κ-Bungarotoxin: Complete Amino Acid Sequence of a Neuronal Nicotinic Receptor Probe†

Gregory A. Grant*

Department of Biological Chemistry and Department of Medicine, Division of Dermatology, Washington University School of Medicine, St. Louis, Missouri 63110

Vincent A. Chiappinelli

Department of Pharmacology, St. Louis University School of Medicine, St. Louis, Missouri 63104

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ABSTRACT: The complete amino acid sequence of κ-bungarotoxin, a neurotoxin isolated from the venom of the banded krait *Bungarus multicinctus*, has been determined by automated Edman analyses of the intact protein and peptides derived from digests with trypsin and chymotrypsin. κ-Bungarotoxin consists of a single polypeptide chain of 66 amino acids with a molecular weight of 7313. It contains 10 cysteinyl residues, presumably arranged in 5 disulfide bonds, and is completely devoid of methionine and tryptophan. The amino acid sequence of κ-bungarotoxin shows greatest homology to the curaremimetic postsynaptic long neurotoxins of which α-bungarotoxin is also a member. However, there are some striking differences between κ-bungarotoxin and other members of this group which may explain its unusual ability to block neuronal acetylcholine receptors.

Venoms of elapid and hydrophid snakes contain a number of basic, curaremimetic polypeptides known as postsynaptic neurotoxins [molecular weights 6600–8000; p*I*'s ≥8.0; reviewed in Karlsson (1979)]. These neurotoxins bind specifically and with high affinity to the acetylcholine recognition sites on nicotinic receptors found in vertebrate muscle and in the electric organs of certain marine species, blocking the action of acetylcholine at these receptors (Lee, 1979). Available amino acid sequence data indicate that the postsynaptic neurotoxins are a family of polypeptides with close structural homology.

κ-Bungarotoxin is a neurotoxin recently isolated from the venom of the banded krait *Bungarus multicinctus* (Chiappinelli, 1983) and is distinguished from the major postsynaptic neurotoxin in this venom, α-bungarotoxin, by several features. Following sodium dodecyl sulfate (SDS)[1]–polyacrylamide gel electrophoresis of the purified toxins, κ-bungarotoxin and α-bungarotoxin appear as single bands of protein with apparent molecular weights of 6500 and 8000, respectively. Isoelectric focusing has also been used to compare the two toxins, with κ-bungarotoxin exhibiting a p*I* of 9.1 and α-bungarotoxin running at p*I* ≥9.5 (Chiappinelli, 1983).

The unusual physiological properties of κ-bungarotoxin are quite distinct from those of α-bungarotoxin and most other postsynaptic neurotoxins. κ-Bungarotoxin blocks nicotinic transmission in ciliary and sympathetic ganglia of the chick at doses of 40–140 nM (Chiappinelli, 1983; Dryer & Chiappinelli, 1983; Chiappinelli & Dryer, 1984). Intra- and extracellular recordings indicate that the blockade is specific for neuronal nicotinic receptors present on ganglia neurons. κ-Bungarotoxin also blocks nicotinic transmission in the rat superior cervical ganglion, with higher doses of the toxin (1.5 μM) required for a complete blockade of the rat. κ-Bungarotoxin does not appear to block neuromuscular acetylcholine receptors. In sharp contrast to these observations, α-bungarotoxin (1–10 μM) has no detectable effect on neurotransmission in ciliary and sympathetic ganglia of the chick or in the rat superior cervical ganglion (Brown & Fumagalli, 1977; Carbonetto et al., 1978; Chiappinelli, 1983) even though α-bungarotoxin is one of the most potent blockers of nicotinic receptors found in muscle and electric organs. Thus, while most postsynaptic neurotoxins tested do not appear to bind to the physiologically detected neuronal nicotinic receptor, κ-bungarotoxin can be used as a probe to study this receptor both physiologically and biochemically.

The reason that κ-bungarotoxin is a potent antagonist at the neuronal nicotinic receptor remains to be determined. As an initial exploration of this question, the present report describes the complete amino acid sequence of κ-bungarotoxin and compares this sequence with those of previously characterized snake venom toxins. The results indicate that while κ-bungarotoxin shows considerable structural homology with other postsynaptic neurotoxins, it is nonetheless an exceptional member of this toxin family. Several regions of the κ-bungarotoxin molecule are unique when compared to the previously sequenced postsynaptic neurotoxins. The possible physiological significance of these unusual regions is discussed.

Materials and Methods

Protein Preparation. κ-Bungarotoxin was purified from crude lyophilized *Bungarus multicinctus* venom (Miami Serpentarium, Miami, FL; lot BM8910STLZ) as previously described (Chiappinelli, 1983).

S-Carboxymethylation. After reduction with dithiothreitol, the resultant thiol groups of κ-bungarotoxin were alkylated with iodo[1-^{14}C]acetic acid according to Grant et al. (1980). The S-carboxymethylated protein was desalted and brought

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* Correspondence should be addressed to this author at the Department of Biological Chemistry, Washington University School of Medicine.

[1] Abbreviations: TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate.

