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Maurotoxin, a four disulfide bridge toxin from *Scorpio maurus* venom: purification, structure and action on potassium channels

R. Kharrat[a,*], P. Mansuelle[b], F. Sampieri[b], M. Crest[c], R. Oughideni[b], J. Van Rietschoten[b], M.F. Martin-Eauclaire[b], H. Rochat[b], M. El Ayeb[a]

[a]*Laboratoire des Venins et Toxines, Institut Pasteur de Tunis, 13 Place Pasteur, B.P. 74, 1002 Tunis-Bélvédère, Tunisia*
[b]*UMR 6560, Université de la Méditerranée CNRS, Laboratoire de Biochimie, Ingénierie des Protéines, IFR Jean Roche, Faculté de Médecine Nord, Bd. P. Dramard, 13916 Marseille Cedex 20, France*
[c]*Laboratoire de Neurobiologie, CNRS-UPR 9024, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France*

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Abstract A new toxin acting on K^+ channels, maurotoxin (MTX), has been purified to homogeneity from the venom of the chactoid scorpion *Scorpio maurus*. MTX is a basic single chain 34 amino acid residue polypeptide, amidated at its C terminal, and crosslinked by four disulfide bridges. It shows 29–68% sequence identity with other K^+ channel toxins, and presents an original disulfide pattern, the last two half-cystine residues (31–34) being connected. Although the first three disulfide bonds have not been defined experimentally, modelling based on the structure of charybdotoxin favored two combinations out of six, one of which has two bridges (3–24 and 9–29) in common with the general motif of scorpion toxins. The last bridge would connect residues 13 and 19. MTX inhibits the binding to rat brain synaptosomal membranes of both [^{125}I]apamin, a SK_{Ca} channel blocker (IC_{50} 5 nM), and [^{125}I]kaliotoxin, a Kv channel blocker (IC_{50} 30 pM). MTX blocks the Kv1.1, Kv1.2 and Kv1.3 currents expressed in *Xenopus* oocytes with IC_{50} of 45, 0.8 and 180 nM, respectively. MTX represents a member of a new class of short toxins with 4 disulfide bridges, active on voltage-dependent K^+ channel and also competing with apamin for binding to its receptor.

Key words: Scorpion toxin; Potassium channel

1. Introduction

Venoms from scorpions, snakes, anemones and bees contain numerous polypeptide toxins, binding with high affinity to different specific ion channel membrane proteins. These toxins have proved to be important tools for studying ion channel specificity and functioning [1–3]. In scorpion venoms, two main kinds of toxins have been characterized [3]: those active on voltage dependent sodium channels, responsible for the high toxicity of the venoms, and those active on potassium channels, either voltage dependent or calcium activated. The sodium channel toxins are made of 60–70 residues, crosslinked by four disulfide bridges. The potassium toxins are smaller, less than 38 residues and only three disulfide bridges. However, all scorpion toxins have in common a structural core made of an α-helix linked by three covalent bridges to an antiparallel two-stranded β-sheet [4].

We report in the present paper the purification, from the venom of the chactoid *Scorpio maurus*, of a new toxin named maurotoxin (MTX) with new physicochemical and pharmacological properties. MTX is a small toxin (34 residues) cross-linked by four disulfide bridges. MTX blocks the Kv1.1, Kv1.2 and Kv1.3 channels and competes with both apamin and kaliotoxin for binding to rat brain synaptosomes. These properties are unique for a scorpion toxin.

2. Materials and methods

The venom obtained by electrical stimulation of animals collected in the area of Beni Kheddach in Tunisia was extracted with water (50 mg/ml). The suspension was clarified by centrifugation at 11 000×*g* for 15 min. The supernatant was loaded on a Sephadex G-50 column (1.7×150 cm) equilibrated with 0.1 M ammonium acetate buffer, pH 8.2. Column effluent was monitored at 280 nm. Fractions were tested for toxic activity by intracerebroventricular (i.c.v.) injection to male C57BL/6 mice weighing 20 g. The fourth fraction was purified by HPLC on a C18 column (4.6×250 mm, 5 μm particle size), eluted with a linear 5–25% gradient of 0.1% TFA/acetonitrile in 0.1% TFA/water in 25 min at a flow rate of 1 ml/min. Monitoring was done at 215 nm. The fraction containing the major toxic activity on mice was treated on a C8 column (4.6×250 mm, 5 μm) under the same conditions but with a shallower gradient (1–20% in 40 min). The toxic peak was collected, lyophilized and stored at −80°C until use. The LD_{50} values are calculated as described previously [5].

