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Calitoxin, a Neurotoxic Peptide from the Sea Anemone *Calliactis parasitica*: Amino Acid Sequence and Electrophysiological Properties†

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ABSTRACT: We have isolated a new toxin, calitoxin (CLX), from the sea anemone *Calliactis parasitica* whose amino acid sequence differs greatly from that of other sea anemone toxins. The polypeptide chain contains 46 amino acid residues, with a molecular mass of 4886 Da and an isoelectric point at pH 5.4. The amino acid sequence determined by Edman degradation of the reduced, S-carboxymethylated polypeptide chain and tryptic and chymotryptic peptides is Ile-Glu-Cys-Lys-Cys-Glu-Gly-Asp-Ala-Pro-Asp-Leu-Ser-His-Met-Thr-Gly-Thr-Val-Tyr-Phe-Ser-Cys-Lys-Gly-Gly-Asp-Gly-Ser-Trp-Ser-Lys-Cys-Asn-Thr-Tyr-Thr-Ala-Val-Ala-Asp-Cys-Cys-His-Glu-Ala. No cysteine residues were present in the peptide. Similarly to other sea anemone toxins, calitoxin interacts, in crustacean nerve muscle preparations, with axonal and not with muscle membranes, inducing a massive release of neurotransmitter that causes a strong muscle contraction. The low homology of CLX with RP II and ATX II toxins has implications regarding the role played by particular amino acid residues.

The sea anemone *Calliactis parasitica* or *Calliactis tricolor* is a coelenterate normally present along the European coasts of the Atlantic Ocean. It grows on shells of the gastropod mollusc *Buccinum undatum* that are occupied by hermit crabs (Paguridae). In the Mediterranean Sea, *Calliactis parasitica* lives in symbiosis with the crab *Pagurus bernhardus* or *Pagurus policaris*.

The advantages postulated for anemone-bearing pagurids are camouflage, assistance in capturing prey, protection from

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predators, and shell reinforcement (McLean & Mariscal, 1973). In a study of the protection afforded by *Calliactis* in the predation of hermit crabs by *Octopus*, Ross (1971) found that all crabs without the sea anemone were rapidly ingested by *Octopus*, while those with *Calliactis* were spared. In addition, *Octopus* that approached a *Calliactis* reacted as though they had received a punishing stimulus.

We report the primary structure of a neurotoxic peptide, which we have called "calitoxin" (CLX),[1] purified from the nematocysts of *Calliactis*. We suggest that CLX plays a protective role in the *Calliactis*/hermit crab symbiosis.

Electrophysiological studies indicate that CLX induces spontaneous action potentials in crustacean motor axons that result in a massive transmitter release at the synaptic terminals. Despite differences in amino acid sequence, the observed effects are similar to those reported for other anemone toxins (Schweitz et al., 1985).

Experimental Procedures

Materials. Sequence-grade trypsin and carboxypeptidases A and B were from Boehringer Mannheim; chymotrypsin, carboxypeptidase P, tetrodotoxin (TTX), and Tween 20 were from Sigma; reagents for amino acid analyses and for sequence determination were from Pierce; hexafluoroacetone was from Merck.

Assay for Toxicity. Samples of crude extract, column fractions, and the purified toxin were lyophilized, dissolved in distilled water, and tested for activity by injecting 0.1 mL into the hemocoel of the crab *Carcinus mediterraneus* at the base of one of the walking legs. The toxic unit corresponded to the minimum amount of toxin causing paralysis within 1 min from injection in a 10-g crab.

Calitoxin Purification. The sea anemones, *Calliactis parasitica*, collected in the Bay of Naples, were stirred for 1 h at room temperature in distilled water (1:1 w:v). The resulting suspension was centrifuged at 18000 rpm for 1 h with a SS 34 Sorvall rotor. The pellet was treated as before until no activity was present in the supernatant. The combined supernatants, diluted with distilled water to 4 $m\Omega^{-1}$ and adjusted to pH 8.8, were chromatographed on a column of QAE-Sephadex A-25 equilibrated with 0.01 M Tris-HCl, pH 7.8, containing 0.06 M NaCl. The toxic activity was eluted with 0.2 M ammonium acetate, pH 5.0. After lyophilization, the toxic material was further purified by gel filtration on a column of Sephadex G-50 superfine equilibrated with 0.1 M ammonium acetate, pH 5.0, and eluted with the same buffer. The active fractions were combined and fractionated further by RP-HPLC on a Beckman instrument using a semipreparative column (1 × 25 cm, 5-μm particle size, ODS). The elution was performed with a 20-min linear gradient (from 5 to 60% of solvent B) made up by solvent A (0.1% TFA) and solvent B (acetonitrile with 0.1% TFA) at a flow rate of 3 mL/min. The homogeneity of CLX was ascertained by way of analytical RP-HPLC using the same elution system, and by chromatofocusing.

