New polypeptide components purified from mamba venom

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Abstract New polypeptide components have been isolated from *Dendroaspis angusticeps* venom using chromatography. Two polypeptides containing 59 and 57 amino acids, called 'DaE1' and 'DaE2' respectively, have been purified to homogeneity and fully sequenced. Spectrometric analysis yielded masses of 6631.5 and 6389.0 Da, respectively. The polypeptides share 98 and 95% identity, respectively, with trypsin inhibitor E (DpE) of *Dendroaspis polylepis polylepis*. 'DaE' polypeptides inhibit Kv1.1 channels with an IC_{50} value in the range of 300 nM. They can be considered as new dendrotoxins, albeit with fairly low affinity as compared to α-DTX. 'DaE' polypeptides do not affect Kir2.1 channels.

Key words: Snake; Mamba; Venom; Toxin; Polypeptide; Potassium

1. Introduction

Mambas are snakes of the Elapidae family belonging to the genus *Dendroaspis*. At present, only four species endemic to Africa are known: *Dendroaspis angusticeps* (Eastern green mamba), *Dendroaspis polylepis polylepis* (black mamba), *Dendroaspis viridis* (Western green mamba) and *Dendroaspis jamesoni kaimosae* (Jameson's mamba). In 1979, it was found that *D. angusticeps* venom contains components that can facilitate the release of acetylcholine from nerve endings [1]. Subsequently, a polypeptide (named dendrotoxin, DTX) was isolated from the same venom and assumed to be responsible for the facilitatory actions [2]. In 1988, four different polypeptides from the venom of *D. angusticeps* were identified, designated as α-, β-, γ- and δ-DTX. The amino acid composition of α-DTX indicated that it was identical to DTX [3]. Two similar polypeptides, called toxin I and K, have also been isolated from *D. polylepis polylepis* [4].

Dendrotoxins are potent and selective neurotoxins because of the following observations: (i) intracerebroventricular (*i.c.v.*) injection of nanograms dendrotoxin per gram body weight in rats induces strong epileptiform convulsions [5], (ii) autoradiography on rat brain slices shows that ^{125}I-labeled dendrotoxins bind specifically and with high affinity to different regions of the brain [6,7], and (iii) voltage clamp experiments provide evidence for a selective block of voltage-dependent (e.g. Kv1.1-type) K^+ channels [7–12]. In addition, the functional site of α-DTX important for the binding to Kv1-type K^+ channels on rat brain synaptosomal membranes has been elucidated and includes six major binding residues, all located in the N-terminal region, with Lys5 and Leu9 being the most important [13]. From a structural point of view, dendrotoxins are homologous to Kunitz serine protease inhibitors (e.g. BPTI), although dendrotoxins are weak inhibitors of trypsin on the one hand, and, on the other hand, the protease inhibitors do not block K^+ channels [14,15].

While dendrotoxins are beyond doubt the most studied polypeptides from mamba venom thus far, a variety of other polypeptides (6–8 kDa) also exists. In a very recent review, an attempt was made to classify all the known polypeptides from mamba venoms in distinct structural groups [16]. The first group is composed of three kinds of polypeptides: 'true' dendrotoxins, protease inhibitors, with weaker activity on K^+ channels, and calcicludines, blockers of high-voltage activated Ca^{2+} channels. The second group contains short and long α-neurotoxins, muscarinic toxins, anticholinesterases, 'angusticeps-type' polypeptides (with low toxicity and unknown properties) and calciseptines (specific L-type Ca^{2+} channel blockers). In a third group are classified polypeptides of miscellaneous structures, sometimes with unknown function (e.g. dendroaspis natriuretic peptide (DNP), short neurotoxins-like (mambin and homologous structures), long polypeptides (DpA) and ribonucleases (DaN3)).

Given that mamba venom contains a myriad of bioactive polypeptides in the range of 6000–8000 Da, the objective of this work was to discover new Kv- and Kir-type channel-specific toxins. Here we report the purification of two new polypeptide components from *D. angusticeps* venom. These two polypeptides contain 59 and 57 amino acids, are called 'DaE1' and 'DaE2', respectively, have been sequenced and found to share 98 and 95% identity, respectively, with trypsin inhibitor E (DpE) of *D. polylepis polylepis* venom purified in 1978 [17]. Although less potent than α-DTX, 'DaE' polypeptides inhibit Kv1.1 channels with an IC_{50} value in the range of 300 nM. In contrast, they do not affect Kir2.1 channels.