Electrophoresis of the toxin in nondenaturing conditions was performed essentially according to Reisfeld et al. [6]. The dialyzed samples (10 μg) were loaded on a 15% (w/v) polyacrylamide gel and run, using 0.35 M alanine buffer pH 4.5 on a small slab gel unit (Hoefer Scientific instruments, model SE 200s from San Francisco, CA, USA). Molecular weights of proteins were determined by SDS-PAGE analysis under non-reducing conditions as previously described [7] with a stacking gel of 3% (w/v) (pH 6.8) and a running gel of 15% (w/v) polyacrylamide (pH 8.8). Both types of gel were fixed and stained with Coomassie blue and dried under vacuum.

Amino acid analyses were performed on a Beckman 6300 apparatus after hydrolysis of the samples in 6 N HCl, 1% phenol, at 110°C for 24 h in evacuated tubes using a Picotag work station (Waters).

Sequence determination was performed on native protein, and *S*-pyridylethylated derivative (5 nmol) obtained by reduction of the disulfide bridges with dithioerythritol (60-fold molar excess over disulfide bridges in 250 mM Tris-HCl, 4 mM EDTA, 6 M guanidine, pH 8.5, under nitrogen at 40°C for 20 h in the dark, followed by *S*-alkylation with 2 μl of 4-vinylpyridine for 20 min at room temperature. Desalting of *S*-alkylated protein was done by HPLC on a microbore column. Also, the alkylated protein (1 nmol) was digested with trypsin (5% w/w) for 20 h in 12 μl 125 mM NH_4HCO_3, pH 8.0 at 37°C. The peptides were purified on a 140 HPLC system (Applied Biosystem) equipped with a C18 PTH-column (2.1×220 mm, 5 μm). After an isocratic step of 3 min, the elution gradient was 2–88% of solvent B (30% water, 0.1% TFA in acetonitrile) in solvent A (0.1% TFA in water) for 60 min, 200 μl/min. Automatic Edman degradation was performed on an Applied Biosystem 476 A sequencer.

In order to identify PTH-cystine derivatives that appear in the course of sequencing of native MTX, an assay was performed on 1 nmol of DL-cystine loaded on the sequencer filter and subjected to Edman degradation in exactly the same way as a peptide sample. By HPLC, in the standard conditions for PTH identification, the

*Corresponding author.

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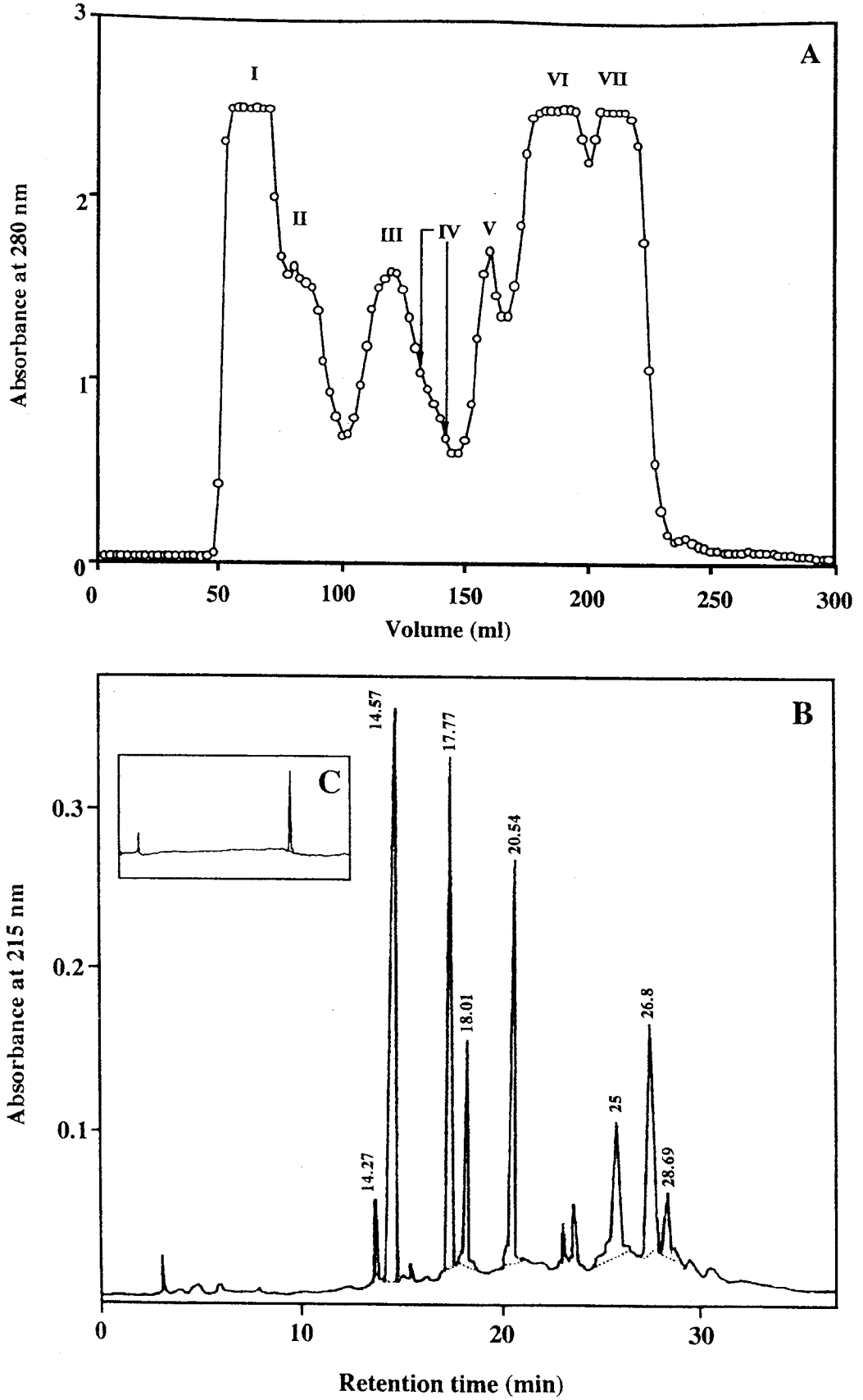

