Kaliotoxin, a Novel Peptidyl Inhibitor of Neuronal BK-Type Ca\(^{2+}\)-activated K\(^{+}\) Channels Characterized from *Androctonus mauretanicus mauretanicus* Venom*

Marcel Crest‡, Guy Jacquet, and Maurice Gola
From the Laboratoire de Neurobiologie, Centre National de la Recherche Scientifique, 31, Chemin Joseph-Aiguier, 13402 Marseille Cedex 9, France

Halim Zerrouk and Abdellah Benslimane
From the Institut Pasteur du Maroc, Laboratoire de Purification des Proteines, Casablanca, Morocco

Hervé Rochat, Pascal Mansuelle, and Marie-France Martin-Eauclaire
From the Laboratoire d'Ingenierie des Proteines, URA 1455, Centre National de la Recherche Scientifique, Faculté de Médecine, 13326 Marseille Cedex 3, France

A peptidyl inhibitor of the high conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (KCa) has been purified to homogeneity from the venom of the scorpion *Androctonus mauretanicus mauretanicus*. The peptide has been named kaliotoxin (KTX). It is a single 4-kDa polypeptide chain. Its complete amino acid sequence has been determined. KTX displays sequence homology with other scorpion-derived inhibitors of Ca\(^{2+}\)-activated or voltage-gated K\(^{+}\) channels: 44% homology with charybdotoxin (CTX), 52% with noxiustoxin (NTX), and 44% with iberiotoxin (IbTX). Electrophysiological experiments performed in identified nerve cells from the mollusc *Helix pomatia* showed that KTX specifically suppressed the whole cell Ca\(^{2+}\)-activated K\(^{+}\) current. KTX had no detectable effects on voltage-gated K\(^{+}\) currents (delayed rectifier and fast transient A current) or on L-type Ca\(^{2+}\) currents. KTX interacts in a one-to-one way with KCa channels with a \(K_d\) of 20 nM.

Single channel experiments were performed on high conductance KCa channels excised from the above *Helix* neurons and from rabbit coeliac ganglia sympathetic neurons. KTX acted exclusively at the outer face of the channel. KTX applied on excised outside-out KCa channels induced a transient period of fast-flicker block followed by a persistent channel blockade. The KTX-induced block was not voltage-dependent which suggests differences in the blockade of KCa channels by KTX and by CTX. Comparison of KTX and CTX sequences leads to the identification of a short amino acid sequence (26–33) which may be implicated in the toxin-channel interaction. KTX therefore appears to be a useful tool for elucidating the molecular pharmacology of the high conductance Ca\(^{2+}\)-activated K\(^{+}\) channel.

Potassium-selective channels are extraordinarily diverse as regards to their gating mechanism, pharmacology, ionic conductance properties, and regulation. They are involved in a number of physiological processes such as neuronal electrical activity, muscle contraction, secretory processes, cell proliferation, and cell volume regulation (for reviews see Refs. 1–4).

Toxins isolated from the venom of insects, scorpions, snakes, and other species are useful tools for probing the structural differences between these channels and evaluating their physiological contribution to the cell behavior (5, 6).

Within the wide class of Ca\(^{2+}\)-activated K\(^{+}\) channels, two main subtypes have been recognized. These channels differ not only in their unitary conductance and gating mechanisms, but also in their affinity to toxins: charybdotoxin (CTX), a protein isolated from the venom of the scorpion *Leiurus quinquestriatus* (7), is a potent inhibitor of the high conductance Ca\(^{2+}\)-activated BK channel and apamin, which has been isolated from the bee venom, specifically blocks small-sized Ca\(^{2+}\)-activated SK channels (8–10). Owing to its high selectivity for BK-type channels, CTX has been intensively used to investigate the structure and function of BK channels in a variety of membranes. CTX blocks BK channels in muscles (11), epithelia (12), and neurons (13). More recently, it has been reported that CTX also binds to voltage-dependent K\(^{+}\) channels in synaptosomes (14), dorsal root neurons (15), lymphocytes (16), and myotubes from *Drosophila* mutants (17). The high affinity binding site for CTX therefore seems to be shared by various voltage- or Ca\(^{2+}\)-activated K\(^{+}\) channels, which may have structural homologies (18, 19).

