Br. J. Pharmac. (1984), **82**, 709–716

Structure-activity studies of homologues of short chain neurotoxins from Elapid snake venoms

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1 Three neurotoxin homologues (CM10 and CM12 from *Naja haje annulifera* and S_5C_{10} from *Dendroaspis jamesoni kaimosae*) and two short neurotoxins (CM14 from *Naja haje annulifera* and erabutoxin b from *Laticauda semifasciata*) were examined by circular dichroism (c.d.) and tested for neuromuscular activity on chick biventer cervicis nerve-muscle preparations.

2 All three homologues had acetylcholine receptor blocking activity, as they abolished responses to indirect stimulation, acetylcholine and carbachol but had no effect on responses to direct muscle stimulation. CM10 was only about 5 times less potent than the short neurotoxin CM14; S_5C_{10} and CM12 were respectively 30 and 300 times less active. The block induced by the three homologues, but not by the neurotoxins, was readily reversed by washing.

3 CM10 and CM12 had virtually identical c.d. spectra which were closely similar to those of the neurotoxins. The spectrum of S_5C_{10} indicated changes in the environment of tyrosine-25 and in the position of tryptophan-29. These alterations could distort the 3-dimensional arrangement of the residues postulated to form the receptor binding site.

4 The results with CM10 and CM12 highlight a role for the first loop (residues 6–16) in the binding of neurotoxins to acetylcholine receptors, in addition to the previously postulated reactive site.

Introduction

The homologous postsynaptically acting neurotoxins present in Elapidae snake venom provide an excellent data set for structure-activity studies (Low, 1979; Karlsson, 1979). Thirty-three short neurotoxins (60–62 amino acid residues) and 31 long neurotoxins (66–73 amino acid residues) have been sequenced and all of them share many common features (Dufton & Hider, 1983). Several of these proteins have been subjected to X-ray crystallographic studies, (Tsernglou & Petsko, 1976; Kimball *et al.*, 1979; Walkinshaw *et al.*, 1980; Kistler *et al.*, 1982), and ^{1}H nuclear magnetic resonance studies (Arseniev *et al.*, 1976; Inagaki *et al.*, 1980; Hider *et al.*, 1982; Hosur *et al.*, 1983). As a result a clear view of their 3-dimensional structure in solution is emerging (Figure 1). Some of the conserved residues, for instance half cystine, glycine and proline lie towards the globular region of the molecule and presumably they are responsible for determining the tertiary structure. Emerging from this globular region are three relatively exposed β-sheeted loops and it is this portion of the protein which possesses the determinants of neurotoxicity (Low, 1979; Dufton & Hider 1983). Indeed, 14 amino acid residues have been highlighted which are now generally considered to be intimately associated with the reactive site common to these toxins (Ishikawa *et al.*, 1977). It is clear from the combined results of spin labelling (Tsetlin *et al.*, 1982), fluorescence (Tsetlin *et al.*, 1982) and monoclonal antibody studies (Boulain *et al.*, 1982) that residues centred on loops 2 and 3 are directly associated with receptor interaction and several proposals concerning the relative juxtaposition of these residues to form a mimic of a cholinoceptor ligand have been made (Dufton & Hider, 1977; Low, 1979; Tamiya *et al.*, 1980; Menez *et al.*, 1982).

In addition to these established neurotoxins, there is a group of closely related proteins with similar structures but greatly reduced toxicity. Because of their marked structural similarity, these molecules have been termed short neurotoxin homologues (Joubert, 1975; Joubert & Taljaard, 1979; Dufton & Hider, 1983). However, no pharmacological properties have been demonstrated for them apart from