Fig. 1. Purification of MTX form scorpion *Scorpio maurus* venom. A: Chromatography on Sephadex G-50. Fractions I–VII were collected. B: Chromatography of fraction IV on C18 RP-HPLC. C: Chromatography of peak eluting at 14.57 min (1B) on C8-RP-HPLC (see Section 2).

a V S C T G S K D C Y A P C R K Q T G C P N A K C I N K S C K C Y G C *

b C Y G C *

c V S - T G S K D - Y A P - R K Q T G C P N A K C I N K S C K - Y G -

Fig. 2. Amino acid sequence of MTX. 'a' corresponds to Edman degradation of reduced and *S*-alkylated MTX. Cysteine residues were identified as PTH-*S*-pyridylethylcysteine. 'b' corresponds to the degradation of the C-terminal peptide obtained by trypsic hydrolysis of *S*-alkylated MTX. 'c' corresponds to degradation of the native protein: – means no PTH for this step, C corresponds to PTH-cystine (see Section 2). '*' means that MTX is C-terminal amidated.

PTH-cystine derivatives gave two peaks, one coeluting with PTH-Tyr and the second eluting 0.66 min later. The main derivative was quantified as 77 pmol of tyrosine, whereas the peak surface of the second derivative was five times smaller. When sequencing the native protein, these two peaks were both carefully looked for and quantified at each step.

Differently disulfide-paired models of MTX were built with the INSIGHT II, HOMOLOGY, and DISCOVER programs of Biosym Technologies, starting from the 3-dimensional structure of charybdotoxin [8]. All models were energy-minimized until the RMS derivative fell under 0.01 kcal/mol.

Electrospray mass spectrometry of native protein and C-terminal peptide was performed on a VG-Bio-Q (Bio-Tech) in the positive mode [9]. The peptide and protein were dissolved at 5 μM concentration in water/methanol (50:50 v/v) containing 1% acetic acid.

Mono ^{125}I-labeled apamin and [^{125}I]kaliotoxin ([^{125}I]KTX) were prepared and used for competition assays with MTX on rat brain synaptosomal fraction (P2) as described [10,11].