In the present paper, we describe the purification, the sequence and the molecular effects on high conductance BK-type neuronal channels of a new K\(^{+}\) channel inhibitor purified from a pool of *Androctonus mauretanicus mauretanicus* venom.

**EXPERIMENTAL PROCEDURES**

**Materials**

The venom of *Androctonus mauretanicus mauretanicus* was obtained by manually stimulating the animals. Two-hundred scorpions

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‡ To whom correspondence should be addressed: Laboratoire de Neurobiologie, CNRS, LNB4, 31, Chemin Joseph-Aiguier, 13402 Marseille Cedex 09, France.

1 The abbreviations used are: CTX, charybdotoxin; KTX, kaliotoxin; IbTX, iberiotoxin; NTX, noxiustoxin; TTX, tetrodotoxin; TEA, tetraethylammonium; EGTA, ethylenebis(oxyethylenenitrilo)tetracetic acid; BSA, bovine serum albumin; KCa, calcium-activated potassium channel; BK, high conductance KCa channel; HPLC, high-performance liquid chromatography.
Electrophysiological Tests

High-performance Liquid Chromatography
A Millipore/Waters Associates system was used, including two model 510 pumps, a U6K injector, an automated gradient controller, a 490 spectrophotometric detector, and a data integrator/recorder module. Reverse-phase HPLC was carried out at 25 °C on a Beckman 4 × 250-mm analytic column packed with 5 μm of Lichrosphere 100 RP-18. Solvent A was 0.1% trifluoroacetic acid (v/v) in water, and solvent B was acetonitrile. Additional details concerning all the chromatographic steps are given in the text and figure legends. A Gilson (France) model 202 fraction collector was used with Corning glass tubes at the detector output. The fractions pooled were lyophilized twice in order to eliminate the solvents. Samples for toxicity assays were lyophilized in the presence of 0.1% BSA.

Polyacrylamide Gel Electrophoresis
Polyacrylamide gel electrophoresis of basic proteins was performed at pH 4.1 on 20% homogeneous Phast-Gel using a Phast-System (Pharmacia, Sweden) as defined in the Pharmacia application file No. 200. Proteins were stained with Coomassie Blue under nonde-naturating conditions using the Pharmacia development technique No. 200 for native polyacrylamide gel electrophoresis.

Reduction and S-Carboxymethylation
The toxin was reduced with dithioerythritol and S-alkylated with iodoacetic acid as described previously (20). The reduced carboxymethylated toxin (RCM-toxin) was desalted by performing dialysis against 50 mM ammonium bicarbonate, pH 8.0.

Amino Acid Analysis
Acid hydrolysates with 6 N HCl were carried out on 1-nmol samples of RCM-toxin for 20 and 70 h at 110 °C and under vacuum using a Pico-Tag work station from Millipore/Waters Associates. The amino acid composition was calculated from the analyses made on a Beckman 6300 amino acid analyzer.

CNBr Cleavage at Methionine
The RCM-toxin was cleaved by CNBr (21). CNBr-cleaved peptides were separated by HPLC on column prepacked with 5 μm of Lichrosphere 100 RP-18. Solvent A was 0.1% trifluoroacetic acid (v/v) in water, and solvent B was acetonitrile. Additional details concerning all the chromatographic steps are given in the text and figure legends. A Gilson (France) model 202 fraction collector was used with Corning glass tubes at the detector output. The fractions pooled were lyophilized twice in order to eliminate the solvents. Samples for toxicity assays were lyophilized in the presence of 0.1% BSA.

Invertebrate Neurons—Two groups of identified neurons were selected on the basis of their various potassium currents (24): (a) U cells (25), these cells generate purely calcium-dependent spikes, and most of the outward current flows through Ca2+-dependent potassium channels (KCa); (b) P cells possess a calcium- and sodium-dependent spike (26). The outward repolarizing current flows in almost equal amounts through Ca2+-dependent (KCa) and voltage-activated (KV) potassium channels. The KCa channel in P cells has similar gating and pharmacological properties to those of the U cell KCa channel. It displays, however, a long-lasting Ca2+-dependent inactivation which is absent in U cells (27). P cells also have A channels.