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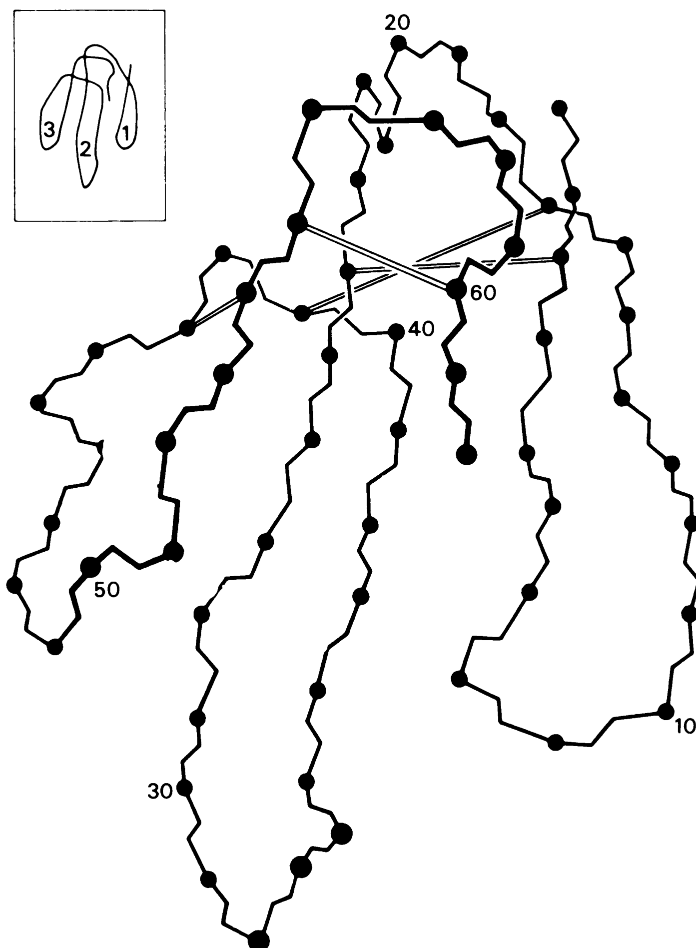

Figure 1 Schematic representation of the backbone of short neurotoxins, based on the X-ray structure of erabutoxin b (Tsernoglou & Petsko, 1976); inset: loop nomenclature.

their ability to kill mice. It has only been inferred that they bind to cholinoceptors to cause respiratory paralysis. Before they can be considered suitable for structure-activity studies it should be established whether or not their limited toxicity is in fact associated with an affinity for the nicotinic receptor. Because of their potential use in facilitating the distinction between the various proposals for toxin reactive sites, we have investigated this problem using an *in vitro* muscle preparation. We describe the properties of three toxin homologues, namely CM10 and CM12 (*Naja haje annulifera*) and S_5C_{10} (*Dendroaspis jamesoni kaimosae*), comparing them with two established short neurotoxins, CM14 (*Naja haje annulifera*) and erabutoxin b (*Laticauda semifasciata*).

Methods

Isolation and purification of toxin homologues

Desiccated *Naja haje annulifera* and *Dendroaspis jamesoni kaimosae* venoms were supplied by D. Muller, 215 Brakston Drive, Blairgowrie, Johannesburg, 2001, South Africa. Fractionation of the crude venoms by gel filtration and ion exchange chromatography has been previously described for both venoms (Joubert, 1975; Joubert, 1978). Purification of proteins CM10, CM12, CM14 and S_5C_{10} was achieved as previously reported (Joubert, 1975; Joubert & Taljaard, 1979). Erabutoxin b was a kind gift from Professor N. Tamiya, Tohoku University, Sendai, Japan.

Structural analysis

Secondary structure prediction analysis was performed using the methodology of Dufton & Hider (1977) and Hider & Ragnarsson, (1981), utilizing parameters determined by Levitt (1978).

Circular dichroism (c.d.) measurements were made using a Jasco J40CS. The results are expressed in terms of molar absorptivity (ΔE) based on molecular weights, the units are $mol^{-1} cm^{-1}$. Absorption spectra of the same solutions were recorded with a Cary 17 spectrophotometer.

Chick biventer cervicis nerve-muscle preparation

Biventer cervicis nerve-muscle preparations were isolated from chicks aged 4–10 days. Experimental conditions were identical to those described by Harvey & Karlsson (1982). Toxin concentrations are expressed as $\mu g\, ml^{-1}$. As the molecular weights are almost identical, comparison of potencies on this basis is valid.