2. Materials and methods

2.1. Venom purification

Venom of *D. angusticeps* was purchased from Sigma (USA); 50 mg was dissolved in 100 mM ammonium acetate, pH 7 (Merck, Germany). After vortexing, the sample was clarified by centrifugation at 14 000 rpm for 15 min and its supernatant was then applied onto a Superdex 30 prep grade HiLoad 16/60 FPLC column (Pharmacia LKB Biotech, Sweden) equilibrated with 100 mM ammonium acetate, pH 7. The material was eluted with the same buffer at a flow rate of 0.2 ml/min. Absorbance of the eluate was monitored at 280 nm and

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4 ml fractions were collected automatically. The fraction of interest was recovered, lyophilized and applied on a Mono S HR 5/5 cation exchange FPLC column (Pharmacia, Sweden) equilibrated with 50 mM phosphate buffer, pH 6.8. Separation was performed using a linear gradient of 0–100% 1 M NaCl (supplemented with 50 mM phosphate buffer, pH 6.8). The flow rate was 1 ml/min and the absorbance was measured at 214 nm. Desalting of the material was performed with a PepRPC HR 5/5 C_2/C_{18} reversed phase FPLC column (Pharmacia, Sweden) equilibrated with 0.1% trifluoroacetic acid (TFA) in distilled water. Separation was performed by using a linear gradient of 0–60% acetonitrile (supplemented with 0.1% TFA) for 40 min. The flow rate was 0.5 ml/min and the absorbance was measured at 214 nm. Fractions containing the material of interest were recovered, dried (Speed Vac Plus, Savant, USA), and applied to a 208TP5415 C_8 reversed phase HPLC column (Vydac, USA) using the same reversed phase solutions as above. Separation was performed by using a linear gradient of 0–30% acetonitrile (supplemented with 0.1% TFA) between 4 and 6 min, followed by a linear gradient of 30–40% between 6 and 15 min.

2.2. Mass and sequence determination

The masses of the two polypeptides were determined by electrospray mass spectrometry which was performed on a Bio-Q quadrupole mass spectrometer equipped with an electrospray ionization source (Micromass, Altrincham, UK). 20 μl of sample solution, diluted in 50% acetonitrile in 0.5% formic acid, were injected manually in the loop of the Rheodyne injector and pumped to the source at 6 μl per min. The solvent was delivered by a 140 A Solvent Delivery System (Applied Biosystems, Foster City, CA, USA). Scans of 12 s over the mass range of 400–1600 AMU were collected over 2 min. Calibration of the instrument was performed with 50 pmol of horse myoglobin (Sigma, USA). Mass determination of the internal peptides was performed by the technique of matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF-MS) carried out on a TofSpec SE time-of-flight instrument using a Nitrogen laser (337 nm) (Micromass, Wythenshawe, UK). Scans were accumulated over 20–70 laser shots, using a cyano hydroxycinnamic acid as matrix. External calibration was realized using both angiotensin II and bovine insulin (Sigma, USA). To avoid N-terminally blocked polypeptides through cyclization of an N-terminal glutamine, the polypeptides have been incubated with pyroglutamate aminopeptidase (Boehringer Mannheim, Germany) subsequently for 18 h at 4°C and 4 h at 25°C in a 100 mmol sodium phosphate buffer of pH 8, containing 10 mmol EDTA, 5 mmol dithioerythritol and 5% glycerol, at an enzyme/substrate ratio of 1/50. The cysteines in DaE1 and DaE2 have been alkylated with 3-bromopropylamine (Sigma, USA) as described by Jue and Hale [18]. Internal peptides of the alkylated DaE1 and DaE2 have been obtained by enzymatic cleavage with endoproteinase Glu-C (protease V8) (Boehringer Mannheim, Germany). The toxins were incubated overnight at room temperature in 20 mM ammonium bicarbonate of pH 8 at an enzyme/substrate ratio of 1/40. Separation of the peptides resulting from the enzymatic cleavage was performed on a Sephasil C_{18} sc 2.1/10 reversed phase column, installed on a SMART chromatographic system (Pharmacia, Uppsala, Sweden) using gradient elution. Solvent A was 0.1% trifluoracetic acid in water and solvent B was 0.08% trifluoracetic acid in 70% acetonitrile. Polypeptide purification after alkylation has been performed on a C_{18} YMC ODS-AQ, 2×100 mm column (YMC, New York, USA), installed on the SMART system, using the same solvents as for the peptide separation. N-terminal and peptide sequence analyses were performed on an automatic 476 A pulsed liquid sequenator equipped with an on-line analyzer for PTH derivatives (Applied Biosystems, Foster City, CA, USA).