The voltage-dependent Kv1.1, Kv1.2 and Kv1.3 channels were expressed in *Xenopus* oocytes by injection of 0.2 ng of cRNA/oocyte. cDNAs were cloned from rat brain and were a generous gift of S. Alper (Beth Israel Hospital, Harvard Medical School, Boston, MA, USA). The plasmids were transcribed using the Ambion Megascript kit (Ambion, Texas, USA). The cRNAs were stored frozen at 1 mg/ml in water. Defolliculated oocytes were stored in a ND95 medium supplemented with gentamicin 0.1 U/ml (Boehringer, Germany). The currents were recorded 2 days after injection and oocytes were bathed in a calcium free ND96 medium. Macroscopic Kv currents were recorded with the two electrode voltage clamp method using intracellular electrodes filled with KCl (3 M) and a GeneClamp 500 amplifier (Axon Instruments, California, USA). The holding potential was set at −80 mV, leak and capacitive currents were subtracted during analysis.

3. Results

3.1. Purification and chemical characterization of MTX

Water extract (100 OD units at 280 nm) of the venom of *Scorpio maurus* eluted in seven fractions from the Sephadex G-50 column (Fig. 1A). Toxic activity tested by i.c.v. injection to mice was found in the fourth fraction containing 3 OD units. HPLC on a C18 column of this fraction resulted in several components (Fig. 1B). The major active peak (18%) eluting at 14.57 min was shown to be homogeneous by rechromatography on a C8 column (Fig. 1C). It was named maurotoxin (MTX) and represented about 0.6% of the venom water extract. Purity of the MTX was established by gel electrophoresis at pH 4.5 (one band, the most basic protein of the venom), SDS-PAGE (one fraction with a M_r of 3.2–3.8 kDa), mass spectrometry giving a single component with a M_r of 3612.26 ± 0.17.

The amino acid sequence determined by Edman degradation on 400 pmol of *S*-alkylated MTX and on 700 pmol of native MTX is shown in Fig. 2. Hydrolysis of 1 nmol of *S*-alkylated MTX by trypsin followed by C18 HPLC and sequence analysis of the obtained fractions confirmed the overall sequence of MTX and particularly its C-terminal end (Fig. 2). Amino acid composition analyses of native and alkylated MTX are in agreement with the established amino acid sequence (data not shown).

The calculated molecular mass of MTX was 3612.20 for MTX-amide and 3613.18 for MTX with a free carboxylic acid. The experimental M_r of MTX was 3612.26 ± 0.17 (see above). Moreover, the C-terminal peptide, obtained by trypsin proteolysis of *S*-pyridylethylated MTX, had a M_r of 653.6 as determined by mass spectrometry analysis, which is compatible with a CYGC-NH_2 sequence (calculated M_r = 653.83). Thus, it was concluded that MTX is C-terminal amidated.

When sequencing the native protein, no PTH derivative was observed at steps 3, 9 and 13 and peaks corresponding to cystine derivatives (see Section 2) appeared at steps 19, 24 and 29. It was not possible to draw any conclusions for steps

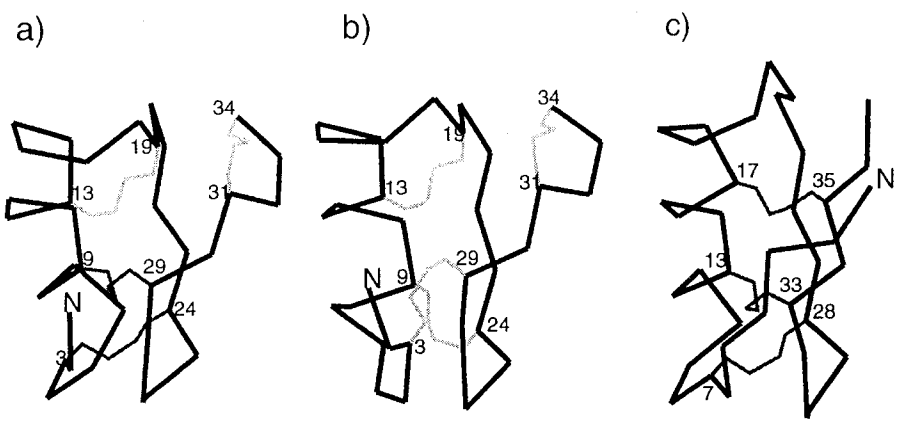

Fig. 3. MTX modeling based on the NMR structure of charybdotoxin. Out of the six possible disulfide pairings deduced from native MTX sequencing data, two models 'a' and 'b' were selected, representing the most likely structures (see Section 3). 'c' is the 3-dimensional structure of charybdotoxin [8].

