Volume 320, number 3, 189–192 FEBS 12276 April 1993

Characterization of a new leiurotoxin I-like scorpion toxin

PO_5 from *Androctonus mauretanicus mauretanicus*

H. Zerrouk[b], P. Mansuelle[a], A. Benslimane[b], H. Rochat[a] and M.F. Martin-Eauclaire[a]

[a]*Ingénierie des Protéines CNRS, URA 1455, Laboratoire de Biochimie, Faculté de Médecine Nord, Bd. Pierre-Dramard, 13916 Marseille Cédex 20, France* and [b]*Institut Pasteur du Maroc, Laboratoire de Purification des Protéines, 1, Place Charles-Nicole, BP 120 Casablanca, Morocco*

Received 1 February 1993

Three novel peptide inhibitors of the SK_{Ca} channels were purified to homogeneity from the venom of the scorpion *Androctonus mauretanicus mauretanicus* using one step of RP-HPLC and competition assays with [^{125}I]apamin to rat brain synaptosomes PO_1, PO_2 and PO_5 have $K_{0.5}$ of 100, 100 and 0.02 nM, respectively, for the apamin binding site. The sequence of PO_5 was established and compared to that of other scorpion toxins active on K^+ channels: it contains 31 residues and has a free carboxyl end. It shares sequence similarity with apamin and leiurotoxin I.

Scorpion toxin; Structure; Potassium channel; Apamin

1. INTRODUCTION

Scorpion venoms are known to contain toxic polypeptides that specifically alter the gating properties of the voltage-dependent Na^+ channel [1,2]. Efforts to characterize minor venom components have recently led to the discovery of some toxins able to act on K^+ channel structures [3,4]. The wide diversity of K^+ channels has been particularly studied during the past decade and there has been much work on toxins affecting different types of K^+ channels. Some scorpion toxins are useful probes for elucidating the mechanism of action and the pharmacology of the different channels. Six toxin polypeptides active on K^+ channels have been isolated from the venom of the Moroccan scorpion *Androctonus mauretanicus mauretanicus*: one toxin (KTX) is specific for the large conductance Ca^{2+} activated K^+ channels (BK_{Ca}) of invertebrate neurons and rabbit sympathetic neurons [5]. The five other peptides compete for binding to rat brain synaptosomes membranes with radioiodinated apamin ([^{125}I]apamin), a derivative of a toxin from the honey bee venom *Apis mellifera* [6,7], indicating that these toxins interact specifically with the small conductance Ca^{2+}-activated K^+ channel (SK_{Ca}). We report the chemical and biological characterizations of one of these polypeptides. Its sequence contributes to the analysis of toxin structure–activity relationships and the understanding of its mode of action.

Correspondence address: M.F. Martin-Eauclaire, URA 1455 CNRS, Laboratoire de Biochimie, Faculté de Médecine Nord, Bd. Pierre-Dramard, 13326 Marseille Cédex 15, France.

2. MATERIALS AND METHODS

The venom of *Androctonus mauretanicus mauretanicus* was obtained by manually stimulating the animals (every 100 μl of venom yielded 8 24 mg of lyophilised protein). UV grade acetonitrile was from Fisons Scientific (UK), trifluoroacetic acid (TFA) was from Baker (UK) and all the other analytical reagents were from Merck (Germany) Bovine serum albumin (BSA) and apamin were from Sigma Carboxypeptidase P (CPase P) was from Boehringer (Germany). The water used to prepare solvents and buffers was purified with the Milli/Ro/Milli Q system from Millipore. Reverse-phase HPLC was carried out, at 25°C, on a Merck 4 × 250 mm analytical column prepacked with a lichrosphere 5 μm 100 RP-18 as previously described [5]. Additional details concerning all the chromatographic steps are given in the text and figure legends. Samples for biological assays were lyophilized in the presence of 0.1% BSA. Electrophoresis was performed at pH 8.5 on homogeneous high density Phast-gel using a Phast-system (Pharmacia, Sweden) with migration towards the cathode. The peptides (200 pg) were stained with silver using the Pharmacia development technique File 211. The toxin was reduced with dithioerythritol and S-alkylated with iodoacetic acid as described previously [8]. The reduced carboxymethylated toxin (RCM-toxin) was desalted by chromatography on RP-18 HPLC columns. Electrospray mass spectrometry was performed by Neosystem Laboratoire (Strasbourg, France). Automated Edman degradation was performed on a Beckman 890 MM sequencer and the characterization of phenylthiohydantoin derivatives was carried out by C_{18}-HPLC as described [9]. One nmol of PO5 was digested with CPase P (15% by weight) according to the manufacturers' instructions. Toxicity in vivo was tested on male C57Bl/6 mice by intracerebroventricular injections (i.c.v.) as described [7]. Mono ^{125}I-labelled apamin was obtained and used in competition assays with rat brain synaptosomal fraction (P_2) as described [10].

