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# On the site by which α-dendrotoxin binds to voltage-dependent potassium channels: site-directed mutagenesis reveals that the lysine triplet 28–30 is not essential for binding

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**Abstract** We constructed a synthetic gene encoding the published amino acid sequence of DTx from *Dendroaspis angusticeps*, a ligand of voltage-dependent postassium channels that facilitates neurotransmitter release. We expressed it in *Escherichia coli* as a fusion protein secreted in the culture medium. The recombinant DTx was generated in vitro by chemical treatment and recovered as two isoforms. One of them (rDTx), like the venom toxin, has an N-terminal pyroglutamate whereas the other (rQDTx) has a free N-terminal glutamine. Chromatographic differences between rDTx and natural DTx led us to re-examine the amino acid sequence of natural DTx. In contrast to what was previously published, position 12 was an Asp and not Asn. Despite this difference, rDTx and DTx had similar toxicity in mice and binding affinity to synaptosomes, suggesting that residue 12 is not important for DTx function. Nor is the N-terminal residue implicated in DTx function since rDTx and rQDTx also had similar biological activities. We also synthesized and expressed a mutant of the DTx gene in which the lysine triplet 28–30 was changed into Ala-Ala-Gly. The two resulting recombinant isoforms exhibited only small decreases in biological activity, excluding the possibility that the positively charged lysine triplet 28–30 of DTx is directly involved in the toxin functional site.

*Key words:* Potassium channel; Recombinant snake toxin; Site directed mutagenesis

## 1. Introduction

Dendrotoxins are small proteins (57–60 amino acids) isolated from mamba venoms which facilitate the release of neurotransmitters at peripheral (1–3) and central [4] synapses by blocking certain voltage-dependent potassium channels to which they bind with high affinity. They constitute important tools for isolating and studying subtypes of potassium channels (for review see [5]). Seven dendrotoxins have been purified from venoms of the snakes belonging to the *Dendroaspis* genus. Their amino acid sequences have been determined either totally or partially [6–9] and the three-dimensional structures of α-dendrotoxin (DTx) from *Dendroaspis angusticeps* [10] and dendrotoxins I and K (DpI and DpK) from *Dendroaspis polylepis* [11,12] have been solved. All these proteins are structurally similar to Kunitz-type serine protease inhibitors, such as the bovine pancreatic trypsin inhibitor BPTI [10,13].

The site by which these toxins interact with their target is not known. However, on the basis of amino acid sequence comparisons between dendrotoxins and non-toxic homologues, it was often proposed [14–16] that the conserved positively charged residues, and in particular the lysine triplet found in position 28–30 in DTx and DpI, might interact with a corresponding negatively charged region on the potassium channel. Consistent with an electrostatic interaction between the two partners, it has been shown recently that the DTx binding site on rat brain potassium channel is located in an extracellular domain which includes an essential negatively charged residue ([17,18], for review see [19]).

The aim of this work was to probe the functional role of the lysine triplet 28–30 of DTx by protein engineering. This was achieved in three steps. First, we constructed a synthetic gene encoding the published amino acid sequence of DTx from *Dendroaspis angusticeps* [7], expressed it at a fusion protein in *E. coli* according to a convenient approach which previously proved to be efficient for producing correctly folded disulfide-containing proteins [20,21]. Second, the recombinant DTx was generated by chemical cleavage of the fusion protein and examined regarding its physicochemical and biological properties under both in vivo and in vitro conditions. Third, we synthesized an additional gene in which the lysine triplet 28–30 of DTx was replaced by Ala-Ala-Gly, and generated the recombinant mutant using the same expression system employed for the wild-type DTx. Examination of the biological properties of the mutant provides evidence that the lysine triplet is not essential for binding of DTx to potassium channels.

## 2. Materials and methods

### 2.1. Materials

Enzymes were purchased from Boehringer-Mannheim. Oligonucleotides were synthesized on an Applied Biosystems 381A. Amino acid composition analyses were performed using an Applied Biosystems device (420A Derivatizer and 130A Separation system) and N-terminal sequencing using the Applied Biosystems sequencer (477A Protein Sequencer) on line with the PTH analyser (120A Analyser). Masses were determined using an electrospray mass spectrometer (Analytica, Bran-

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Expression of DTx was previously described in a preliminary report abstract of the Xth Meeting on Toxinology which was held in Paris on 2–4 September 1992.


