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AMINO ACID RESIDUES FORMING THE INTERFACE OF A NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR WITH κ-BUNGAROTOXIN: A STUDY USING SINGLE RESIDUE SUBSTITUTED PEPTIDE ANALOGS

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κ-Bungarotoxin is a high affinity antagonist of neuronal nicotinic acetylcholine receptors of the α3 subtype. Three sequence segments of the α3 subunit that contribute to forming the binding site for κ-bungarotoxin were previously located using synthetic peptides corresponding to the complete α3 subunit, i.e., α3(1-18), α3(50-71) and α3(180-201). Here we use single residue substituted peptide analogs of the α3(50-71) sequence, in which amino acids are sequentially replaced by Gly, to determine which residues are important for κ-bungarotoxin binding activity. Although no single substitution obliterated κ-bungarotoxin binding, several amino acid substitutions lowered the affinity for κ-bungarotoxin -- i.e., two negatively charged residues (Glu51 and Asp62), and several aliphatic and aromatic residues (Leu54, Leu56, and Tyr63). These results indicate that the interface of the α3 subunit with κ-bungarotoxin involves primarily hydrophobic interactions, and a few negatively charged residues.

The venom of the Formosan banded krait, *Bungarus multicinctus*, contains two closely related polypeptide toxins that bind with high affinity to nicotinic acetylcholine receptors -- α-bungarotoxin and κ-bungarotoxin. The latter toxin is also referred to as Toxin F, bungarotoxin 3.1, and neuronal bungarotoxin (1-5). α-Bungarotoxin blocks nicotinic transmission at the neuromuscular junction and a few cholinergic responses in the mammalian brain (6-8), whereas κ-bungarotoxin inhibits nicotinic acetylcholine receptors of the autonomic ganglia and both excitatory and inhibitory nicotinic transmission in various regions of the brain (9-15). Molecular genetic approaches have revealed extensive heterogeneity of the neuronal nicotinic acetylcholine receptors, as indicated by the cloning of multiple cDNAs for α (α2, α3, α4, α5) and β (β2, β3, β4) subunits (16-24), and expression of their transcripts in *Xenopus* oocytes (18, 20, 22, 25, 26). The nicotinic acetylcholine receptors formed by the α3 and α4 subunits are sensitive to κ-bungarotoxin, when coexpressed with the β2 subunit, whereas neither the α2, α3 or α4 nicotinic acetylcholine receptor subtypes are blocked by α-bungarotoxin (18, 20, 22). Interestingly, the α3β4 complex is insensitive to κ-bungarotoxin (22), indicating that the β subunit is also able to

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Abbreviations: αBGTBP (α-bungarotoxin binding protein), and HPLC (high pressure liquid chromatography).

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affect the ligand binding properties of neuronal nicotinic acetylcholine receptors. The sequences for two neuronal subunits corresponding to chick brain α-bungarotoxin binding proteins have recently been reported, designated αBGTBP α1 (or α7) and αBGTBP α2 (27, 28), and a homomeric receptor complex formed by expression of the αBGTBP α1 subunit in *Xenopus* oocytes has been shown to be sensitive to α-bungarotoxin (28).

Our laboratory has used synthetic peptides, corresponding to the deduced amino acid sequences of the cloned cDNAs, to map the α-bungarotoxin and κ-bungarotoxin binding sites on different nicotinic acetylcholine receptor α subunits (29-33). An α-bungarotoxin binding sequence on the *Torpedo* electric organ and vertebrate muscle α1 subunits has been shown to be between residues 173-204 by several laboratories, using synthetic and biosynthetic peptides (34-40). We have identified two sequence segments that contribute to forming the α-bungarotoxin binding site on the *Torpedo* α1 subunit at positions 55-74 and 181-200 (29, 30). The amino acid residues within the sequence *Torpedo* α1(181-200) that are crucial for α-bungarotoxin binding have been identified by using single residue substitution analogs of the α1(181-200) peptide (31). We have found that the κ-bungarotoxin binding site on the α3 subunit also involves several peptide loops, i.e., sequence segments α3(1-18), α3(50-71) and α3(180-201) (33). In the present study, we determine what residues within this sequence α3(50-71) are important for κ-bungarotoxin binding by sequentially substituting each amino acid of the sequence with Gly, and assessing the change in affinity of the substituted peptide for [^{125}I]κ-bungarotoxin. Although no single substitution obliterates κ-bungarotoxin binding activity, several amino acid substitutions effectively reduced the apparent affinity of the α3(50-71) peptide sequence for κ-bungarotoxin.