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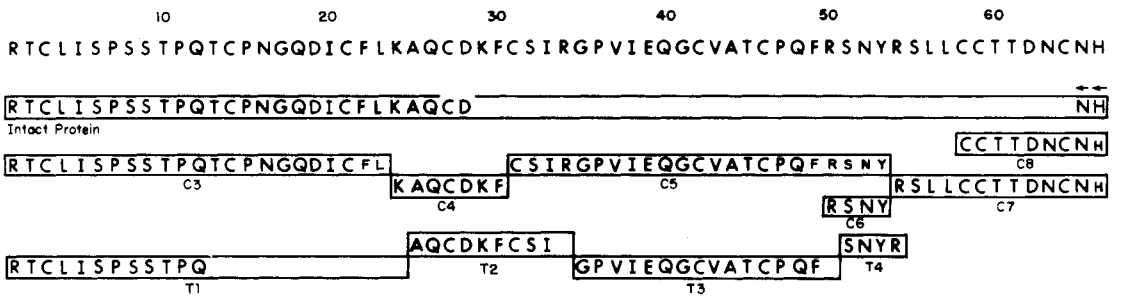

FIGURE 1: Summary proof of the primary structure of κ-bungarotoxin. Each enclosed bar designates an isolated peptide. Only those peptides on which sequence analysis was performed are included. Large letters indicate positive identification, and small letters indicate identification deduced from amino acid composition data. Gaps in the overlines indicate tentative Edman identification of that residue. Reverse arrows indicate positive identification by carboxypeptidase analysis. Letters below the bars designate tryptic (T) and chymotryptic (C) peptides.

to 50 mM ammonium bicarbonate, pH 8.5, by ultrafiltration with a YM2 membrane in an Amicon-stirred ultrafiltration cell.

Enzymatic Digests. Cleavages with bovine chymotrypsin (Worthington) and TPCK-treated bovine trypsin (Worthington) were performed by incubating S-carboxymethylated κ-bungarotoxin in 50 mM ammonium bicarbonate, pH 8.5, with 2% (w/w) protease at 37 °C. Protease, 1% (w/w), was added at zero time and again at 1 h, and the sample was allowed to incubate overnight. The reaction was stopped by acidification to pH 2–3 with 1 N HCl. Carboxypeptidase A (Worthington) digestion of S-carboxymethylated κ-bungarotoxin was performed in 50 mM ammonium bicarbonate buffer, pH 8.5, at room temperature. Aliquots were removed at timed intervals, mixed with an equal volume of 1 N HCl, dried by vacuum centrifugation, and analyzed directly on an amino acid analyzer (Allen, 1981; Ambler, 1972).

Peptide Purification. All peptides were purified by reverse-phase HPLC. After acidification of the proteolytic digests, they were loaded directly onto a 4.6 mm × 25 cm C-18 μBondapak reverse-phase column (Waters) which had been equilibrated in 0.05% TFA. The columns were washed with 0.05% TFA for 5 min and then developed with a linear gradient from 0.05% TFA to acetonitrile made 0.05% in TFA. The chromatography was monitored by the absorbance at 214 nm, and small aliquots were removed from each fraction for liquid scintillation counting. Peptides were recovered by pooling the appropriate fractions and drying under a stream of nitrogen. The peptides were redissolved in 50% TFA for sequencing, and in the case of the chymotryptic peptides, 10–20% were removed for amino acid analysis. All other parameters are as described in the figure legends except that in the reruns of the tryptic peptide pools the slope of the gradient was half that of the initial chromatography depicted in Figure 3.

Amino Acid Analysis. Samples were hydrolyzed in 6 N HCl in evacuated sealed glass tubes for 18–24 h at 110 °C. Hydrolysates were dried by rotary evaporation, redissolved in 0.05 N HCl, and analyzed on a Waters HPLC amino acid analyzer. No corrections were made for partial hydrolytic destruction of serine, threonine, or *S*-(carboxymethyl)cysteine.

Sequence Analysis. All polypeptides were sequenced by automated Edman degradation on an Applied Biosystems 470A protein sequencer. Samples were typically loaded at the 0.5–1-nmol level. The phenylthiohydantoins, after automatic conversion from the phenylthiazolinones, were identified by reverse-phase HPLC analysis on an Altex Ultrasphere OD-S-PTH column by a modification of a previous procedure (Grant et al., 1983). The mobile phase was 7.5 mM sodium acetate, pH 5.4, and the modifier was a 17/3 mixture of methanol/acetonitrile. The column was run at 34 °C with

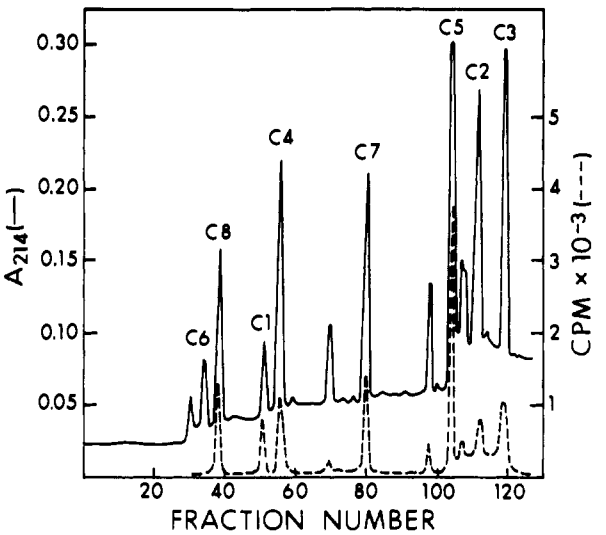

FIGURE 2: HPLC purification of chymotryptic peptides. The column was eluted with a 0.25%/min linear gradient of acetonitrile made 0.05% in TFA. The flow rate was 1 mL/min, and 1-min fractions were collected. Other details are described in the text.

