Homologous κ-Neurotoxins Exhibit Residue-Specific Interactions with the α3 Subunit of the Nicotinic Acetylcholine Receptor: A Comparison of the Structural Requirements for κ-Bungarotoxin and κ-Flavitoxin Binding†

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ABSTRACT: κ-Flavotoxin (κ-FTX), a snake neurotoxin that is a selective antagonist of certain neuronal nicotinic acetylcholine receptors (AChRs), has recently been isolated and characterized [Grant, G. A., Frazier, M. W., & Chiappinelli, V. A. (1988) *Biochemistry 27*, 1532–1537]. Like the related snake toxin κ-bungarotoxin (κ-BTX), κ-FTX binds with high affinity to α3 subtypes of neuronal AChRs, even though there are distinct sequence differences between the two toxins. To further characterize the sequence regions of the neuronal AChR α3 subunit involved in formation of the binding site for this family of κ-neurotoxins, we investigated κ-FTX binding to overlapping synthetic peptides screening the α3 subunit sequence. A sequence region forming a "prototope" for κ-FTX was identified within residues α3(51–70), confirming the suggestions of previous studies on the binding of κ-BTX to the α3 subunit [McLane, K. E., Tang, F., & Conti-Tronconi, B. M. (1990) *J. Biol. Chem. 265*, 1537–1544] and α-bungarotoxin to the *Torpedo* AChR α subunit [Conti-Tronconi, B. M., Tang, F., Diethelm, B. M., Spencer, S. R., Reinhardt-Maelicke, S., & Maelicke, A. (1990) *Biochemistry 29*, 6221–6230] that this sequence region is involved in formation of a cholinergic site. Single residue substituted analogues, where each residue of the sequence α3(51–70) was sequentially replaced by a glycine, were used to identify the amino acid side chains involved in the interaction of this prototope with κ-FTX. Substitution of several aliphatic (L_{54}, L_{56}, and L_{65}), aromatic (W_{55}, W_{60}, W_{67}, and Y_{63}), and positively charged (K_{57}, K_{64}, K_{66}, and K_{68}) residues drastically reduced the ability of the peptides to interact with κ-FTX. Comparison of the results of the present study with those obtained previously for the binding to the same prototope of the related neuronal toxin, κ-BTX, suggests that, like the interaction of peripheral AChRs with the family of the α-neurotoxins, the interaction of neuronal AChRs with toxins of the κ-neurotoxin family involves aromatic and aliphatic residues of general importance. Individual binding preference of specific toxins may involve charged amino acid residues at the toxin/AChR interface.

The subunits of nicotinic acetylcholine receptors (AChRs)[1] belong to a family of homologous proteins (Stroud et al., 1990; Betz et al., 1990a,b; Galzi et al., 1991; McLane et al., 1993). *Torpedo* electric organ and vertebrate muscle AChRs are pseudosymmetric pentameric complexes formed by four different subunits (α, β, γ or ε, and δ) (Raftery et al., 1980; Conti-Tronconi et al., 1982a,b; Mishina et al., 1986), whereas the neuronal AChRs identified so far are pentamers formed by one (αx), two (αx,βy), or three (αx,αy,βz) types of subunits (Deneris et al., 1988; Wada et al., 1988; Duvoisin et al., 1989; Lindstrom et al., 1989; Couturier et al., 1990a; Anand et al., 1991; Cooper et al., 1991; Luetje & Patrick, 1991; Bertrand et al., 1992; Conroy et al., 1992). Neuronal AChRs, in addition to having alternative subunit stoichiometries, are composed of several different subunit subtypes, i.e., at least eight different α subunits and five different β subunits [reviewed in Connolly (1989), Luetje et al. (1990b), and Deneris et al. (1991)]. The many possible combinations of α and β subunits result in a broad spectrum of possible neuronal with distinct pharmacological properties.

Two neurotoxins extensively used to distinguish AChR subtypes are α-bungarotoxin (α-BTX) and κ-bungarotoxin (κ-BTX)[2] from the venom of the banded krait, *Bungarus multicinctus* [reviewed in Chiappinelli (1989, 1991)]. α-BTX is an antagonist of vertebrate muscle and fish electric organ AChRs; κ-BTX is a potent inhibitor of the α3β2 neuronal AChRs.

Although the cholinergic sites seem to be at the interface of two AChR subunits (Blount & Merlie, 1989; Blount et al., 1990; Pedersen & Cohen, 1990; Middleton & Cohen, 1991; Gu et al., 1991; Sine & Claudio, 1991; Czajkowski & Karlin, 1991), the α subunit itself contributes important structural elements for cholinergic site formation. Even after denaturation the α subunit of the *Torpedo* AChR is able to bind α-bungarotoxin (Haggerty & Froehner, 1981), indicating that a continuous peptide sequence forms the binding site for this toxin. This has allowed our laboratory and other laboratories

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; α-BTX, α-bungarotoxin; κ-BTX, κ-bungarotoxin; κ-FTX, κ-flavitoxin; IAA, iodoacetamide; PBS, phosphate-buffered saline.