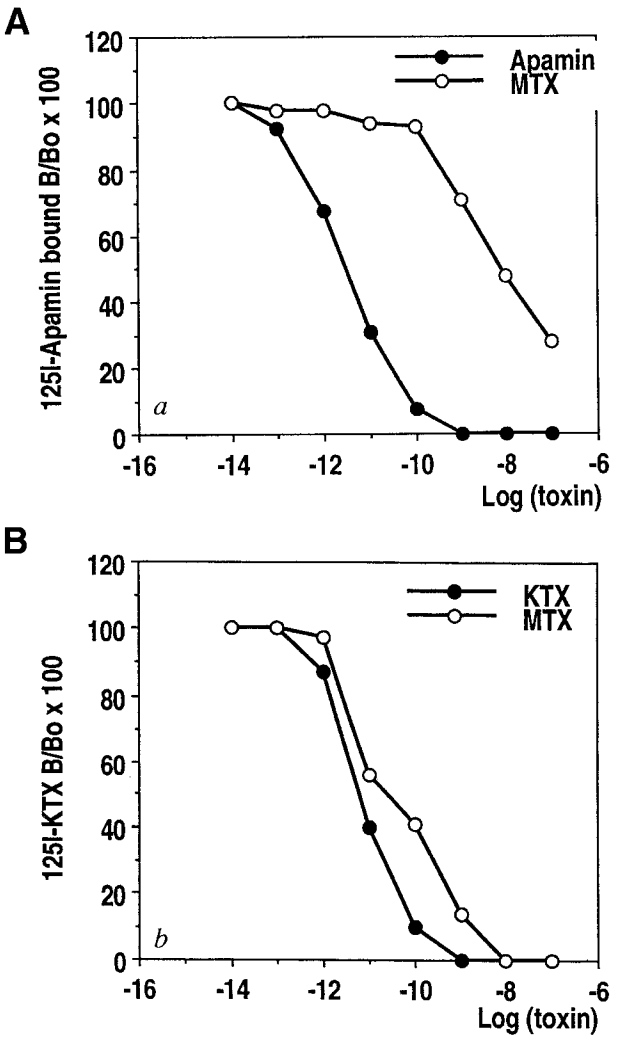

Fig. 4. Competitive binding experiments on rat brain synaptosomes. A: A 10 pM solution of [^{125}I]apamin was incubated, in a final volume of 500 μl, with rat brain synaptosomes (160 μg of protein) and various concentrations of either apamin or MTX. Incubation at 0°C, for 1 h in a 25 mM Tris-HCl 10 mM KCl pH 7.2 buffer was stopped by centrifugation (11 000×*g*, 5 min) and the pellets were washed three times in the same cold buffer. B: A 40 pM solution of [^{125}I]KTX (1–37) was incubated in a final volume of 200 μl with synaptosomes (30 μg of protein) and various concentrations of either KTX or MTX. Incubation at 25°C, for 1 h in a 25 mM Tris-HCl 50 mM NaCl 0.1% BSA pH 7.4 buffer was stopped by centrifugation (11 000×*g*, 5 min) and the pellets washed twice with 1 ml of cold washing buffer (20 mM Tris-HCl 150 mM NaCl 0.1% BSA pH 7.4). The radioactivity bound to the membranes was counted. Bo is the binding of [^{125}I]toxin in the absence of competitor, B is the binding in the presence of one of the native toxins. Non-specific binding was less than 10% for [^{125}I]apamin and less than 25% for [^{125}I]KTX (1–37). The values are the means of triplicate (A) or duplicate (B) experiments. The standard error of the mean of the data was around 5%.

31 and 34, because of the low signal/noise ratio. From these results, we could assume that the first three half-cystines at positions 3, 9, 13 were paired with the half-cystines 19, 24 and 29. Therefore, the fourth bridge was predicted to be between half-cystines 31 and 34. From these data, there remained only six different possible pairings for the three first disulfide bridges. Models corresponding to these pairings were built taking the charybdotoxin 3-dimensional structure as template [8]. Only two of these models had conserved their α-helix and the commonly conserved pattern found in scorpion toxins. They also gave the best score in Ramachandran plot after energy minimization. The two models correspond to disulfide pairings 3–24, 9–29, 13–19, 31–34, and 3–29, 9–24, 13–19, 31–34 (Fig. 3a,b).