Protein concentration was determined according to Lowry et al. (1951).

Isoelectric Point Determination. The isoelectric point of CLX was determined by using a Mono-P HR 5/20 column (Pharmacia Fine Chemicals). The column was equilibrated with 0.025 M Tris-HCl, pH 7.1, and eluted at a flow rate of 1 mL/min with Polybuffer 74, pH 4.0, according to the manufacturer's specifications. The pH of the fractions was measured with a Beckman Model 3500 pH meter.

Amino Acid Analyses. Purified toxin was hydrolyzed in vacuo at 110 °C for 20 and 70 h with 6 N HCl, while peptides derived from proteolytic cleavages were hydrolyzed in vacuo for 20 h. After the removal of HCl over NaOH in vacuo, the hydrolyzates were filtered and analyzed on a Beckman Model 119 CL or on an LKB Model 4400 amino acid analyzer (Stein & Moore, 1963). For tryptophan determination, the samples were hydrolyzed with 50 μL of 3 N mercaptoethanesulfonic acid (Penke et al., 1974). The hydrolyzates were brought to pH 2.2 by adding 2 volumes of 2 N NaOH and analyzed on the Beckman 119 CL, using 0.35 M sodium citrate, pH 5.25, as eluting buffer, at a column temperature of 75 °C. For (carboxymethyl)cysteine (CMCys) determination, samples were hydrolyzed either with 3 N MESNA or with 6 N HCl in the presence of 0.05% thioglycolic acid. The amino acid analyses were performed on the Beckman analyzer, and for optimal separation of CMCys, the pH of the first buffer was lowered to 3.15.

Analysis for Free SH. The spectrophotometric determination of thiol groups was carried out in 0.1 M Tris-HCl, pH 8.5, containing 6 M guanidine hydrochloride, with Nbs_2 as a reagent (Riddles et al., 1979). Free sulfhydryls were also determined by performing a carboxymethylation reaction in the absence of reducing agent. The toxin (10 nmol) was added to 100 μL of 0.1 M Tris-HCl, pH 8.5, containing 2 mM Na_2EDTA, 6 M guanidine hydrochloride, and a 20-fold molar excess of iodoacetamide over the total $^1/_2$-Cys content. The mixture was incubated for 60 min, after which the toxin was desalted by RP-HPLC, hydrolyzed, and analyzed for CMCys.

S-Carboxymethylation of Calitoxin. Fully reduced and S-carboxymethylated CLX was prepared by treatment with a 5-fold molar excess of 1,4-dithiothreitol over the total $^1/_2$-Cys content, in 0.1 M Tris-HCl, pH 8.5, containing 2 mM Na_2-EDTA and 6 M guanidine hydrochloride. After incubation for 4 h at 37 °C, the toxin was further treated with a 15-fold molar excess of iodoacetic acid for 45 min at room temperature, in the dark. Desalting was done by RP-HPLC.

Enzymic Digestions. Samples of reduced and S-carboxymethylated CLX were incubated with trypsin and chymotrypsin at a final enzyme:substrate ratio of 1:100 (w/w) in 1% NH_4HCO_3 for 4 h at 37 °C.

S-Carboxymethylated CLX was incubated with CPA, prepared according to Seizinger et al. (1985) at a final enzyme:substrate ratio of 1:50 (w/w) in 1% NH_4CO_3.

Digestions with CPP were performed for 6 h and overnight in 50 mM sodium acetate, pH 4, containing 0.2 mg/mL Tween 20 at a final enzyme:substrate ratio of 1:20 (w/w). Free amino acids were extracted in 5 volumes of acetone/0.2 N HCl (3:1 v/v) and analyzed.

Time course digestions of peptides with CPB and/or CPA were performed as already described (Jones, 1986).