[2] κ-Bungarotoxin (κ-BTX) is also referred to as Bgt 3.1 (Ravdin & Berg, 1979), toxin F (Loring et al., 1984), and neuronal bungarotoxin (Lindstrom et al., 1987).

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to study the binding of α-BTX and κ-BTX to synthetic AChR sequences, as representative structural elements of the native AChR. Using this approach, we have identified the sequence regions and the individual residues that form cholinergic sites on α subunits of several AChRs (see below).

Like any approach that uses a sequence region excised from the structural context of the cognate native protein, the use of synthetic peptides has important caveats. First, a functional domain formed by residues from several different sequence regions of a protein or from more than one subunit may not be able to bind the ligand as an isolated sequence. Second, even if a given short sequence region contributes the critical residues to form a binding site, the corresponding synthetic peptide may fold in a way incompatible with binding of the ligand. Thus, negative results may obscure the importance of a given sequence region for formation of ligand-binding sites. On the other hand, a positive result might be obtained for a peptide when in fact the corresponding sequence region is inaccessible to the ligand in the native protein due to obstruction by the surrounding sequence of the same subunit or of other subunits. For instance, some monoclonal antibodies (mAbs) specific for the *Torpedo* AChR main immunogenic region (MIR) recognize the synthetic sequence α(67–76), which contains important constituent elements of the MIR from both the *Torpedo* and the human muscle α subunit, but do not cross-react with the native human AChR (Tzartos et al., 1988, 1990). Similarly, although coexpression in *Xenopus* oocytes of α3 and β2 neuronal subunits forms a κ-BTX-sensitive AChR, expression of the α3/β4 subunit pair results in an AChR that does not bind κ-BTX (Deneris et al., 1988; Luetje et al., 1990a,b). Also, an α-BTX-binding "prototope"[3] has been identified on the rat α5 subunit (McLane et al., 1990b), which may in fact be a "structural" subunit that does not bind cholinergic ligands when part of a native neuronal AChR molecule (Conroy et al., 1992).

Despite these caveats, synthetic peptide studies have demonstrated reliable predictive value. For instance, the location of the MIR on the muscle and *Torpedo* α subunits was correctly identified by using synthetic peptides, as verified by expression studies of mutant AChRs (Barkas et al., 1987; Lindstrom et al., 1988). Studies employing synthetic and biosynthetic peptides identified the sequence region of the α subunit of peripheral AChRs surrounding the vicinal cysteines at 192 and 193 as contributing to form the α-BTX-binding site (Neumann et al., 1986; Ralston et al., 1987; Wilson et al., 1988; Wilson & Lentz, 1988; Lentz & Wilson, 1988; Conti-Tronconi et al., 1988, 1989, 1990, 1991; Greismann et al., 1990; McLane et al., 1991b; Barkas et al., 1987; Aronheim et al., 1988; Ohana & Gershoni, 1990; Ohana et al., 1991), and suggested several residues in that sequence region as important to interaction with α-BTX (Conti-Tronconi et al., 1991): these results have been confirmed by *Xenopus* oocyte expression studies of mutated AChRs [e.g., see Tomaselli et al. (1991)].

Our laboratory has used synthetic peptide libraries corresponding to the deduced amino acid sequences of AChR α subunits to map the binding sites for α-BTX and κ-BTX, and to determine the structural requirements for the binding specificity of different AChR subtypes (Conti-Tronconi et al., 1988, 1989, 1990, 1991; McLane et al., 1990a,b, 1991a,b,c,d). We have shown that, in addition to the sequence region α180–200, a second sequence segment of the *Torpedo* α subunit, corresponding to residues α(55–74), also binds α-BTX (Conti-Tronconi et al., 1990). The synthetic sequence α(55–74) of the *Torpedo* α subunit also binds anti-AChR mAbs which compete with α-BTX for native AChR binding. On the other hand, the homologous sequence regions from the vertebrate AChR do not bind α-BTX effectively, and its identification as part of the AChR surface contributing—or in close proximity—to the cholinergic site has not been confirmed by other experimental approaches [e.g., see Barkas et al. (1987), Dennis et al. (1988), and Gazi et al. (1990)].

κ-BTX binds to several synthetic regions of the rat α3 subunit—peptides α3(1–18) and α3(51–70) predominantly, and the overlapping peptides α3(180–199) and α3(183–201) weakly (McLane et al., 1990a). The latter two peptides are homologous to the sequence region which in peripheral AChRs is best recognized by α-BTX (Conti-Tronconi et al., 1990). α-Najatoxin does not appear to recognize this sequence region (Wahlsten, Conti-Tronconi, and Raftery, unpublished experiments), in spite of the ability of α-najatoxin to fully compete with α-BTX for binding to native peripheral AChRs. Therefore, homologous snake neurotoxins may recognize partially overlapping sites, and the use of different toxins able to bind to the same AChR subtype may yield complementary, but unique information.

