A Venom-derived Neurotoxin, CsTx-1, from the Spider Cupiennius salei Exhibits Cytolytic Activities

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Background: CsTx-1, an ICK motif containing neurotoxin, acts as L-type Ca\textsuperscript{2+}-channel inhibitor.

Results: The partial α-helical C terminus of CsTx-1 exhibits cytolytic activity toward prokaryotic and eukaryotic cell membranes.

Conclusion: CsTx-1 is one peptide with different domains for ion channel inhibition and cytolytic activity.

Significance: Shown is an important new mechanism for the evolution of spider venomous peptides.

CsTx-1, the main neurotoxic active peptide in the venom of the spider Cupiennius salei, is composed of 74 amino acid residues, exhibits an inhibitory cysteine knot motif, and is further characterized by its highly cationic charged C terminus. Venom gland cDNA library analysis predicted a prepropeptide structure for CsTx-1 precursor. In the presence of trifluoroethanol, CsTx-1 and the long C-terminal part alone (CT1-long) exhibit an α-helical structure, as determined by CD measurements. CsTx-1 and CT1-long are insecticidal toward Drosophila flies and destroys Escherichia coli SBS 363 cells. CsTx-1 causes a stable and irreversible depolarization of insect larvae muscle cells and frog neuromuscular preparations, which seem to be receptor-independent. Furthermore, this membrane-lytic activity could be measured for Xenopus oocytes, in which CsTx-1 and CT1-long increase ion permeability non-specifically. These results support our assumption that the membrane-lytic activities of CsTx-1 are caused by its C-terminal tail, CT1-long. Together, CsTx-1 exhibits two different functions; as a neurotoxin it inhibits L-type Ca\textsuperscript{2+} channels, and as a membrane-lytic peptide it destroys a variety of prokaryotic and eukaryotic cell membranes. Such a dualism is discussed as an important new mechanism for the evolution of spider venomous peptides.

Spiders evolved some 300 million years ago (1). With currently 42,055 species, spiders represent the second most abundant group of terrestrial organisms after the insects (2). The majority of spiders rely on the potency of their venom for immediate prey immobilization or to repel aggressors. For fast-paralyzing or killing a prey item, spiders very successfully developed a variety of multicomponent venoms in which components usually act synergistically. It seems that araneomorph spiders have evolved a much greater variety of different substance combinations, which provide likewise immediate paralysis of prey than the ancient mygalomorph spiders. Additive interactions between different venom compounds of the same group or synergistic interactions between different venom compound groups, such as ions, low molecular mass compounds, enzymes, neurotoxins, small cationic peptides, and α-helical small cationic peptides, have been identified recently (for review, see Ref. 3).

The venom strategy of species in the wolf spider superfamily such as Lycosa singoriensis (4–6), Oxyopes takobius (7–9), and Cupiennius salei (3, 10–13) is based on synergistic interactions between low molecular mass compounds, neurotoxins, and α-helical small cationic peptides with cytolytic activities (3). Moreover, first results indicate that two different venomous functions can even be combined within one peptide. The spider Cheiracanthium punctorium, also from this superfamily, contains a large two-domain modular protein (CpTx-1a; 15.1 kDa) forming a putative amphipathic structure that exhibits a pronounced insecticidal and cytolytic effect. This protein is composed of two similar domains, both exhibiting the putative inhibitory cysteinestopeptide knot (ICK) motif and additional C-terminal putative α-helical parts (14).

CsTx-1 (ω-cetenin toxin-Cs1a (UniprotKB P81694)) represents the prevalent and most active neurotoxic peptide in the C. salei venom (10, 15). The peptide is composed of 74 amino acid residues, exhibits an inhibitory cysteine knot motif and additional C-terminal putative α-helical parts (14).