The three major K+ current components (KCa, KV, and A) were specifically identified by performing either intracellular injections of EGTA (KCa current) or intracellular injections of TEA+ (KV current) or bath applications of 10 mM 4-aminopyridine (A current) (for details, see Ref. 27).

In most experiments, the inward Na+ current was suppressed by either adding 10−4 M tetrodotoxin (TTX) or bathing the cell in a Na+-free saline. Ca2+ currents and Ca2+-dependent currents were suppressed by adding 1 mM Cd2+ to the bath.

After ganglion excision, the connective tissue was softened by a 5–10-min treatment with 1 mg/ml protease (Sigma type XIV). No attempt was made to isolate cell bodies from the axon trunk. Cells were continuously perfused. The normal physiological saline contained: NaCl, 75 mM; KCl, 5 mM; CaCl2, 8 mM; MgCl2, 8 mM; Tris, pH 7.5, 5 mM.

Sympathetic Neurons—These neurons were selected because they have a high density of KCa channels with a high unitary conductance (28). Details of the experimental procedure will be published elsewhere. In brief, coeliac ganglia were excised and placed in a Ringer saline buffered with bicarbonate-CO2, and the connective sheet was softened by a short (15–20 min) protease treatment as used with invertebrate neurons. This treatment was followed by a 2–3-h cleaning of the neuron surface with a gentle stream of Ringer saline. With this procedure, most of the ganglionic connectivity was preserved.

Electrophysiological Recordings—Helix nerve cells were studied under conventional two-electrode voltage clamp and patch clamp conditions, and sympathetic neurons under patch clamp conditions, as described previously (26, 29). Voltage clamp data were sampled at 0.5–1 kHz through a 16-bit A/D converter and stored on floppy disks. The holding potential was set at −50 mV (−90 mV for evoking A currents). Patch clamp data were low-pass filtered at 2–5 kHz using a 6-pole Bessel filter and continuously recorded in a video cassette recorder after 16-bit digitization at 44 kHz with a pulse code modulator (Biologic, France). Stored data were further digitized at 1–5 kHz and transferred to an OMEGA M28 PC computer for further analysis. All voltages given are from the bath potential. For outside-out patches, electrodes were filled with a KCl-rich saline (80 mM for Helix and 150 mM for rabbit), 10−4 to 10−1 M free calcium adjusted by EGTA buffer (30), 1 mM MgCl₂, and 5 mM Tris, pH 7.5.

Lysylized toxins and venoms were dissolved in 1 ml of normal saline supplemented with 1 mg of BSA. These stock solutions were diluted at the desired concentration with salines containing 1 mg/ml BSA.

RESULTS

Purification and Characterization of Kalitoxin

The venom was dialyzed against distilled water in order to eliminate salts and small peptides (PM < 3500). Part of the dialyzed venom was diluted in the standard Helix Ringer saline in the presence of 1 mg/ml BSA. Electrophysiological tests were performed under voltage clamp conditions in the U cell group. In these cells, pulse depolarizations at positive levels successively activated a Ca2+-current and a Ca2+-dependent K+ current (27).

The dialyzed venom almost completely blocked the U cell outward current without having any noticeable effects on the inward Ca2+ current (Fig. 1B) (n = 5). The venom concentration used in these experiments was in the range of those used for Lophotoxus peruvianus (7) and Centruroides noxius (51) that proved to effectively block Ca2+-dependent K+ channels and voltage-dependent K+ channels, respectively.

These data indicated that the venom from A. mauretanicus...
might contain a charybdoxin-like toxin. By pooling several adjacent peaks from the HPLC chromatogram, we first determined that the fraction active on the *Helix* Kca channel was located only in the early peaks (retention time: 40–60 min; Fig. 1A). The following electrophysiological experiments were therefore performed in order to identify the active toxin located within peaks numbered 1–6 in Fig. 1A. These peaks were then individually checked. To prevent protein denaturation and adhesion to the recipient wall, lyophilized fractions were diluted (approximately 0.5 μg/ml) in BSA-containing *Helix* salines, stored, and used in plastic flasks.