A new κ-neurotoxin, κ-flavitoxin (κ-FTX), has been characterized from *Bungarus flaviceps* venom (Chiappinelli et al., 1987). It shares 82% sequence identity with κ-BTX (Grant et al., 1988): the different residues are clustered in two sequence regions believed to interact with the AChR (Grant et al., 1988). κ-FTX and κ-BTX are mutually competitive for their binding to ganglionic AChR(s) which most likely include(s) the α3 subunit (Chiappinelli et al., 1987). Given the similar pharmacology and significantly diverged sequences of κ-FTX and κ-BTX, it is of interest to identify the sequence regions of the α3 subunit recognized by κ-FTX, for comparison with those recognized by κ-BTX.

Single substitution analogues of the sequence region α3(51–70) identified amino acid residues critical for κ-BTX binding to that prototope (McLane et al., 1990a). Given their sequence differences, κ-FTX and κ-BTX might differ in the interaction with individual residues on the AChR surface. A comparison of κ-FTX and κ-BTX binding specificities offered the unique opportunity for us to look at the importance of amino acid substitutions on the *toxin*, and to assess the effect of these amino acid replacements on the binding specificity to a prototope sequence.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Characterization. We used 32 overlapping peptides, as indicated along the abscissa of Figure 1, 18–20 residues long, corresponding to the complete sequence of the neuronal α3 subunit, and 20 single residue substituted analogues of the sequence α3(50–71), in which each amino acid of this sequence was sequentially substituted with glycine. The characterization, actual sequence and codes of the peptides, and their alignment on the α3 sequence were reported previously (McLane et al., 1990a, 1991a).

Radiolabeling of κ-FTX and κ-BTX. κ-FTX was purified from *Bungarus flaviceps* venom (Miami Serpentarium) as previously described (Chiappinelli et al., 1987). κ-BTX and α-BTX were purchased from Biotoxins, Inc. (St. Cloud, FL).

[3] "Prototope" is a term that was originally coined by House and Kemp (1987) for a peptide corresponding to the regulatory domain of protein kinase C, and subsequently used by Lentz and co-workers (Wilson et al., 1988) for a toxin-binding peptide of the AChR. It refers to a continuous peptide sequence that is able to form an independent ligand-binding site in the absence of surrounding structural elements, by analogy with the continuous epitope recognized by antibodies.

κ-FTX and κ-BTX were radiolabeled with carrier-free Na^{125}I by the chloramine T method as previously described (McLane et al., 1990a, 1991a). The specific activities of ^{125}I-κ-FTX and ^{125}I-κ-BTX were 110 and 320 Ci/mmol, respectively.

Peptide-Binding Assays. The binding of ^{125}I-κ-FTX and ^{125}I-κ-BTX to peptides was assessed by a solid-phase assay as previously described (McLane et al., 1990a, 1991a), with modifications to eliminate the high nonspecific binding observed for ^{125}I-κ-FTX. 96-well plastic plates (Nunc) were treated overnight at 4 °C with 20 μg/mL poly(DL-alanyllysine) (ICN) in 0.1 M $NaHCO_3$ and washed with 10 mM NaH_2PO_4/100 mM NaCl, pH 7.2 (phosphate-buffered saline, PBS). Peptides (500 μg/mL in 10 mM KH_2PO_4 buffer, pH 7.0) were added to plates (50 μg of peptide/well), an equal volume of 0.25% glutaraldehyde was added, and the plates were incubated overnight at 4 °C. The wells were washed with PBS 3 times and then with PBS containing 0.05% Tween 20 (PBS/Tween). Nonspecific binding was reduced by preincubation with 10 mg/mL cytochrome *c* in 10 mM KH_2PO_4, pH 7.0 ("blocking solution"), for 1–2 h at room temperature. ^{125}I-κ-FTX and ^{125}I-κ-BTX [(1–3) × 10^6 cpm] were added at concentrations of 0.1–0.2 μM in blocking solution. The plates were incubated overnight at 4 °C, as incubation at room temperature for shorter periods of time resulted in a high level of nonspecific binding by κ-FTX. Following incubation with toxins, the wells were rapidly washed 4 times with PBS/Tween. Bound ^{125}I-κ-FTX and ^{125}I-κ-BTX was removed with 150 μL of 1% sodium dodecyl sulfate. Using this protocol, nonspecific background binding of ^{125}I-κ-FTX and ^{125}I-κ-BTX to wells without peptide was greatly reduced, while the specific binding of ^{125}I-κ-BTX to the synthetic peptides was comparable to that previously reported (McLane et al., 1990a, 1991a). To test the specificity of peptide binding, wells that had been preblocked with cytochrome *c* were incubated with or without unlabeled κ-FTX (2 μM) in blocking solution at room temperature for 3 h. Wells were rapidly washed with PBS/Tween, incubated with ^{125}I-κ-FTX (0.1 μM) for 30 min at room temperature, and washed as described above. The role of free cysteine residues of the peptides in the interaction with ^{125}I-κ-FTX was determined by treatment of the peptides with 5 mM iodoacetamide for 30 min at 4 °C prior to the blocking step.