The abbreviations used are: ICK, inhibitory cysteine knot; CsTx-1, ω-cetenin toxin-Cs1a; CsTx-2b, ctenitoxin-Cs2b; TFE, 2,2,2-trifluoroethanol; SBS, souchier bactériologique de Saclay; NMDG, N-methyl-D-glucamine; contig, group of overlapping clones.
residues with an amidated C terminus and 4 disulfide bridges adopting the ICK motif. CsTx-1 blocks L-type Ca\(^{2+}\) channels in mammalian neurons at nanomolar concentrations. Furthermore, CsTx-1 produces a slow voltage-independent block of mid/low and high voltage-activated Ca\(^{2+}\) channels in cockroach neurons (16). Previous investigations showed that the loss of the highly positively charged C-terminal 13 amino acid residues, resulting in CsTx-2a (citenitoxin-Cs2a; Ser-1–Arg-61) or of the last 14 amino acid residues (CsTx-2b; citenitoxin-Cs2b; Ser-1–Phe-60) dramatically reduces its insecticidal activity (17). Nevertheless, the synthetic C-terminal cationic peptide (CT1-short, Gly-62–Lys-74) exhibits neither insecticidal nor bactericidal activity at up to millimolar concentrations (17). Obviously, 13–14 amino acid residues fragments are too short to expect membranolytic activities. However, the secondary structure prediction of the C-terminal last 30 amino acid residues of CT1-long (Gly-45–Lys-74) reveals a possible α-helical structure. This could indeed indicate that CsTx-1 is a peptide with two structurally different domains exerting two different biological functions. To explore the relationship of structure and function of CsTx-1 and its shorter variant CsTx-2a, transcriptomic investigations into possible polymorphisms, especially in its C-terminal part, are essential.

Here, we report on the cDNA assembly of CsTx-1 with respect to the peptide structure and its functional properties. Also, the effects of CsTx-1, CT1-long, CT1-short, and CsTx-2a have been investigated on different membrane systems and bioassays. Our results show that in addition to its published L-type Ca\(^{2+}\) channel blocking activity (16), CsTx-1 additionally exhibits cytolytic activity.

**EXPERIMENTAL PROCEDURES**

**Spider Maintenance, Venom Collection, and Peptide Purification**—Spider breeding, venom collection, and purification of CsTx-1 by reverse phase-HPLC in a four-step protocol were done as previously described (15). CsTx-2a was obtained by digesting CsTx-1 with coagulation factor Xa as reported (17). CT1-short and CT1-long were synthesized using Fmoc (N-(9-fluorenyl)methoxycarbonyl) solid phase chemistry and were purified by GeneCust (Laboratoire de Biotechnologie du Luxembourg S.A.). The concentrations of CsTx-1, CsTx-2a, CT1-long, and CT1-short were determined in duplicate by amino acid analysis.

cDNA Library of Venom Glands of C. salei—From 20 adult female spiders, venom glands were prepared after milking at different time intervals (24, 48, and 62 h and 8 and 14 days), stored in RNAlater (Qiagen), and sent on dry ice to SKULDTECH (Montferrent, France) to generate the cDNA library by 454 sequencing. CsTx-1 was identified in the venom gland cDNA library (202,877 ESTs; 34,107 consensus sequences; 98% assembly) using the SKULDTECH generated data base screening with BLASTp and analysis of the cDNA sequences.

**Circular Dichroism (CD) Measurements**—For CD measurements, samples (40 μM) were dissolved in a 5 mM Na\(_2\)HPO\(_4\)/NaH\(_2\)PO\(_4\), pH 7.2, and 150 mM NaF or in same buffer containing 50% (v/v) 2,2,2-trifluoroethanol (TFE). Measurements were performed with a Jasco J-715 spectropolarimeter in a Suprasil R 110-QS 0.1-cm quartz cell (Hellma Analytics) in the range of 178–260 nm at 20 °C. Three independent measurements were recorded per sample, and each spectrum was the average of three scans to improve the signal-to-noise ratio. All spectra were corrected for buffer or buffer/TFE blank measurements. Secondary structure content was deconvoluted using Dichroweb server, applying the analysis program CDSSTR and reference set 1 (18–20).

**Insecticidal Activity**—Drosophila melanogaster were used to determine the insecticidal activity of CT1-short and CT1-long. Four different peptide concentrations of CT1-short between 200 and 500 pmol/mg fly (injected in a total volume of 0.05 μl of insect ringer) and four different peptide concentrations of CT1-long between 36 and 130 pmol/mg fly were tested on each of 20 flies, and 20 flies were used as controls (0.05 μl of insect ringer only). Calculations of the lethal doses LD\(_{50}\) (50% of the test flies died of intoxication 24 h post injection) were performed as described elsewhere (17).