The active fraction was found to be located in the peak labeled 3 in the HPLC chromatogram; within 1 min of perfusion (1.5 ml/min) this peak selectively blocked (*n* = 3) the U cell Kca current (Fig. 1C). Adherent peaks (Fig. 1C) had no blocking effects, which ruled out the possibility that the blockade might be attributable to cross-contamination and unspecific effects of the solvents used to separate the venom proteins.

A reverse-phase chromatography of peak 3 resulted in an active major fraction (eluting at 50 min) (Fig. 2A) which gave a single band under polyacrylamide gel electrophoresis in homogeneous Phast-gel 20% (not illustrated). The electrophoretic mobility suggested the existence of a small basic peptide. The purified peptide was analyzed to determine its amino acid content after reduction and carboxymethylation (Table I). Tyrosine and tryptophane were lacking. The molecular mass calculated from the amino acid composition (37 residues) was 4024 Da, which was almost the same as that obtained with the other K+ channel peptide inhibitors characterized so far. The amino acid composition was very different, however, from that of the previous peptides (see below).

The LD50 of the pure peptide (after intracerebroventricular injection into the mouse) was 1.2 μg/kg. The active peptide amounted to about 1% (in optical density units at 215 nm) of the dialyzed venom loaded onto the column at the first reverse-phase chromatographic step. This peptide was called kalitoxin (KTX) due to its ability to block potassium (or sodium)-selective channels.

Automated Edman degradation of 2 nmol of RCM-KTX led to the identification of the 29 amino-terminal residues (*line a* in Fig. 2C). The initial Edman degradation yield was 25%, and the repetitive yield was 90% for the first 21 steps. To further determine the amino acid sequence, 6 nmol of RCM-KTX were cleaved at the methionine level by a BrCN treatment, and the resulting peptide mixture was separated by reverse-phase HPLC (data not shown). Edman degradation
of these peptides made it possible to identify residues 24–28 and 30–37 (lines b and c in Fig. 2C). The complete amino acid sequence agreed with the amino acid composition of KTX shown in Table I. An attempt at sequencing KTX from its carboxyl-terminal end with carboxypeptidase Y was unsuccessful.

**Blockade of Ca²⁺-activated K⁺ Currents in Helix Neurons by Kaliotoxin**

The toxin applied to a U cell had TEA-like effects; the Ca²⁺-dependent U cell spike lengthened, its repolarizing phase was delayed, and the fast phase of the post-spike hyperpolarization was suppressed (Fig. 2B). These effects were partly reversible upon washing. They were similar to those induced by charybdotoxin on *Aplysia* neurons (32) and pyramidal cells (33).

Evidence that the spike lengthening resulted from a specific blockade of the Ca²⁺-dependent component of the repolarizing current is provided by the data in Fig. 3A and B, which were obtained under voltage clamp conditions in two different U cells. Fig. 3A1 shows a set of currents induced by pulse depolarizations at potentials ranging from −10 to +90 mV. The outward current at large positive potentials had two distinct phases: a fast phase, the amplitude of which increased almost linearly with the pulse potential, and a slower phase, which was particularly prominent at +70 and +90 mV. Just after recording the current set in Fig. 3A1, the cell was impaled with a third microelectrode filled with 0.7 M EGTA. Intracellularly injected EGTA (20–50 nA for 2–5 min) specifically suppressed the slow current component, thus unmasking the fast phase of voltage-gated K⁺ current (Fig. 3A2). The EGTA-sensitive current (Fig. 3A3) had similar properties to those of the Ca²⁺-activated K⁺ current originally described (34) in molluscan neurons (35). Its activation rate decreased characteristically at positive potentials approaching the equilibrium potential for calcium ions.

In spite of its reduced unitary conductance (40–60 picosiemens) (29), the main properties of the KCa channel in *Helix* nerve cells were similar to those of the large BK channels (36). This channel was blocked by relatively low concentrations of extracellularly applied TEA⁺ (Kd ≈ 2 mM) and by charybdotoxin (Kd ≈ 50 nM). It was insensitive to amamine (100 nM), to the Mast cell degranulating peptide (200 nM), and to the venom from *Dendroaspis angusticeps* and *Dendroaspis polyolepis*.