MATERIALS AND METHODS

Peptides. Peptides were synthesized by manual parallel synthesis (41). The purity was assessed by HPLC analysis on a C18 column and amino acid composition analysis of all peptides, and gas-phase sequencing of randomly selected peptides as previously described (30-33). The sequences and codes are shown in Figure 1.

[^{125}I]κ-bungarotoxin. κ-Bungarotoxin was purified from *Bungarus multicinctus* venom, radiolabelled with carrier-free [^{125}I], and calibrated as previously described (33). For some experiments commercial κ-bungarotoxin was used (Biotoxins, St. Cloud, FL). The specific activity of [^{125}I]κ-bungarotoxin was 40-50 Ci/mmol.

Binding Assays. Peptides (25 μg in 10 mM potassium phosphate pH 7.4) were added in triplicate to wells of 96-well Nunc Immunolon plates and incubated overnight at 4°C. The plates were washed twice with 10 mM sodium phosphate (pH 7.4) containing 100 mM NaCl and 0.1% Tween-20. The plates were blocked with 10 mg/ml cytochrome c in 10 mM potassium phosphate pH 7.4 for 2 hours at room temperature prior to addition of [^{125}I]κ-bungarotoxin solutions. Nonspecific binding, which accounted for less than 25% of the total binding, was determined by preincubating replicate wells with 10 μM unlabelled κ-bungarotoxin for 2 hours at room temperature prior to addition of [^{125}I]κ-bungarotoxin (0.01-1 μM). After 4 hours at room temperature, the wells were washed 5 times with 10 mM sodium phosphate (pH 7.4) containing 100 mM NaCl and 0.1% Tween-20 and bound radioactivity was removed by addition of 2% sodium dodecyl sulfate (0.2 ml/well) and counted in a gamma counter. Scatchard analysis was performed using the programs EBDA and LIGAND (42, 43). Experiments were repeated three times to insure an accurate determination of the Kd.

RESULTS AND DISCUSSION

Synthetic peptides corresponding to the sequence segment 51-70 of the rat nicotinic acetylcholine receptor α3 subunit, which contains an important structural element of the κ-bungarotoxin binding site (32), and single residue substitution analogs of this sequence segment were used in this study (Figure 1). The peptides were tested for their ability to bind [^{125}I]κ-bungarotoxin using solid phase assay described above, that we have previously used to map the α-bungarotoxin and κ-bungarotoxin binding sequences on neuronal nicotinic acetylcholine receptor α subunits (32, 33). The role of each amino acid residue within of the sequence α3(51-70) for κ-bungarotoxin binding activity was assessed by determining the affinity of each single residue substituted peptide analog relative to the native sequence. The concentration of [^{125}I]κ-bungarotoxin was varied between 0.01-1 μM, and Scatchard analysis was performed after correction for nonspecific binding. The results of a typical experiment (n=3) are shown in Fig. 2. The values obtained for the Kds in different experiments are summarized in Table I.

Although no single substitution obliterated κ-bungarotoxin binding activity, several substitutions lowered the affinity for κ-bungarotoxin relative to the peptide corresponding to the native sequence, suggesting that these residues are involved in κ-bungarotoxin binding. Amino acid substitutions that resulted in a 3 to 5-fold increase in the apparent Kd included Glu51, Leu54, Leu56, Asp62, and Tyr63. A slight decrease (2 to 3-fold) in the affinity for κ-bungarotoxin was observed for amino acid substitutions of Trp60, Trp67 and Ser70. The Bmax values, which merely reflect the ability of peptides to bind to the plastic matrix of the solid support in an active conformation, varied substantially between different peptides, but seemed to increase when charged amino acids were substituted (See Fig. 2).