Results

Secondary structure of toxin homologues

The secondary structure of the short neurotoxins is characterized by extensive β-sheet (Dufton & Hider, 1983). In this study, this is exemplified by erabutoxin b, as shown by its circular dichroism spectrum (Figure 2). The same pattern of structuring, as revealed by circular dichroism, is shown by the three homologues CM10, CM12 and S_5C_{10} (Figure 2). Furthermore, on heating, the backbone of these molecules collapses over the range 70–80°C, a range typical for many short neurotoxins (Dufton & Hider, 1983). In view of the homologous cystine links and similar β-sheet structural tendencies (Figure 3), it appears most likely that the structures of these three proteins are similar to that demonstrated for erabutoxin b (Tsernoglou & Petsko, 1976; Kimball *et al.*, 1979; Inagaki *et al.*, 1980). In particular, CM10 and CM12 show very similar profiles (Figure 2) to those typical of short neurotoxins over the entire wavelength range 190–320 nm. They both produce a positive band in the 230 nm region and a negative band at 270–290 nm. These bands are associated with the simultaneous presence of tyrosine and tryptophan. The 280 nm band is less negative in the spectra of CM10 and CM12 than in the erabutoxin b spectrum and this is associated with the presence of two tyrosines in the homologues as compared to the single tyrosine in erabutoxin b. These two absorptions (at 230 and 280 nm) are characteristic of *elapidae* neurotoxins, (Menez *et al.*, 1976; Drake *et al.*, 1980; Dufton & Hider, 1983) and indicate that the highly conserved tryptophan-29 occupies a similar environment in the homologues and in the neurotoxins. As might be expected for isotoxins dif-

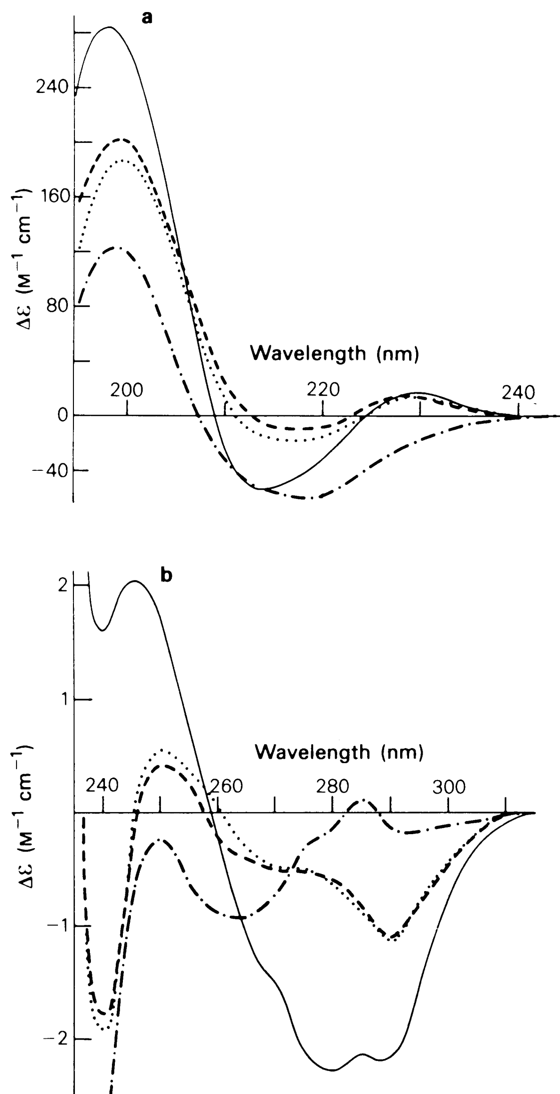

Figure 2 Circular dichroism spectra of four of the toxins tested. Molar absorptivity (Δε) is plotted as a function of wavelength (a) for u.v. range associated with protein secondary structure and (b) near u.v. range indicative of constrained aromatic residues. Erabutoxin b, (——); CM10, (----); CM12, (.......); and S_5C_{10} (-.-.-.). Solutions were in Tris-HCl (20 mM, pH 7.4). The c.d. of erabutoxin b at 198 nm falls at the extreme of a range of Δε values for short neurotoxins (280–150 $M^{-1} cm^{-1}$) (Dufton & Hider, 1983).