2.3. Expression in oocytes

For in vitro transcription, plasmids were first linearized either with *Nhe*I (for IRK1/pGEMHE2), or with *Pst*I 3′ to the 3′ non-translated β-globin sequence of RCK1/pGEMHE. Next the 'Riboprobe Gemini System' was used (Promega, USA) with T7 RNA polymerase and a cap analogue diguanosine triphosphate. Stage V–VI *Xenopus laevis* oocytes were isolated by partial ovariectomy under anesthesia (tricaine, 1 g/l). Anaesthetized animals were then kept on ice during dissection. The oocytes were defolliculated by treatment with 2 mg/ml collagenase (Boehringer Mannheim, Germany) in zero calcium ND-96 solution (see Section 2.5) for 45 min. Between 2 and 24 h after defolliculation, oocytes were pressure injected with 50 nl of 1–100 ng/μl mRNA. The oocytes were then incubated in ND-96 solution at 18°C for 1–4 days.

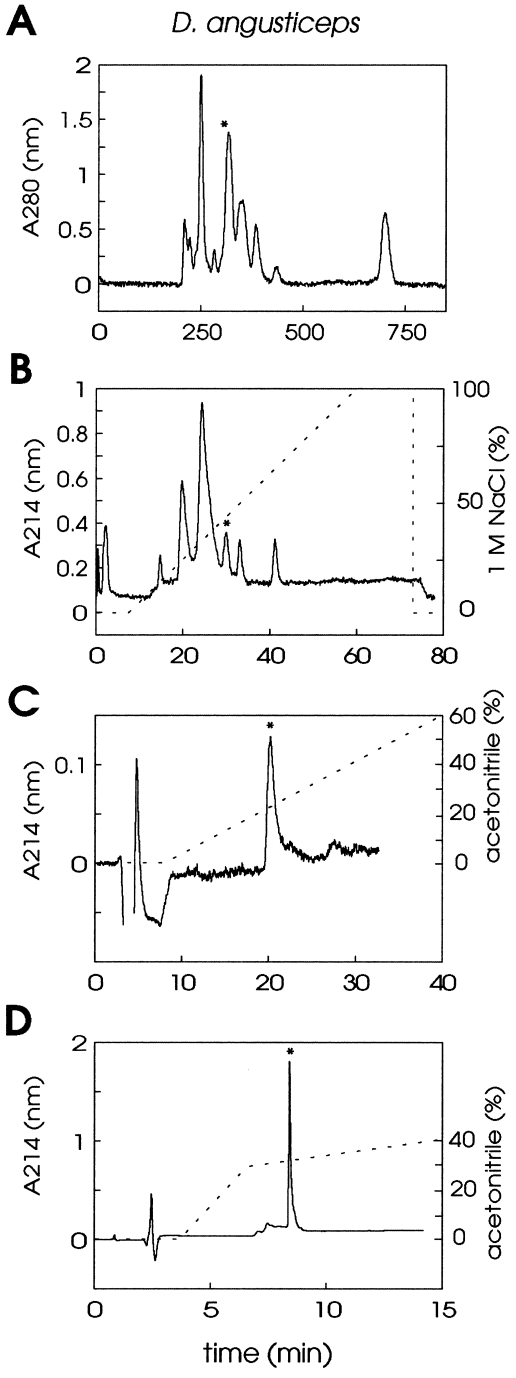

Fig. 1. Purification of DaE1 and DaE2 from *D. angusticeps*. A: Crude venom fractionated by FPLC gel filtration as indicated in Section 2. The labeled fraction (*) was recovered and lyophilized. B: Second purification step using a Mono S HR 5/5 cation exchange column. The labeled fraction (*) was recovered and desalted on a PepRPC HR 5/5 C_2/C_{18} reversed phase column (C). The fraction containing the material of interest was recovered, dried, and applied to a 208TP5415 C_8 reversed phase column for final purification (D).