PEPTIDE	SEQUENCE
Native	ETNLWLKQIWNDYKLKWKPS
E51	**G**TNLWLKQIWNDYKLKWKPS
T52	E**G**NLWLKQIWNDYKLKWKPS
N53	ET**G**LWLKQIWNDYKLKWKPS
L54	ETN**G**WLKQIWNDYKLKWKPS
W55	ETNL**G**LKQIWNDYKLKWKPS
L56	ETNLW**G**KQIWNDYKLKWKPS
K57	ETNLWL**G**QIWNDYKLKWKPS
Q58	ETNLWLK**G**IWNDYKLKWKPS
I59	ETNLWLKQ**G**WNDYKLKWKPS
W60	ETNLWLKQI**G**NDYKLKWKPS
N61	ETNLWLKQIW**G**DYKLKWKPS
D62	ETNLWLKQIWN**G**YKLKWKPS
Y63	ETNLWLKQIWND**G**KLKWKPS
K64	ETNLWLKQIWNDY**G**LKWKPS
L65	ETNLWLKQIWNDYK**G**KWKPS
K66	ETNLWLKQIWNDYKL**G**WKPS
W67	ETNLWLKQIWNDYKLK**G**KPS
K68	ETNLWLKQIWNDYKLKW**G**PS
P69	ETNLWLKQIWNDYKLKWK**G**S
S70	ETNLWLKQIWNDYKLKWKP**G**

Figure 1. Single Amino Acid Substitution Analogs of the Sequence Segment α3(50-71).
The sequence and code for each peptide is indicated, and the position of amino acid substituted by Gly is indicated in **bold**.