RESULTS

Mapping of the Binding Site(s) for κ-FTX on the Rat α3 AChR Subunit Using a Synthetic Peptide Library: Comparison with κ-BTX. The ability of ^{125}I-κ-FTX to bind to synthetic peptides corresponding to the α3 sequence was tested using a solid-phase assay (McLane et al., 1990a, 1991a). This assay was modified to reduce the otherwise high nonspecific binding of ^{125}I-κ-FTX. A typical experiment, in which the entire α3 subunit sequence was scanned for ^{125}I-κ-FTX binding, is shown in Figure 1. The results with ^{125}I-κ-FTX (black bars) are compared with those obtained with ^{125}I-κ-BTX (white bars). Both ^{125}I-κ-FTX and ^{125}I-κ-BTX consistently bind to the sequence segment α3(51–70). As previously demonstrated, ^{125}I-κ-BTX also binds the amino-terminal segment α3(1–18), but ^{125}I-κ-FTX does not convincingly recognize this sequence. ^{125}I-κ-FTX binds several other α3 subunit peptides corresponding to residues 66–85, 111–130, 153–171, and 302–321, and the overlapping peptides 334–353, 349–368, and 364–383 (Table I). The level of ^{125}I-κ-FTX binding to these sequences is relatively low [~20–35% of that observed for α3(51–70)]. Preincubation of these peptides with excess unlabeled κ-FTX reduced the binding of ^{125}I-κ-FTX to levels found for nonspecific binding to the solid support in the absence of peptide, resulting in a >10-fold reduction in ^{125}I-κ-FTX binding to peptide α3(51–70) and a 3–4-fold reduction in ^{125}I-κ-FTX binding for other peptides. Preincubation with α-BTX did not significantly reduce ^{125}I-κ-FTX binding to peptides

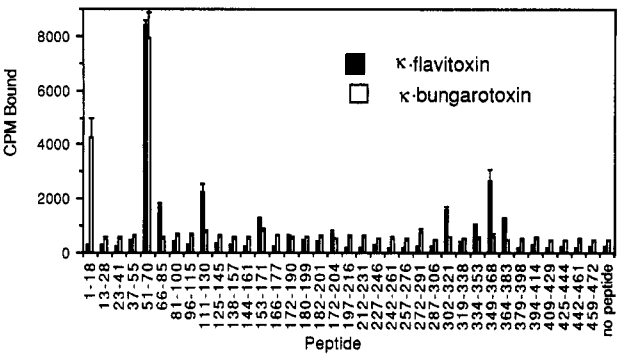

FIGURE 1: Mapping the binding sites of ^{125}I-κ-FTX and ^{125}I-κ-BTX on the AChR α3 subunit using a synthetic peptide library. Synthetic peptides corresponding to overlapping sequences of the deduced amino acid sequence for the rat α3 AChR subunit (Boulter et al., 1986) were tested for ^{125}I-κ-FTX and ^{125}I-κ-BTX binding as described under Experimental Procedures. The columns represent the mean of triplicate determinations, and the error bars are standard deviations.

Table I: ^{125}I-κ-FTX Binding to α3 Synthetic Sequences

amino acid residues	sequence	% rel binding[a]
1–18	SEAEHRLFQYLFEDYNEI	10 ± 8
13–28	EDYNEIIRPVANVSHP	7 ± 3
51–70	ETNLWLKQIWNDYKLKWKPS	≪100
66–85	KWKPSDYQGVEFMRVPAEKI	22 ± 2
111–130	KYTGEVTWIPPAIFKSSCKI	34 ± 9
153–171	KAKIDLVLIGSSMNLKDYW	22 ± 10
172–190	ESGEWAIKAPGYKHEIKYN	10 ± 2
180–199	APGYKHEIKYNCCEEIYQDI	10 ± 6
183–201	YKHEIKYNCCEEIYQDITY	10 ± 5
302–321	THTMPTWVKAVFLNLLPRVM	21 ± 2
334–353	PKTRTFYGAELSNLNCFSRA	19 ± 10
349–368	CFSRADSKSCKEGYPCQDGT	29 ± 7
364–383	CQDGTCGYCHHRRVKISNFS	25 ± 12
no peptide		7 ± 4

[a] The column reports the binding of ^{125}I-κ-FTX to the different peptides indicated, relative to binding to peptide α3(51–70), which was arbitrarily considered as 100%. For each experiment, the average cpm of triplicate samples was divided by the average cpm obtained for biding to peptide α3(51–70). The values reported are the mean of the percent relative binding obtained in three independent experiments ± the standard deviation.