The recordings in Fig. 3B were obtained from another U cell subjected to the same voltage program as in the series in Fig. 3A. KTX mimicked the effects of intracellular EGTA. The KTX-sensitive current (Fig. 3B) had similar properties to those of the KCa-sensitive current, i.e., KTX blocked the Ca²⁺-activated K⁺ component of the outward current with apparently no effect on the voltage-gated K⁺ component.

**Blockade of Single KCa Channels by KTX**

*KCa Channels Excised from Helix Neurons—* KTX were excised from *Helix* U cells in either the inside-out or outside-out configuration. Pipettes were filled with a KC1-rich saline (80 mM KC1) supplemented with 0.1 mM CaCl₂, 1 mM MgCl₂, 5 mM Tris. Several criteria were used to identify the excised channels as Ca²⁺-activated K⁺ channels. 1) KCa channels had a voltage-dependent opening probability which increased e-fold (e = 2.72) per ≈15 mV depolarization (29); 2) KCa channels in outside-out patches bathed in the physiological *Helix* saline were reversibly blocked by adding 5 mM TEA⁺ to the bath; 3) with inside-out patches, the bath saline was replaced by the above KC1-rich saline in which the Ca²⁺ concentration was varied from nominally 0 (by adding 1 mM EGTA) to 10⁻⁴ M. KCa channel openings were prevented by the saline containing EGTA. Using these criteria, we found that kaliotoxin (50–100 nM) applied to the cytoplasmic face of the patch had no effect on the opening probability of KCa channels.

The recordings in Fig. 4 were obtained on an outside-out patch fitted with two KCa channels. In controls, the channel had long lasting openings at 0 mV, the duration of which increased at positive patch potentials (+20 mV). KTX (40 nM) decreased the channel opening probability, mainly by reducing the opening time duration (n = 3). Only brief openings persisted in the presence of KTX. The blockade was partly relieved after 15–20-min washing with the physiological saline.

**KCa Channels Excised from Rabbit Sympathetic Neurons—** The KCa channels from sympathetic neurons have a large unitary conductance of 160–200 picosiemens under symmetrical ionic (150 mM KC1) conditions (28). The experiment illustrated in Fig. 5 was performed in an outside-out patch that contained one channel of this type. The patch pipette, filled with a KC1-rich (150 mM) saline, was bathed in the physiological Ringer saline.

With 10⁻⁴ M Ca²⁺ in the pipette and at 0 mV transmembrane potential, the channel had a high probability of opening (P₀ ≈0.80); long-lasting periods of opening were interspersed with...
Kaliotoxin Blocks $\text{Ca}^{2+}$-activated $\text{K}^+$ Channels

The sensitivity of the Helix KCa channel to KTX was determined on the macroscopic KCa current in voltage-clamped U cells. The Fig. 6A shows a set of KCa currents induced by a +20 mV pulse in the presence of increasing concentrations of KTX (from 0 to 50 nM). At the end of the experiment, the cell was injected with EGTA, the EGTA-resistant currents (mainly $\text{Ca}^{2+}$ and KV currents) were subtracted from the currents recorded in the presence of KTX. $A_b$, relative KCa current (from series $A$) versus KTX concentration (○), and (from another cell) versus CTX concentration (△). The continuous and dotted curves were based on the theoretical dose-response curve for a bimolecular reaction. $B_1$, the blockade induced by 50 nM KTX was independent of the voltage pulse (range: from -10 to +70 mV). Insets, currents in a U cell before and after application of 50 nM KTX. $B_2$, the channel blockade was also independent of the holding potential. Data from a U cell in the presence of 50 nM KTX.

Dose Dependence of KCa Current Block by KTX

The data in Fig. 3 indicate that kaliotoxin apparently had no effects on $\text{Ca}^{2+}$ channels or on voltage-gated $\text{K}^+$ channels. This point was then definitely confirmed by the experiments illustrated in Fig. 7.

