 mV in 10-mV increments. (B, bottom panel) The fraction of unblocked current, I/I_0, is plotted as a function of toxin concentration for $AgTX_1$ (circles), $AgTX_2$ (triangles), and ChTX (squares). Curves through the data points (least-squares fit) correspond to $I/I_0 = K_i/(K_i + [T])$ where [T] is toxin concentration and K_i is the inhibition constant equal to 0.16, 0.64, and 227 nM for $AgTX_1$, $AgTX_2$, and ChTX, respectively. The unblocked fraction of current was measured by holding the oocyte membrane at −70 or −80 mV and stepping for 50 ms to 0 mV in the absence and presence of the indicated concentration of toxin. Each point is the mean ± SE (or range of mean) of between two and seven measurements.

have named these novel peptides agitoxin (AgTX) 1, 2, and 3, respectively.

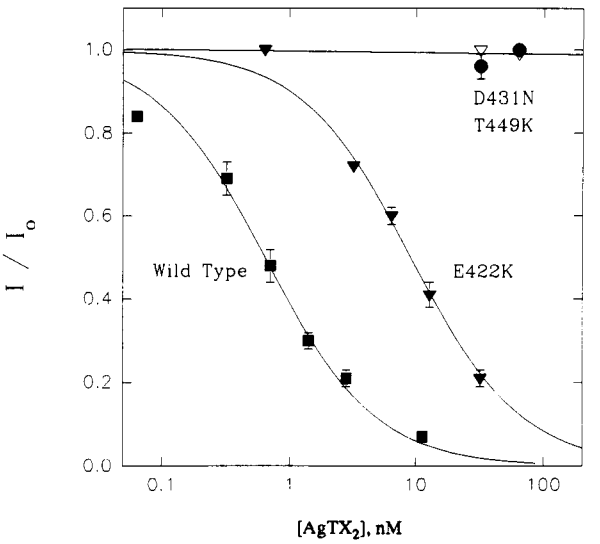

FIGURE 4: Effect of *Shaker* K^+ channel mutations on inhibition by $AgTX_2$. The fraction of unblocked current, I/I_0, is plotted as a function of $AgTX_2$ concentration for the wild-type *Shaker* K^+ channel (squares) and point mutants Glu422Lys (E422K; filled triangles), Asp431Asn (D431N; circles), and Thr449Lys (T449K; empty triangles). The inhibition constant (K_i) for wild-type and E422K channels is 0.64 and 9.1 nM, respectively. Mutants D431N and T449K are insensitive to toxin over the concentration range shown. Recordings were made as described in Figure 3B.

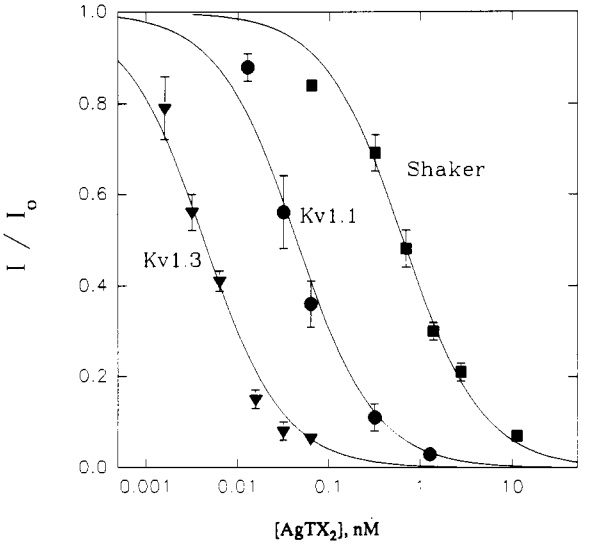

FIGURE 5: Sensitivity of three voltage-dependent K^+ channels to $AgTX_2$. The fraction of unblocked current as a function of toxin concentration was measured in *Shaker* (squares; K_i = 0.64 nM), Kv1.1 (circles; K_i = 0.044 nM), and Kv1.3 (triangles; K_i = 0.0043 nM) K^+ channels. The curves are fit (least-squares) according to the equation in the Figure 3 legend. The fraction of unblocked current was measured during 100-ms steps to 0 mV for Kv1.1 and Kv1.2 channels. Conditions were otherwise identical to those described in the Figure 3 legend.

No sequence was obtained from the N-terminus of peaks R_2 and R_3 (Figure 1B), indicating a blocked N-terminus. Mass spectroscopy, amino acid analysis, and internal sequencing showed that R_2 is identical to a previously described toxin, Lq2 (data not shown; Lucchesi et al., 1989). Due to small quantities of peak R_3, we did not pursue the characterization of this peptide further.