Table I also reports the sequences of the peptides that bind ^{125}I-κ-FTX and/or ^{125}I-κ-BTX. Several of the peptides that are bound by ^{125}I-κ-FTX at low levels contained cysteine residues; i.e., α3(111–130) and α3(334–353) each contain one cysteine, whereas α3(349–368) and α3(364–383) both contain three cysteinyl residues. Given the possibility of disulfide exchange as a nonspecific type of interaction between ^{125}I-κ-FTX and these cysteine containing peptides, we were interested in determining the effects of sulfhydryl modification on ^{125}I-κ-FTX binding. Peptides that were bound to plates were treated with iodoacetamide (IAA) to carbamylmethylate free sulfhydryl residues of the peptides α3(111–130), α3(334–353), α3(349–368), and α3(364–383) prior to blocking and incubation with ^{125}I-κ-FTX. As shown in Figure 2, treatment with IAA did not affect ^{125}I-κ-FTX binding to any peptide to an extent discernible above random fluctuations in repeated assays.

The most conservative conclusion of the data presented above is that ^{125}I-κ-FTX binds specifically to the peptide α3(51–

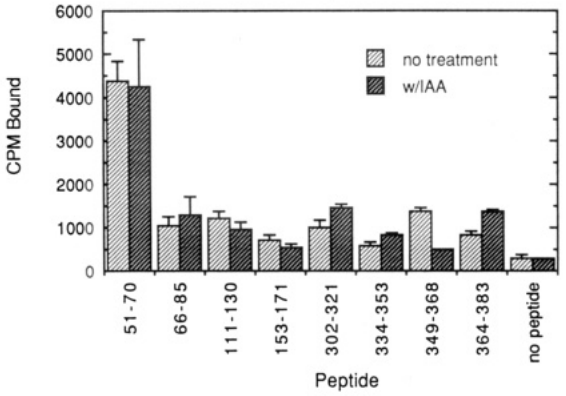

FIGURE 2: Effect of alkylation of peptide sulfhydryl groups with iodoacetamide. Selected peptides from the synthetic α3 subunit peptide library that was used to map the binding sites of ^{125}I-κ-FTX (Figure 1) were treated with iodoacetamide for 30 min at room temperature as described under Experimental Procedures. The binding of ^{125}I-κ-FTX to IAA-treated ("w/IAA") and untreated peptides was tested as described in Figure 1. The columns represent the mean of triplicate determinations, and the error bars are standard deviations.

70), and probably the overlapping sequence α3(66–85). The low-level binding of ^{125}I-κ-FTX to several additional peptides is not due to a sulfhydryl exchange effect, but may involve a tendency of ^{125}I-κ-FTX to interact in a nonspecific manner with hydrophobic and charged residues that are dispersed throughout these low-level-binding peptides. We cannot rule out the possibility that any of these additional peptides represent sequence segments that contribute to forming the binding site for κ-FTX in the intact receptor: but given the relative levels of binding compared to α3(51–70), it seems unlikely that they represent the main determinants for κ-FTX binding to the α3 AChR subunit.

Specificity of the Interaction of ^{125}I-κ-FTX Binding to Peptide α3(51–70): Amino Acid Residue Requirements Using Single-Substitution Analogues. In order to further examine the specificity of the interaction of ^{125}I-κ-FTX with peptide α3(51–70), and to determine if differences in the requirement for individual residues exist for ^{125}I-κ-FTX and ^{125}I-κ-BTX, we determined the effect of single amino acid substitutions on ^{125}I-κ-FTX binding. We used single-substitution analogues, in which each amino acid residue of the sequence α3(51–70) was sequentially replaced by glycine. These peptide analogues were tested for their ability to bind ^{125}I-κ-FTX, using the solid-phase assay described above. The results of a typical experiment are depicted in Figure 3, and the results of three experiments are summarized in Table II. The effects of amino acid substitutions of α3(51–70) on ^{125}I-κ-BTX binding, which we have reported previously (McLane et al., 1991a), are also summarized in Table II for comparison.

^{125}I-κ-FTX binding was more sensitive to amino acid substitutions than that of ^{125}I-κ-BTX (McLane et al., 1991a). A number of amino acid residue replacements resulted in large or moderate reductions in ^{125}I-κ-FTX binding. Consistent large reductions in ^{125}I-κ-FTX binding [~70–80% of that of the unsubstituted α3(51–70) sequence] included the following substitutions: (i) leucines at positions 54, 56, and 65; (ii) tryptophans at positions 55, 60, and 67; (iii) tyrosine at position 63; and (iv) lysine at positions 57, 64, 66, and 68. Moderate reductions in ^{125}I-κ-FTX binding (~30–50%) were observed for peptides with the following substitutions: T_{52}, N_{53}, Q_{58}, I_{59}, N_{61}, P_{69}, and S_{70}. Replacement of E_{51} or D_{62} did not affect ^{125}I-κ-FTX binding.