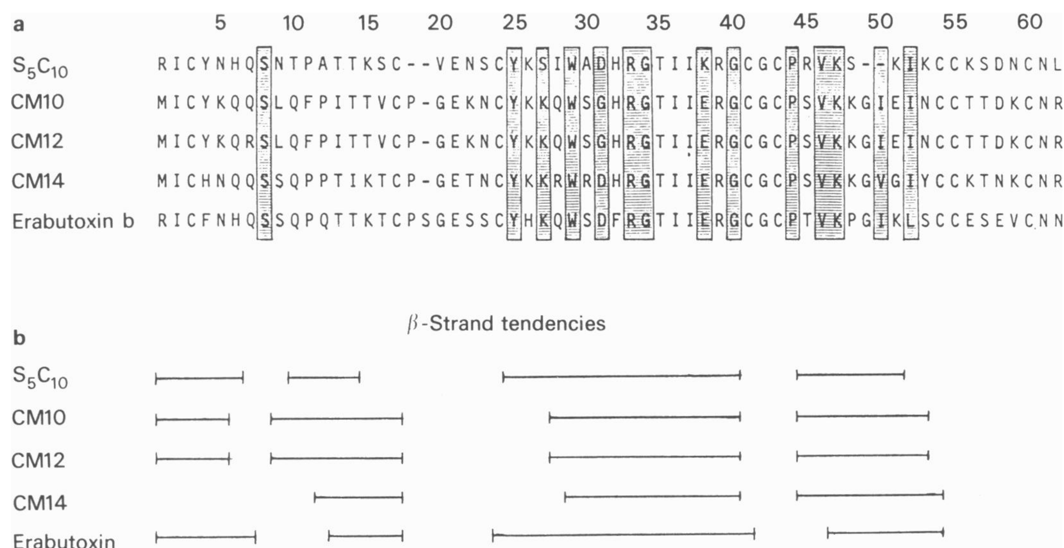

Figure 3 Toxin sequences (a) and regions of β-strand tendencies (b). The sequences have been aligned with respect to the position of the half cysteine residues. The IUPAC one letter notation for amino acids is used (*Eur. J. Biochem.*, **5**, 151–153, 1968). The positions thought to be important for neurotoxicity (Ishikawa *et al.*, 1977) have been enclosed. The β-strand tendencies were predicted for S_5C_{10}, CM10, CM12 and CM14 but were obtained by X-ray crystallography for erabutoxin b (Kimball *et al.*, 1979).

fering by a single substitution, the c.d. spectra of CM10 and CM12 are virtually identical. In contrast, S_5C_{10} lacks a positive band in the 230 nm region and has a weak absorption associated with tryptophan over the 280–300 nm region. These differences from the typical neurotoxin c.d. spectrum (Figure 2) imply that tryptophan-29 and other residues on the central loop are orientated in a manner not typical of neurotoxins. Indeed, the entire c.d. spectrum of S_5C_{10} at room temperature corresponds to that of a

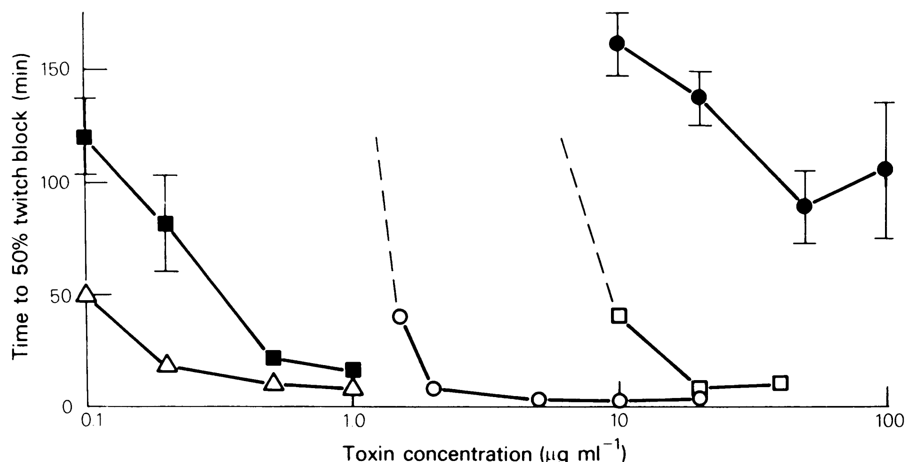

Figure 4 Time to 50% block of responses to indirect stimulation in chick biventer cervicis nerve-muscle preparations. (○), CM10; (●), CM12; (■), CM14; (□), S_5C_{10}; (△), erabutoxin b. Each point represents the mean of 6–12 experiments; standard errors are indicated by the vertical lines, unless smaller than the symbols. Broken lines indicate the approximate relationship at the concentration at which some preparations were blocked but others were not.