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ford, USA). Dichroic spectra were recorded on a Jobin-Yvon CD6 dichograph. SDS-PAGE were performed using the PhastSystem from Pharmacia. Expression vector pEZZ 18 [22,23] was generously provided by Prof. Mathias Uhlén (Royal Institute of Technology, Stockholm) and the bacterial host for expression was *E. coli* HB 101 [24]. Biolafitte fermentor (St. Germain en Laye, France) and culture medium from Difco (USA) were used for production of recombinant proteins. Natural DTx used as a standard was either a gift from Dr. Evert Karlsson (Uppsala University, Sweden) or was purified in our laboratory from venom provided by Latoxan (Rosans, France).

*2.2. Molecular biology*

DNA manipulation, transformation and plasmid purification were performed according to published procedures [25]. Sequences of both the synthetic genes and the expression vector were checked by single- and double-stranded sequencing [26,27].

*2.3. Synthetic gene assembly*

A 213 bp DNA fragment encoding the published DTx sequence [7] was synthesized as follows. Twelve oligomers ranging in length from 26 to 48 nucleotides were synthesized and purified [25]. Six duplexes resulting from annealing of complementary oligonucleotides (100 pmol of each) were ligated three × three to generate two segments having, respectively, 119 and 94 bp. These two segments were finally ligated to yield the complete synthetic gene flanked by *Eco*RI and *Pst*I restriction sites. It was purified, excised and then inserted into M13mp18 to assess its nucleotide sequence. The strategy to construct the mutant gene was similar except that one duplex was substituted by a mutated one in which the codons for the three consecutive Lys at positions 28–30 of the toxin were replaced by codons encoding the triplet Ala-Ala-Gly.

*2.4. Expression vector construction*

Following biological amplification in bacteria, the two synthetic genes were excised from M13mp18 and inserted in the expression vector pEZZ 18 (Pharmacia) using *Eco*R1 and *Pst*1 restriction sites. The resulting vectors were checked by double-stranded sequencing and used to transform *E. coli* HB101 strain for protein production.

*2.5. Production and purification of the fusion recombinant proteins*

Transformed bacteria were grown according to [20] in 4 or 12 l of culture medium. When the $A_{600nm}$ reached 25, the culture medium was centrifuged and the resultant supernatant clarified by filtration through a 0.22 μm filter and concentrated by ultrafiltration on a membrane with a cut-off weight of 10 kDa. The fusion proteins were purified using an IgG-Sepharose 6FF column (Pharmacia) according to [21] followed by a chromatographic step onto a MonoS 5/5 column (Pharmacia) to which we applied a 35 min linear gradient from 0 to 70% of eluent B (eluent A, 10 mM ammonium acetate, pH 5.6; eluent B, 1.5 M ammonium acetate, pH 5.6). Fusion proteins were then cleaved by cyanogen bromide in HCl 0.1 N at room temperature for 24 h and the resulting recombinant toxins were finally purified onto a MonoS 5/5 column using a 60 min linear gradient from 20 to 70% of eluent B (Eluent A, 10 mM ammonium acetate, pH 7.3. Eluent B, 1.5 M ammonium acetate, pH 7.3) and a flow rate of 0.8 ml/min.

*2.6. Binding to rat brain synaptosomes*

Recombinant proteins were used in competition binding assays with [$^{125}$I]-dendrotoxin I (DpI) (100–250 Ci/mmol) or [$^{125}$I]α-dendrotoxin (DTx) (Amersham; 2000 Ci/mmol) as radioactive tracers. Rat brain synaptosomes were prepared from the brains of 200–250 male Sprague–Dawley rats as described in [28]. For the binding assay, synapatosomal membranes were incubated for 30 min at room temperature with 1 nM of labelled toxin in presence of increasing concentrations ($10^{-11}$ to $10^{-7}$ M) of competitor. $K_i$ values were calculated following the Cheng and Prusoff method [29].

*2.7. Chick biventer cervicis preparations*

Twitch tension experiments were performed using chick biventer cervicis and associated nerves, isolated from 4- to 8-day-old chicks killed by exposure to anaesthetic halothane [30]. Isolated muscles were mounted in a 10 ml tissue bath containing physiological salt solution at 33°C with a resting tension that produced a maximum twitch. The motor nerve was stimulated every 10 s with pulses of 0.2 ms duration and a voltage greater than that which produced a maximal twitch. Details are given in [31].