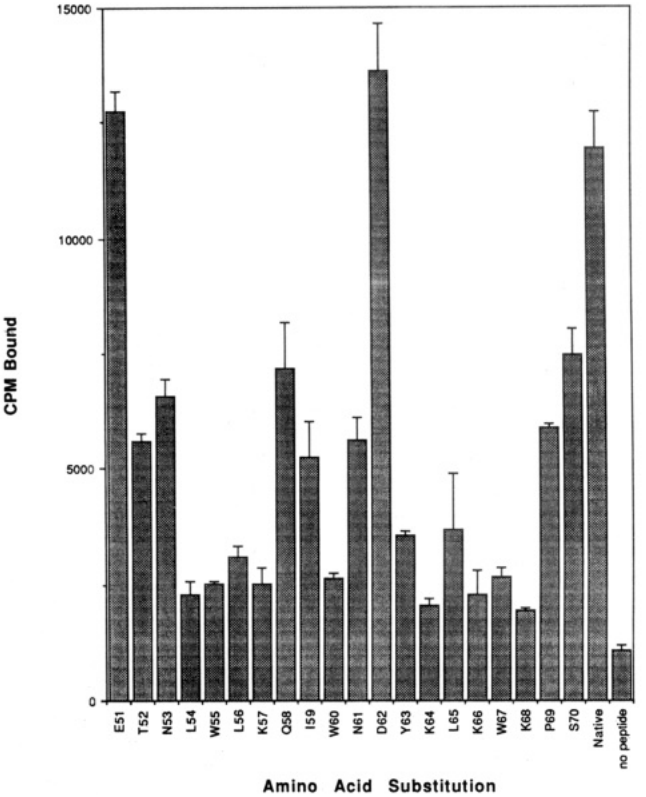

FIGURE 3: Effect of single amino acid substitutions of the peptide sequence α3(51–70) on ^{125}I-κ-FTX binding. Single amino acid substitution analogues corresponding to the sequence α3(51–70) in which each amino acid was sequentially replaced by glycine were tested for ^{125}I-κ-FTX binding, as described for Figure 1. The amino acid of the sequence α3(51–70) that was replaced by glycine is indicated along the horizontal axis. The columns represent the mean of triplicate determinations, and the error bars are standard deviations.

DISCUSSION

In the present study, we have used a synthetic peptide library corresponding to overlapping sequence segments of the entire rat α3 AChR subunit to map the binding site(s) of a scarce neurotoxin specific for neuronal AChRs, κ-FTX. We identified one peptide sequence, α3(51–70), that appears to form a major prototope of the κ-FTX-binding site of the α3 subunit. Other peptides were found to bind ^{125}I-κ-FTX at lower levels, and might reflect nonspecific binding of this toxin to hydrophobic sequence regions, or selective charge interactions. In a previous study using the same α3 subunit peptide library, we identified two peptides, α3(51–70) and α3(1–28), that bound ^{125}I-κ-BTX, a related κ-neurotoxin (McLane et al., 1990a). The differences in the binding patterns for these two homologous toxins indicated that the residue contacts forming the toxin/receptor interfaces might also differ.

To further study the differences in the structural requirements for ^{125}I-κ-FTX and ^{125}I-κ-BTX binding, we tested the ability of ^{125}I-κ-FTX to bind to a panel of peptide analogues of the sequence segment α3(51–70), in which each amino acid residue was sequentially replaced by glycine. The binding of κ-FTX was more sensitive to substitutions of the α3(51–70) sequence than that of κ-BTX (McLane et al., 1991a). Several amino acids were found to be critical for ^{125}I-κ-FTX binding. Amino acid substitutions that disrupted ^{125}I-κ-FTX binding included hydrophobic (L_{54}, L_{56}, and L_{65}), aromatic (W_{55}, W_{60}, W_{67}, and Y_{63}), and positively charged (K_{57}, K_{64}, K_{66}, and K_{68}) residues. Other substitutions had moderate effects on ^{125}I-κ-FTX binding (T_{52}, N_{53}, Q_{58}, I_{59}, N_{61}, P_{69}, and S_{70}), whereas substitution of negatively charged residues (E_{51}

Table II: Effect of Single-Residue Substitution of the Sequence Region α3(51–70) on the Binding of ^{125}I-κ-FTX and ^{125}I-κ-BTX

residue substituted[a]	binding rel to unsubstituted sequence α3(51–70)	
	^{125}I-κ-FTX[b]	^{125}I-κ-BTX[c]
E_{51}	1.14 ± 0.8	**0.33**
T_{52}	0.64 ± 0.15	1.03
N_{53}	0.60 ± 0.07	0.83
L_{54}	**0.22 ± 0.02**	**0.36**
W_{55}	**0.24 ± 0.04**	0.78
L_{56}	**0.30 ± 0.04**	**0.26**
K_{57}	**0.22 ± 0.02**	0.62
Q_{58}	0.70 ± 0.09	1.07
I_{59}	0.48 ± 0.07	2.06
W_{60}	**0.26 ± 0.03**	0.45
N_{61}	0.52 ± 0.06	1.16
D_{62}	1.18 ± 0.06	**0.20**
Y_{63}	**0.32 ± 0.03**	**0.21**
K_{64}	**0.20 ± 0.03**	0.66
L_{65}	**0.34 ± 0.04**	1.03
K_{66}	**0.20 ± 0.01**	0.78
W_{67}	**0.24 ± 0.04**	0.45
K_{68}	**0.19 ± 0.03**	0.86
P_{69}	0.63 ± 0.14	0.96
S_{70}	0.72 ± 0.11	0.42