2.4. Electrophysiological recordings and analysis

Whole-cell currents from oocytes were recorded using the two-microelectrode voltage clamp technique (GeneClamp 500, Axon Instru-

ments, USA). Resistances of voltage and current electrodes were kept as low as possible (voltage electrode: 0.1–0.5 MΩ; current electrode: 0.1 MΩ) and were filled with 3 M KCl. Current records were sampled at 1 ms intervals and filtered at 0.5 kHz, using a 4-pole low-pass Bessel filter. To eliminate the effect of the voltage drop across the bath grounding electrode, the bath potential was actively controlled, as measured near the outside surface of the oocyte by means of a two-electrode virtual-ground circuit (also called 'bath clamp'). Leak current amplitudes were only in the range of 1% of the amplitude of time- and voltage-dependent currents. In non-injected or H_2O-injected oocytes ($n = 20$), endogenous currents observed in the tested voltage range amounted only to approximately 1% of the amplitude of RCK1 and IRK1 currents. All experiments were performed at room temperature (19–23°C).

2.5. Solutions

The ND-96 solution contained (in mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.5, supplemented with 50 mg/ml gentamicin sulfate (only for incubation).

3. Results and discussion

Crude venom from *D. angusticeps* was fractionated by means of gel filtration, ion exchange, and two reversed phase steps as detailed in Section 2 and shown in Fig. 1. The masses of two polypeptides of interest were determined by electrospray mass spectrometry. Spectrometric analysis of the two polypeptides from *D. angusticeps*, which we named 'DaE1' and 'DaE2', yielded masses of 6631.5 and 6389.0 Da, respectively.

To avoid the presence of N-terminally blocked polypeptides through cyclization of the N-terminal glutamine (dendrotoxins indeed often appear N-terminally blocked), the two polypeptides have been incubated with pyroglutamate aminopeptidase as detailed in Section 2. The cysteine residues present in DaE1 and DaE2 have been alkylated with 3-bromopropylamine according to Jue and Hale [18]. Enzymatic cleavage with endoproteinase Glu-C (protease V8) of the aminopropylated toxins DaE1 and DaE2 resulted in 10 peptide fragments (Table 1). Mass spectrometric data of these peptides revealed an interesting finding: the difference between the experimentally measured masses of peptides E4a and E4b, E5a and E5b, and E6a and E6b, respectively, invariably totaled 241.4 Da. This mass equals the mass of an N-terminal di-peptide composed of a Leu and Gln residue, which we have been able to confirm during sequence analysis (see also further). Furthermore, all the mass spectrometric data for the 10 different peptides confirm the calculated masses and respective sequence positions in the toxin. For instance, peptide E1 corresponds with the sequence stretch Lys52–Gly59, has an experimental mass of 1007.2 Da and a calculated mass of 1006.1 Da. Peptide E6a corresponds with sequence position Leu1–Gly38 (see toxin DaE1), has an experimental mass of 4558.3 Da and a calculated mass of 4557.1 Da. In contrast, peptide E6b corresponds with sequence position Hys3–Gly38 (see toxin DaE2), has an experimental mass of 4316.8 Da and a calculated mass of 4315.8 Da.

N-terminal and peptide sequencing permitted unequivocal assignment of 59 residues for DaE1 and 57 residues for DaE2 (Fig. 2). Both peptides contain six Cys residues and only differ from each other N-terminally with respect to the presence or absence of residues Leu–Gln in DaE1 and DaE2, respectively. The measured molecular masses of the two peptides, 6631.5 and 6389.0 Da respectively, fit to the obtained amino acid sequences. The differences between the measured and the predicted masses only amount to 0.02% and 0.03% respectively (predicted masses for DaE1 and DaE2 with the Cys residues in the oxidized state: 6633 Da and 6391 Da respectively).