Table 1 A comparison of the relative toxicities of toxins as judged by their influence on chick biventer cervicis nerve-muscle preparations and LD_{50} values in mice

Toxin	*Equieffective concentration* ($\mu g\, ml^{-1}$)[1]	*Ratio*	*LD_{50}* ($\mu g\, g^{-1}$)[2]	*Ratio*
Erabutoxin b	0.1	1	0.1 (i.m.)	1
CM14	0.3	3	0.1 (s.c.)	1
CM10	1.5	15	5.0 (s.c.)	50
S_5C_{10}	10	100	5.5 (s.c.)	55
CM12	100	1000	62 (s.c.)	620

[1] Concentration required to achieve 50% twitch block in chick biventer cervicis muscles in 50 min.
[2] In mice. These values were taken from Tamiya & Arai, 1966 (erabutoxin b); Joubert, 1975 (CM10, CM12 and CM14); Joubert & Taljaard, 1979 (S_5C_{10}).

short neurotoxin at higher temperatures (Dufton & Hider, 1983). This conformation change, which is characterisitic of all *elapidae* neurotoxins, involves residues on the central loop (Drake *et al.*, 1980; Dufton & Hider, 1983).

Neuromuscular blocking activity of toxin homologues

All three homologues reduced responses of chick biventer cervicis preparations to indirect stimulation. Twitch blockade was slower with the lower concentrations of homologues (Figure 4). With CM10 there was an especially sharp division between effective and ineffective concentrations. Thus at 1 $\mu g\, ml^{-1}$ 6 preparations out of 11 did not block at all, whereas the average time to achieve 50% blockade in the other 5 preparations was 19 ± 7 min. At concentrations that blocked twitch responses, the 3 homologues also reduced contracture responses to acetylcholine and carbachol but not to KCl. The relative potency of the homologues together with the two short neurotoxins, CM14 and erabutoxin b was measured by recording the time taken to cause 50% inhibition of the twitch response to indirect stimulation (Figure 4). S_5C_{10} and CM12 are 100–1000 times less effective at the neuromuscular junction than the short neurotoxin erabutoxin b (Table 1). In contrast, CM10 was found to be only about 5 times less potent than CM14.

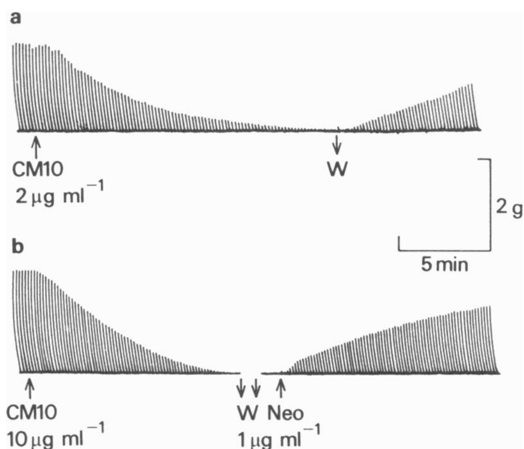

Figure 5 Reversibility of the twitch blockade induced by a neurotoxin homologue. (a) CM10 (2 $\mu g\, ml^{-1}$) abolished responses to indirect stimulation. A 20s wash (at arrow marked W) led to a rapid recovery. (b) CM10 (10 $\mu g\, ml^{-1}$) abolished twitch responses. After 3 x 20s washes there was only a very small recovery which was greatly increased by addition of neostigmine (Neo).