Peptide Separation. Peptide separation and desalting of modified CLX were carried out by RP-HPLC with a Beckman instrument, using an analytical column (0.5 × 25 cm; 5-μm particle size; ODS). The elution was performed at a flow rate of 1 mL/min with a linear gradient of acetonitrile containing 0.1% TFA. The column was equilibrated at 5% acetonitrile/0.1% TFA, and the elution was obtained by raising the

[1] Abbreviations: Asp(OMe), O^4-methyl ester of aspartic acid; CLX, calitoxin; CPA, carboxypeptidase A; CPB, carboxypeptidase B; CPP, carboxypeptidase P; CMCys, (carboxymethyl)cysteine; CMCys(OMe), O^6-methyl ester of (carboxymethyl)cysteine; Na_2EDTA, disodium ethylenediaminetetraacetate; EJP, excitatory junction potential; Glu(OMe), O^5-methyl ester of glutamic acid; MESNA, mercaptoethanesulfonic acid; Nbs_2, 5,5'-dithiobis(2-nitrobenzoic acid); PTH, phenylthiohydantoin; RP-HPLC, reversed-phase high-pressure liquid chromatography; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TTX, tetrodotoxin.

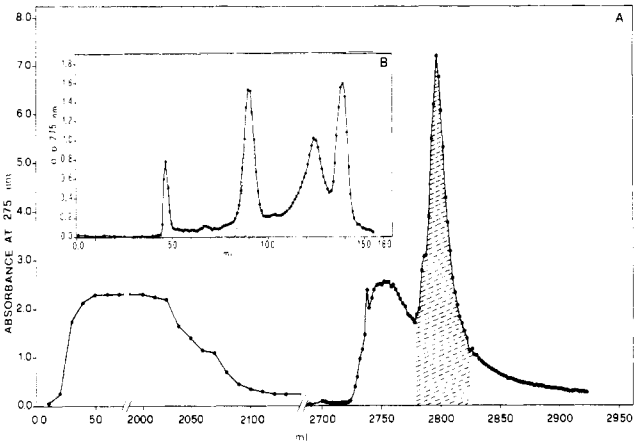

FIGURE 1: (A) QAE-Sephadex A-25 chromatography of crude toxin. The crude extract was applied on a column (2.7 × 20 cm) of QAE-Sephadex A-25 previously equilibrated with 0.01 M Tris-HCl pH 7.8, containing 0.06 M NaCl. Elution was carried out with 0.2 M ammonium acetate, pH 5.0, after washing with about 700 mL of 10 mM Tris-HCl, pH 7.4. The hatched area indicates toxic fractions. (B) Purification of CLX on a column of Sephadex G-50 superfine. The hatched area shows toxic fractions. Column was 1.5 × 73 cm. Sample applied, 28 mg.

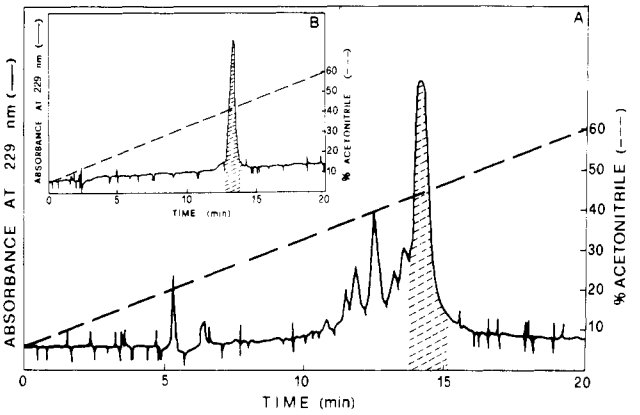

FIGURE 2: (A) Chromatographic purification of CLX by an RP-HPLC semipreparative column. Solvent A, 0.1% TFA; solvent B, acetonitrile with 0.1% TFA. CLX was eluted with a linear gradient from 5 to 60% solvent B in 20 min. Sample applied, 3 mg; OD at 229 nm; range of absorption, 0–2 ODU. The hatched area indicates the purified CLX. (B) Analytical chromatography of purified CLX by RP-HPLC. Conditions are described under Experimental Procedures. Sample applied, 20 μg; range of absorption, 0–0.2 ODU at 229 nm.

concentration of acetonitrile to 50% over 90 min. Peptides in the effluent were monitored by their absorbance at 210 nm. Numbering of peptides follows their order in the sequence, starting from the amino terminus.