When the amino acid sequences of DaE1 and DaE2 were compared with other known proteins, (Swiss Prot and BLAST data bank searches), the following observations could be: DaE1 and DaE2 clearly belong to the first 'structural' group of mamba polypeptides composed of 'true' dendrotoxins, protease inhibitors and calcicludines. These polypeptides are basic, contain 57–60 residues in total of which there are six Cys residues, and display a high degree of homology in their C-termini (residues 39–54). DaE1 and DaE2 share 98 and 95% identity, respectively, with DpE, which is trypsin inhibitor E [17]. It is interesting to note that all the residues of DpE and DaE1 are identical, except at position 55 (His in DpE vs. Arg in DaE1). As compared with other toxins of this group, the following degrees of identity were calculated for DaE1: 53% with α-DTX [3], 63% with δ-DTX [3,20], 61% with γ-DTX [3],

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                    10        20        30        40        50        60
α-DTX     ZPRRKLCILHRDPGRCYDKIPAFYYNQKKKQCERFDWSGCGGNSNRFKTIEECRRTCIG-  59
DTX I     ZPLRKLCILHRNPGRCYQKIPAFYYNQKKKQCEGFTWSGCGGNSNRFKTIEECRRTCIRK 60
δ-DTX     --AAKYCKLPVRYGPCKKKIPSFYYKWKAKQCLPFDYSGCGGNANRFKTIEECRRTCVG- 57
γ-DTX     --AAKYCKLPVRYGPCKKKIPSFYYKWKAKQCLFLDYSGCGGNANRFKTIEECRRTCVG- 57
DTX K     --AAKYCKLPLRIGPCKRKIPSFYYKWKAKQCLPFDYSGCGGNANRFKTIEECRRTCVG- 57
Dv 14     --AAKYCKLPVRYGPCKKKIPSFYYKWKAKQCLYFDYSGCGGNANRFKTIEECRRTCVG- 57
DpB       --RPYACELIVAAGPCMFFIPAFYYSKGANKCYPFTYSGCRGNANRFKTIEECRRTCVV- 57
DpE       LQHRTFCKLPAEPGPCKASIPAFYYNWAAKKCQLFHYGGCKGNANRFSTIEKCRHACVG- 59
DaE1      LQHRTFCKLPAEPGPCKASIPAFYYNWAAKKCQLFHYGGCKGNANRFSTIEKCRRACVG- 59
DaE2      --HRTFCKLPAEPGPCKASIPAFYYNWAAKKCQLFHYGGCKGNANRFSTIEKCRRACVG- 57
          ↑↑                                                      ↑
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Fig. 2. Primary structure of DaE1 and DaE2. The complete amino acid sequences of DaE1 and DaE2 are given and compared with those of similar dendrotoxins, after manual alignment of the Cys residues (black boxes). DaE1 and DaE2 contain 59 and 57 amino acids, respectively. They share 98 and 95% identity, respectively, with DpE [17]. DpE and DaE1 are identical, except at position 55 (see single arrow). DaE1 and DaE2 differ only in the first two N-terminal residues (see double arrow). White boxes indicate sequence identity. The total number of residues for each polypeptide, varying between 57 and 60, is summarized on the right. Other abbreviations: α- and γ-DTX [3], δ-DTX [3,20], DTX I and K [4], Dv 14 (unpublished, cited in [15]), and DpB [19].