The paralysis produced by all three homologues, in contrast to that produced by CM14 and erabutoxin b, was easily reversed by washing (Figure 5). CM10-, but not CM14-induced blockade could also be reversed by the anticholinesterase, neostigmine (Figure 5).

Discussion

The three neurotoxin homologues blocked responses of an isolated nerve-muscle preparation to both nerve stimulation and exogenously applied nicotinic agonists, indicating a postjunctional site of action. As they did not block responses to direct muscle stimulation by KCl their postjunctional activity is not an effect on muscle contractility, but must be on the acetylcholine receptors. Thus, we have demonstrated that these neurotoxin homologues do indeed bind to the same target as the *elapidae* neurotoxins, although with reduced affinity.

These are important observations as they render the homologues suitable for realistic structure-activity studies, particularly as their overall conformation is similar to that of the neurotoxins. The relative efficiencies of the homologues as antagonists follows the sequence CM10 > S_5C_{10} > CM12 (Table 1). Indeed, CM10 possesses a potency just less than that of the short neurotoxin CM14 isolated from the

same venom. Although their relative toxicities *in vivo* follow the same order, the difference between the CM10 and CM12 LD_{50} values is much less than the differences in their potency on the isolated nerve-muscle preparation, an aspect which highlights the difficulties of using LD_{50} values in structure-activity studies.

The residues identified by Ishikawa *et al.* (1977) as being conserved throughout the short neurotoxins and therefore possibly associated with neurotoxicity are indicated in Figure 3. It is clear that, with the exception of glycine-31, they are conserved in both CM10 and CM12. In contrast there are several differences found in the S_5C_{10} sequence, namely serine-27, lysine-28 and two deletions in the critical 46–52 segment. We can consider how these changes affect the secondary structure and receptor binding ability of S_5C_{10}. The serine substitution for lysine at position 27 is unlikely to be solely responsible for the loss in receptor affinity as there are several potent neurotoxins, such as Toxin b, *Aipysurus laevis*; 4.9.3, *Dendroaspis viridis*; and Toxin a, *Ophiophagus hannah*, which also lack a positive residue at this position (Ishikawa *et al.*, 1977; Dufton & Hider, 1977). However, there are two other differences in the sequence of S_5C_{10} that are probably significant.

Firstly, it has been established by both X-ray and 'H n.m.r. studies on neurotoxins that the conserved tyrosine-25 is sited at the origin of the central loop and that it participates in an extensive hydrophobic interaction with the adjacent residues 27 and 28, with glutamate-38 also acting as a hydrogen bond acceptor for tyrosine-25 (Dufton & Hider, 1983). Both these residues are different in S_5C_{10}: serine-27 is unable to form extensive hydrophobic interactions with tyrosine-25 and lysine-38 is unable to act as a hydrogen bond acceptor for the phenolic hydroxyl function of tyrosine-25. Thus, a different local structure can be predicted for this region, and evidence for this is provided by the c.d. spectrum of S_5C_{10}. Tyrosine has been found to be intimately associated with the c.d. band at 230 nm (Woody, 1978), and the lack of a positive band in this region of the S_5C_{10} spectrum is indicative of a change in the environment of the tyrosine residue. Another toxin homologue, 4.9.6. isolated from the venom of *Dendroaspis viridis*, gives a similar c.d. spectrum and has alanine substituted at position 38 (Helliwell, 1978).

Secondly, the structure of S_5C_{10} differs from those of typical short neurotoxins by having 10, rather than 12, residues in the third loop (residues 44–53). Therefore, this segment will not extend as far as in typical neurotoxins. As a consequence it will be impossible for the same intimate interaction to occur between the conserved lysine-47 and valine-50 in the third loop and tryptophan-29 Kimball *et al.*, 1979). Tryptophan-29 in S_5C_{10} can, therefore, be predicted to be more exposed to the solvent than is the case for short neurotoxins. This is compatible with the atypical aromatic c.d. spectrum of S_5C_{10}. The c.d. spectrum lacks a 228 nm band and the $\Delta\varepsilon$ value in the 280–300 nm range is very weak. This is similar to the spectrum of a partially perturbed neurotoxin (Drake *et al.*, 1980), representing a local conformational change involving an increased exposure of tryptophan-29 to the aqueous medium (Dufton & Hider, 1983).