*2.8. Peptide sequencing of natural α-DTx*

100 μg of natural DTx were denatured, reduced and blocked with 4-vinylpyridine as recommended by Applied Biosystems. They were then cleaved for 6 h at 37°C by 5 μg of endoproteinase Lys-C (Boehringer-Mannheim) in 200 μl of 25 mM Tris-HCl, pH 8.5; 1 mM EDTA. Resulting peptides were separated by reverse-phase HPLC onto a $C_{18}$ column (Vydac; 10 mm, 250 × 4.6 mm) using a 50 min linear gradient from 0 to 50% of eluent B (eluent A, TFA 0.1% in water; eluent B, TFA 0.1% in water/acetonitrile 50:50). Each fraction was then submitted to amino acid composition analysis and Edman degradation.

*2.9. Central toxicity and $LD_{50}$ determination*

For lethal dose 50 ($LD_{50}$) determination, groups of five to sixteen female Swiss mice (23.4 ± 3.0 g) were injected intracerebroventricularly (i.c.v.) under light diethyl ether anaesthesia with 2 to 10 ng of toxin per g of body weight (2 to 10 μl in solution in NaCl 0.9%). $LD_{50}$ was established after 48 h survival.

For central toxicity comparison, DTx and recombinant mutated DTx (twice the $LD_{50}$ of α-DTx in 10 μl) were injected i.c.v. to groups of eight animals (22.9 ± 1.3 g). The animals were allowed to recover consciousness and the times from injection of samples to the onset of symptoms (epileptiform activity, abnormal posturing.) and to death were measured.

## 3. Results and discussion

*3.1. Synthetic genes and expression vector construction*

The first objective of this work was to design an appropriate expression vector for the wild-type DTx. The nucleotide sequence of a synthetic gene encoding recombinant DTx was therefore designed on the basis of its published amino acid sequence [7] using usable codons in *E. coli* [32]. Since natural DTx possesses a pyroglutamate at its N-terminal end, we introduced a codon for glutamine at the first position of the synthetic gene with the view to generate a pyroglutamic acid in the recombinant DTx, under acid conditions. Since there was no methionine in the sequence of DTx, we introduced a cyanogen bromide cleavage site in the fusion proteins by inserting a methionine codon at position −1 of the gene encoding the recombinant toxin. We also flanked our construction with restriction sites (including *Eco*RI and *Pst*I) in order to clone it into pEZZ 18 [22,23], a vector which is well suited for producing disulfide-containing proteins in *E. coli* [20,21]. The resulting expression vector ZZ-DTx encodes the protein A signal peptide followed by two IgG binding domains (15 kDa) linked to the recombinant toxin moiety (7 kDa) by a six amino acid peptide (Val-Asp-Ala-Asp-Ser-Met).

It was previously proposed that conserved positively charged residues might contribute to the establishment of the interaction between DTx and potassium channels [14–16]. In particular, the lysine triplet 28–30 was suggested to play some critical function in this interaction. As shown on Fig. 1, this triplet is localized at the base of the molecule which clearly adopts the well-known pear shape of BPTI [10]. We substituted the lysine triplet by the sequence Ala-Ala-Gly (AAG), and this choice resulted from an examination of a chart of amino acid sequences of various dendrotoxins and their non-toxic homologues [5,33]. The recombinant mutant was produced in *E. coli* from a synthetic gene encoding DTx in which the three lysines were mutated into AAG, according to an approach similar to that used for production of the wild-type DTx. The resulting expression vector was called ZZ-DTx-AAG.

