A Molluscivorous *Conus* Toxin: Conserved Frameworks in Conotoxins†

David R. Hillyard,‡ Baldomero M. Olivera,*,§ Scott Woodward,‡ Gloria P. Corpuz,∥ William R. Gray,§ Cecilia A. Ramilo,§,∥ and Lourdes J. Cruz§,∥

Marine Science Institute and Department of Biochemistry, University of the Philippines, Manila, Philippines, and Department of Biology and Howard Hughes Medical Institute, University of Utah, Salt Lake City, Utah 84112

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ABSTRACT: We purified and characterized a 27 amino acid toxin from a snail-hunting *Conus* venom, *Conus textile*. This toxin causes convulsive-like activity in snails and causes subordinate lobsters to assume an exaggerated dominant posture. The sequence of this peptide is Trp-Cys-Lys-Gln-Ser-Gly-Glu-Met-Cys-Asn-Leu-Leu-Asp-Gln-Asn-Cys-Cys-Asp-Gly-Tyr-Cys-Ile-Val-Leu-Val-Cys-Thr. The sequence was confirmed by determining the nucleotide sequence of a cDNA clone coding for the peptide. The conservation of Cys residues compared to the ω-conotoxins from piscivorous *Conus* venom suggests that toxins from different cone venoms may use only a few "Cys-motifs" as conserved structural backbones for targeting to a variety of receptors in different animals.

The snail-hunting cone snails are among the most striking animals in nature; their shells are decorated with elaborate tent markings, long admired by shell collectors. A typical snail-hunting *Conus* is the Cloth-of-Gold cone *Conus textile*.

Our laboratories have previously characterized toxins in the venom of a fish-hunting *Conus*, *Conus geographus*, the geography cone (Olivera et al., 1985; Cruz et al., 1985). From this venom, we isolated a set of paralytic peptides which the geography cone snail uses to immobilize its fish prey. These fall into three classes: the α-conotoxins, the μ-conotoxins, and the ω-conotoxins which inhibit the nicotinic acetylcholine receptor, the presynaptic voltage-sensitive Ca channel, and the muscle voltage-activated sodium channel, respectively. A common characteristic of all of these toxins is that they are relatively small peptides (13–30 amino acids), extensively disulfide bonded.

The toxins of the snail-hunting cones remain uncharacterized; we describe a toxin from the snail-hunting cone, *C. textile*, and demonstrate that it is also a small, disulfide-rich peptide. However, this toxin, the "King-Kong peptide" (see biological activity below), does not appear to act on a target homologous to the receptors for the conotoxins characterized from piscivorous *Conus* venoms, but has novel biological activity in invertebrate systems. DNA cloning was used to confirm the primary structure of the King-Kong peptide: the nucleotide sequence from a King-Kong cDNA clone validated the peptide sequence. In the report below, we describe in detail the purification and physiological and biochemical characterization of the King-Kong peptide.

Experimental Procedures

Materials. Specimens of *Conus textile* were collected from the sea around the islands of Marinduque and Cebu in the Philippines. Venom was extracted from the specimens as described previously (Cruz et al., 1976). Venom ducts were quick-frozen in liquid nitrogen after dissection from *C. textile* and stored at −70 °C until used for cloning experiments.

Preparation of Crude Venom Extract. Lyophilized *C. textile* venom was taken up in 0.2 M NH_4OAc, pH 7.5 (to make a 20% suspension), and soaked for 30 min over ice with occasional stirring. The solution was centrifuged for 10 min at 12000*g*, and the supernatant was saved. The pellet was resuspended in the same buffer, sonicated for 3 × 15 s at 60–70 W, and then centrifuged. The second pellet was resuspended in the same buffer, sonicated, and centrifuged again. All supernates were combined and kept over ice until further use.

Bio-Gel P-6 Chromatography of Crude Venom Extract. The crude extract was applied to a preequilibrated Bio-Gel P-6 column and eluted with 0.2 M NH_4OAc, pH 7.5. Five-milliliter fractions were collected at a flow rate of 0.5 mL/min. The absorbance of eluates was monitored at 280 nm. Fractions corresponding to an elution volume of 100–120 mL were pooled, concentrated, bioassayed, and used for further purification of HPLC.

Purification of the Peptide by HPLC. The Bio-Gel P-6 fraction was chromatographed on an analytical VYDAC reverse-phase C18 column in several batches. Peptides were eluted with a gradient of acetonitrile in 0.1% TFA as indicated in Figure 1B at 1 mL/min. The largest UV-absorbing peak (shaded area) which eluted from 78.1 to 80.3 min was repurified on the same column by using the same solvent system. The purity of the isolated peptide was checked with an isocratic run (0.1% TFA/46.8% CH_3CN) at a flow rate of 1 mL/min on the VYDAC RP C18 column as shown in Figure 1C.