NH_2-Terminal Determination. The procedure described by Tarr (1982) was used for the determination of the NH_2-terminal residue of the reduced, S-carboxymethylated CLX. Identification and quantitation of phenylthiohydantoins were carried out by RP-HPLC as described (Pucci et al., 1983).

Sequence Analysis. (*A*) *Manual Method.* Samples (5–10 nmol) were subjected to manual degradation with phenyl isothiocyanate using the procedure reported for small peptides (Tarr, 1982; Kuhn & Crabb, 1986). Phenylthiohydantoins were identified as described above. In the separation system used, PTH-Asp(OMe), PTH-Glu(OMe), and PTH-CMCys-(OMe) eluted, in this order, between PTH-Tyr and PTH-Met, a region free of other PTH derivatives.

(*B*) *Automated Sequencing.* Samples were subjected to automated Edman degradation in a Beckman 890 C spinning cup sequencer (Edmann & Begg, 1967). The 0.1 M Quadrol program (double coupling, single cleavage) was used in the presence of polybrene.

(*C*) *Electrophysiological Methods.* For the electrophysiological experiments, the closer muscle of the walking legs of the crab *Eriphia spinifrons* was used. The legs were obtained by inducing autotomy. The axons innervating the closer muscle were isolated in the meropodite. After exposure of the fibers of the closer muscle, the leg was fixed in a small chamber (25-mL volume) containing natural seawater at 18 °C (Rathmayer & Erxleben, 1983). Excitatory junction potentials (EJPs), elicited by stimulation of the "fast" excitatory motor axon with a single or train of pulses, were recorded intracellularly from a muscle fiber using conventional electrophysiological techniques. The toxin, dissolved in distilled water, was added to the preparation. Final concentrations ranged between 2×10^{-7} and 5×10^{-5} M.

RESULTS

Calitoxin Purification. Fractionation of crude extract obtained on a column of QAE-Sephadex A-25 is shown in Figure 1A. The paralyzing activity eluted between 2780 and 2840 mL. This step enhanced the activity about 5-fold. Figure 1B shows the elution diagram of the toxic material further purified on a column of Sephadex G-50 superfine (10-fold). The combined, lyophilized fractions were further purified by RP-HPLC (Figure 2A). Figure 2B shows CLX (purified 30-fold) chromatographed on an analytical RP-HPLC. At this stage of purification, the toxic unit corresponds to 0.2 μg of CLX.

Table I: Amino Acid Composition of Sea Anemone Toxin CLX[a]

amino acid	CLX composition	amino acid	CLX composition
Asp	4.89 (4)	Ile[c] (▲)	1.03 (1)
Asn	(1)	Leu (▲)	1.05 (1)
Thr[b]	4.18 (4)	Tyr[b] (▲)	1.95 (2)
Ser[b]	4.23 (4)	Phe (▲)	1.00 (1)
Glu	3.16 (3)	Trp (▲)	0.85 (1)
Gln		Lys	2.73 (3)
Pro	0.93 (1)	His	1.96 (2)
Gly	4.62 (5)	Arg	
Ala	3.71 (4)		
CMCys	5.68 (6)	total	46
Val[c] (▲)	2.02 (2)	hydrophobic % (▲)	20
Met (▲)	0.93 (1)	charged %	26

[a] Values presented are means of four analyses, calculated on the basis of Phe taken as 1. The numbers in parentheses are from sequence data. [b] Values extrapolated at zero time. [c] Values at 70 h.

Structural Characterization of Calitoxin. The isoelectric point of CLX was at pH 5.40 ± 0.02, a value similar to that of toxins isolated from *Anemonia sulcata* (Cariello & D'Aniello, 1975).

The amino acid composition of CLX is reported in Table I. Six $^1/_2$-Cys residues were found in the S-carboxymethylated protein. No free SH groups were found, either with the spectrophotometric method with Nbs_2 or after treating the protein with iodoacetamide under denaturing, nonreducing conditions.

Sequence Studies. End group determinations on the S-carboxymethylated chain showed that the NH_2-terminal residue was Ile, whereas after 120 min of incubation with CPA, the only amino acid detected was Ala (0.75 mol/mol of protein). Longer incubation times released no other amino acids. CPP digestion at pH 4 released Ala and Glu (0.64 and 0.25 mol/mol, respectively).