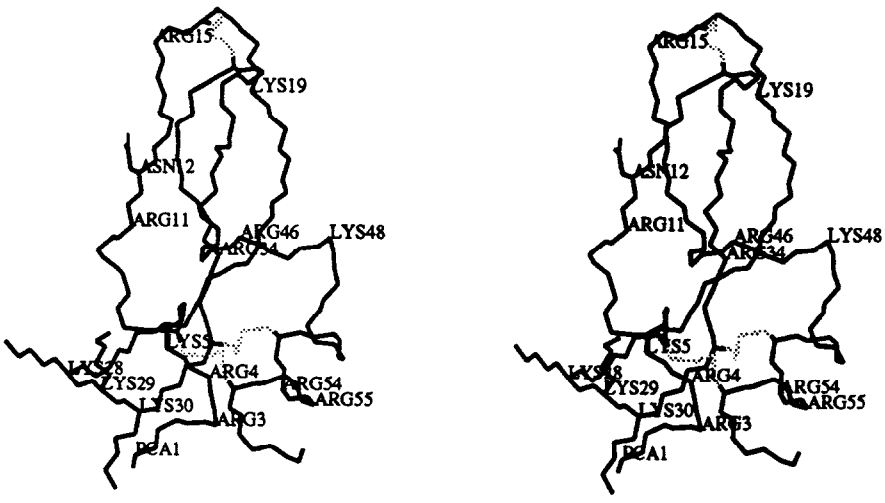


Fig. 1. Stereo view of the structure of DTx. The figure shows the structure of the toxin according to [10]. The side chains of mutated residues (positions 12, 28, 29 and 30), as well as all additional positively charged residues, are shown. The disulfide bridges are stippled.

*3.2. Production of the fusion proteins*

The recombinant fusion proteins resulting from expression of ZZ-DTx and ZZ-DTx-AAG were mainly found in the culture medium of bacterial suspensions, as expected for pEZZ expression vectors [22,23]. Chromatography on an IgG Sepharose column yielded proteins having the molecular weight (22 kDa) expected for the fusion proteins. These proteins were, however, contaminated by lower molecular weight proteins which result from degradation of fusion proteins, as previously discussed [20,21]. These side products were removed by cation-exchange chromatography as described in section 2. As indicated by amino acid composition analyses, we found 1 mg and 1.5 mg fusion proteins per l of culture for ZZ-DTx-AAG and ZZ-DTx, respectively.

*3.3. Purification and characterization of recombinant toxins*

Recombinant DTx and DTx-AAG resulted from cyanogen bromide cleavage of fusion proteins with a yield close to 65%. They were purified by cation-exchange chromatography. As shown in Fig. 2, however, each recombinant protein was recovered as two isoforms (peaks 1 and 2) having the same molecular weight and amino acid compositions but, as indicated by Edman degradation, differing at their N-terminal residue. One isoform has its N-terminal blocked (peak 1) while the other possesses an N-terminal glutamine (peak 2). Mass determination revealed an 18 unit mass difference between the two isoforms of the wild-type and mutated recombinant DTxs. This is in agreement with the loss of an $-NH_3^+$ moiety and with the presence of a pyroglutamate residue formed by acidic cyclisation of the N-terminal glutamine in the lighter isoforms. Finally, sequencing was investigated until position 33 for the unblocked mutant toxin, and we thus assessed the effectiveness of the introduced mutations. Therefore, two isoforms of each recombinant protein were produced. They were called rDTx and rDTx-AAG for proteins having an N-terminal pyroglutamate residue and rQDTx and rQDTx-AAG for proteins having a free N-terminal glutamine residue. Since the circular dichroic spectra of the natural, recombinant wild-type and mutated DTxs were superimposable (not shown), we conclude that all these proteins have a similar content in secondary structure, suggesting that they all adopt a folding similar to that of the natural DTx.

*3.4. Chemical differences between natural and recombinant wild-type toxin*

While purifying rDTx, we observed that this protein eluted from the cation-exchange column later than DTx isolated from the venom, indicating its more basic nature. This was surprising since their amino acid compositions and masses were similar, within the experimental errors. We thus anticipated that this elution difference could be associated with an amino acid sequence difference not detectable by amino acid composition analyses such as Asn/Asp or Gln/Glu. This prompted us to re-investigate the amino acid sequence of DTx isolated from the venom of *Dendroaspis angusticeps*. For this purpose, we generated five toxin fragments using endoproteinase Lys C which were purified by RP-HPLC and characterized by amino acid composition analysis and sequencing. These fragments correspond to the published sequence of DTx [7] from residues 6–59, except that residue 12 was found to be an aspartate instead of the expected asparagine. Clearly, the natural DTx that we used as a control is an isoform of that described by Joubert and Taljaard [7] and Karlsson (cited in [2]). Therefore, DTx, as it is isolated from the venom of a *Dendroaspis angusticeps* from East Africa, has an amino acid sequence that is distinct from the one to which we currently refer to. Determination of new isoforms of toxins is not so surprising because of the great diversity of toxic and non-toxic homologous proteins

Table 1
Inhibition of radioactive toxin binding to rat brain synaptosomal membranes by natural and recombinant dendrotoxins

| Toxin | $K_i$ (nM) |
|---|---|
| DTx | 1.14 ± 0.15 |
| rDTx | 1.37 ± 0.36 |
| rQDTx | 0.22 ± 0.01 |
| DTx | 0.62 ± 0.12 |
| rDTx-AAG | 2.13 ± 0.06 |
| rQDTx-AAG | 4.76 ± 0.24 |

$K_i$ values are calculated from Fig. 2 as described in section 2.