[a] The peptides correspond to the sequence segment α3(50–71) where the amino acid residue substituted by glycine is denoted by the single-letter abbreviation and the position number of that residue within the sequence. [b] The relative binding of ^{125}I-κ-FTX to the single-residue-substituted peptides is compared to the native sequence, arbitrarily assigned as 1.0. The "relative binding" for each peptide was determined by dividing the mean cpm bound by the cpm obtained for the native peptide. The data for each experiment were collected in triplicate. The values presented are the mean "relative binding" calculated for three experiments, ± the standard deviation. Large reductions in binding are in boldfaced characters. [c] The relative binding of ^{125}I-κ-bungarotoxin to the single-residue-substituted peptides in compared to the native sequence (arbitrarily assigned as 1.0). The values presented in this table represent the relative K_ds that were reported previously (McLane et al., 1991a) and are given here for the sake of comparison. Large reductions in the apparent affinity for ^{125}I-κ-BTX are in boldfaced characters.

or D_{62}) had no effect. In contrast, ^{125}I-κ-BTX binding to α3(51–70) is relatively sensitive to replacement of these negatively charged residues (see Table II). In common with ^{125}I-κ-FTX, ^{125}I-κ-BTX is sensitive to substitution of leucines (L_{54}, L_{56}) and tryptophans (W_{55}, W_{60}, W_{67}), but the replacement of lysine residues (K_{57}, K_{64}, K_{66}) is much better tolerated by κ-BTX.

Although both κ-FTX and κ-BTX appear to interact with hydrophobic and aromatic amino acid residues, differences seem to exist in the manner in which these homologous toxins bind with the α3(51–70) prototope. These differences primarily involve the requirement for charged amino acid side chains; i.e., whereas the binding of ^{125}I-κ-FTX appears to be stabilized by electrostatic interactions with positively charged lysine residues, the binding of ^{125}I-κ-BTX appears to require negatively charged groups. By examining the amino acid sequences of these homologous neurotoxins, we may gain insight into the reason for these different charge requirements. For this purpose, the amino acid sequences of κ-FTX (Grant et al., 1988) and κ-BTX (Grant & Chiappinelli, 1985) are given in Figure 4. The sequences of κ-FTX (top) and κ-BTX (bottom) are numbered and aligned, and the amino acid residues that differ between the two neurotoxins are indicated by asterisks.

κ-FTX and κ-BTX are both highly basic peptides (p*I* 8.8 and 9.1, respectively), and their sequences differ at 12 of 66 positions. The residue differences cluster in two regions, region I (positions 23–33) and region II (positions 43–54), which are believed to be of functional importance (Grant et al., 1988).

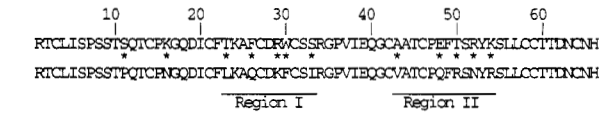

FIGURE 4: Comparison of the amino acid sequences of κ-FTX and κ-BTX. The amino acid sequences reported for κ-FTX (top) and κ-BTX (bottom) are given as the single-letter amino acid codes (Grant et al., 1988; Grant & Chiappinelli, 1985). The positions are numbered, and amino acid residues that differ in the two toxins are indicated by asterisks.

Region I of the α-neurotoxins is involved in the interaction with the peripheral AChR α subunit (Love & Stroud, 1986; Basus & Scheek, 1988; Basus et al., 1988). Within region I of the κ-neurotoxins, the amino acid substitutions are primarily conservative, and do not result in differences in charge distribution. It may be this region that determines the overall binding specificity of κ-FTX and κ-BTX for the sequence segment α3(51–70). The amino acid substitutions of region II, on the other hand, result in different overall charge, and differences in the spatial arrangement of charged groups. In particular, a glutamate at position 48 of κ-FTX, which is glutamine in κ-BTX, may determine the relative importance for positively charged groups of the α3(51–70) sequence for binding. On the other hand, the different spatial arrangements of an arginine residue (positions 50 and 52 of κ-BTX and κ-FTX, respectively) may account for the unique sensitivity of κ-BTX binding to substitution of negatively charged residues in the peptide α3(51–70).