The KV current set in Fig. 7A was obtained from a Helix P cell subjected to pulse depolarizations from a -50 mV holding potential. A brief application of KTX resulted in full recovery, whereas prolonged KTX applications were poorly reversible. The fraction of KCa current blocked by KTX appeared to be independent of both the potential pulse level (range: -10 to +70 mV in Fig. $6_{B1}$) and on the holding potential (Fig. $6_{B2}$). These results contrast with the voltage-dependent blockade of the channel induced by CTX in Aplysia neurons (92).

Kaliotoxin Does Not Affect $\text{Ca}^{2+}$ Currents or Voltage-dependent $\text{K}^+$ Currents

The data of Fig. 5 indicate that kaliotoxin did not affect the $\text{Ca}^{2+}$ current for a set of Helix neurons (n = 3). A brief application of KTX resulted in full recovery, whereas prolonged KTX applications were poorly reversible.

Dose Dependence of KCa Current Block by KTX
Kaliotoxin Blocks Ca\textsuperscript{2+}-activated K\textsuperscript{+} Channels

Helix nerve cells. To relieve the inactivation of A channels, the holding potential was set to \(-90\) mV conditioning potential applied in order to remove A current and Ca\textsuperscript{2+}-dependent current were suppressed by adding 1 mM Cd\textsuperscript{2+} to the bath saline. KTX had no effect on the Ca\textsuperscript{2+} current set in Fig. 7B which shows two superimposed current traces obtained in response to the attached voltage program: a brief voltage pulse at \(+10\) mV, aimed at inducing an entry of Ca\textsuperscript{2+} ions, specifically suppressed the slow KCa component of the total outward current induced by a test pulse applied 0.8 s later. The current set in Fig. 8B represents the current part suppressed by the brief Ca\textsuperscript{2+} entry. This current has similar properties to those of the U cell KCa current including a characteristic slowing down at large positive potentials. The series in Fig. 8A was obtained from a P cell. In control saline (Fig. 8A1) the outward current showed the two phases typical of KV and KCa current. KTX specifically suppressed the slow KCa component (Fig. 8A4), thus unmasking the fast-activating KV component (Fig. 8A3).

**DISCUSSION**

We have demonstrated here that the venom from *A. mauretanica mauretanica* contains a peptide kaliotoxin that specifically blocks Ca\textsuperscript{2+}-activated potassium channels present in nerve cells from two different animal species, the rabbit and the mollusc *H. pomatia*. In spite of differences in their unitary potential, the cell was bathed in the physiological saline; Ca\textsuperscript{2+} current and Ca\textsuperscript{2+}-dependent current were suppressed by adding 1 mM Cd\textsuperscript{2+} to the bath saline. KTX had no effect on the KV current induced by either moderate or large depolarizations.

The Ca\textsuperscript{2+} current set in Fig. 7B was from a U cell bathed in a saline containing TEA\textsuperscript{+} (20 mM) and 20 mM TTX (aKV) and 20 mM TTX (aKV) and KTX (50 nM) and most of the KV current (KV \approx 10 mM). The fact that KTX had no effect on the Ca\textsuperscript{2+} current ruled out the possibility that the blockade of the KCa current may have resulted merely from that of the Ca\textsuperscript{2+} channels.

The sensitivity of the A current to KTX was assessed in P cells. To relieve the inactivation of A channels, the holding potential was set at \(-90\) mV. The other K\textsuperscript{+} currents were blocked by 20 mM TEA\textsuperscript{+} which had no effect on the A channels. The A current was then identified by assessing its voltage-gated KV currents from a U cell bathed in normal saline containing 10 mM TTX and 20 mM TEA\textsuperscript{+}. C, fast transient A currents from a P cell; A currents were elicited by pulse depolarizations at the levels indicated from a \(-90\) mV conditioning potential applied in order to remove A current inactivation. KTX (right-hand series) had no detectable effects on these currents.

Two KCa Channel Subtypes in Helix Neurons

Are Blocked by KTX

It has been shown that two KCa channels are present in Helix nerve cells (27). In most neurons the macroscopic KCa current in these cells persisted during prolonged depolarizations, i.e. there was no evidence for the existence of inactivating processes (29). In a few nerve cells, exemplified by the P cell group, the macroscopic KCa current displayed a pronounced fast relaxation upon step depolarization. We have demonstrated 1) that both currents have identical gating and pharmacological properties and 2) that the KCa inactivation in P cells is Ca\textsuperscript{2+}-dependent (26).