**Antimicrobial Activity**—Antimicrobial activity of CsTx-1, CsTx-2a, CT1-long, and CT1-short against Escherichia coli ATCC 25922, E. coli souchier bactériologique de Saclay (SBS) 363, Staphylococcus aureus ATCC 29213, and Trypanosoma brucei brucei MiTat1.2 (221) were determined as described in Kuhn-Nentwig et al. (21).

**Insect (Calliphora vicina) and Frog (Rana temporaria) Neuro-muscular Preparations and Electrophysiological Experiments**—Late third stage larvae of C. vicina (22, 23) were used in all experiments. After dissection, the internal organs and the ventral ganglion were removed so that the preparation consisted only of muscles attached to the cuticle. The segmental nerves were stimulated through the suction electrode. Recordings of the resting membrane potential were made by glass intracellular microelectrodes from ventral longitudinal fibers. The resting membrane potential of muscle fibers was measured in several cells in control and after 30 and 60 min of continuous perfusion with saline at room temperature (22 °C). Saline was composed of 172 mM NaCl, 2.5 mM KCl, 0.6 mM CaCl\(_2\), 4 mM MgCl\(_2\), 5 mM HEPES, pH 7.2. Different concentrations of CsTx-1 and albumin (0.01%, Sigma) were added to the bath. By nerve stimulation, excitatory postsynaptic currents were evoked and recorded by a conventional two-electrode voltage clamp (Axoclamp-2B amplifier, Axon Instruments), and the data were filtered at 2 kHz.

To investigate the ionic nature of the current induced by CsTx-1 on C. vicina muscle fibers, the cells were clamped by conventional two electrode method at −70 mV. Three series of experiments were performed: (i) in saline (172 mM NaCl); (ii) 95% of Na\(^+\) substituted by sucrose; (iii) 95% of Na\(^+\) substituted by N-methyl-d-glucamine (NMDG) chloride. Changes in holding current and input resistance were simultaneously recorded before and up to 30 min after application of 100 nM CsTx-1. Periodically (approximately, each 5 min) a value of membrane potential by temporal reduction of current to zero level was estimated. The glass microelectrodes were filled with KCl and had a resistance of 10–15 megaohms.

Frog muscle (musculus sartorius) preparations of R. tempora-raria were placed into a 1.5-ml plastic chamber and super-fused with saline at 22 °C. Saline was composed of 117 mM NaCl, 2.5 mM KCl, 0.6 mM CaCl\(_2\), 4 mM MgCl\(_2\), 5 mM HEPES, pH 7.2.
Different concentrations of CsTx-1 and albumin (0.01%, Sigma) were added to the bath.

Frog (Xenopus laevis) Oocyte Preparations and Electrophysiological Experiments—Female X. laevis were kept under a 12-h day/night cycle. The animals were anesthetized by immersion until loss of all reflexes (~10–15 min) in prechilled water containing 0.2% ethyl 3-aminobenzoate methane sulfate (A5040; Sigma). The female frogs were then laid on wet tissues placed on an ice bed (ventral face up) and kept wet by covering the animal with soaked tissue. The nose of the animal was exposed to air to enable breathing. Through a small abdominal incision an inner diameter of 0.4 mm from the surface of the oocyte (25). Perfusion was stopped for 5 min to perform electrophysiological experiments on oocytes exposed to the toxin. 100 μl of a toxin were applied directly to the bath (volume 200 μl).

RESULTS

cDNA Structure of CsTx-1—Scanning our venom gland cDNA library, we analyzed several contigs to elucidate the complete cDNA sequence encoding CsTx-1. The cDNA sequence starts with a 5′-UTR of 71 bps followed by an ORF of 369 bps and a 3′-UTR of 102 bps. The predicted polypeptide consists of the signal peptide comprising 20 amino acid residues followed by an acidic prosequence of 27 amino acid residues, the premature peptide of 75 amino acid residues, and the stop codon.