On the basis of structural analysis of α-neurotoxins, the α-toxin/α AChR subunit interface is thought to involve primarily hydrophobic and hydrogen-bonding interactions, and only a few charged amino acids (Love & Stroud, 1986). Further support for the involvement of aromatic residues in the binding site for cholinergic ligands is the demonstration that acetylcholine binds to a synthetic receptor formed exclusively by aromatic residues that accommodates the quaternary ammonium ion via cation/π-electron interactions (Doughtery & Stouffer, 1990), and by the finding that the region of the acetylcholinesterase molecule likely to accommodate the positively charged moiety of acetylcholine is a gorge lined with ~14 aromatic residues (Sussman et al., 1991). Furthermore, treatment of *Torpedo* AChR with different affinity ligands [e.g., nicotine and acetylcholine photoaffinity analogues, lophotoxin, and [^{3}H]-*p*-(dimethylamino)benzenediazonium fluoroborate (DDF)] consistently yielded labeled aromatic residues (Y_{93}, W_{149}, Y_{190}, and Y_{198} of the α subunit) (Abramson et al., 1989; Dennis et al., 1988; Galgi et al., 1990; Cohen et al., 1991; Middleton & Cohen, 1991). Aromatic amino acids have been implicated in the binding of α-neurotoxins to the sequence segment surrounding the vicinal cysteines-192 and -193 of the α subunits of the AChRs from *Torpedo* electric organ (Conti-Tronconi et al., 1991; Tzartos & Remoundos, 1990; Chaturvedi et al., 1992) and vertebrate muscle (Wilson & Lentz, 1988; Ohana & Gershoni, 1990; McLane et al., 1991b), and of α-BTX-binding neuronal AChRs from chick brain (McLane et al., 1991c,d). We have previously shown that κ-BTX binding to the α3(51–70) sequence is sensitive to substitutions involving tryptophan residues, and have reconfirmed this trend in the present study with κ-FTX.

In addition to aromatic residues, substitution of several aliphatic amino acids (L_{54}, L_{56}, and L_{65}) influences κ-FTX binding. Aliphatic amino acids in the sequence regions α(180–200) and α(55–74) have also been implicated in α-BTX binding to *Torpedo* α subunit (Conti-Tronconi et al., 1991). Aliphatic

amino acid residues are also important for binding of α-BTX to the sequence region 180–199 of the neuronal α5 subunit sequence.

The structural requirements for α-neurotoxin binding to the peripheral AChRs, as predicted from the X-ray structure of α-BTX, indicate that the toxin/receptor interface is composed of residues that are hydrophobic or aromatic, and these structural predictions have been borne out by studies employing synthetic peptides. The results reported here and previously (McLane et al., 1991a) on the binding of κ-FTX and κ-BTX to the α3 prototope α3(51–70) support the notion that a similar mechanism may apply to the interaction of κ-neurotoxins with neuronal AChRs. Despite the common structural features of κ-BTX-, κ-FTX-, and α-BTX-binding peptides, differences in the ability of the sequence segments α(50–71) [or α(55–74)] and α(181–200) in forming prototopes for these toxins are observed. For instance, whereas α1(181–200) is the principal prototope identified for α-BTX, and α1(55–74) binds α-BTX poorly, κ-BTX binds α3(50–71) with higher affinity than α3(180–199) (Conti-Tronconi et al., 1990; McLane et al., 1990a). In addition, whereas κ-BTX binds both α3(1–18) and α3(50–71), κ-FTX only appears to recognize α3(50–71), as presented here. At present, however, we are not able to determine what structural differences confer these different preferences in binding. Hopefully, using recombinant toxins and site-directed mutagenesis answers to these questions will be resolved.

We have summarized in the introduction the several caveats of the experimental approach employed in this study, i.e., the use of a synthetic sequence region excised from the structural context of the cognate protein as a representative structural element of the binding site of the native protein. Great caution must be exercised in extrapolating the results of low-affinity binding to synthetic sequences to the very high affinity binding sites of the native, heterooligomeric complexes of the intact AChR. The relative contribution to binding of κ-FTX or related toxins of an individual sequence segment observed in our experiments using peptides may not be representative of its accessibility or conformation in the native receptor, or the actual importance of that sequence region for high-affinity toxin binding to native AChRs. On the other hand, identification of potential sequence regions that might contribute to forming the cholinergic-binding site on different AChR subtypes will make it possible to use targeted mutagenesis and expression of functional AChRs to test the actual importance of these sequence regions in formation of the high-affinity sites in the intact receptor.

In conclusion, we have mapped the binding site of κ-FTX on the rat α3 subunit and identified the critical amino acid residues involved in the interaction of κ-FTX with one binding sequence—α3(51–70). This exercise has confirmed our previous results which identified this same sequence region as forming a binding site for κ-BTX (McLane et al., 1990a, 1991a). The ability of κ-FTX to bind to the same sequence as κ-BTX, and the differences in amino acid residues demonstrated to be critical for κ-FTX and κ-BTX binding, provides us with additional information as to the interfacial amino acid residues in the interaction between the κ-neurotoxins and the α3 neuronal AChR. This type of information could ultimately be used for the rational design of peptide toxins that are specific for a given AChR subtype, but which are not naturally occurring, by protein engineering.

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