3.2. Biological activity

MTX was toxic with a LD_{50} of 100 ng/20 g mouse as determined by i.c.v. injection. MTX competed fully with [^{125}I]apamin and [^{125}I]KTX for their binding to rat brain synaptosomal membranes with IC_{50} of 5 nM and 30 pM respectively (Fig. 4). In the same conditions, the IC_{50} for native apamin and KTX were respectively 5 and 10 pM. These results suggest that MTX recognizes both apamin and kaliotoxin receptor sites with high affinity. MTX blocked the Kv1.1, Kv1.2 and Kv1.3 currents with various affinities. The most potent effect concerned Kv1.2 with an IC_{50} of 0.8 ± 0.2 nM ($n = 6$). Fig. 5 describes the blockade of Kv1.2 currents by incremental MTX concentrations (0.2, 1 and 5 nM). The blockade was fully reversible in 40 min. Similar experiments performed with Kv1.1 and Kv1.3 currents gave IC_{50} of 45 ± 6 nM ($n = 4$) and 180 nM ($n = 2$), respectively (not illustrated).

4. Discussion

MTX is a new basic single chain polypeptide, composed of 34 amino acid residues, crosslinked by four disulfide bridges. It has been purified to homogeneity from *Scorpio maurus* venom. MTX is amidated at its C-terminal end. MTX is a newly characterized scorpion toxin with 8 half-cystine residues acting on K^+ channels. Electrophysiological experiments on the MTX induced blockade of Kv1.1, KV1.2 and Kv1.3 currents revealed pharmacological properties different from KTX. Kaliotoxin has been described as a potent blocker of Kv1.3 (IC_{50} of 0.4 nM) and Kv1.1 (IC_{50} of 46 nM), whereas it has a low affinity ($IC_{50} > 1$ mM) to Kv1.2 [12,13]. The MTX induced blockade mimicked the kaliotoxin effects on Kv1.1, whereas it blocked Kv1.3 with a IC_{50} (180 nM) 500 times higher than kaliotoxin. Interestingly, MTX appears to be a potent blocker of the Kv1.2 current (IC_{50} of 0.8 nM), apparently more active than other scorpion toxins active on Kv1.2 [12]. On the basis of these properties, MTX is considered as the first member of a new group of K^+ channel toxins. It presents (Fig. 6) 38–68% sequence identity with toxins acting on voltage dependent K^+ channels, and 29% identity with toxins acting on apamin sensitive potassium channels, leiurotoxin I and P05 [14,15]. Like these toxins, MTX lacks four or five amino acid residues at its N-terminal part, and is able to compete with [^{125}I]apamin on rat brain synaptosomes. MTX presents two basic residues at positions 20–21 (Fig. 6) that could participate in the apamin-like activity and play the same function as the doublet in positions 13–14 of P05 [16,17]. If sequence alignments are in accordance with conformations, the positions 20–21 would be located at the end of an α-helix, as in apamin [18]. MTX also has the two basic residues at positions 13 and 20, considered important for LTX in its apamin-like activity [14]. In contrast, for P01, a scorpion toxin with a low affinity for the apamin site (in the μM range), positions 13, 20 and 21 are occupied by Glu, Ser and Thr residues, respectively [19].

Six of 8 cysteine residues of MTX (3, 9, 13, 24, 29 and 31)