Automatic sequence analyses of S-carboxymethylated CLX showed that the first two amino acid residues were Ile-Glu. Figure 3 shows the separation, by RP-HPLC, of tryptic

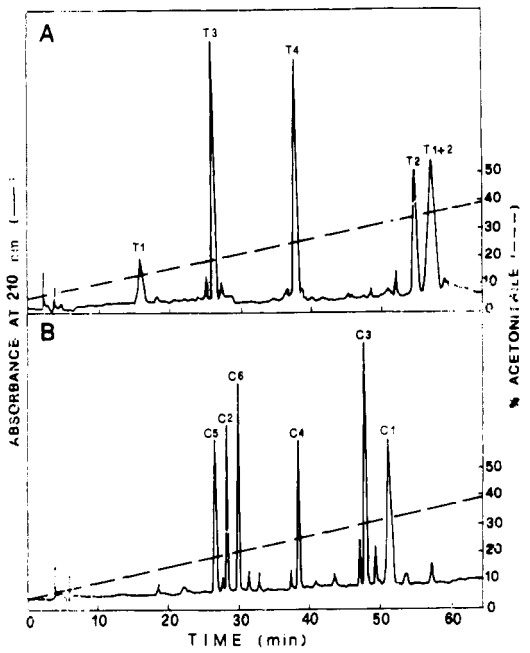

FIGURE 3: Separation by RP-HPLC of tryptic (A) and chymotryptic (B) peptides of CLX. The column was equilibrated with 0.1% TFA containing 5% acetonitrile and eluted with a linear gradient of acetonitrile (--). The absorbance was monitored at 210 nm (—).

(Figure 3A) and chymotryptic (Figure 3B) peptides obtained from reduced, S-carboxymethylated CLX. Their amino acid compositions were submitted to the scrutiny of the reviewers and will be furnished to the interested reader by writing to the authors.

The sequence determined on the tryptic and chymotryptic peptides of CLX is reported in Table II, while the alignment of tryptic peptides is reported in Figure 4.

Peptide T1 was obtained with a low yield (31%), confirming that cleavage at lysyl residues is slowed down by the presence of an acidic environment (-Glu-CMCys-Lys-CMCys-Glu-; see Figure 4). Furthermore, peptide T1 was identified as the NH_2-terminal peptide, because it contained the only Ile residue present in CLX. The sequence of peptide T2 was obtained manually up to Thr-18 despite the presence of a prolyl residue. The sequence of peptide C1 provided the information that peptide T2 follows T1 in the sequence, as also suggested by the amino acid composition of peptide T1+2. Peptide T4 was positioned at the COOH terminal of CLX, since it does not contain basic amino acids but an alanyl residue at the COOH terminal, as determined by CPA digestion (0.75 mol/mol of peptide).

The positions established for peptides T1, T2, and T4 necessarily determine the position of the fourth tryptic peptide T3.

Table II: Amino Acid Sequence of Tryptic (T) and Chymotryptic (C) Peptides Obtained by Manual (—), Automatic (===) Edman Degradation, and CPase Digestions (←)

PEPTIDE	SEQUENCE RESULTS[a]
T1	Ile-Glu-Cys-Lys
T2	Cys-Glu-Gly-Asp-Ala-Pro-Asp-Leu-Ser-His-Met-Thr-Gly-Thr-(Val, Tyr,Phe,Ser,Cys,Lys)
T3	Gly-Gly-Asp-Gly-Ser-Trp-Ser-Lys
T4	Cys-Asn-Thr-Tyr-Thr-Ala-Val-Ala-(Asp,Cys_2,His,Glu)-Ala
C1	Ile-Glu-Cys-Lys-Cys-Glu-Gly-Asp-(Ala,Pro,Asp,Leu,Ser,His,Met)
C2	Thr-Gly-Thr-Val-Tyr
C3	Phe-Ser-Cys-Lys-Gly-Gly-(Asp,Gly,Ser)-Trp
C4	Ser-Cys-Lys-Gly-Gly-Asp-Gly-Ser-Trp
C5	Ser-Lys-Cys-Asn-(Thr)-Tyr
C6	Thr-Ala-Val-Ala-Asp-Cys-Cys-His-Glu-Ala

[a] The repetitive yields of manual Edman degradation were between 70 and 78%.

The sequence of peptide T2 subsequent to Thr-18 was determined on the chymotryptic peptides C2 and C3. Furthermore, peptides C3 and C5 provided further information on the alignment of tryptic peptides.