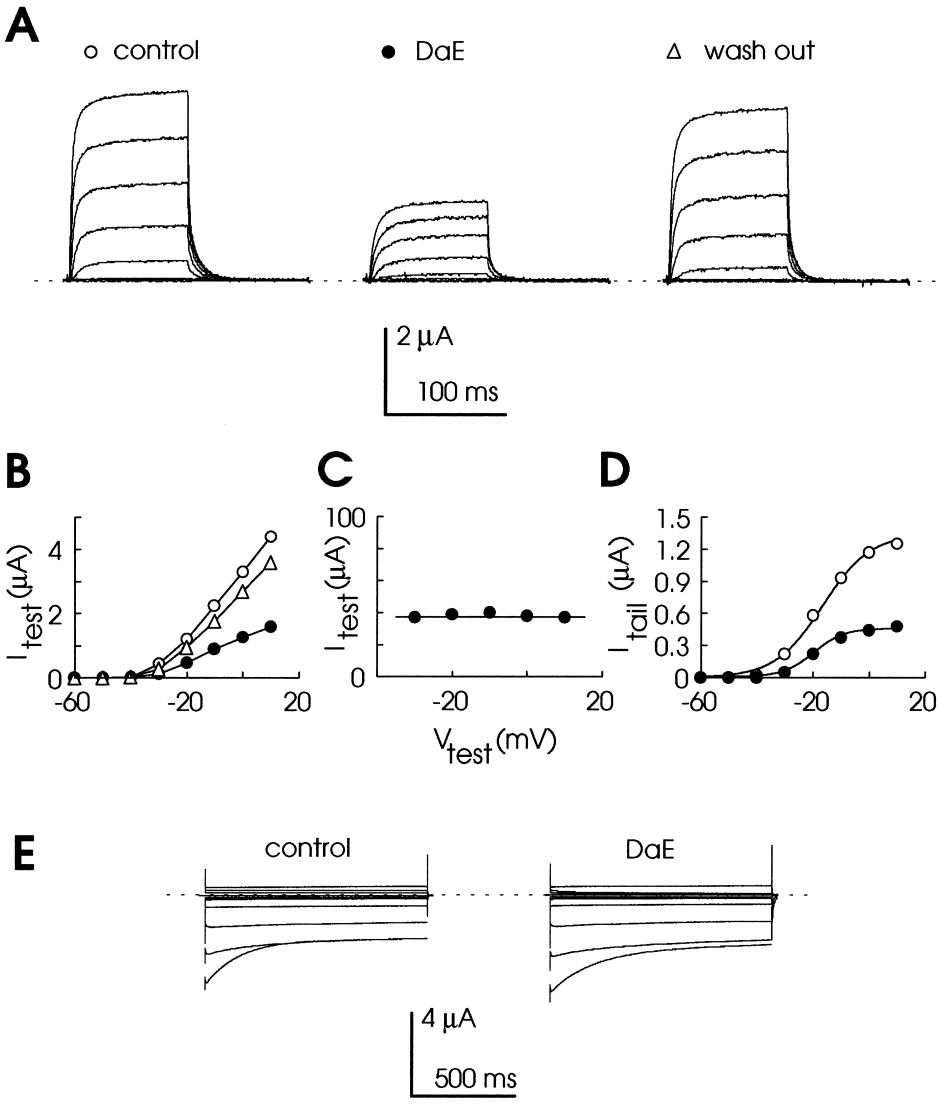

Fig. 3. Electrophysiological characterization of DaE polypeptides. Macroscopic current recordings obtained with the two-microelectrode voltage clamp technique on *X. laevis* oocytes. A: Currents through Kv1.1 (RCK1) channels evoked from a holding potential of −90 mV stepped to test potentials ranging from −60 to +10 mV, with 10 mV increments, in control (○), in the presence of 500 nM DaE (●), and after wash out (△). B: Current–voltage relationship for the experiment illustrated in A. Current amplitudes were measured at the end of each test pulse. C: Remaining current (%) plotted as a function of the test potential in the presence of 500 nM DaE, yielding a horizontal line indicative for the absence of voltage-dependent block. D: The presence of 500 nM DaE does not shift the steady-state action curve of Kv1.1 ($V_{1/2} = -16.0 \pm 3.1$ mV in control ($n = 10$) and -16.5 ± 4.2 mV in the presence of DaE ($n = 6$)). E: Currents through Kir2.1 (IRK1) channels evoked from a holding potential of 0 mV stepped to test potentials ranging from −140 to +60 mV, with 20 mV increments. 500 nM DaE is without effect. Recordings were in symmetrical K^+ solutions.