a constant flow rate of 1.5 mL/min according to the following program (time, modifier): 0 min, 7.5%; 3 min, 7.5%; 4 min, 22%; 9 min, 27%; 10 min, 44%; 18 min, 45%; 20 min, 48%; 22 min, 48%; 24 min, 7.5%. All PTH-amino acids, including PTH-*S*-(carboxymethyl)cysteine, were well resolved. In addition, the phenylthiohydantoin of *S*-[^{14}C](carboxymethyl)-cysteine was identified by liquid scintillation counting.

Results

A summary of the sequencing strategy as well as the complete sequence of κ-bungarotoxin is presented in Figure 1. Automated Edman degradation of the intact carboxymethylated protein yielded the amino-terminal sequence of the first 26 residues and tentative identification of residues 27 and 28. The remaining sequence was determined from a complete set of chymotryptic peptides and a partial set of overlapping tryptic peptides. The carboxy terminus was verified by carboxypeptidase A analysis.

Chymotryptic Peptides. Approximately 16 nmol (100 μg) of S-carboxymethylated κ-bungarotoxin was digested with bovine chymotrypsin, and the resulting peptides were separated by reverse-phase HPLC. This separation is shown in Figure 2, and the compositions of the peptides are shown in Table I. Eight major peptides (C1–C8) were obtained from this digest, and all peptides for which sequence information was obtained are included in Figure 1. Compositional analysis of peptides C1 and C2 clearly shows that they are derived from that portion of the protein corresponding to peptide C3.

Table I: Amino Acid Compositions[a] of Chymotryptic Peptides of κ-Bungarotoxin

peptide:	C1	C2	C3	C4	C5	C6[d]	C7	C8
residue no.:	1–4	5–23	1–23	24–30	31–53	50–53	54–66	58–66
Asp (D) + Asn (N)		2.2 (2)	2.1 (2)	1.2 (1)	1.2 (1)	0.7 (1)	3.1 (3)	3.0 (3)
Thr (T)	0.8 (1)	1.8 (2)	2.8 (3)		0.9 (1)		1.9 (2)	1.9 (2)
Ser (S)		2.9 (3)	3.3 (3)		2.2 (2)	1.0 (1)	1.1 (1)	
Glu (E) + Gln (Q)		2.3 (2)	2.3 (2)	1.2 (1)	3.1 (3)			
Pro (P)		2.5 (3)	2.6 (3)		1.9 (2)			
Gly (G)		1.4 (1)	1.2 (1)		2.4 (2)			
Ala (A)				1.2 (1)	1.0 (1)			
$^1/_2$-Cys (C)[b]	0.5 (1)	0.8 (2)	1.4 (3)	0.7 (1)	1.2 (3)		0.6 (3)	1.3 (3)
Val (V)		0.3 (0)			1.0 (2)[e]			
Met (M)								
Ile (I)		1.3 (2)	1.5 (2)		0.7 (2)[e]			
Leu (L)	1.0 (1)	1.2 (1)	2.0 (2)				1.9 (2)	
Tyr (Y)					0.9 (1)	0.7 (1)		
Phe (F)		1.0 (1)	1.2 (1)	1.1 (1)	1.0 (1)			
His (H)							1.0 (1)	0.8 (1)
Lys (K)				1.5 (2)				
Arg (R)	1.0 (1)	0.4 (0)	1.2 (1)		1.4 (2)	1.0 (1)	1.0 (1)	
Trp (W)	ND[c] (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)
no. of residues	4	19	23	7	23	4	13	9
yield (nmol)	7	6	2	12	12	3	7	4

[a] By amino acid analysis after 24-h acid hydrolysis and calculated from sequence (in parentheses). [b] Determined as *S*-(carboxymethyl)cysteine. [c] ND, not determined. [d] Corrected for 1 nmol of contaminating C8. [e] Contains a Val-Ile sequence.

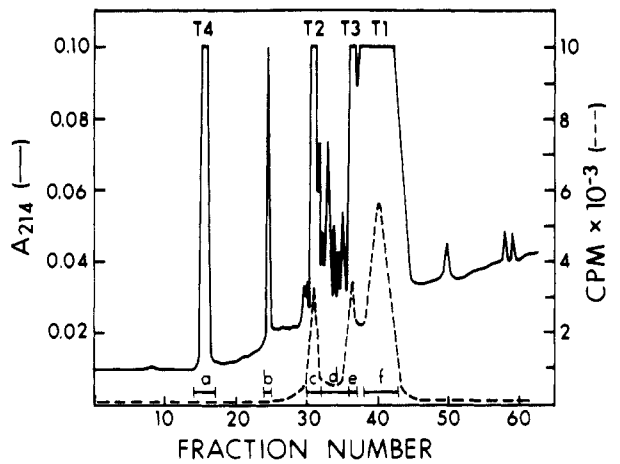

FIGURE 3: HPLC purification of tryptic peptides. The column was eluted with a 1%/min linear gradient of acetonitrile made 0.05% in TFA. The flow rate was 1 mL/min, and 1-min fractions were collected. Other details are described in the text.