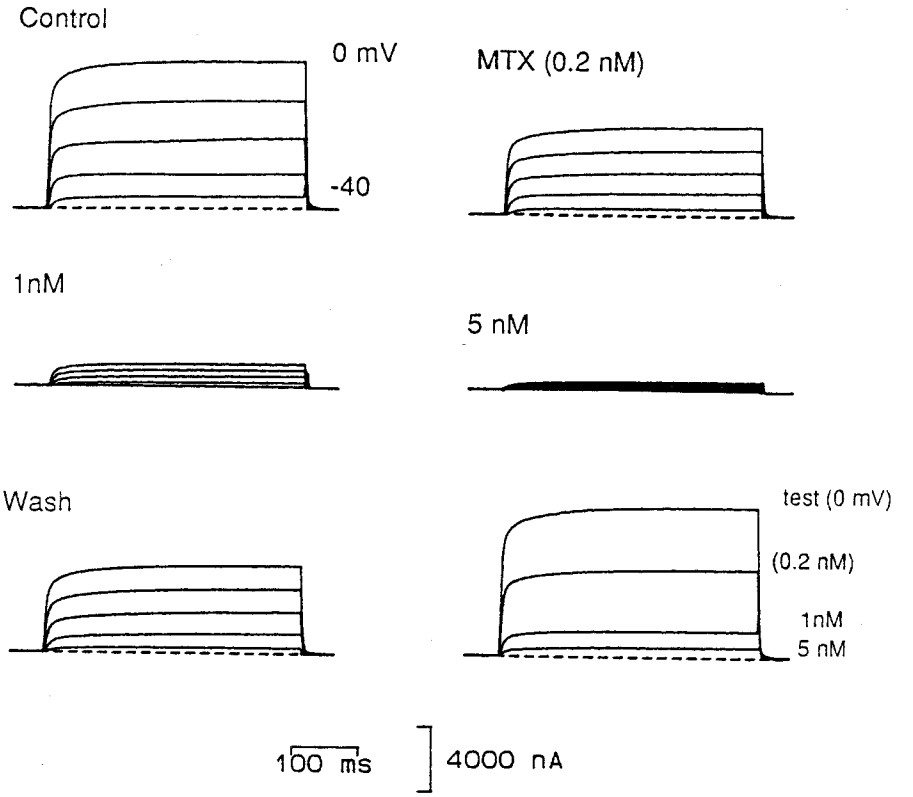

Fig. 5. Blockade of the Kv1.2 current in *Xenopus* oocytes induced by MTX. Control panel: Kv1.2 currents produced in voltage clamped oocytes by a series of voltage steps from −40 mV up to 0 mV. Application of MTX (0.2, 1, and 5 nM) decreased currents by 31%, 85% and 100%, respectively. The 5 nM induced blockade is partly reversed by a 18 min wash. Dose-response effects of MTX are determined by repeating a test pulse (0 mV) during application of incremental MTX concentrations.

occupy conserved positions of the common sequential motif which was reported to be shared by all scorpion toxins including those crosslinked by four disulfide bridges [4], i.e. [...]C[...]CXXXC[...]GXC[...]CXC[...]. However, with two bridges connecting successive half-cystine residues (Cys-13–19 and Cys-31–34), the covalent structure of MTX has no equivalent in other scorpion neurotoxins. MTX has in common with PI1, a toxin active on *Shaker* B K^+ channels [20], eight half-cystines in homologous positions and also the absence of the conserved glycine residue in position 28 (Fig. 6). In position 29 MTX presents a similar alanine residue as in noxiustoxin and margatoxin. These particularities may play a role in the unexpected disulfide organization. Data based on careful examination of PTH-Cys obtained during native MTX sequencing suggested that the first three half-cystines were bridged to the next three half-cystines: thus the pairing of Cys-31 and -34 was deduced. Six MTX models corresponding to the possible pairings of the three other disulfide bonds between Cys-3, -9, -13 and Cys-19, -24 and -29 were built taking the charybdotoxin as a template. The two best models are shown in Fig. 3a,b. We could not discriminate between these two models on purely geometric or energetic criteria. It should be noticed, however, that model a was the only one showing as many as two homologous disulfides with charybdotoxin and thus fitted the best with the overall scorpion neurotoxin family structure. The disulfide pairings of MTX in model a were Cys-3–24, Cys-9–29, Cys-13–19, and Cys-31–34. In the charybdotoxin molecule, we observed that substitution of Gly-26 (homologous of Gly-22 in MTX) into an Ala residue could occur only at the expense of disturbing the structure, since there is no room for the Ala side-chain methyl group, which otherwise would bump the Cys-17 side-chain. In the model of MTX, however, it seems that Ala-22 could find its place since the Cys-13 (homologous of Cys-17 in charybdotoxin) side-chain is oriented differently, due to the pairing 13–19 instead of 13–31. In fact, the Cys-13 habitual partner (Cys-31) is making a bridge with the near residue Cys-34, which has no equivalent in other scorpion neurotoxins. Moreover, during model minimization, the initial almost straight-shaped α-helix bent progressively with the Pro-12 residue as a hinge; this could afford additional accommodation for the Ala residue, as observed in the 3-dimensional structure determination of noxiustoxin [21]. When a synthetic MTX was constructed and its disulfide pairing determined, the covalent structure deduced was identical to model a [22].

The first bridge brings together the two regions where sequence similarity is the highest within the longest toxins (Fig. 6). This clustered region comprising the homologous amino acid residues in the N-terminal sequence (positions 6–11 of Fig. 6 for KTX and MTX) and in the C-terminal sequence (positions 30–34) is suggested to be involved in the binding of KTX to K^+ channels [23]. It notably contains the lysine residue (position 30 of Fig. 6) which was shown to be an essential element for the activity of charybdotoxin [24]. The competition between MTX and [^{125}I]KTX in binding to rat brain synaptosomal membranes can be associated more precisely with these homologous amino acid residues.