Finally, peptide C6 confirmed part of the sequence of peptide T4 and provided the sequence of the last six residues of CLX.

Physiological Studies. Application of CLX (final concentration in the bath between 10^{-7} and 10^{-5} M) to neuromuscular preparations of the crab *Eriphia spinifrons* had no effect on membrane potentials of the muscle fibers (V_m = –62.8 mV ± 3; n = 8). However, clear effects were seen on the excitatory junctional potentials elicited by stimulation of the motor axon. Figure 5 shows typical results (1 μM CLX). The excitatory junction potentials (EJPs) were recorded intracellularly from fibers of the closer muscle. Figure 5a is a control EJP of 6-mV amplitude upon stimulation of the fast excitatory axon to this muscle, with a single pulse. The first effects of the toxin were observed 5 min after its application. The amplitude of the EJP upon single pulse stimulation was greatly increased (3-fold in the experiment showed in Figure 5b). Often, the augmented EJP generated a graded electrically excited active muscle response. The amplitude ranged from several millivolts (Figure 5b) to an overshoot of 20–30 mV of zero potential (Figure 5c). The large postsynaptic responses elicited twitch contraction of the muscle (see mechanical artifact in Figure 5b). The second effect of CLX on this preparation started 5–10 min after its application. Single

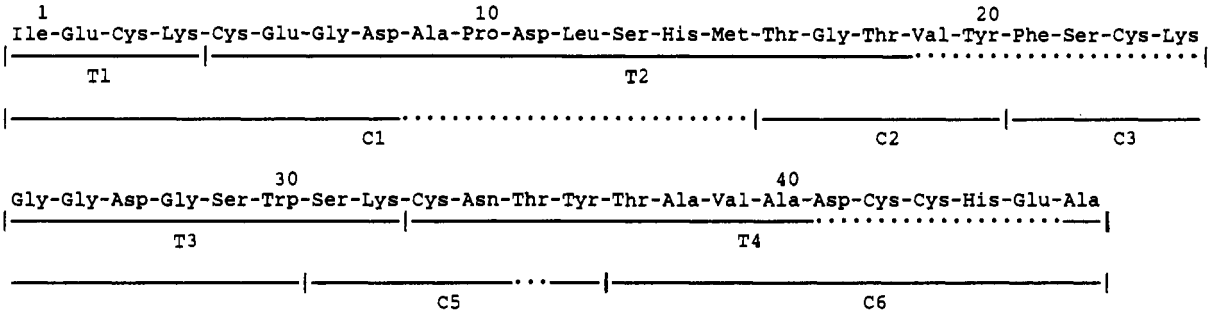

FIGURE 4: Complete amino acid sequence of CLX. Dots denote amino acid residues not sequenced. Peptides T1+2 (positions 1–24) and C4 (positions 22–30) are not indicated.

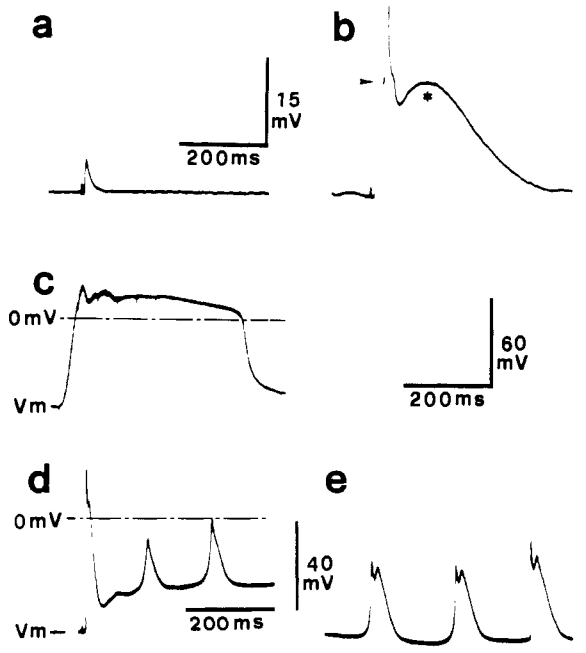

FIGURE 5: Effects of CLX on neuromuscular transmission in the closer muscle of *Eriphia*. (a) Control EJP after a single pulse to the fast excitatory axon. (b) EJP 5 min after CLX application. The toxin caused a graded electrical response (arrowhead) followed by a contraction artifact (asterisk). The synaptic inflection was retouched. (c) Large EJP causing an overshoot and a long-lasting depolarization of the muscle fibers. The dashed line indicates zero potential. (d) Six minutes after CLX application, a single pulse produced repetitive response to the fast axon (the first EJP elicited an overshooting axon potential). The dashed line indicates zero mV. (e) Spontaneous responses in the absence of stimulation.