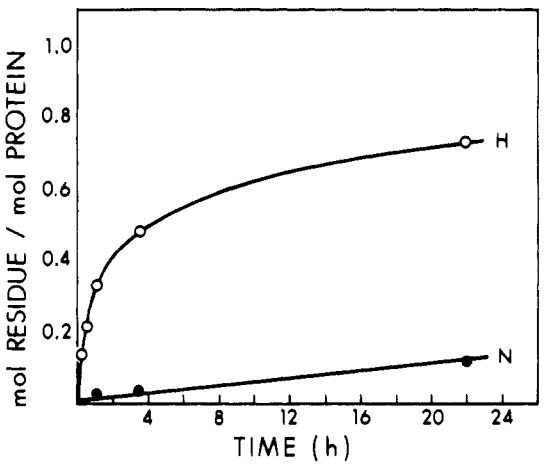

FIGURE 4: Results of carboxypeptidase A treatment of κ-bungarotoxin. Aliquots were removed at the indicated times and analyzed for released amino acids. Details are described in the text.

Therefore, sequence analysis of C1 and C2 was not performed since it would not contribute any additional information over that already obtained from the analysis of C3. The remaining peaks (unlabeled) in Figure 2 were also not investigated further after compositional analysis. The peak at fractions 69–70 appeared to contain a mixture of at least two peptides corresponding to residues 54–57 and residues 23–30. The peaks at fractions 97–99 and fractions 106–108 appeared to contain low-level mixtures of peptides whose identities could not be discerned from compositional data. The peak at fraction 30 contained only a trace of amino acids.

Tryptic Peptides. Approximately 10 nmol (65 μg) of S-carboxymethylated κ-bungarotoxin was digested with bovine trypsin, and the resulting peptides were separated by reverse-phase HPLC as shown in Figure 3. The fractions were pooled as designated by the bars. Pools a and b were analyzed directly, while pools c–f were rechromatographed on the same reverse-phase system with a linear gradient of 0.5%/min. Single major peaks were obtained from pools c, e, and f, while pool d failed to yield significant amounts of material upon rechromatography. Four major peptides (T1–T4) were obtained from this digest and are shown in Figure 1. These peptides were subjected directly to sequence analysis. Compositional analysis was not performed because of the relatively low levels of material available. A tryptic peptide corresponding to residues 55–66 was not found. The nature of pool f suggests that more than one peptide may be present. Therefore, a peptide composed of residues 55–66 may have been present in this pool but not recovered in sufficient quantity after rechromatography. There was no evidence for peptides produced from cleavage at Lys-29. The acidic nature of the residues on either side of Lys-29, which would tend to significantly reduce the rate of cleavage by trypsin, is consistent with this observation. Finally, sequence analysis of pool b (Figure 3) failed to yield any sequence information.

Carboxypeptidase A. The results of carboxypeptidase A treatment of S-carboxymethylated κ-bungarotoxin are shown in Figure 4. The relatively rapid release of a histidinyl residue followed by a much slower release of an asparaginyl residue is consistent with the known specificity of carboxypeptidase A (Ambler, 1972). Although asparagine is normally released slowly, it is probably being released slower than usual in this case due to the penultimate (carboxymethyl)cysteine residue. In addition, it is not unexpected that (carboxymethyl)cysteine is not released at all over the time course of the experiment.

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                                                    10        20        30        40        50        60        70        80
                                                     |         |         |         |         |         |         |         |
 1 Bungarus multicinctus: kappa bungarotoxin  66-5  RTCL---ISPSSTPQT---CPNGQDICFLKAQCDKFCSIRGPVIEQGCVATCPQFRSNYRSLLCCTTDNCNH

 2 Bungarus multicinctus: alpha bungarotoxin  74-5  IVCHTTATIPSS-AVT---CPPGENLCYRKMWCDAFCSSRGKVVELGCAATCPSKKPYEEVT-CCSTDKCNHPPKRQPG
 3 Notechis scutatus scutatus: toxin III-4    73-5  LICV---MGPKT-PRT---CPRGQNLCYTKTWCDAFCSSRGKVVELGCAATCPIAKSYEDVT-CCSTDNCNPFPVRPRHPP
 4 Ophiophagus hannah: toxin B                73-5  TKCY---VTPDATSQT---CPDGQDICYTKTWCDGFCSSRGKRIDLGCAATCPKVKPGVDIK-CCSTDNCNPFPTWKRKH
 5 Naja haje anchietae: toxin III             72-5  IRCF---ITPDVTSQA---CPDGQNICYTKTWCDNFCGMRGKRVDLGCAATCPTVKPGVDIK-CCSTDNCNPFPTRERS
 6 Dendroaspis viridis: toxin 4.7.3.and 4.9.3 72-5  RTCY---KTPSVKPET---CPHGENICYTETWCDAWCSQRGKREELGCAATCPKVKAGVGIK-CCSTDNCDPFPVKNPR
 7                      toxin V               72-5  RICY---KTPSVKPET---CPHGENICYTETWCDAWCSQRGKRVELGCAATCPKVKAGVGIK-CCSTDNCNPFPVWNPR
 8 Naja melanoleuca: toxin b                  71-5  IRCF---ITPDVTSQI---CADG-HVCYTKTWCDNFCASRGKRVDLGCAATCPTVKPGVNIK-CCSTDNCNPFPTRNRP
 9                   toxin 3.9.4              71-5  KRCY---RTPDLKSQT---CPPGEDLCYTKKWCDAWCTSRGKVIELGCVATCPKVKPYEQIT-CCSTDNCNPHP-KMKP
10 Astrotia stokesii: toxin b                 70-5  LSCY---LGVKH-SQT---CPPGENVCFVKTWCDGFCNTRGERIIMGCAATCPTAKSGVHIA-CCSTDNCNIYA-KWGS
11 Laticauda semifasciata:toxin LS III        66-5  RECY---LNPHD-TQT---CPSGQEICYVKSWCNAWCSSRGKVLEFGCAATCPSVNTGTEIK-CCSADKCNTYP