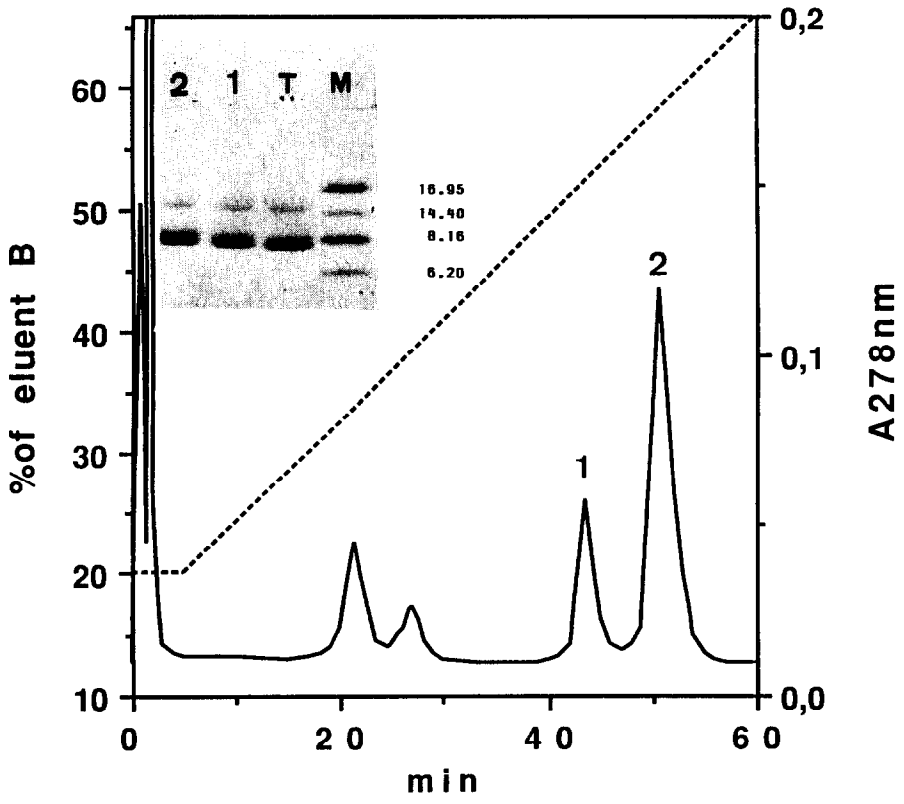


Fig. 2. Purification of recombinant DTx. Products resulting from cleavage by CNBr of the fusion protein were separated onto a MonoS 5/5 column (Pharmacia). Eluent A, ammonium acetate 10 mM, pH 7.3. Eluent B, ammonium acetate 1.5 M, pH 7.3. Flow rate 0.8 ml/mim. The inset shows an SDS-PAGE (20% acrylamide) of fractions 1 and 2. Lane M, molecular mass markers (from top to bottom) 16.95, 14.4, 8.16 and 6.2 kDa. Lane T, natural DTx. Lane 1, protein from fraction 1; lane 2, from fraction 2. The samples were boiled for 5 min in sample buffer containing 2.5% SDS and 5% 2-mercaptoethanol and then incubated for 30 min with 10 mM iodoacetamide at 20°C before loading. The gel was stained with Coomassie blue.

already described in the venoms of the snakes of the mamba genus [5,6,33]. More than 70 peptides in the molecular weight range 6000–9000 Da have been recently reported in the venom of *Dendroaspis polylepis* [34].