3. RESULTS AND DISCUSSION

The venom was dialyzed against distilled water to eliminate salts and peptides with MW < 1,000. One single step of reverse-phase HPLC chromatography (Fig.

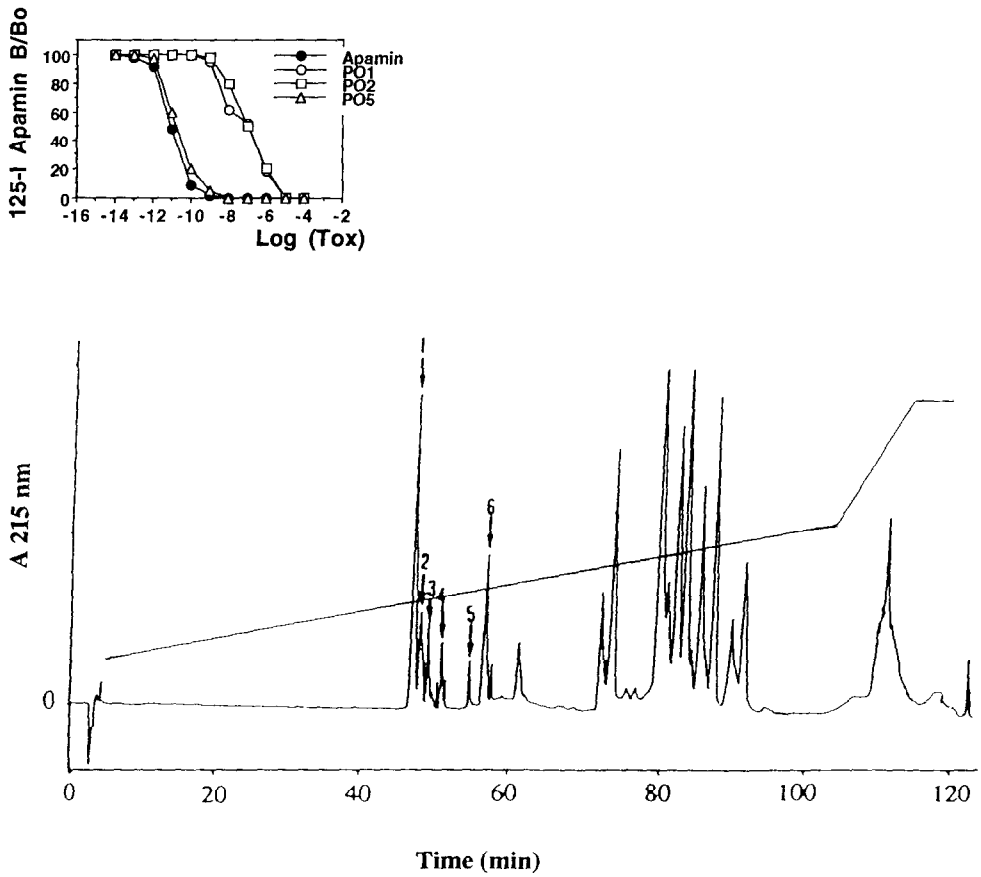

Fig. 1. Purification of the toxins. Reverse-phase HPLC on Lichrospher 100 RP-18 (5 μm) of *Androctonus mauretanicus mauretanicus* dialyzed venom (0.8 mg) Solvent A, 0.1% TFA; solvent B, acetonitrile/0.1% TFA; linear gradient from 5 to 45% B in 100 min; flow rate 1 ml/min; absorbance unit full scale at 215 nm = 1. Arrows indicate the fractions able to compete with [^{125}I]apamin bound to its receptor site on rat brain synaptosomal membranes. Inset. Competitive inhibition of [^{125}I]apamin binding to rat brain synaptic membrane by PO_5. B_0 is the binding of [^{125}I]apamin (10 pM) in the absence of competitor and B is the binding in the presence of the indicated concentrations of native PO_5 (160 μg of P2 membranes in 500 μl of 20 mM Tris, 10 mM KCl medium, pH 7.5) The bound radioactivity was determined in the pellet after centrifugation. Points are the mean of at least two experiments consisting of quadruplate assays