As a final confirmation that the determined amino acid sequence corresponds to the active component of the preparation, two of the three toxins were synthesized in *E. coli* as part of a fusion protein. Peptides were cleaved and purified: chemical characteristics of these recombinant toxins are shown

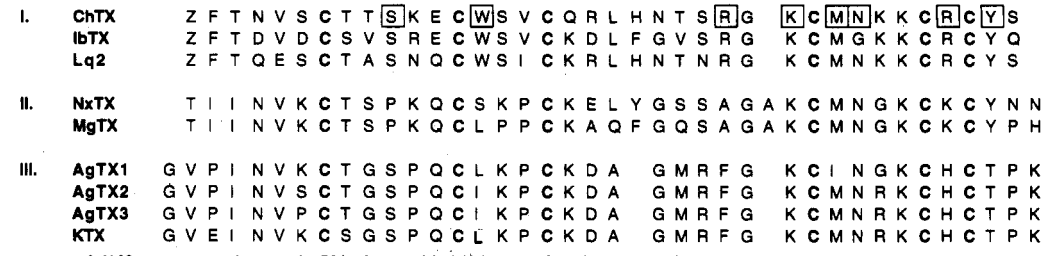

FIGURE 6: Alignment of different scorpion toxin K^+ channel inhibitors. On the basis of amino acid sequence, these toxins fall into three distinct groups. Charybdotoxin (ChTX) (Gimenez-Gallego et al., 1988), iberiotoxin (IbTX) (Galvez et al., 1990), and Lq2 (Lucchesi et al., 1989) fall together into the first related group. Noxiustoxin (NxTX) (Possani et al., 1982) and margatoxin (MgTX) (Garcia-Calvo et al., 1993) form a second group, and agitoxins 1–3 ($AgTX_{1-3}$) together with kaliotoxin (KTX) (Romi et al., 1993) define a third group of closely related toxins. The sequences have been aligned with respect to the six cysteine residues which are in boldface type. The number of amino acids between the third and fourth cysteine residues is conserved within each group but is not conserved between the groups. The residues in ChTX that are critical for interaction with the maxi-K channel are boxed (Stampe et al., 1994).

Table 3: Profile of Sensitivity of Several Voltage-Activated K^+ Channels to $AgTX_1$, $AgTX_2$, and ChTX[a]

	$AgTX_1$	$AgTX_2$	ChTX
Shaker	0.16	0.64	227
Kv1.1	136	0.044	1500
Kv1.3	1.7	0.004	0.19
Kv1.6	149	0.037	22
Kv2.1	>2000	>2000	>2000

[a] The inhibition constant, K_i (nM), is reported for each toxin–channel pair. For *Shaker*, Kv1.1, and Kv1.3 channels, K_i was determined by fitting a curve (equation in Figure 3) to the data points as shown in Figures 3–5. For Kv1.6 and Kv2.1 channels, blockade was measured at several toxin concentrations (three–six measurements), and K_i was determined according to $K_i = (I/I_0)[T]/(1 - I/I_0)$ where I/I_0 is the fraction of unblocked current at toxin concentration [T]. No blockade was observed for the Kv2.1 channels at toxin concentrations of 100 nM. Five percent blockade would have been easily detectable, setting a limit of $K_i > 2000$ nM.

in Table 2. The recombinant toxins displayed the same activity against *Shaker* K^+ channels as their native counterparts.

Biophysical Characterization of the Shaker Channel Toxins. Due to limited quantities of purified native toxin, all biophysical studies were carried out using recombinant toxins. Figure 3A (top panel) shows $AgTX_2$ blockade of *Shaker* K^+ channels (inactivation gate removed) expressed in *Xenopus* oocytes. Addition of 0.65 nM toxin blocks roughly half the K^+ current elicited by voltage steps between –40 and 40 mV, and the effect is fully reversible. Figure 3B (bottom panel) shows the sensitivity of *Shaker* K^+ channels to three toxins: $AgTX_1$, $AgTX_2$, and ChTX. $AgTX_1$ and $AgTX_2$ both inhibit *Shaker* channels with high affinity (K_i = 0.16 and 0.64 nM, respectively) while ChTX inhibits with much lower affinity (K_i = 227 nM). The toxin $AgTX_3$, which differs from $AgTX_2$ by only a single substitution of Ser to Pro at position 7 (Figure 2B), inhibited the *Shaker* K^+ channel with the same affinity as $AgTX_2$. [These experiments were done using native $AgTX_3$ (data not shown).]

Figure 4 shows the effect of several mutations in the toxin binding region of the *Shaker* K^+ channel on blockade by $AgTX_2$. When residue Glu422 is mutated to Lys (E422K), the toxin affinity is reduced by a factor of 14. When Asp431 is mutated to Asn (D431N) or when Thr449 is mutated to Lys (T449K), the channel becomes insensitive to blockade. Furthermore, if the D431N mutant and wild-type subunits are coexpressed in the same oocyte, one finds for both $AgTX_1$ and $AgTX_2$ that only a single wild-type subunit (in the tetrameric channel) is necessary to observe an intact toxin binding site (data not shown). These findings are quantitatively consistent with the results of past studies using a preparation where the active component was thought to be ChTX (MacKinnon & Miller, 1989; MacKinnon et al., 1990; MacKinnon, 1991). The present study indicates that one of these new toxins accounted for the activity against the *Shaker* K^+ channel.