### 3.5. *Biological properties of recombinant toxins*

In a first series of experiments, we investigated the capacity of recombinant DTxs to compete with radioactive tracers for binding to rat brain synaptosomal membranes. We used radioactive DTx or its homolog, DpI, as radioactive tracers, and found similar $K_i$ values for unlabelled DTx. Either tracer could therefore be used to probe the relative affinity of DTx and its mutants. As shown in Fig. 3, binding of these tracers to synaptosomes is fully inhibited by all recombinant DTxs. More precisely, Table 1 shows that DTx and rDTx which only differ by the nature of the residue at position 12 (aspartate in natural DTx and asparagine in rDTx) are characterized by similar $K_i$ values. This result indicates not only that the recombinant protein is biologically active but also that residue 12 is unlikely to be critical for the binding of DTx to potassium channels. rDTx-AAG was characterized by an approximately 4 fold increase in the $K_i$ value as compared to DTx. Such a variation in a $K_i$ value is too small to be indicative of the effective participation of the mutated residues to the establishment of the interactions between the protein and a specific partner as shown, for instance, in the case of an antibody–antigen complex [35]. More likely, such residues are likely to be localized in the vicinity of the interacting zone [36]. As a further illustration of the possible fluctuation of $K_i$ values, we found that changing the pyroglutamic residue at the N-terminus of rDTx and rDTx-AAG into a positively charged glutamine, as in rQDTx and rQDTx-AAG, induced a decrease and an increase in $K_i$ values, by factors of 5 and 2, respectively (see Table 1). The reason for such a fluctuation is unclear. Therefore, on the basis of competition experiments, we conclude that the three charges at positions 28–30, as well as the asparagine-12 or the N-terminus positive charge, are unlikely to be implicated in the contact area.

In a second step, the effect of DTxs and the mutants on twitch tension of indirectly stimulated chick biventer cervicis nerve muscle preparations were measured. All recombinant DTxs increased tension in a manner similar to that of the natural DTx (not shown). These results agree with the binding inhibition experiments and confirm that the lysine triplet is not essential for the binding to potassium channels. As a control, we checked that there was no change in postsynaptic sensitivity and therefore that the observed effect presumably resulted from blockage of neuronal potassium channels.

Finally, central neurotoxicity of natural and mutated DTxs were assayed by intracerebroventricular (i.c.v.) injections. Minimum lethal doses of DTx by i.c.v. injection have been reported in rat and chick [37,38] (2.5 ng and 19 ng/g of body weight, respectively) but not in mice. We found the $LD_{50}$ to be approximately equal to 5 ng/g, a value 5000 times lower than