MTX shows maximum sequence identity with PI1 (Fig. 6). However, the disulfide pairing of these two toxins appears to be different, only two of the bridges being identical. This

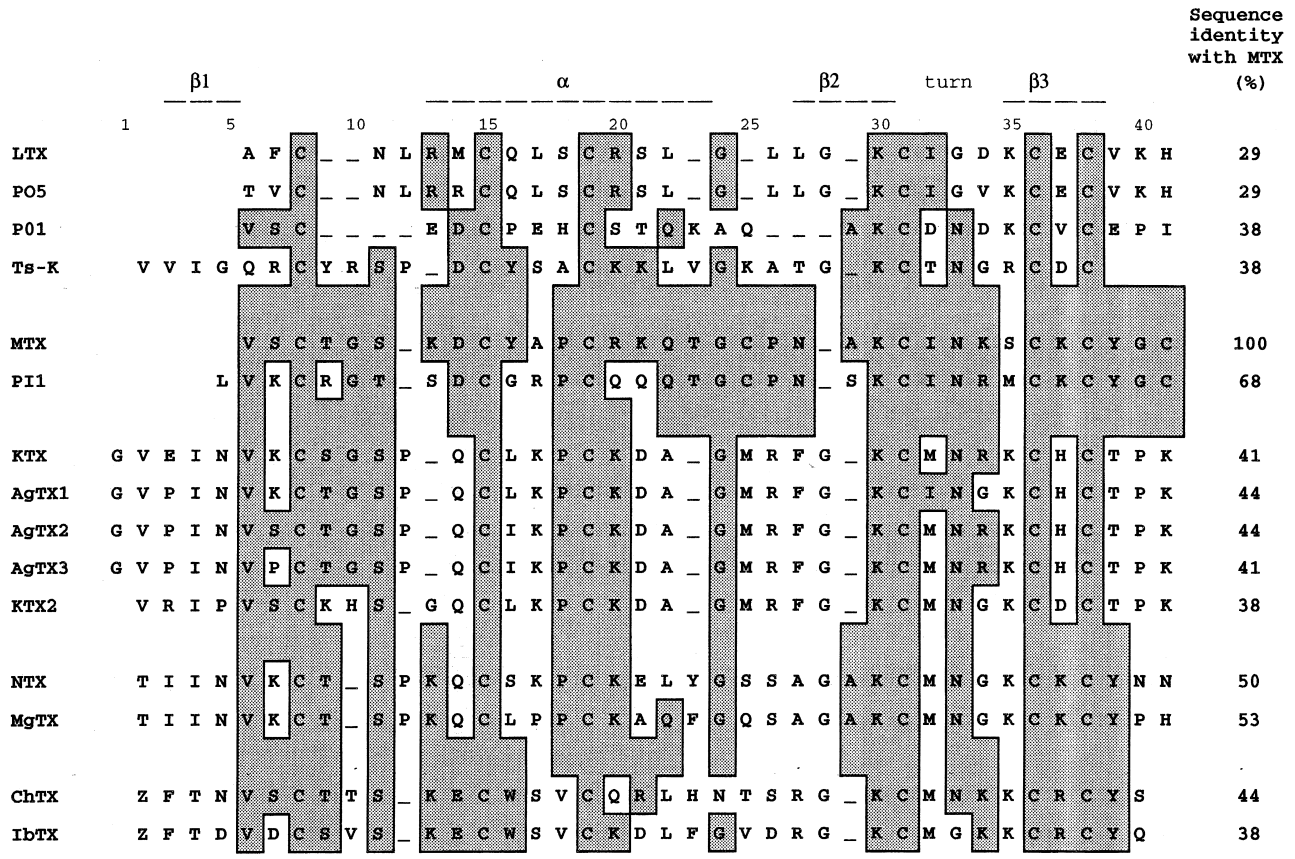

Fig. 6. Sequence comparison of different scorpion toxin K^+ channel inhibitors. On the basis of amino acid sequence, MTX may represent a link between voltage dependent potassium channel toxins and apamin-like toxins, and be an element of a new group. The sequences, taken from [14,15,20,25,26], have been aligned with respect to the six half-cystine residues. The conformational elements presented at the top of the sequences refer to the structure of charybdotoxin [8]. The percentage similarity to MTX is indicated. Boxed residues in all other sequences than MTX are identical or homologous to those in the MTX sequence.

finding may be of interest regarding the scorpion toxin structure-activity relationships.

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