12 Laticauda semifasciata: erabutoxin a       62-4  RICF---NQHSSQPQTTKTCPSGESSCVNKQWSDF----RGTIIERGC--GCPTVKPGIKLS-CCESEVCNN
13 Laticauda laticaudata: laticotoxin a       62-4  RRCF---NHPSSQPQTNKSCPPGENSCVNKQWRDH----RGTITERGC--GCPTVKPGIKLT-CCQSEDCNN
14 Naja mossambica mossambica: toxin III      62-4  LNCH---NQMSAQPPTTTRCSRWETNCVKKRWRDH----RGVKTERGC--GCPTVKKGIQLH-CCTSDNCNN
15 Naja haje annulifera: toxin CM-10          61-4  MICY---KQQSLQFPITTVCP-GEKNCVKKQWSGH----RGTIIERGC--GCPSVKKGIEIN-CCTTDKCNR
16 Hemachatus haemachates: toxin IV           61-4  LECH---NQQSSQTPTTQTCP-GETNCVKKQWSDH----RGSRTERGC--GCPTVKPGIKLK-CCTTDRCNK
17 Enhydrina schistosa: toxin 4               60-4  MTCC---NQQSSQPKTTTNCA--ESSCVKKTWSDH----RGTRIERGC--GCPQVKPGIKLE-CCHTNECNN
18 Pelamis platurus: pelamitoxin a            60-4  MTCC---NQQSSQPQTTTNCA--ESSCVKKTWSDH----RGTRIERGC--GCPQVKSGIKLE-CCHTNECWN
19 Aipysurus laevis: toxin a                  60-4  LTCC---NQQSSQPKTTTDCA--DNSCVKKTWQDH----RGTRIERGC--GCPQVKPGIKLE-CCKTNECNN
20 Dendroaspis viridis: toxin 4.11.3          60-4  RICY---NHQSTTPATTKSC--GENSCVKKTWSDH----RGTIIERGC--GCPKVKRGVHLH-CCQSDKCNN

21 Naja haje haje: toxin CM-2                 61-4  FTCF---TTPSDTSET---CPDGQNICYEKRWNSH----QGVEI-KGCVASCPEFESRFRYLLCCRIDNCNK
22 Dendroaspis agusticeps: toxin 2            60-4  MICY---SHKTPQPSATITC--EEKTCYKKSVRKL----PAVVAGRGC--GCPSKEMLVAIH-CCRSDKCNE
23 Dendroaspis viridis: toxin 4.9.6           60-4  MICY---SHKTPQNSATITC--EEKTCYKFVTKLP-----GVILARGC--GCPKKEIFRKSIHCCRSDKCNE
24 Hemachatus haemachates: toxin CM-1b        57-4  LECY----QKS---KVVT-CQPEQKFCYSDTMTFF---PNHPVYLSGCTF-CRTDES-GER--CCTTDRCNK

25 Naja melanoleuca: toxin 3.2                61-4  IKCH-----NTPLPFIYKTCPEGNNLCFKGTLKFP----KKITYKRGCADACPKTSALVKYV-CCNTDKCN
26 Naja naja annulifera: toxin CM-13a         61-4  LKCH-----NTQLPFIYKTCPEGKNLCFKTTLKKL---PLKIPIKRGCAATCPKSSALLKSV-CCNTDKC
27                       toxin CM-4a          60-4  LECN-----QLIP-IAHKTCPEGKNLCYKMFMVST----STVPVKRGCIDVCPKNSALVKYV-CCNTDRCN
28 Naja naja nivea       toxin VII-1          60-4  LKCH-----KLVPP-VWKTCPEGKNLCYKMFMVST----STVPVKRGCIDVCPKDSALVKYV-CCSTDKCN
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FIGURE 5: Comparison of κ-bungarotoxin to related snake venom toxins. Sequence 1 is that of κ-bungarotoxin determined in this study. Sequences 2–20 are curaremimetic postsynaptic long (2–11) and short (12–20) neurotoxins. Sequences 21–24 comprise a group of relatively nontoxic short toxins, and sequences 25–28 are cytotoxins. The numbers separated by a dash which appear just before the sequences indicate the number of amino acids in the protein and the number of disulfide bonds, respectively. Gaps (indicated by dashes) have been placed in the sequences to optimize homology. The numbering at the top indicates total positions rather than the number of actual residues.