Three different posttranslational processing steps are involved in the maturation process of CsTx-1: 1) cleavage of the signal peptide, 2) limited proteolysis of the acidic propeptide at the processing quadruplet motif (PQM: 44EQAR47) according to the EtoR rule (26), and 3) additionally, a C-terminal amidation taking place in which Gly-75 is removed, and Lys-74 is simultaneously amidated (27) (Fig. 1). Remarkably, the codons encoding the different amino acid residues of the mature peptide CsTx-1 are highly conserved. Screening 782 EST sequences encoding the mature CsTx-1 and focusing on the C-terminal part, two silent mutations by substitution in the third codon position encoding mature CsTx-1 and concentrating on the C-terminal part, two silent mutations by substitution in the third codon position encoding mature CsTx-1 and 3) additionally, a C-terminal amidation taking place in which Gly-75 is removed, and Lys-74 is simultaneously amidated (27) (Fig. 1). Remarkably, the codons encoding the different amino acid residues of the mature peptide CsTx-1 are highly conserved. Screening 782 EST sequences encoding the mature CsTx-1 and focusing on the C-terminal part, two silent mutations by substitution in the third codon position encoding mature CsTx-1 and focusing on the C-terminal part, two silent mutations by substitution in the third codon position, the CD spectra of CsTx-1 were recorded in sodium phosphate buffer adopting mainly a β-sheet, β-turn,
and unordered conformation (Fig. 2, Table 1). These findings are consistent with the secondary structure of ICK motif-containing peptides (14). The addition of TFE induces pronounced spectral changes of CsTx-1. In TFE solution the peptides are considered to adopt α-helical structures, and the TFE-induced helicity of the peptides is a measure of their helix propensity (29). The α-helical structure content of the peptide increases from 2 to 42% with a simultaneous decrease of the β-sheet from 38 to 19% and unordered structure content from 40 to 18%. Only a minor increase of the α-helical structure with simultaneously minor transformations of the β-sheet, β-turn, and unordered structure content is visible in CsTx-2a after TFE addition (Fig. 2, Table 1).

The prediction of α-helical structures (ExPASy (30)) for CsTx-1 resulted in the identification of a putative α-helical segment (Ala-52 to Lys-65) in the C-terminal cysteine-free part of CsTx-1 (Fig. 3a). As expected, CT1-short exhibits a non-α-helical conformation even in the presence of TFE (Fig. 2, Table 1). CD measurements of CT1-long in PBS buffer suggest a non-α-helical structure (Fig. 2, Table 1). However, the addition of TFE resulted in a high α-helical conformation of CT1-long (66%) and simultaneously decreases of the β-sheet from 28 to 16% and unordered structure content from 48 to 11% (Fig. 2, Table 1).

Insecticidal Activity of CsTx-1, CsTx-2a, CsTx-2b, CT1-short, and CT1-long—Truncation of the last 13 C-terminal amino acids of CsTx-1 (CsTx-2a) decreases its insecticidal activity about 7-fold, and a further truncation of Arg-61 (CsTx-2b) provokes an activity loss of about 190-fold (17). CT1-short is not insecticidal up to a concentration of 500 pmol/mg fly. Remarkably, CT1-long exhibits an insecticidal activity with an LD₅₀ of 82.64 pmol/mg fly (Table 2).

Antimicrobial Activity of CsTx-1, CT1-short, and CT1-long—No bactericidal activity of CsTx-1 (250 μM), CT1-long (149 μM), and CT1-short (250 μM) against E. coli (ATCC 25922) and S. aureus (ATCC 29213) was observable. Nevertheless, CT1-long (149 μM) reduced the growth of S. aureus 4-fold when compared with the bacterial control group without peptide. Surprisingly, CsTx-1 destroys the E. coli mutant SBS 363 in a concentration of 31.25 μM and CT1-long in one-third of this concentration. Furthermore, CT1-long exhibits a trypanocidal activity in a concentration of 5 μM. CT1-short is, up to a concentration of 250 μM, neither bactericidal nor trypanocidal.

Effects of CsTx-1 on Calliphora and Frog Neuromuscular Preparations—Spontaneous and nerve evoked postsynaptic currents of C. vicina late third stage larvae were unaffected by CsTx-1 at concentrations between 50 and 200 nM. Depolarizing effects of CsTx-1 on C. vicina larvae and frog neuromuscular preparations were investigated at 50–900 nM. Fly muscle fibers were depolarized at 100 nM, whereas frog muscle fibers exhibit this effect only in a 3-fold higher concentration (300 nM) of the peptide. The drop of the resting membrane potential for both types of muscle fibers was irreversible and could not be removed by long-lasting washing (30–60 min) (Table 3). In the presence of 300 nM CsTx-1, the depolarization of fly muscle is about 33% and was accompanied with muscle contractions that ceased at a very low (~30 mV) membrane potential.