Amino Acid Analysis. Peptide samples were hydrolyzed in vacuo with 6 N HCl/1% phenol for 18 h at 105 °C. Amino acid analysis was done by reverse-phase HPLC of phenylthiocarbamyl derivatives (Heinrikson & Meredith, 1984; Bidlingmeyer et al., 1984).

Peptide Sequencing. The purified King-Kong peptide was reduced and carboxymethylated as previously described (Cruz et al., 1987) and then analyzed in a spinning-cup sequencer according to the method of Tarr et al. (1978). Phenylthiohydantoin derivatives were identified by HPLC using a gradient slightly modified from that of Hunkapiller and Hood (1978).

Cloning. The clone for the King-Kong peptide was identified from a cDNA library made from poly(A^+) RNA from *Conus textile* venom duct using the Okayama–Berg procedure (Okayama & Berg, 1983). Details of cloning and sequencing

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‡ Howard Hughes Medical Institute, University of Utah.
§ Department of Biology, University of Utah.
∥ University of the Philippines.

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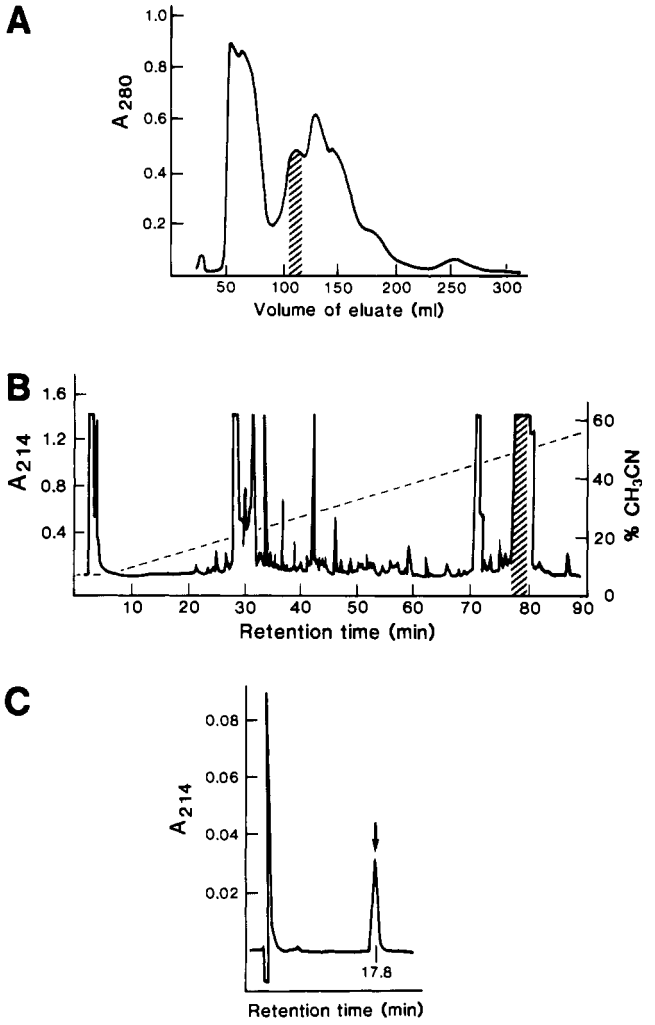

FIGURE 1: Purification of King-Kong peptide from *Conus textile* venom. (A) Crude extract of the venom containing 184.4 mg of protein was chromatographed on a Bio-Gel P-6 column (1.4 × 105 cm) using 0.2 M NH_4OAc, pH 7.5, as eluant. (B) Fractions corresponding to the shaded area in (A) were pooled for HPLC on a VYDAC RP C18 column. The gradient of acetonitrile in 0.1% TFA is indicated by the dashed line. The most abundant component (shaded area) was purified by using the same column and solvent system. (C) Isocratic run of purified King-Kong peptide at 46.8% CH_3CN/0.1% TFA on a VYDAC RP C18 column.

will be described in an accompanying publication (S. Woodward et al., unpublished experiments).