Assignment of the Complete Sequence. The first 26 residues of κ-bungarotoxin were unambiguously identified by automated sequence analysis of the intact S-carboxymethylated protein. This sequence was extended to residue 30 by overlap with C4 and to residue 33 by overlap with T2. The overlap of peptide C5 with T2 further extended the sequence to residue 48, and peptide T3 allowed assignment of residue 49. Amino acid composition analysis of C5 shows that the remaining residues in C5 correspond exactly to peptide C6. Placement of C6 at positions 50–53 is also consistent with the cleavage specificity of chymotrypsin. Peptide T4 extends the sequence to position 54 by overlap and is also consistent with the placement of Arg-50 (trypsin specificity) and peptide C6. The remaining residues, positions 55–66, are contained within peptide C7 which overlaps with T4 at residue 54 and is consistent with the cleavage specificity of chymotrypsin. Peptide C8 is a subset of C7, and the compositions of C7 and C8 both indicate that the C-terminal residue is a histidine. Identification of the carboxy-terminal residue as histidine is verified by the results of the carboxypeptidase experiment which also confirms the placement of C7 and C8 as the carboxy-terminal peptides. Finally, the sequence of κ-bungarotoxin as determined in this manner agrees exactly with the amino acid composition determined independently by amino acid analysis as shown in Table II.

Table II: Amino Acid Composition of κ-Bungarotoxin

amino acid	composition calcd[a]	composition from sequence	amino acid	composition calcd[a]	composition from sequence
Asp	7.4	7	Met	0	0
Thr	6.0	6	Ile	3.8	4
Ser	6.3	6	Leu	4.3	4
Glu	6.2	6	Tyr	1.0	1
Pro	5.3	5	Phe	3.0	3
Gly	3.2	3	His	0.9	1
Ala	2.1	2	Lys	1.7	2
$^1/_2$-Cys	ND[b]	10	Arg	3.8	4
Val	1.7	2	Trp	ND	0
					66[c]

[a] Amino acid composition was determined after 24-, 48-, and 72-h hydrolyses. Serine and threonine were extrapolated to zero-time hydrolysis. Valine and isoleucine were estimated from the 72-h value. Based on a molecular weight of 6800. [b] ND, not determined. [c] Total.

Discussion

The complete sequence of κ-bungarotoxin has been determined from peptides generated by trypsin and chymotrypsin in conjunction with automated Edman analysis and carboxypeptidase A digestion of the intact protein. The complete κ-bungarotoxin structure contains 66 amino acid residues, is devoid of methionine and tryptophan, and has a calculated molecular weight of 7313. Table II shows the comparative amino acid composition of κ-bungarotoxin as determined by amino acid and sequence analysis and verifies that the sequence as determined accounts for all amino acids in the protein.

Although the overlaps at residues 49–50 and 53–54 are minimal, the proteolytic patterns and compositional data leave no doubt that the sequence presented is the only possible interpretation of the data. Furthermore, this sequence displays excellent overall homology to that of a wide variety of postsynaptic neurotoxins. Figure 5 compares the structure of κ-bungarotoxin to other snake venom toxins which comprise four structurally related subfamilies—long neurotoxins (1–11), short neurotoxins (12–20), short toxins (21–24), and cytotoxins (25–28)—found in the families Elapidae and Hydrophiidae. So far, no curaremimetic postsynaptic neurotoxins or homologous cytotoxins have been isolated from the venoms of other families.

The long and short neurotoxins block transmission at vertebrate neuromuscular junctions and in electric fish electric tissue by binding with high affinity to nicotinic acetylcholine receptors. In general, the blockade produced by the short neurotoxins, while relatively potent, is more reversible than that produced by the long neurotoxins, α-bungarotoxin being the most irreversible of the long neurotoxins. The cytotoxins, also referred to as cardiotoxins, direct lytic factors, cobramines, and membrane-active polypeptides, comprise a group of highly basic proteins structurally related to the curaremimetic neurotoxins but which are characterized functionally as affecting a great variety of cells, causing persistent depolarization of

the cell membrane and consequently impairing both the function and the structure of the cell. Finally, the short toxins, as they are referred to here, are a group of proteins isolated from the venom of elapids and hydrophids which show substantial homology to the other toxins but which generally display very little toxicity in themselves.

Approximately 90 venom proteins belonging to this homologous group of toxins have been sequenced (Lee, 1979; NBRF protein sequence data base[2]). Figure 5 is not, therefore, a comprehensive list of these proteins but has been constructed to be representative of the features of this group. κ-Bungarotoxin is of particular interest not only because of its unusual functional properties but also because it displays some striking structural deviations from the general trends. In the following discussion, the observations made concerning homology will apply to all known sequences and not necessarily just to those listed in Figure 5.