The KCa component in P cells could therefore be easily and reversibly separated from the KV component by just producing a brief Ca\textsuperscript{2+} entry. This is depicted in the inset in Fig. 8B which shows two superimposed current traces obtained in response to the attached voltage program: a brief voltage pulse at \(+10\) mV, aimed at inducing an entry of Ca\textsuperscript{2+} ions, specifically suppressed the slow KCa component of the total outward current induced by a test pulse applied 0.8 s later. The current set in Fig. 8B represents the current part suppressed by the brief Ca\textsuperscript{2+} entry. This current has similar properties to those of the U cell KCa current including a characteristic slowing down at high positive potentials. The series in Fig. 8A was obtained from a P cell. In control saline (Fig. 8A1) the outward current showed the two phases typical of KV and KCa current. KTX specifically suppressed the slow KCa component (Fig. 8A4), thus unmasking the fast-activating KV component (Fig. 8A3).

**Fig. 7.** KTX had no effect on either Ca\textsuperscript{2+} currents or voltage-dependent K\textsuperscript{+} currents. A, voltage-gated KV currents from a *Helix* P cell. Ca\textsuperscript{2+} currents and Ca\textsuperscript{2+}-activated K\textsuperscript{+} currents were suppressed by adding 1 mM Cd\textsuperscript{2+} to the normal saline. B, Ca\textsuperscript{2+} currents from a U cell bathed in normal saline containing 10 mM TTX and 20 mM TEA\textsuperscript{+}. C, fast transient A currents from a P cell; A currents were elicited by pulse depolarizations at the levels indicated from a \(-90\) mV conditioning potential applied in order to remove A current inactivation. KTX (right-hand series) had no detectable effects on these currents.

**Fig. 8.** KTX blocks the inactivating KCa current in *Helix* P cells. A1, set of outward K\textsuperscript{+} currents in a P cell depolarized at \(-30\) to \(+70\) mV, in 20-mV steps. Two current components are present in P cells: a fast activating voltage-gated KV current and a slowly rising Ca\textsuperscript{2+}-dependent KCa current. Cell bathed in normal saline. A2, same series in the presence of 50 nM KTX. The slow component was almost fully suppressed. A3, KTX-sensitive currents obtained by subtracting series A2 from series A1. B, inactivating KCa current in P cells. Experiment performed in another P cell. Inset: a conditioning pulse (+10 mV, 40 ms) applied 0.8 s before a test pulse suppressed most of the slow Ca\textsuperscript{2+}-dependent K\textsuperscript{+} component. The set of currents (same voltage levels as in series A) is the component suppressed by the conditioning pulse. These currents have similar properties to those of the KTX-sensitive current (slow onset at large depolarizations).
conductance, KCa channels in Helix neurons (unitary conductance under symmetrical ionic conditions: 80 picosiemens) and in rabbit sympathetic neurons (160-200 picosiemens) belong to the wide group of Ca²⁺-dependent voltage-gated potassium channels generally referred to as BK (big) channels. The intermediate-conductance channel in Helix neurons is sometimes referred to as IK channel, although IK channels in GH3 cells (38) have properties that lie between those of BK and SK (small-sized) channels, whereas Helix channels have the same pharmacological and gating properties as the BK-type (29).

KTX at concentrations ranging from 10 to 250 nM did not affect either voltage-gated K⁺ channels (delayed rectifier and fast transient A channels) or L-type Ca²⁺ channels in effecting either voltage-gated K⁺ channels (delayed rectifier and BK-type channels). It has been reported, however, that CTX blocks Ca²⁺-insensitive, V-dependent K⁺ channels in synapses (14, 39), in dorsal root ganglion cells (15), and in lymphocytes (16, 40). Whether KTX has similar effects still remains to be established.