Furthermore, three different series of experiments under voltage clamp conditions were performed to elucidate the effects of CsTx-1 (100 nM) on fly muscle cells: (i) in saline (172 mM NaCl), (ii) where 95% of Na⁺ was substituted by sucrose, and (iii) where 95% of Na⁺ was substituted by NMDG, which is known to block a high diversity of Na⁺, K⁺, Ca²⁺, and other ion channels (31). In the presence of 172 mM NaCl an increasing inward current, a decreasing cell input resistance (Fig. 4a), and a strong depolarization were observed after application of CsTx-1 (Fig. 4b). Increasing the Na⁺ concentration to 277 mM did not intensify the depolarizing effect of CsTx-1. However, a 10-fold elevation of Ca²⁺ from 0.6 to 6 mM in the bathing...
solution substantially damped the depolarizing effect of CsTx-1. Interestingly, an unspecific blockade of Ca\(^{2+}\) channels by 5 mM Co\(^{2+}\) diminished the depolarizing effect of CsTx-1 (Fig. 4d). After replacement of Na\(^+\) (172 mM) with sucrose the depolarizing effect was very small. In contrast, CsTx-1 induced a strong depolarization in the presence of NMDG alone (Fig. 4B). A clear drop of the cell input resistance was observed in the presence of Na\(^+\) or NMDG alone when compared with the input resistance in the presence of sucrose (Fig. 4c).

Effects of CsTx-1 on Xenopus Oocyte Plasma Membranes—We investigated the possible effects of these peptides on the permeability of Xenopus oocytes. The membrane potential was maintained at −80 mV, and the oocytes were exposed to different concentrations of CsTx-1. Submicromolar concentrations (0.05−0.5 μM) induce ion currents amounting to 8−32 μA (Fig. 5a). The current showed a variability of up to 10-fold in amplitude and often a lag phase of 10−60 s upon exposure to CsTx-1. Furthermore, we analyzed the effect of pH and divalent cations on the membrane permeability induced by CsTx-1 (0.5 μM).

### TABLE 1

Estimation of secondary structure of CsTx-1, CsTx-2a, CT1-long, and CT1-short by circular dichroism

| Peptides in solution | Conditions | α-Helix | β-Sheet | Turns | Unordered | Total | NRMSD* |
|----------------------|------------|---------|---------|-------|-----------|-------|--------|
| CsTx-1               | PBS        | 2       | 38      | 18    | 40        | 98    | 0.083  |
| CsTx-1               | TFE        | 42      | 19      | 21    | 18        | 99    | 0.022  |
| CsTx-2a              | PBS        | 2       | 41      | 19    | 37        | 98    | 0.086  |
| CsTx-2a              | TFE        | 8       | 36      | 22    | 35        | 100   | 0.074  |
| CT1-long             | PBS        | 1       | 28      | 23    | 48        | 99    | 0.019  |
| CT1-long             | TFE        | 66      | 16      | 7     | 11        | 100   | 0.005  |
| CT1-short            | PBS        | −2      | 30      | 21    | 49        | 97    | 0.010  |
| CT1-short            | TFE        | 2       | 31      | 22    | 45        | 99    | 0.023  |

| * Normalized root mean square deviation, calculated by DICHROWEB server /CDSSTR, reference set 1 (19, 20).
| ^ PBS, 5 mM sodium phosphate, 150 mM sodium fluoride, pH 7.2.
| ^ TFE, 5 mM sodium phosphate, 150 mM sodium fluoride, pH 7.2, 50% trifluoroethanol.