The most invariant feature of these four groups of proteins, and the one which forms the main basis for their relatedness, is the presence of four completely conserved disulfide bonds involving the cysteinyl residue at positions 3, 20, 27, 48, 52, 64, 65, and 70 in Figure 5. The four disulfide bonds are formed between Cys-3 and Cys-27, Cys-20 and Cys-48, Cys-52 and Cys-64, and Cys-65 and Cys-70. In addition, Gly-47 is completely conserved. No other feature of primary structure is common to all four groups, although Pro-53 occurs in all known sequences except one (sequence 24 in Figure 5) which is a relatively inactive variant and, with only a few exceptions, there is an asparaginyl residue at position 71. The short neurotoxins range in length from 60 to 62 residues, while the long neurotoxins range from 66 to 74 residues and are distinguished by the presence of an additional disulfide bond between Cys-33 and Cys-37. This disulfide is absent in the other three subfamilies. The cytotoxins and short toxins range in length from 57 to 61 residues.

If κ-bungarotoxin is not considered for the moment, among the long and short neurotoxins, Trp-32, Arg-40, and Gly-41 are also invariant. In addition, position 34 is usually occupied by an aspartyl residue. In the three known exceptions to this, where the aspartyl residue has been replaced either by an asparaginyl residue (sequence 11 in Figure 5) or by a glycyl residue (sequence 15 in Figure 5), the toxicity of the proteins is low, although it is as yet unclear whether or not this can be ascribed strictly to this difference. The short neurotoxins are also conserved at Ser-11, Tyr-28, Glu-45, Arg-46, Val-55, Lys-56, and Gly-58, while the long neurotoxins are conserved at Gly-23, Ala-50, Thr-51, Ser-66, and Asp-68. In addition, an aromatic residue, either a Phe or a Trp, is always found at position 36 between the extra disulfide of the long neurotoxins. Finally, the long neurotoxins are also characterized by a four-residue insertion in the vicinity of positions 36–39, which contains one cysteinyl residue of the fifth disulfide, a two-residue insertion at positions 49–50, and what amounts to a three-residue deletion shortening the loop formed by Cys-3 and Cys-27 relative to the short neurotoxins at positions 17–19 in Figure 5.

It is obvious from these observations that κ-bungarotoxin belongs to the group known as long neurotoxins. κ-Bungarotoxin contains 10 cysteinyl residues corresponding to the 10 conserved cysteinyl residues that form the 5 disulfides of the long neurotoxins. By virtue of this homology, it is probable that κ-bungarotoxin contains these same disulfide bonds. κ-Bungarotoxin also displays the characteristic insertions at positions 36–39 and 49–50 and the deletions at positions 17–19. In addition, Gly-23, Ala-50, Thr-51, Asp-68, and the aromatic residue at position 36 are conserved. κ-Bungarotoxin displays an average of 47% homology with the long neurotoxins, 35% homology with the short neurotoxins, and 25% homology with the cytotoxins. With the exception of toxin CM-2 from the Egyptian cobra (sequence 21 in Figure 5) which displays an unusually high degree of homology (48%) with κ-bungarotoxin, the other relatively nontoxic short toxins display an average homology of only 23%.

Some striking differences between κ-bungarotoxin and the other long neurotoxins are evident, and these may perhaps pertain to its unusual properties. Most notable of these is the lack of the otherwise invariant tryptophanyl residue at position 32. In κ-bungarotoxin, it has been replaced by a glutaminyl residue. Along with Arg-40 and Gly-41, Trp-32 is considered to be important for the neurotoxicity of the curaremimetic neurotoxins. With one exception, this tryptophanyl residue is missing in the noncuraremimetic short toxins and cytotoxins. Chemical modification of Trp-32, which is exposed on the surface of the protein in the long and short neurotoxins (Low, 1979a,b) invariably results in a loss of toxicity (Karlsson et al., 1973; Ohta & Hayashi, 1974a; Seto et al., 1970; Karlsson, 1979; Low, 1979b). However, the long neurotoxins lose toxicity to a much smaller degree than the short neurotoxins. It has been proposed (Low, 1979a,b), partly as a result of crystallographic studies of erabutoxin b, that the longer C-terminal chain of the long neurotoxins can at least partially compensate for the modification of Trp-32 in maintaining the proper structure for acetylcholine receptor binding by virtue of its proposed interaction with one edge of the active site (positions 40–47 in Figure 5). In the short neurotoxins, it appears that this function is normally served by the residues at positions 3–20 by virtue of the increased length of the Cys-3 to Cys-27 loop in this region. This idea is supported by both naturally occurring and experimentally produced shortened C-terminal tails in long neurotoxins (Low, 1979b; Karlsson, 1979). In this regard, it is interesting to note that κ-bungarotoxin lacks Trp-32, does not have a lengthened C-terminal region as in other long neurotoxins, and displays very little, if any, ability to block neuromuscular postsynaptic acetylcholine binding (V. Chiappinelli, unpublished results), although it is quite effective in blocking neuronal acetylcholine binding (Dryer & Chiappinelli, 1983).

It is also interesting to note that, with the exception of κ-bungarotoxin, the length of the polypeptide chain following the last cysteinyl residue (Cys-70) shows a certain correlation to the toxin's pharmacological properties. In all of the 22 known sequences of long neurotoxins, 4 or more residues follow Cys-70; in all of the 33 known sequences of short neurotoxins, only 2 residues follow Cys-70; and in all of the 27 known sequences of cytotoxins, only a single residue, or no residue in one case, is found after Cys-70. The importance of this observation to the mode of action is not clear at this time, but it appears that κ-bungarotoxin is the only exception so far noted. In view of the proposal that the length of the C-terminal tail may somehow affect binding to the acetylcholine receptor (Low, 1979a,b), it may be significant in explaining the properties of κ-bungarotoxin.