Results

Purification of the King-Kong Peptide and Preliminary Characterization of Biological Activity. Crude *Conus textile* venom was extracted and purified by using Bio-Gel P-6 chromatography, followed by HPLC on a VYDAC reverse-phase C18 column twice, first using an acetonitrile gradient followed by an isocratic run; these steps are shown in Figure 1 and described in detail under Experimental Procedures. Although both the crude venom ($LD_{50} \sim 36$ mg/kg) and the Bio-Gel P-6 fraction (see Figure 1A) elicited convulsive-like symptoms upon intracerebral injection into mice, these symptoms are not due to the King-Kong peptide but a different peptide in the venom which will be described elsewhere. The purified King-Kong peptide exhibited no activity in mice but was biologically active when tested on garden snails and on crustacean systems (see below). The final purified toxin showed the chromatography pattern in Figure 1C; unless otherwise indicated, all experiments described in this report were done with this fraction.

Table I: Amino Acid Analysis of King-Kong Peptide

amino acid	pmol	mol/mol
Asp	508	3.91 (4)[a]
Glu	390	3.00 (3)
Ser	141	1.09 (1)
Gly	295	2.27 (2)
Thr	182	1.40 (1)
Tyr	146	1.13 (1)
Val	198	1.52 (2)
Met	146	1.12 (1)
Cys	621	4.78 (6)
Ile	97	0.75 (1)
Leu	468	3.60 (3)
Lys	94.5	0.73 (1)
Trp	ND[b]	(1)

[a] Values in parentheses indicate the number of residues found by sequence analysis (see Table II). [b] ND, not determined.

The purified toxin was tested for activity in using intracerebral and intraperitoneal injection into mice; no apparent biological activity could be detected by these assays. A variety of invertebrates were also tested. In collaboration with Dr. E. Kravitz, the purified peptide was found to be active on lobsters. A very peculiar behavior was found when the peptide was injected into a lobster which exhibited subordinate behavior in the presence of a larger lobster; normally, subordinate animals walk with their head down toward the substrate, tail up. After injection of the peptide, the subordinate lobster assumed an exaggerated dominant stance, even in the presence of the dominant lobster. It would walk around in a typical dominant posture, head up high with a peculiar curvature of the tail almost looking scorpion-like (hence, the trivial name "King-Kong peptide"; i.e., it converts a subordinate lobster into a "King-Kong" lobster; we are indebted to Professor Kravitz for coining this colorful name).

The peptide is also active on molluscs, causing a convulsive-contractile-like movement in garden snails, the biological activity first used to identify the peptide. A more refined analysis of the physiological activity of this peptide was carried out (Lev-Ram et al., 1987; V. Lev-Ram, unpublished results); when injected into the R15 neuron from the abdominal ganglion of Aplysia, the toxin has complex effects enhancing both a slow inward Ca current often called I_{nsr} (negative slope resistance current; Adams & Levitan, 1985) and also an inward rectifying potassium current (Benson & Levitan, 1983). These complex effects on Aplysia R15 neuron need to be further defined and similar physiological experiments carried out in other systems. From this preliminary physiological characerization, the King-Kong peptide appears to have quite a different type of physiological activity from those of the α-, ω-, or μ-conotoxins that have been characterized from the fish-hunting cone snails.

Structural Determination of the King-Kong Peptide. An amino acid analysis was carried out on purified King-Kong peptide; these results are shown in Table I. A sequence analysis of the King-Kong peptide carried out with a Beckman spinning cup sequencer (see Experimental Procedures) showed a 27 amino acid peptide with 6 Cys residues; the results are shown in Table II.

In order to determine whether the peptide has a blocked C-terminal end, a fast atom bombardment mass spectrometry analysis was carried out. The observed value for MH^+ was 3035.30; the predicted value, given the sequence in Table II, is 3035.22 if the carboxyl terminus were free, while if it were blocked with an amide group, a predicted value of 3034.21 should have been obtained. Thus, we conclude that the C-terminal end of the King-Kong peptide is not blocked.

Table II: Sequence Analysis of King-Kong Peptide

step	assigned residue	yield (nmol)
1	Trp	5.43
2	Cys	3.89
3	Lys	5.06
4	Gln	4.47
5	Ser	2.54
6	Gly	2.10
7	Glu	3.00
8	Met	2.92
9	Cys	2.04
10	Asn	1.68
11	Leu	2.41
12	Leu	1.79
13	Asp	1.84
14	Gln	1.35
15	Asn	0.82
16	Cys	1.00
17	Cys	1.59
18	Asp	0.91
19	Gly	0.56
20	Tyr	0.78
21	Cys	1.00
22	Ile	1.06
23	Val	0.93
24	Leu	0.56
25	Val	0.68
26	Cys	0.57
27	Thr	0.23