### TABLE 2

Biological activities of CsTx-1, CsTx-2a, CsTx-2b, CT1-long, and CT1-short

| Peptide       | LD\(_{50}\) Drosophila | EC\(_{50}\) T. brucei brucei MiTat1.2(221)* | E. coli SBS 363b | S. aureus ATCC 29213^ |
|---------------|------------------------|------------------------------------------|-----------------|---------------------|
| CsTx-1        | 0.35                   | ND                                       | 15.63–31.25     | > 250               |
| CsTx-2a       | 2.58                   | ND                                       | ND              | ND                  |
| CsTx-2b       | 66.51                  | ND                                       | 4.66–9.32       | > 149^              |
| CT1-long      | 82.64                  | 5.01                                     | > 250           | > 250               |
| CT1-short     | > 500                  | > 40                                     | 0.313–0.625     | 0.157–0.313         |

* 1 × 10^7 cells/ml.
^ 6.9 × 10^7 cfu/ml.
^ 2.7 × 10^7 cfu/ml.
^ The growth of S. aureus is 4-fold reduced when compared with the control group without peptide.
^ Ref. 45.
^ Ref. 21.
Decreasing pH 7.4 to 6.4 was without significant effect. In contrast, at pH 8.4 the conductance induced by CsTx-1 amounted to only ~30% of that at pH 7.4.

To exclude a contribution of the endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) channel to the conductance increase, experiments in Ca\(^{2+}\)-free medium (Online resource 1, M6) were performed. It should be noted that the concentration of Ca\(^{2+}\) in the medium is crucial for the size of the induced permeability increase (Table 4). Decreasing the Ca\(^{2+}\) concentration from 1 mM (Online resource 1, M1) to below 10\(^{-9}\) M (Online resource 1, M6) resulted in an about 5–10-fold enhancement of the permeability increase induced by CsTx-1 despite the presence of 5 mM divalent cation Mg\(^{2+}\). In a medium containing 40 mM divalent cation Ba\(^{2+}\) (Online resource 1, M5), 0.5 \(\mu\)M CsTx-1 failed to increase the membrane permeability.

To determine the relative permeability of different ions, current induced by continuous voltage ramps from ~80 to +80 mV were monitored in the absence and presence of 0.5 \(\mu\)M CsTx-1 (Fig. 6). Such experiments were repeated in media of different ion compositions (not shown), and reversal potentials (\(E_r\)) were determined (Table 4). From these values, relative ion permeabilities were determined using the Goldman-Hodgking-Katz voltage equation (32). The following relative permeabilities were found: Cl\(^{-}\) (1) > K\(^{+}\) (0.8) > Na\(^{+}\) (0.7) > choline\(^{+}\) (0.6) > methansulfonate\(^{-}\) (0.2); small anions are preferred to cations.

Identification of the Domain of CsTx-1 Responsible for the Permeability Increase—Several fragments of CsTx-1 were used for this purpose. Applying CsTx-2a and CT1-short alone at a concentration of 0.5 \(\mu\)M or 5 \(\mu\)M to oocytes did not induce a permeability increase. Additionally, a combination of CsTx-2a and CT1-short at a concentration of 1 or 5 \(\mu\)M did not increase the oocyte membrane permeability. Remarkably, 5 \(\mu\)M CT1-long induced an increase in membrane permeability (Fig. 5b).

**DISCUSSION**

**Insecticidal and Antimicrobial Activity of CsTx-1 and CT1-long**—The inhibitory activity of CsTx-1 toward L-type Ca\(^{2+}\) channels in mammalian neurons as well as on mid/lows and high voltage-activated Ca\(^{2+}\) channels in cockroach neurons clearly defines the neurotoxic activity of CsTx-1 (16). This insecticidal activity is strongly dependent on the intact structure of CsTx-1 (Table 2), and the last 14 or 13 C-terminal amino acids (CT1-short) have been postulated to be important for the toxicity (10, 17). The cationic C-terminal part of CsTx-1 could act as an anchor, and the inhibition of ion channels could be the result of a direct contact of the ICK-containing structure of CsTx-1 with the target ion channel. In the same way an interaction of CsTx-1 with the ion channel surrounding lipid layer is also thinkable. Such a case could be shown for GsMTx4, a specific inhibitor for pro- and eukaryotic stretch-activated mechanosensitive channels acting via bilayer tension (33, 34). The neurotoxic activity of the ICK structure of CsTx-1 is then further synergistically assisted by the pore-forming activity of the peptide C-terminal \(\alpha\)-helical part.