pulses to the fast axon elicited repetitive firing of the axon, generating large EJPs and dopolarization of the muscle fibers (Figure 5d). Starting after 7–10 min, the axon also produced spontaneous action potentials in the absence of stimulation leading to large EJPs (Figure 5e).

Within the time limits of this preparation (over 3 h), the effects of CLX could not be washed out. The CLX effects could be blocked by TTX (1.6×10^{-7} M).

Similarly to ATX I and ATX III, CLX did not affect the squid giant motor fiber system. In contrast with the *Anemonia* toxins, CLX had no effect on the frog spinal cord.

Discussion

This paper describes the purification, the amino acid sequence, and the electrophysiological effects of CLX, a new toxin from the sea anemone *Calliactis parasitica*.

Sea anemone toxins have been divided into three classes according to amino acid sequence (Schweitz et al., 1985). The "short" toxin ATX III from *Anemonia sulcata* belongs to the third class. Of the other two classes, one comprises *Radianthus paumotensis* toxins, and the other comprises *Anemonia sulcata* toxins (ATX I, ATX II, and ATX V), AP-A and AP-B from *Anthopleura xantogrammica*, AP-C from *Anthopleura elegantissima*, and AFT I and AFT II from *Anthopleura fuscoviridis*.

The CLX sequence is different from those of the toxins of these three classes (Figure 6). In fact, the homology is only 33% with RP II, and it drops to 26% and 22% for ATX I and ATX II, respectively. Even taking into account the conservative substitutions, the degree of homology reaches, at most, 50%.

Similarities are essentially present in the NH_2-terminal sequence up to residue 24. Similarities in the second half of the chain would be higher if we consider the presence of two

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          1                   10                  20
ATXI      G A A C L C K S D G P N T R G N S M S G T I W V F
                P
ATXII     G V P C L C D S D G P S V R G N T L S G I I W L A
              I
ATXV      G V P C L C D S D G P S V R G N T L S G I L W L A
AP-A      G V S C L C D S D G P S V R G N T L S G T L W L Y
AP-B      G V P C L C D S D G P R P R G N T L S G I L W F Y
AP-C      G V P C L C D S D G P S V R G N T L S G I L W L A
AFTI      G V A C L C D S D G P N V R G N T L S G T I W L A
AFTII   G G V P C L C D S D G P S V R G N T L S G I I W L A
RPII      - A S C K C D D D G P D V R S A T F T G T V D F D
CLX       - I E C K C E G D A P D L S H - - M T G T V Y F S