In Figure 5 and Table 3, we investigate the specificity of $AgTX_1$, $AgTX_2$, and ChTX for several different voltage-activated K^+ channels. $AgTX_2$ inhibits the Kv1.3 K^+ channel with an extraordinarily high affinity (K_i = 4 pM), and all of the *Shaker*-like (Kvl) K^+ channels so far tested are sensitive. $AgTX_1$ shows high-affinity blockade of *Shaker* and Kv1.3, but it only weakly inhibits Kv1.1 and Kv1.6. The more distantly related Kv2.1 voltage-activated K^+ channel is completely insensitive to blockade by these toxins. In addition, maxi-K channels from smooth muscle and *slowpoke* Ca-activated K^+ channels are insensitive to $AgTX_1$ and $AgTX_2$ (K_i > 200 nM; data not shown).

DISCUSSION

In this study, we have identified and studied three new members of the family of K^+ channel inhibitors from scorpion venom. The three toxins are very closely related to one another in their amino acid sequences, and they are all potent inhibitors of the *Shaker* K^+ channel. These toxins comigrate with ChTX on reversed-phase HPLC, and because they lack Trp and Tyr residues, they absorb minimally at 280 nm. One of these toxins accounted for the activity against the *Shaker* K^+ channel in previous studies (MacKinnon & Miller, 1989; MacKinnon et al., 1988, 1990; MacKinnon, 1991). This conclusion is further supported by the demonstration that specific mutations in the *Shaker* channel effect blockade quantitatively in the manner previously described (MacKinnon et al., 1990). The chemical identity of the toxins described here is firmly established since full function has been achieved in biosynthetic versions of $AgTX_1$ and $AgTX_2$.

In Figure 6 we have aligned all members of the K^+ channel inhibitors from scorpion venom. We have excluded the distantly related leiurotoxin which is active against apamin-sensitive K^+ channels (Chicchi et al., 1988). These toxins fall into three distinct groups. ChTX, IbTX, and Lq2 are very similar in that they begin with a pyroglutamate residue in the first position and overall they display around 70% amino acid identity (Garcia et al., 1991). Noxiustoxin (NxTX) and margatoxin (MgTX) form a second group (Garcia-Calvo et al., 1993). These two toxins have almost 80% identical residues, but they show only 40% identity when compared to members of the first group. Finally, $AgTX_1$, $AgTX_2$, and $AgTX_3$, together with the previously described KTX, form a third group of toxins that are 80–90% identical, but are only 40–50% identical to members of groups 1 and 2 (Crest et al., 1992).

What do these separate groups, defined on the basis of amino acid sequence, mean in terms of channel specificity? It is clear that there is significant overlap in specificity. For example, members from all three groups block Kv1.3 with

high affinity [Table 3 and Garcia-Calvo et al. (1993)]. Perhaps one trend is emerging: the high-conductance Ca^{2+}-activated K^+ channels are inhibited with high affinity by group 1 but not by group 2 and group 3 toxins (Garcia et al., 1991). However, the closely related KTX is reported to inhibit a smaller conductance Ca^{2+}-activated K^+ channel in *Helix* neurons but not voltage-dependent K^+ channels in the same preparation (Romi et al., 1993). Perhaps a more direct connection between function (channel inhibition) and the toxin groups as defined above will become apparent after more information on channel inhibition is obtained.

So far the solution structure of two of the group 1 toxins, ChTX and IbTX, has been solved, and they are nearly identical (Bontems et al., 1991a,b; Lambert et al., 1990; Johnson & Sugg, 1992). There is a β sheet formed by three antiparallel strands and a single helix: the helix and β sheet are linked by two of the three disulfide bridges. Given the conserved placement of the cysteine residues in the amino acid sequence of all of these toxins, we expect that group 2 and group 3 toxins will have similar three-dimensional structures to the group 1 toxins. However, there are some differences in the amino acid sequences that may well lead to subtle structural differences that could have important functional consequences. For example, group 2 and group 3 toxins have a high proline content, and some of these prolines are located in the helix region.

Eight residues that play a critical role in the interaction of ChTX with a maxi-K channel have been identified in previous studies (Stampe et al., 1994). Even conservative mutations involving these residues (shown in Figure 6) weaken toxin inhibition by at least 8-fold. Of these critical residues, only Lys27, which is thought to interact directly with K^+ in the ion conduction pore, is fully conserved in all toxins (Park & Miller, 1992). Five out of the eight critical residues are not conserved in the group 3 toxins. Met29, when substituted by Ile in ChTX, decreased affinity for the maxi-K channel by 1000-fold, but Ile is present in the corresponding position of $AgTX_1$. Clearly, the specific interactions on the contact surface will be different in these toxins.

The number of scorpion toxin K^+ channel inhibitors continues to grow, and undoubtedly more toxins and perhaps new groups will be identified in the future. These inhibitors are turning out to be as diverse as their K^+ channel targets.

ACKNOWLEDGMENT

We thank C. Dunnwiddie, E. Simpson, and J. Jacobs for sharing their unpublished data with us.

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