Table III: Nucleotide Sequence of Presumptive King-Kong Peptide Coding Region

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Asn  Lys  Arg  Trp  Cys  Lys  Gln  Ser  Gly  Glu  Met  Cys  Asn
AACAAGAGGTGGTGCAAACAAAGCGGTGAAATGTGTAAT

Leu  Leu  Asp  Gln  Asn  Cys  Cys  Asp  Gly  Tyr  Cys  Ile
TTGTTAGACCAAAACTGCTGCGACGGCTATTGCATA

Val  Leu  Val  Cys  Thr  ---
GTACTTGTCTGCACCTAA-----
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Cloning of King-Kong Peptide cDNA. In order to confirm the structural assignments shown above, we have made a cDNA library of poly(A^+) RNA from *Conus textile* venom duct. Mixed oligonucleotide probes were made from the King-Kong sequence, and candidate clones were identified. As will be detailed elsewhere (S. Woodward et al., unpublished results), the King-Kong peptide transcript is in fact a member of a larger family of transcripts that share highly conserved sequences. One member of this family has proven to be consistent with a transcript coding for the King-Kong peptide. The nucleotide sequence of the presumed peptide coding region is shown in Table III. It will be observed that the nucleotide sequence of this cDNA clone agrees both with the sequence shown in Table II and also with the assignment of a free carboxyl group at the C-terminal end of the peptide. If the peptide were amidated, a GXX sequence should have been obtained following the coding sequence for King-Kong (where X is either Lys or Arg); the fact that the C-terminal threonine is followed by a stop codon (TAA) is consistent with a free carboxyl terminus. At the N-terminal end of the peptide, a typical proteolytic cleavage site with two consecutive basic amino acids is observed. Such potential proteolytic cleavage sites have been found in many peptide precursors. Thus, the cDNA sequence validates the amino acid sequence assignment shown in Table II.

Discussion

In this work, we have described the purification and biochemical characterization of the first toxin from a snail-hunting cone venom, that of *Conus textile*. It is clear that snail-hunting cones have pursued a biochemical strategy similar to that of the piscivorous *Conus* species, i.e., to use small peptides rich in cysteine residues. The King-Kong peptide clearly has novel biological activity, quite different from any of the major paralytic toxins so far characterized from *Conus geographus* venom. Even the preliminary physiological data indicate that this peptide has different specificity from the α-, μ-, and ω-conotoxins characterized from *Conus geographus*. Although the precise molecular target of the King-Kong peptide has not yet been identified, the purification and structural determination should make the chemical synthesis of this peptide possible and allow a biochemical and physiological characterization of the receptor target.

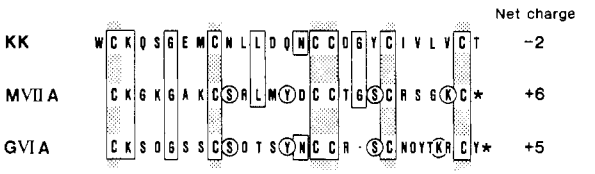

FIGURE 2: Comparison of King-Kong peptide to ω-conotoxins. The sequences of *Conus textile* King-Kong peptide, ω-conotoxin MVIIA (from *Conus magus*), and ω-conotoxin GVIA (from *Conus geographus*) are shown, aligned to maximize the number of identities in the three sequences. Boxed residues are identities between ω-conotoxins and the King-Kong peptide; circled residues are identities between the two ω-conotoxins not shared by the King-Kong peptide. Except for O (*trans*-4-hydroxyproline), the standard one-letter abbreviations for amino acid residues are used. An asterisk indicates that the α-carboxyl group is amidated.

Automated peptide sequencing revealed that the King-Kong peptide is 27 amino acids with 6 Cys residues and no modified amino acids. Both C and N terminii are free. In order to confirm the peptide structure, we independently determined the sequence of a cDNA clone and obtained a nucleic acid sequence completely consistent with the 27 amino acid primary structure assignment in Table II. The nucleic acid sequence also confirmed that the C terminus of the King-Kong peptide is not blocked by an amide group, in contrast to all of the paralytic toxins so far characterized from the fish-hunting cone venom *Conus geographus* (α-, μ-, and ω-conotoxins from that venom are all amidated at the C terminus). This general approach to confirming the sequence of medium-sized peptides (such as the conotoxins) has a number of advantages. Often only small amounts of peptide can be obtained from a particular venom, and although some amino acid sequencing information is easy to obtain, completing the sequence can be difficult with uncertainties often persisting, particularly for the C-terminal end of the peptide. However, once a cDNA library has been made from venom-producing tissue (in the case of *Conus*, the venom duct), this can serve as a permanent confirmatory sequencing tool, for which material will not be limiting. Such a combination of protein chemistry and recombinant DNA technology should make definitive assignments of peptide sequences in the *Conus* system much more facile.