Several biological activities of CT1-long support the proposed combined acting mechanism. The insecticidal activity of CT1-long and CsTx-2a are comparable, whereas CT1-long is only about 3-fold less active when compared with the cytolytic peptide cupiinnin 1a (Table 2). Especially for CsTx-1 and CT1-long, the bactericidal activity depends strongly on the lipopolysaccharide (LPS) chain length that is connected to the outer membrane of Gram-negative bacteria. In contrast to E. coli ATCC 22592, which was not susceptible below 250 \(\mu\)M toward CsTx-1 and CT1-long, the E. coli mutant SBS 363 exhibits a high susceptibility. CsTx-1 was only 3-fold less bactericidal than CT1-long. Access to the negatively charged phospholipids of the outer membrane is more pronounced toward shorter LPS chains in the case of E. coli SBS 363 (35). When compared with the bactericidal activity of cupiinnin 1a, CT1-long is 15-fold less active, and CsTx-1 is 50-fold less active. Differences in the activity toward Gram-negative and Gram-positive bacteria may reflect different access to negatively charged membrane structures due to peptide size and its amphipathic domain.

**Target Specific or Broad Cytolytic Effects on Excitable Membrane Systems?**—CsTx-1 causes irreversible and concentration-dependent depolarization of fly larvae or frog muscle fibers, resulting in fly larvae muscle contractions and subsequent damage of the fibers. However, spontaneous and nerve-evoked postsynaptic currents of fly larvae muscle fibers were unaffected. To elucidate more in detail of a proposed membranolytic effect of CsTx-1, voltage clamp experiments revealed that after CsTx-1 application, the transmembrane current increased with a simultaneous drop of the cell input resistance that was also measured when Na\(^{+}\) was substituted by NMDG. In contrast, when Na\(^{+}\) was substituted by sucrose, no depolarization was measured. Thus, we have reliable evidence that CsTx-1 increases unspecifically the permeability of a membrane for ions because the rather large organic cation NMDG becomes able to enter a cell. These findings are similar to the results of Vassilevski et al. (14) concerning Cpt-1x-1, which also increased the membrane permeability of frog muscle fibers in a comparable manner.

A reduced depolarization effect caused by increasing Ca\(^{2+}\) or Co\(^{2+}\) ion concentrations may be explained by occupying negatively charged membrane structures that prevent an attraction of the cationic C terminus of CsTx-1 and possibly the induction of the \(\alpha\)-helix. Thus, positively charged divalent cations seem to protect the membrane from the toxin.

**Function of the C-Terminal \(\alpha\)-Helical Part of CsTx-1**—Similar as shown above for excitable membranes, CsTx-1 also...
increases the permeability of Xenopus oocyte plasma membranes. No permeability increase was detected when administering CsTx-2a, CT1-short, or the combination of CsTx-2a and CT1-short. This confirmed previously performed insect bioassays which clearly demonstrated that CT1-short has to be covalently linked to CsTx-2a to cause toxicity (17). Remarkably, CT1-long alone induces membrane permeability even though about a 10-fold higher concentration than CsTx-1 is needed. These results and the above mentioned CD measurements of CsTx-1, CsTx-2a, CT1-long, and CT1-short confirm our hypothesis that without the last 13 C-terminal cationic amino acids no helix formation is possible. Depending on membrane access and structure, CsTx-1 seems to be

![Graphs and Tables]
more successful in increasing the membrane permeability of oocyte membranes, whereas CT1-long is more successful in E. coli SBS 363.

To exclude a contribution of the endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) channel to conductance increase, experiments in Ca\(^{2+}\)-free medium (Online resource 1, M6) were performed. Under Ca\(^{2+}\)-free conditions, this channel is not activated. Interestingly, the permeability increase was even larger in this medium as compared with the medium containing 1 mM Ca\(^{2+}\). In medium containing a large concentration of the divalent cation Ba\(^{2+}\) (Online resource 1, M5), the effect of CsTx-1 was completely blocked, which is similar to the findings described for fly larvae muscle fibers. An exception was Mg\(^{2+}\) that was present at 5 mM in the Ca\(^{2+}\)-free medium. The permeability increase for monovalent ions induced by CsTx-1 has relatively low ion selectivity, but small anions are preferred over cations.