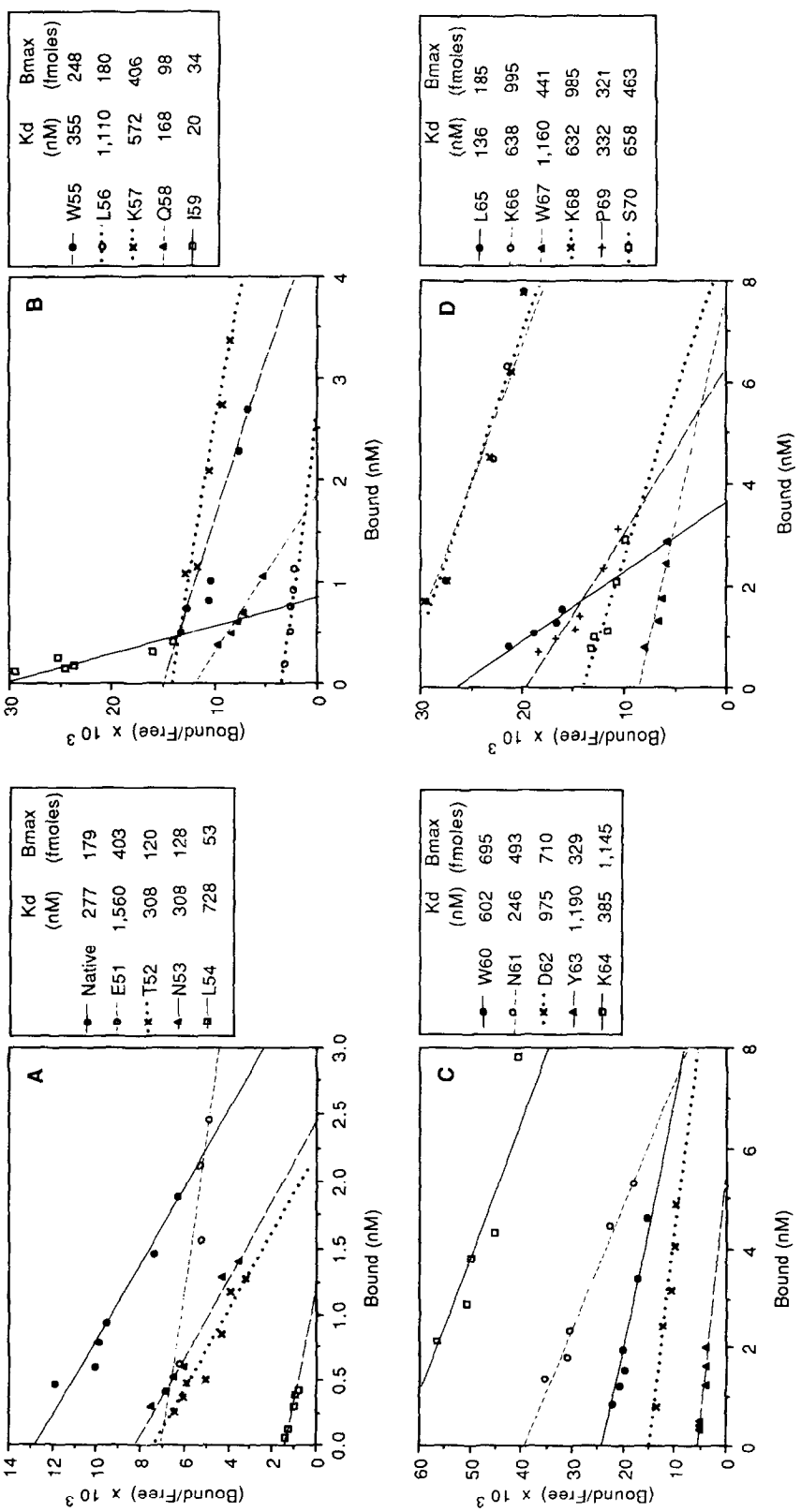

Figure 2. Scatchard Analysis of [^{125}I]κ-Bungarotoxin Binding to Peptides. For each peptide, the total radioactivity bound and the nonspecific radioactivity bound (after blocking with 10 μM unlabelled κ-bungarotoxin) were determined at each concentration of κ-bungarotoxin. Specific binding, the total minus the nonspecific bound was used to calculate an apparent Kd using the ligand-binding analysis programs EBDA and LIGAND (42, 43).

TABLE I. Scatchard Analysis of Single Amino Acid Substitution Analogs of the Sequence Segment α3(51-70)

Peptide	Kd (nM)	Relative to Native (fold increase in Kd)
Native	326 (65)	1.0
E51	984 (53)	3.0
T52	317 (67)	1.0
N53	391 (85)	1.2
L54	905 (42)	2.8
W55	420 (39)	1.3
L56	1,263 (166)	3.9
K57	530 (87)	1.6
Q58	304 (171)	0.9
I59	158 (122)	0.5
W60	720 (91)	2.2
N61	281 (120)	0.9
D62	1,645 (202)	5.0
Y63	1,570 (194)	4.8
K64	492 (106)	1.5
L65	316 (113)	1.0
K66	420 (64)	1.3
W67	723 (98)	2.2
K68	378 (75)	1.2
P69	341 (111)	1.0
S70	771 (120)	2.4

Kds were obtained by Scatchard analysis of data points in triplicate for each concentration, and the average Kd of three experiments is given with the standard deviations in parentheses.

Our results indicate that the amino acid residues, which are crucial for interaction of the α3 subunit with κ-bungarotoxin, include i) negatively charged residues (Glu51 and Asp62), which may reflect the requirement for electrostatic interactions with several positively charged amino acid side chains on the binding surface of κ-bungarotoxin, and ii) aliphatic and aromatic residues (Leu54, Leu56, and Tyr63, and perhaps, Trp60 and Trp67). Although the structure of κ-bungarotoxin has not been reported, its sequence and charge properties are homologous to α-bungarotoxin, for which structural data are available (44-46). A model for the binding interface of α-bungarotoxin has been proposed that involves primarily hydrophobic and hydrogen bonding

interactions, with the participation of only a few charged groups (44). Further support for the role of aromatic residues in formation of the cholinergic site has been indicated by the binding of acetylcholine to a completely synthetic receptor comprised primarily of aromatic rings, which accommodate the quaternary ammonium group of acetylcholine through cation-π electron interactions (47). The importance of complementary aromatic side chains on the *Torpedo* α1 subunit sequence segment 181-200 in conferring high affinity binding of α-bungarotoxin has been suggested by the lower affinities of peptides corresponding to the homologous sequences of vertebrate muscle α1 subunits from different species for which one or more of the aromatic residues is nonconservatively replaced (37, 40, McLane et al., unpublished). The results reported here indicate that κ-bungarotoxin binding to the α3 subunit has a similar requirement for aromatic amino acids.

In conclusion, we demonstrate that synthetic peptides corresponding to single amino acid substitution analogs can be used to identify the important amino acid side chains that are involved in the binding of ligands. The success in using this approach to define the α-bungarotoxin and κ-bungarotoxin binding sites on different nicotinic acetylcholine receptor α subunits has relied on the very high affinity of these toxins for the native receptor, and the ability of continuous sequence segments of the α subunits to form independent toxin binding sites in the absence of other surrounding peptide loops of the native α subunit and from other subunits of the nicotinic acetylcholine receptor complex. The ability to rapidly synthesize single site mutants of these toxin binding sequences has provided a powerful method to determine the important structural features of these binding sites.

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