Surprisingly, the cysteine residues in the *Conus textile* King-Kong peptide can be aligned exactly with those in a set of toxins previously characterized from fish-hunting cone venoms, the ω-conotoxins which inhibit voltage-sensitive Ca channels. As shown in Figure 2, the Cys residues in the King-Kong peptide and ω-conotoxin MVIIA are in precisely coincident positions; this may reflect a conservation of the disulfide-bonding pattern. It is noteworthy that the overall identity of amino acid residues when these 2 peptides are aligned (10 out of 25 compared to ω-conotoxin MVIIA) is almost as great as the number of identical residues when ω-conotoxins from 2 different fish-hunting cone venom species

are optimally aligned (12 out of 25 for ω-conotoxin GVIA compared to ω-conotoxin MVIIA); this would seem to indicate that the King-Kong peptide is closely related to the ω-conotoxins. However, it is clear that the physiological activity must be different, i.e., that the King-Kong peptide does not simply inhibit a homologous molluscan voltage-sensitive Ca channel. The preliminary electrophysiology suggests that, if anything, Ca currents are increased rather than blocked in the presence of the King-Kong peptide. Furthermore, a closer examination of the ω-conotoxins and the King-Kong peptide reveals significant differences in charge and hydrophobicity. The King-Kong peptide has a net negative charge (−2), while ω-conotoxin GVIA and MVIIA have charges of +5 and +6, respectively. In addition, the King-Kong peptide is significantly more hydrophobic than the ω-conotoxins. For example, between the fifth and sixth cysteine residues, both ω-conotoxins have two positively charged amino acids, while the King-Kong peptide has four hydrophobic residues (IVLV). Thus, although there are sequence similarities between the King-Kong peptide and ω-conotoxins, the net negative charge and greatly increased hydrophobic character, as well as the preliminary physiological evidence, all indicate that the King-Kong peptide targets to a receptor fundamentally different from the α-, μ-, and ω-conotoxin targets.

Nevertheless, it is intriguing that the Cys residues are so highly conserved between the King-Kong peptide and the ω-conotoxins. One possibility is that this is a consequence of the evolutionary conservation of ion channels and a disulfide-bonding configuration of the ω-conotoxin type might be used on evolutionarily related (but physiologically different) channels. Thus, we might expect to find (and indeed have found) only a few general "Cys motifs" for the conotoxins. One such Cys motif, the ω-conotoxin motif (C–C–CC–C–C), has been found in a large number of other *Conus* peptides (L. J. Cruz et al., unpublished results); the possibility that conotoxins with conserved Cys motifs bind to evolutionarily related target molecules needs to be explored.

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Registry No. L-Cys, 52-90-4; King Kong peptide, 117144-21-5; King Kong peptide (reduced), 117069-04-2.

REFERENCES

Adams, W. B., & Levitan, I. B. (1985) *J. Physiol. (London) 360*, 51–68.

Benson, J. A., & Levitan, I. B. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3522–3525.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336*, 93–104.

Cruz, L. J., Corpuz, G., & Olivera, B. M. (1976) *The Veliger 18*, 302–308.

Cruz, L. J., Gray, W. R., Yoshikami, D., & Olivera, B. M. (1985) *J. Toxicol., Toxin Rev. 4*, 107–132.

Cruz, L. J., de Santos, V., Zafaralla, G. C., Ramilo, C. A., Zeikus, R., Gray, W. R., & Olivera, B. M. (1987) *J. Biol. Chem. 262*, 15821–15824.

Heinrikson, R. L., & Meredith, S. C. (1984) *Anal. Biochem. 136*, 65–74.

Hunkapiller, M. W., & Hood, L. E. (1978) *Biochemistry 17*, 2124–2133.

Lev-Ram, V., Olivera, B. M., & Levitan, I. B. (1987) *Abstr. Soc. Neurosci. 13*, 792.

Okayama, H., & Berg, P. (1983) *Mol. Cell. Biol. 3*, 280–289.

Olivera, B. M., Gray, W. R., Zeikus, R., McIntosh, J. M., Varga, J., Rivier, J., de Santos, V., & Cruz, L. J. (1985) *Science (Washington, D.C.) 230*, 1338–1343.

Tarr, G. E., Beecher, J. F., Bell, M., & McKean, D. J. (1978) *Anal. Biochem. 84*, 622–627.