                30                  40                  49
ATXI      - - G C P S G W N N C Q G R A - I I G Y C C K Q
ATXII     - - G C P S G W H N C K K H G P T I G W C C K Q
ATXV      - - G C P S G W H N C K K H K P T I G W C C K Q
                                          G
AP-A      P S G C P S G W H N C K A H G P T I G W C C K Q
AP-B      P S G C P S G W H N C K A H G P N I G W C C K K
AP-C      - - G C P S G W H N C K A H G P T I G W C C K Q
AFTI      - - G C P S G W H N C K A H G P T I G W C C K Q
AFTII     - - G C P S G W H N C K A H G P T I G W C C K Q
RPII      E E K T G A V Y T P C D A S W - - V C W C C K R K K
CLX       C K G G D G S W S K C N T Y T A - V A D C C H E A
```

FIGURE 6: Comparison of the amino acid sequence of CLX with other sea anemone toxins. ATX I, ATX II, and ATX V from *Anemonia sulcata*; AP-A and AP-B from *Anthopleura xantogrammica*; AP-C from *Anthopleura elegantissima*; AFT I and AFT II from *Anthopleura fuscoviridis*; RP II from *Radianthus paumotensis*. The numbering scheme adopted for the identification of the amino acid residues refers to the uninterrupted sequence of AP-A toxin. Gaps (-) and/or additions of amino acid residues have been introduced to obtain maximal homology.

short sequence inversions in positions 31–32 and 35–37. Furthermore, five out of six Cys residues are conserved in all toxins in identical sequence positions (Figure 6), providing conserved S–S pairings (Wunderer, 1978; Northon, 1981; Sunahara et al., 1987). The sixth Cys is found in position 29 in most of toxins, while it is in position 45 in RP II and 26 in CLX.

Chemical modification experiments performed on specific amino acid residues of ATX II from *Anemonia sulcata* (Barhanin et al., 1981) have been used to identify the chemical groups responsible for the binding properties and for the toxic action. It has been found that modifications of the ϵ-amino groups of lysines-37, -38, and -48 and of the α-amino group of Gly-1, with loss of positive charges, abolished almost completely the toxicity and caused a consistent decrease in the binding activity; this occurred also with the modification of histidinyl residues 34 and 39. On the other hand, selective modification of His-39 did not alter the toxic and binding activities of ATX II. Modification of the single arginyl residue present in the protein produced a toxin devoid of neurotoxic and binding properties, while modifications of the carboxylic functions of Asp-7, Asp-9, and Gln-49 affected only the toxic but not the binding properties of ATX II. The picture which emerges from such studies is that only the single arginyl residue is responsible for both binding and neurotoxic action, while no single residue is responsible for binding, as also reported for snake neurotoxins (Karlsson, 1979).

With the occurrence of a large number of toxins of known primary structure with a low degree of homology, but with the same activity, it is possible to assess the role played by individual residues, without recourse to chemical modifications. In this respect, CLX is useful for the identification of the amino acid residues directly determining the structural requirements and those involved in the biologial activities of sea anemone toxins.

In addition to the five cysteinyl residues in positions 4, 6, 36, 46, and 47, Pro-11 and Gly-20 are also conserved, while Ala-10 replaces Gly in CLX and Gly-28 is replaced by Lys in RPII (Figure 6). Furthermore, although the relative amount of charged and hydrophobic residues differs with

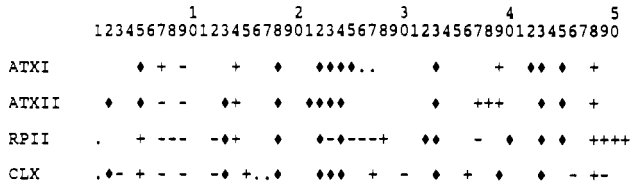

FIGURE 7: Distribution of charged (+/−) and hydrophobic (♦) residues along the polypeptide chains of CLX, ATX I, RP II, and ATX II. ATX II includes also ATX V, AP-A, AP-B, AP-C, AFT I, and AFT II. Numbering is as in Figure 6.

respect to the sequences of other toxins (Sunahara et al., 1987; Schweitz et al., 1985; Reimer et al., 1985), a common, better defined distribution pattern along the peptide chains is observed (Figure 7). From this analysis, we conclude that, contrary to previous reports (Rathmayer, 1979), the NH_2-terminal part can be polar; residue 7 is preferentially Asp although Lys is present in ATX I; residues 18, 22, 24, 33, and 43 are strictly hydrophobic. The central hydrophobic region is shorter than previously reported and can be spaced or flanked by charged residues (RP II).

The COOH-terminal region is always charged. Surprisingly, Arg-14, the modification of which abolishes both binding and toxic action, is absent from CLX. It is replaced by Ser followed by a histidinyl residue. It appears that Asp-9 may play an important role because it is the only "functional" residue that has remained invariant in all the toxins described so far.

Despite the differences in primary structure between CLX and the other sea anemone toxins, their electrophysiological effects on neuromuscular transmission are very similar.

When studied in a neuromuscular preparation of crustaceans, the toxin increased transmitter release, causing repetitive firing of the axons. The effects of CLX observed can be explained by a presynaptic action of the toxin on the motor axon innervating the muscle. This conclusion is supported by two observations: (a) even a high concentration of CLX (5×10^{-5} M) had no effect on the resting membrane potential of the muscle fibers; (b) the presence of TTX completely blocked the effects of CLX.

Similarly to *Anemonia sulcata* toxins, CLX slows down sodium current inactivation, which in turn prolongs the axonal action potentials (Rathmayer, 1976, 1979). The prolongation of the action potential leads to prolonged depolarization of the nerve terminals, thereby causing massive transmitter release.

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