1) led to five fractions inhibiting [^{125}I]apamin binding to rat brain synaptosomes; retention times (RT) were 44.9 min for PO_1, 46.6 min for PO_2, 49.5 min for PO_4, 53.2 min for PO_5 and 55.12 min for PO_6. PO_3 is the previously described KTX [5]. Fractions PO_1, PO_2 and PO_5 were homogeneous as assessed by polyacrylamide gel electrophoresis and RP-HPLC chromatography on a C_{18} column using different elution conditions (not shown). PO_5 was the most toxic fraction to mouse, and the strongest competitor of apamine binding to its site, and was further studied. It amounted to 0.05% by weight of the venom. Its LD_{50}, by intracerebroventricular (i.c.v.) injection to the mouse, was 20 ng or 4.5 pmol for a 20 g animal (as compared to 4 pmol for apamin). The inhibition by PO_5 and also by PO_1 and PO_2 of the binding of the labelled [^{125}I]apamin to its receptor site in rat brain synaptosomes, is shown in Fig. 1 (inset). The derived K_d, calculated from the experimental $K_{0.5}$ (20 pM), was 13 pM, very similar to that of apamin.

The first 30 residues of RCM-PO_5 (0.5 nmol) were identified by automated Edman degradation with an initial degradation yield of 37% and a repetitive yield of 93%. The C-terminal residue was identified as His by CPase P digestion. The molecular mass of PO_5 calculated from the sequence of 31 residues was in perfect accordance with that obtained by electrospray mass spectrometry: 3,415.4 ± 0.6 Da. The level of similarity between PO_5 and leiurotoxin I (LTX I) [11], also known as scyllatoxin [12], a toxin found in the venom of the scorpion *Leiurus quinquestriatus hebraeus*, was very high: 87% sequence identity. LTX I has been found both with a free and with an amidated histidine residue in the C-terminal position, and the two peptides exhibit different chromatographic properties on HPLC [12]. An analog of PO_5 with an amidated C-terminal His was synthesized: sPO_5-NH_2 [13]. The elution of a mixture of the natural and synthetic peptides on C_{18}-RP HPLC gave different retention times (RT of 51.33 min for sPO_5-NH_2 and 53.2 for native PO_5). Moreover, sPO_5-NH_2 was more basic than PO_5 in polyacrylamide gel electropho-

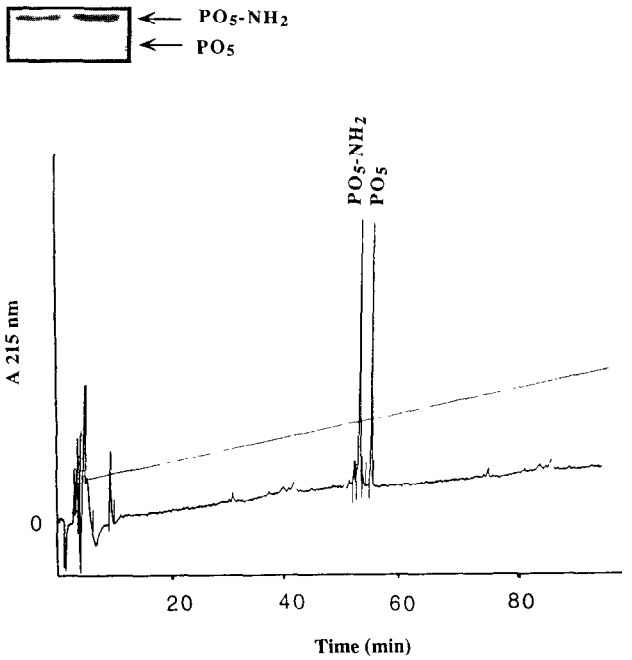

Fig. 2. Co-injection of PO_5 and sPO_5-NH_2 on C18-RP-HPLC (same conditions as above). Inset. Homogeneous gel electrophoresis pH 8.50; migration toward the cathode (up); lane 1 (200 pg); lane 2, mixture of native PO_5 (50 pg) and sPO_5-NH_2 (200 pg)

resis (Fig. 2). Finally, electrospray mass spectrometry (three different experiments) showed that the MW of the two peptides differed by only 1, proving that the C-terminal residue of PO_5 was free (3,414.6 ± 0.7 for sPO_5-NH_2 instead of 3,415.4 ± 0.6 Da for PO_5). None of the four other peptides of the venom able to compete with [^{125}I]apamin for its binding site exhibited the same RT as sPO_5-NH_2, indicating that only the PO_5 with a free C-terminal end was found in the natural secretion.