Secondary Structure of the C-Terminal α-Helical Part of CsTx-1—Secondary structure predictions (ExPASy (30)) reveal an α-helical structure for the C-terminal part of CsTx-1 from Ala-52 to Lys-65 (Fig. 3, a and c). The adjoining highly cationic section seems to be a more random coiled structure. Likewise, we could show by CD measurements that α-helical structures are formed in CsTx-1 and CT1-long after the addition of 50% TFE. In contrast, no α-helical structures were detectable in CsTx-2a and CT1-short after administration of 50% TFE (Fig. 2), which shows the important role of the Gly-62 to Lys-69 segment in helix formation induction of CsTx-1 (Fig. 3, b and c). These results point to a dual role for the cationic C terminus of CsTx-1; first, the attraction of CsTx-1 at negatively charged membranes by the cluster of Lys-67, -68, -69, -71, -72, and -74, and second, simultaneously inducing the formation of an α-helical structure. The hydrophobic face that builds an amphipathic structure is defined mainly by the α-helical structure derived from Met-48, Gly-49, Ala-52, Ile-53, Gly-56, Leu-57, Ile-59, Phe-60, Leu-63, and Phe-64 (Fig. 3, b and c) as predicted by HELIQUEST (36).

Structurally Similar Venomous Peptides—BLASTn and BLASTp results as well as ClustalW 2.1 sequence alignments of CsTx-1 exhibit only for CsTx-9, a further neurotoxically acting peptide from C. salei with 52% sequence similarity (10). Remarkably, the toxin-like structure LsTx-A53 (UniprotKB B6DCP2), identified in a cDNA library of L. singoriensis (6), exhibits also 53% sequence similarity. However, both peptides do not possess such a highly cationic C-terminal part as CsTx-1.

CpTx-1a, a large two-domain modular protein (15.1 kDa (UniprotKB D5GSJ8)) is composed of two similar modules, both exhibiting the putative ICK motif and an additional C-terminal putative α-helical part (14). The second module of this peptide (amino acid residues 65–134) exhibits similarity of only 37% with CsTx-1 (Fig. 3a). Nevertheless, the protein exhibits a secondary structure and insecticidal and cytolytic properties comparable with CsTx-1.

Although we know only few examples of modular or two-domain-containing neurotoxic acting peptides from spider venoms (14, 37, 38), they were also found in some scorpion venoms (39, 40). Scorpine, isolated from the venom of Pandinus imperator, exhibits an α-helical N-terminal domain and a cysteine-stabilized α/β motif located in the C-terminal part. The N-terminal part itself exhibits antimicrobial activity as verified for a synthetic peptide based on this sequence (40). The multifunctional family of the β-KTx polypeptides identified in venoms from different scorpions are, furthermore, such two-domain peptides. They consist of 45–68 amino acids and contain three disulfide bridges. The putative α-helical N-terminal part is followed by the C-terminal region, which is structured according to the cysteine-stabilized αβ motif (41). Different members of this family exhibit both activities: cytolytic in the N-terminal part and K\(_{\text{V}}\)-channel blocking in the C-terminal part (41, 42).

Conclusions—The discovery of cytolytic activity and its localization in the C-terminal part of CsTx-1 in addition to its L-type Ca\(^{2+}\) channel inhibitory effect highlights the evolutionary trend to combine two venomous functions in one compound: ion channel inhibitor and membranolytic activity. This trend is not new or restricted to spiders, as the older arachnid group of scorpions also give some examples as previously assumed (41–43). The strategy of spiders to combine different venom compounds to enhance synergistically the toxicity of single compounds is evolutionarily optimized in the case of CsTx-1 and CpTx-1a (3, 14) with a proposed synergistic interaction even within one peptide. Such mechanisms probably enable spiders to subdue a broader range of prey even if some of them do not express specific ion channels that are targeted by these spider neurotoxins. At the same time this mechanism will impede the development of resistance to a single venom compound. If the combination of two venomous functions in one compound is an evolutionary fascinating strategy, one may ask why no more examples are known. This may be due to the still limited knowledge of spider toxins and their functions, so we encourage...
focusing specifically on such dual function peptides in the future research.

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