It appears likely that the mechanism underlying the channel blockade induced by KTX may differ to some extent from that involving CTX. The binding of both toxins to the channel is a bimolecular process. KTX, however, induces a transient period of fast flickering in the channel openings, which is not observed with CTX (32). This transient period induced by KTX is followed by an almost complete blockade of the channel. The fast flickering period is reminiscent of that induced by tetraalkylammonium ions. According to Guggino et al. (12), KTX and CTX might be classified as fast and slow channel blockers, respectively. Another difference between the blockade induced by KTX or CTX lies in the sensitivity to voltage. The blockade of Ca²⁺-activated K⁺ channels from skeletal muscles (11, 41) and Aplysia neurons (32) by CTX is voltage-dependent. No evidence for this dependence was observed in the whole-cell KCa current in Helix neurons at voltages ranging from −100 to +90 mV. Additional data at the single channel level are required to clear up these points.

For the first time, a potent inhibitor of the high conductance Ca²⁺-activated K⁺ channel has been purified to homogeneity from a scorpion venom obtained by manually stimulating the animals. This mode of extraction prevents the soluble mate-rials from being contaminated by insoluble mucoproteins and small peptides released by the secretory cells during stimulation with electric shocks. Thus, only one HPLC step was necessary to isolate with a very good yield the toxin from the venom of A. mauretanicus mauretanicus.

In Fig. 9A, the KTX amino acid sequence has been aligned with the sequence of other known peptidyl inhibitors of K⁺ channels, CTX (42), NTX (43), and IbTX (44), in accordance with the CLUSTAL alignment program (45). The highest level of similarity was detected between KTX and NTX (52% of homology) and lesser sequence homology was observed between KTX and CTX or IbTX (44% of homology).

The N-terminal end of KTX was not blocked, contrary to those of CTX and IbTX. A nonamidated C-terminal end was detected in CTX (42) and apparently also in NTX and IbTX. When S-carboxymethylated KTX was digested by carboxypeptidase Y, no amino acid was released: the C-terminal Pro present in KTX sequence, however, is a good explanation for this result. Tyr and Trp are lacking in KTX and only one Phe is present (Table I, and Fig. 2C) which makes necessary to use a wavelength of 215 nm to detect it during HPLC runs. This might also explain that CTX is eluted long after KTX when the two toxins are loaded on a 100 RP-18 column using an acetonitrile gradient.

It should be noted that few amino acid positions (boxed residues in Fig. 9A) are conserved among these four polypeptides: 5 residues in addition to half-cystines. In other respects, KTX and CTX appear pharmacologically very close to each other (this work) and different from NTX (19). Fig. 9B shows the hypothetical structure of KTX based on the disulfide linkages in CTX (46). The shaded units in this figure are the invariant residues (in addition to half-cystines, black units) between KTX and CTX. This figure reveals the presence of a cluster of identical or highly conserved positions from residues 26 to 33. We propose that this sequence plays a prominent role in the specific interaction of KTX and CTX with the BK-type Ca²⁺-activated K⁺ channel. This hypothesis seems to be supported by the recent findings obtained with ¹H NMR spectroscopy of CTX in solution (47). In this work, the authors propose that CTX has an α-helix (residues 10-20) linked by two disulfide bridges, to an antiparallel β-sheet made of two short strands (residues 26-28 and 33-35) connected by a turn (residues 29-32). A similar spatial arrangement has been previously found in α and β scorpion toxins acting on voltage-dependent sodium channels (48-50). Both α- and β-toxins have a loop protruding from the structured region, and in α-toxin this loop is five amino acid longer than in β-toxin (49). This structural difference may be related to the ability for these toxins to bind to two distinct sites. It is tempting to suggest that the loop in α- and β-toxins correspond to the one made by the highly conserved residues 29-32 in CTX and KTX. Consequently, we propose that these 4 residues might play a major role in the pharmacological activity of CTX and KTX. This attractive hypothesis implies that
KTX and CTX have a similar three-dimensional structure. Two proline residues, however, are present in positions 12 and 17 in KTX, i.e. in the region corresponding to the α-helix in CTX (residues 10–20). Although proline residues are generally known to destabilize the α-helix structure, α-helices containing proline residues in their sequence have been described (61). More data concerning the three-dimensional structure of KTX and the KTX-channel interaction are required in order to test our hypothesis.

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