The PO_5 amino acid sequence was compared with the sequences of apamin and LTX I, other peptide inhibitors of the SK_{Ca} channel (Fig. 3) and other small scorpion toxins active on K^+ channels of different types (the A-type K^+ channel, the delayed rectifier K^+ channel or the BK_{Ca} channel), i.e. noxiustoxin (NTX), iberiotoxin (IbTX), kaliotoxin (KTX) and charybdotoxin (ChTX) [3–5]. The sequence Arg-Arg-Cys-Gln of the N-terminal part of PO_5 was also found in the C-terminal α-helical part of apamin. The corresponding sequence in LTX I is Arg-Met-Cys-Gln. However, polyclonal antibodies raised against apamin [14] were not able to recognize PO_5 in liquid radioimmunoassay. The adjacent Arg residues were reported to be crucial for the pharmacological activity of apamin [6,7,15]. Furthermore, the binding of the synthetic [^{125}I]Tyr LTX I to its receptor was completely lost after modification of Arg-6 and Arg-13 [16]. Arg-6 and Arg-7 of PO_5 might well be also located in a part of the molecule proposed to be an α-helix in LTX I (from Arg-6 to Gly-16) [17]. The substitution of the Arg-7 of PO_5 by Met in LTX I could explain the lower affinity of LTX I for the apamin binding site (100 pM for LTX I instead of 20 pM for PO_5).

Highly conserved amino acid residues (Fig. 3, residues in bold) are found in PO_5 and the other scorpion toxins active on K^+ channels. The position of the three disulfide bridges of PO_5 has been defined [13]. This is in agreement, for PO_5 and LTX I, with a spatial arrangement of the α-helix and the β-sheet similar to those of ChTX and IbTX, although the β-strand I is missing and the loops connecting the different part of the α-helix and the β-strand II vary in length between the toxins [18–20]. ChTX and IbTX are active on different types of K^+ channels [3]: they have two charged surfaces according to refined models of solution conformations obtained by ^{1}H nuclear magnetic resonance: one is located in the helix and one in the C-terminal region. Among the positively charged amino acids of PO_5, the Lys residues in position 20 and 25 are located in the strongly conserved region. From NMR data of LTX I, an antiparallel β-sheet could be located from Leu-18 with a tight turn at Gly-23–Asp-24 position. The sequence Gly-Lys-Cys XXX Lys-Cys with Lys-Cys located in antiparallel β-sheet and XXX as tight turn, is also conserved in ChTX and IbTX (type 1 β-turn at residues Gly-30–Lys-31). This sequence could be important for the conformation of the toxins. The side chains of these two Lys appear on one of the solvent exposed faces of the β-sheet [18–20]. The interaction between ChTX and its receptor site involves local electric fields, made of both negative charges on the H5 region of the channels, as demonstrated by electrophysiological studies after mutation of the K^+ channel coded by the *Shaker* gene of *Drosophila megalogaster* expressed in *Xenopus* oocytes [21], and positive charges of the toxin. Studies with engineered ChTX suggest that the Lys-27 plays a major role by fixing the toxin to the external

Apamin	CNCKAPETALCARRCQQH*
PO5	TVC-NLRRCQLSCRSL-GLL-**G-KC**IGV**KCEC**VKH
LTX	AFC-NLRMCQLSCRSL-GLL-**G-KC**IGD**KCEC**VKH*
KTX	GVEINVK**C**SGSPQ**C**LKP**C**KDA-GMRF**G-KC**MNR**KC**HCTPK#
NTX	TI-INVK**C**TSPK**QC**SKP**C**KELYGSSAG**AKC**MNG**KC**K**C**YNN
IbTX	ZFT-DVD**C**SVSKE**C**WSV**C**KDLFGVDR**G-KC**MGK**KC**R**C**YQ
ChTX$_1$	ZFT-NVS**C**TTSKE**C**WSV**CQ**RLHNTSR**G-KC**MNK**KC**R**C**YS
ChTX$_2$	ZFT-QES**C**TASN**QC**WSI**C**KRLHNTNR**G-KC**MNK**KC**R**C**YS

Fig. 3. Sequences similarities between PO_5 and other toxins active on K^+ channels. Apamin from bee venom *Apis mellifera*; PO_5 (this work); LTX, leiurotoxin I (scyllatoxin) from *Leiurus quinquestriatus hebraeus*, KTX, kaliotoxin from *Androctonus mauretanicus mauretanicus*, NTX, noxiustoxin from *Centruroides noxius*; IbTX, iberiotoxin from *Buthus tamulus*; $ChTX_1$ and $ChTX_2$, charybdotoxin 1 and 2 from *Leiurus quinquestriatus hebraeus*. *Amidated C-terminal end; LTX can be found with a free or amidated C-terminal, #Sequence of KTX corrected according to [23].

mouth of the K^+ channel, the blockade being achieved by the plugging of the channel pore by the toxin [22].