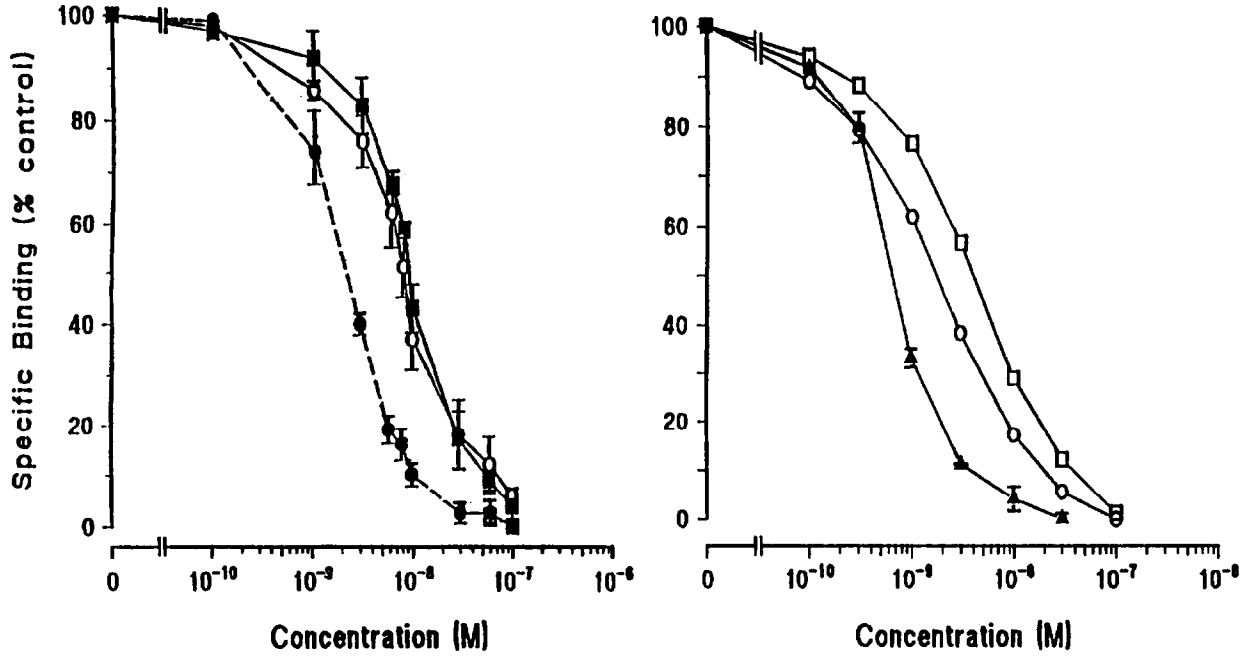


Fig. 3. Competition binding assays to rat brain synaptosomes. Competition between radioactive DTx (1 nM; the specific radioactivity varied from batch to batch between 100 and 2000 Ci/mmol) and various amounts of natural of recombinant DTxs. Left panel: native (■) and recombinant wild-type DTx (rDTx: ○ and rQDTx: ●). The radioactive tracer was [$^{125}$I]dendrotoxin I. Right panel: native (▲) and recombinant mutated DTx (rDTx-AAG, ○; and rQDTx-AAG, □). The radioactive tracer was [$^{125}$I]α-dendrotoxin. Points with error bars are the means ± S.E.M. for three experiments.

that obtained by the peripheral injection route [7]. As expected, the delay between the injection of the toxin into the brain and the appearance of the convulsive symptoms and the death was dose-dependent. Each mutant isoform was therefore injected at a dose equal to twice the $LD_{50}$ of DTx and the delays between i.c.v. injection and appearance of symptoms and death were determined. As shown in Table 2, rDTx-AAG and rQDTx-AAG were both lethal in mice but the onset of symptoms and time of death were slightly later than those observed with DTx and rDTx, and this difference agrees with that observed for $K_i$ values (Table 1). Such a relationship between the $K_i$ values and the minimal lethal doses or between the dose of toxin and the onset of symptoms and death have also been reported in rats by i.c.v. injection of other facilitatory toxins [37,38], and further confirms that the effects observed in vitro, even when they are minor, most probably reflect the more complex events that occur in vivo.

Table 2
Central toxicity of natural DTx, rDTx-AAG and rQDTx-AAG mutants

| Toxin | Onset of symptoms (min) | Death (min) |
|---|---|---|
| DTx | 2.5 ± 1 | 22.5 ± 1 |
| rDTx-AAG | 6.4 ± 2.7 | 39 ± 21 |
| rQDTx-AAG | 7.7 ± 1.5 | 62 ± 21 |

Toxicity after i.c.v. injection into female Swiss mice (lots of eight animals) of natural DTx (9.44 ± 0.81 ng/g injected into 22.9 ± 1.8 g mice), rDTx-AAg (9.08 ± 0.43 ng/g injected into 18.8 ± 0.9 g mice) and rQDTx-AAG mutant (9.07 ± 0.30 ng/g injected into 23.1 ± 0.3 g mice). Average time ± S.E.M. for the onset of symptoms and to death are reported.

## 4. Conclusion

Recombinant toxins and their associated large number of possible mutants constitute important tools to delineate the site by which toxins recognize their complex targets, i.e. receptors, ion channels, etc. [16,36,39–42]. Identification of interacting sites between various potassium channels and specific toxins like charybdotoxin [40–42], DpI [16] or α-dendrotoxin (this work) is now rapidly progressing. All residues of charybdotoxin, a 37 amino acids toxin from scorpion *Leiurus quinquestriatus quinquestriatus* that recognizes $Ca^{2+}$-activated $K^+$ channels, were submitted to individual mutagenesis and it was found that substantial affinity decreases occurred when mutations were made at 8 positions, among which 3 are occupied by positively charged residues, 3 by hydrophobic ones and 2 by residues with hydrogen bonding capacity [40,41]. Since DTx's compete with charybdotoxin in various assays, it was tempting to anticipate that DTx's may also involve positively charged residues. This was all the more an attractive idea as it was specifically suggested that the triplet Lys-28, Lys-29, Lys-30, may be associated with the specific action of the toxins [14–16]. Using recombinant DTx we provide clear evidence, however, that this triplet is unlikely to constitute a major element for the functional properties of DTx. As shown in Fig. 1, however, several other positively charged residues of DTx are possible candidates to establish a binding with $K^+$ channel. Production and characterization of new mutants is now in progress to identify the residues by which DTx establishes such contact. Clearly, comparison of the functional sites of DTx and charybdotoxin should greatly help us to understand the molecular basis for their functional similarities and differences and hence to design drugs with appropriate fine specificities.

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