Tyr-28 is well conserved throughout all types of toxins depicted in Figure 5, but it has been conservatively replaced by a phenylalanyl residue not only in κ-bungarotoxin but also in the long neurotoxin from Stokes sea snake (sequence 10 in Figure 5) and a few cytotoxins. The results of several chemical

[2] National Biomedical Research Foundation, Georgetown University Medical Center, Washington, DC 20007.

modification studies (Ohta & Hayashi, 1974b; Karlsson & Sundelin, 1976; Huang et al., 1973) have shown that this nearly invariant tyrosinyl residue is evidently not essential for neurotoxin function. In fact, the natural variant found in Stokes sea snake venom exhibits a high level of toxicity (Maeda & Tamiya, 1978). It therefore seems unlikely that Phe-28 is involved in the unusual properties of κ-bungarotoxin.

Unlike all of the other long and short neurotoxins, κ-bungarotoxin contains a single residue insertion in the loop formed by the disulfide bond formed between Cys-52 and Cys-64. Furthermore, the invariant seryl residue found at position 66 in all long neurotoxins is replaced by a threonyl residue in κ-bungarotoxin. It is not possible at this time to say if either of these two variations has any effect on function.

The structure and function of the nicotinic receptors found in muscle and electric tissue have been elucidated largely through the use of neurotoxins as probes for these receptors (Kistler et al., 1982; Fambrough, 1979). By comparison, little is known of the neuronal nicotinic receptor (Morley & Kemp, 1981), in part because most neurotoxins examined do not block the function of this receptor. While the recently purified κ-bungarotoxin can be used as a probe for the neuronal nicotinic receptor (Chiappinelli, 1983), its sequence now provides a further opportunity to compare and contrast the properties of various nicotinic receptors. Structurally homologous neurotoxins are now known to be active at one or the other of these receptors in nerve and muscle, indicating that similarities must exist between active sites on the receptors. Nevertheless, the opposite physiological effects of κ-bungarotoxin and α-bungarotoxin are a clear indication that the active site of the neuronal nicotinic receptor is structurally distinct from that of the receptor in muscle. The availability of the unusual κ-bungarotoxin has thus expanded the usefulness of the family of postsynaptic neurotoxins as probes for nicotinic receptors.

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REFERENCES

Allen, G. (1981) in *Sequencing of Proteins and Peptides* (Work, T. S., & Burdon, R. M., Eds.) pp 39–40, Elsevier/North-Holland, Amsterdam.

Ambler, R. P. (1972) *Methods Enzymol. 25*, 143–154.

Brown, D. A., & Fumagalli, L. (1977) *Brain Res. 129*, 165–168.

Carbonetto, S. T., Fambrough, D. M., & Muller, K. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1016–1020.

Chiappinelli, V. A. (1983) *Brain Res. 277*, 9–21.

Chiappinelli, V. A., & Dryer, S. E. (1984) *Neurosci. Lett. 50*, 239–244.

Dryer, S. E., & Chiappinelli, V. A. (1983) *Brain Res. 289*, 317–321.

Fambrough, D. M. (1979) *Physiol. Rev. 59*, 165–227.

Grant, G. A., Henderson, K. O., Eisen, A. Z., & Bradshaw, R. A. (1980) *Biochemistry 19*, 4653–4659.

Grant, G. A., Sacchettini, J. C., & Welgus, H. G. (1983) *Biochemistry 22*, 354–358.

Huang, J. J., Liu, S. S., Ling, K. H., Chang, C. C., & Yang, C. C. (1973) *Toxicon 11*, 39–45.

Karlsson, E. (1979) *Handb. Exp. Pharmacol. 52*, 159–212.

Karlsson, E., & Sundelin, J. (1976) *Toxicon 14*, 295–306.

Karlsson, E., Eaker, D., & Drevin, M. (1973) *Biochim. Biophys. Acta 328*, 510–519.

Kistler, J., Stroud, R. M., Klymkowsky, M. W., Lalancette, R. A., & Fairclough, R. H. (1982) *Biophys. J. 37*, 371–383.

Lee, C. Y. (1979) *Adv. Cytopharmacol. 3*, 1–16.

Low, B. W. (1979a) *Adv. Cytopharmacol. 3*, 141–147.

Low, B. W. (1979b) *Handb. Exp. Pharmacol. 52*, 213–257.

Maeda, N., & Tamiya, N. (1978) *Biochem. J. 175*, 507–517.

Morley, B. J., & Kemp, G. E. (1981) *Brain Res. Rev. 3*, 81–104.

Ohta, M., & Hayashi, K. (1974a) *Biochem. Biophys. Res. Commun. 57*, 973–979.

Ohta, M., & Hayashi, K. (1974b) *Biochem. Biophys. Res. Commun. 56*, 981–987.

Seto, A., Sato, S., & Tamiya, N. (1970) *Biochim. Biophys. Acta 214*, 483–489.