It is tempting to propose, as already suggested [16], that the blockade of K^+ channels by these toxins could be mainly due to the conserved sequence found in the C-terminal part and the antiparallel β-sheet and that the specificity for one or another type of K^+ channel could be due to additional structural elements, some of them contained in the N-terminal α-helicoidal part of the molecule. Future studies using different synthetic analogs of PO_5 and KTX will help to clarify these points and could contribute to the analysis of the wide diversity of K^+ channels.

Acknowledgements. We thank Dr. J.M Sabatier for a gift of synthetic PO_5-NH_2, Dr. P E. Bougis for constant interest and support, Dr. C. Deveau for radioimmunoassays with anti-apamin antibodies, Mrs. B. Céard for performing Phast Gel Electrophoresis.

REFERENCES

[1] Catterall, W.A. (1980) Annu. Rev. Pharmacol. Toxicol. 20, 15–43.
[2] Couraud, F., Jover, E., Dubois, J.N and Rochat, H. (1982) Toxicon 20, 9–13.
[3] Strong, P.N. (1990) Pharmac Ther. 46, 137–142.
[4] Blaustein, M.P., Rogowski, R.S., Sneider, M.J. and Knueger, B.L.K (1991) Mol. Pharmacol 40, 932–942.
[5] Crest, M., Jacquet, G., Gola, M., Zerrouk, H., Benslimane, A., Rochat, H., Mansuelle, P. and Martin-Eauclaire, M F. (1992) J. Biol. Chem. 267, 1640–1647.
[6] Vincent, J.P., Schweitz, H. and Lazdunski, M. (1975) Biochemistry 14, 2421–2525
[7] Granier, C., Pedroso-Muller, E. and Van Rietschoten, J (1978) Eur. J Biochem. 82, 293–299.
[8] Crestfield, A.M., Moore, S. and Stein, W.H. (1963) J. Biol. Chem. 238, 622–627.
[9] Hawke, D., Yuan, P.M. and Shively, J.E. (1982) Anal. Biochem. 120, 302–311.
[10] Seagar, M.J., Granier, C. and Couraud, F. (1984) J. Biol. Chem. 259, 1491–1495.
[11] Chicci, G.G., Gimenez-Gallego, G , Ber, E., Garcia, M.L., Winquist, R. and Cascieri, M.A (1988) J. Biol Chem. 263, 10192–10197.
[12] Auguste, P., Hugues, M., Grave, B., Gesquière, J.C., Maes, P., Tartar, A., Romey, G., Schweitz, H. and Lazdunski, M. (1990) J. Biol. Chem. 265, 4753–4759.
[13] Sabatier, J.M , Zerrouk, H., Darbon, H , Mabrouk, K., Benslimane, H., Rochat, H., Martin-Eauclaire, M.F. and Van Rietschoten, J., Biochemistry (in press).
[14] Defendini, M.L., Bahraoui, E.M., Labbé-Jullié, C , Regnier-Vigouroux, A., El Ayeb, M., Van Rietschoten, J., Rochat, H. and Granier, C. (1990) Mol. Immunol 27, 37–44
[15] Labbé-Jullié, C., Granier, C., Albericio, F , Defendini, M.L., Céard, B., Rochat, H and Van Rietschoten, J. (1991) Eur. J. Biochem. 196, 639–645.
[16] Auguste, P., Hugues, M., Manne, C., Moinier, D., Tartar, A. and Lazdunski, M (1992) Biochemistry 31, 648–654.
[17] Martins, J.C., Zhang, W., Tartar, A , Lazdunski, M. and Baremans, F.A (1990) FEBS Lett. 260, 249–253.
[18] Bontems, F , Roumestand, C., Boyot, P., Quilquin, B., Doljansky, Y., Menez, A. and Toma, F. (1991) Eur. J. Biochem. 196, 19–28.
[19] Bontems, F., Gilquin, B., Roumestand, C., Menez, A and Toma, F. (1992) Biochemistry 31, 7756–7764.
[20] Johnson, B A and Sugg, E E (1992) Biochemistry 31, 8151–8159.
[21] Pongs, O. (1992) Trends Pharmacol. Sci 13, 359–363.
[22] Park, C.S. and Miller, C. (1992) Biochemistry 31, 7749–7755.
[23] Romi, R., Crest, M , Sampieri, F., Zerrouk, H., Van Dorsselaer, A , Mansuelle, P., Jacquet, G., Gola, M., Rochat, H., Martin-Eauclaire, M F. and Van Rietschoten, J., submitted to J. Biol. Chem.