Table 1
Mass spectrometric data for the peptides obtained after Glu-C (E) endoproteinase digestions of the aminopropylated toxins DaE1 and DaE2

Peptide	MALDI experimental mass (MH^+) (Da)	Calculated mass (Da)	Sequence position
E1	1007.2	1006.1	K_{52}–G_{59}
E2	1454.8	1453.5	G_{39}–E_{51}
E3	2072.4	2071.2	L_{34}–E_{51}
E4 **a**	3941.0	3939.4	L_1–Q_{33}
E4 **b**	3699.7	3698.1	H_3–Q_{33}
E5 **a**	5994.4	5992.7	L_1–E_{51}
E5 **b**	5753.0	5751.4	H_3–E_{51}
E6 **a**	4558.3	4315.8	L_1–G_{38}
E6 **b**	4316.8	4557.1	H_3–G_{38}
E7	2053.8	2054.2	G_{39}–R_{55}

All peptides contain at least one modified cysteine. The mass of the aminopropylated cysteine is 57 Da higher than that of a normal cysteine. These modifications are also counted in the calculated mass data. The numbering used for the indication of the sequence position is that of the DaE1 toxin (59 residues). N-terminal peptides derived from DaE1 are indicated as **a**, those obtained from DaE2 are indicated as **b**. The peptides E1, E4 a and b, E6 a and b and E7 are the products of aspecific cleavages.

50% with DTX I [4], 60% with DTX K [4], 63% with Dv 14 (unpublished, cited in [15]), and 56% with DpB [19].

The biological effect of DaE polypeptides is represented in Fig. 3. Application of 500 nM DaE produced an inhibitory effect of approximately 60% on the outward current through Kv1.1 channels, heterologously expressed in *X. laevis* oocytes. The inhibition was voltage-independent, reversible, did not affect the steady-state activation curve, and was characterized by an IC_{50} value of ≈ 300 nM ($n = 6$). Consequently, DaE polypeptides can be considered as new dendrotoxins, although they have fairly low affinity as compared to α-DTX (IC_{50} value Kv1.1 ≈ 1 nM) [12]. Our electrophysiological results are in accordance with the weak affinity as displayed by similar peptides, DaO1 and DaP1, with apparent affinities of only 20–25 nM for ^{125}I-DTX I binding sites in rat brain membranes (unpublished results, but recently reviewed in [16]). The critical residues by which α-DTX binds to rat brain synaptosomal membranes have been determined using a mutational approach based on site-directed mutagenesis and chemical synthesis [13]. Substitutions of Lys5 and Leu9 decrease the affinity of α-DTX more than 1000-fold, and substitutions of Arg3, Arg4, Leu6 and Ile8 by 5- to 30-fold. Based on this, the authors concluded that the functional side of α-DTX includes six major binding residues, all located in the N-terminal region, with Lys5 and Leu9 being the most important. It is interesting to note that DaE polypeptides lack Lys5, but share Arg4 with α-DTX and Leu9 with all dendrotoxins (100% conserved). This partial similarity may explain the low affinity of DaE polypeptides. A preliminary study of this type was also reported for DTX K [21]. Mutant DTX K toxins with residues substituted in the α-helix near the C-terminus (R52A or R53A) yielded binding parameters similar to those of wild-type and native DTX K. Residues located in the β-turn, however, reduced binding with the rank order of decrease being K26A ≫ W25A > K24A = K28A. Furthermore, substitutions in the '3_{10}-helix' located closely to the β-turn, produced equivalent effects (K3A > K6A). In view of these results and as can be seen from Fig. 2, DTX K and DaE polypeptides share residues Lys6 and Trp25. Another nice and recently published paper describes the critical residues constituting the functional interaction surfaces of δ-DTX and its voltage-gated *Shaker* channel receptor [22]. It was shown that δ-DTX uses a triangular patch formed by seven side-chains (Lys3, Tyr4, Lys6, Leu7, Pro8, Arg10 and Lys26) to block the *Shaker* channel. Again, DaE polypeptides share only some of these residues with δ-DTX, viz. Lys6, Leu7 and Pro8, explaining the rather weak affinity for Kv1-type channels.

Finally, we found that DaE polypeptides do not affect inward rectifier K^+ channels, such as Kir2.1 expressed in oocytes, which is in accordance with the selectivity profile of α-DTX, but not of δ-DTX [23].

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