The shortened third loop of S_5C_{10} together with the different tyrosine-25 environment is likely to alter the 3-dimensional array of the residues believed to generate the cholinomimetic site, namely tryptophan-29, arginine-33 and lysine-47. Hence, the neurotoxin homologue S_5C_{10} would not be expected to bind to the acetylcholine receptor with high affinity.

The homologues CM10 and CM12 possess virtually all the residues previously associated with neurotoxicity (Table 1) with the exception of aspartate-31, which in both proteins is replaced by glycine. In view of the similar antagonist properties of the homologue CM10 and the standard short neurotoxin CM14, it would appear that in spite of being conserved throughout the short neurotoxins, aspartate-31 is not essential for interaction with the receptor. Similar conclusions have been reached for cobratoxin where aspartate-31 was modified by conjugation with glycine methyl ester without dramatic loss in activity (Chang *et al.*, 1971). Furthermore, the closely related long neurotoxin LsIII, the binding of which is readily reversible, possesses asparagine at position 31 (Maeda *et al.*, 1974).

Although CM10 has a high affinity for the nicotinic receptor, the same is not true for CM12, which is about 100 times less effective than CM10 on chick biventer muscle. This difference in activity must be a direct consequence of the single substitution of glutamine-7 in CM10 to arginine in CM12. As the substitution fails to produce any major structural change, judging from c.d. measurements, the difference in potency probably results from an interaction with the receptor. The location of this substitution is clearly critical, as the presence of arginine in the same loop at position-11 has no effect on neurotoxicity, as illustrated by the sequence of the powerful short neurotoxin β isolated from *Naja nivea* venom (Botes, 1971). The substitution is adjacent to the highly conserved serine-8 (Table 1), the amide function of which is hydrogen bonded to isoleucine-37 of the central loop of erabutoxin b (Low, 1979). The local positioning of residue-7 has been brought into question (Inagaki *et al.*, 1978; 1981) in so far as histidine-7 in erabutoxin b is located in entirely different environments when observed in the crystal state (Tsernoglou & Petsko, 1976; Kimball *et al.*, 1979)

and in the solution state (Dufton & Hider, 1983). It is conceivable that this first loop is rather flexible and as a result facilitates interaction between the neurotoxin and the receptor. Clearly the presence of a positively charged group at its extremity has a profound influence on the interaction.

A difference between the 33 short neurotoxins and both CM10 and CM12 is the hydrophobic nature of the first loop (segment 6–16). Using values derived by Levitt (1976) the average hydrophobicity of this region for short neurotoxins was calculated to be $+7.1\,\mathrm{kJ\,mol^{-1}}$ ($+22.7$ to $-8.8\,\mathrm{kJ\,mol^{-1}}$), i.e. hydrophilic. In contrast, the values for CM10 and CM12 are -37.4 and $-25.6\,\mathrm{kJ\,mol^{-1}}$, respectively, i.e. hydrophobic. Given that the substitution of asparagine for aspartate-31 is not critical, the difference in hydrophobic properties is likely to be the main reason for the slightly reduced affinity of CM10 for the nicotinic receptor when compared with typical neurotoxins. It may also account for the marked difference in LD_{50} values observed for CM10 and typical neurotoxins, as distribution of the homologue *in vivo* may be complicated by the surface activity of the relatively hydrophobic first loop.

All three homologues were much more easily reversed than short neurotoxins and in this respect they resemble the long neurotoxin LsIII (Harvey & Rodger, 1978). The reason for this interesting property is not clear. It may just result from weak binding affinity, but this appears not to be the case for CM10 and LsIII which block rapidly at quite low concentrations. Previously it was suggested (Harvey & Rodger, 1978) that reversiblility may be associated with a specific area of interaction. From the present results it is tempting to suggest a role for aspartate-31, but more structure-activity studies are essential before any firm conclusions concerning this concept can be made.

Some of this work was supported by a grant from the University of Strathclyde Research and Development Fund. Thanks are also due to Dr Alex Drake (King's College London) for advice on circular dichroism measurements. Correspondence to R.C.H. please.

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(Received December 20, 1983. Revised February 28, 1984.)