Structure–Function Relationships of Curaremimetic Neurotoxin Loop 2 and of a Structurally Similar Segment of Rabies Virus Glycoprotein in Their Interaction with the Nicotinic Acetylcholine Receptor†

Thomas L. Lentz*

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

Received May 22, 1991; Revised Manuscript Received August 29, 1991

ABSTRACT: Peptides corresponding to portions of curaremimetic neurotoxin loop 2 and to a structurally similar segment of rabies virus glycoprotein were synthetically modified in order to gain information on structure–function relationships of neurotoxin loop 2 interactions with the acetylcholine receptor. Binding of synthetic peptides to the acetylcholine receptor of *Torpedo* electric organ membranes was assessed by measuring their ability to inhibit the binding of ^{125}I-α-bungarotoxin to the receptor. The peptides showing the highest affinity for the receptor were a peptide corresponding to the sequence of loop 2 (residues 25–44) of *Ophiophagus hannah* (king cobra) toxin *b* ($IC_{50} = 5.7 \times 10^{-6}$ M) and the structurally similar segment (residues 173–203) of CVS rabies virus glycoprotein ($IC_{50} = 2.6 \times 10^{-6}$ M). These affinities were comparable to those of *d*-tubocurarine ($IC_{50} = 3.4 \times 10^{-6}$ M) and suberyldicholine ($IC_{50} = 2.5 \times 10^{-6}$ M). These results demonstrate the importance of loop 2 in the neurotoxin interaction with the receptor. N- and C-terminal deletions of the loop 2 peptides and substitution of residues invariant or highly conserved among neurotoxins were performed in order to determine the role of individual residues in binding. Residues 25–40 are the most crucial in the interaction with the acetylcholine receptor. Modifications involving Lys-27, Trp-29, Phe-33, Arg-37, and Gly-38 reduced affinity of binding. R37D and F33T modifications reduced the affinity of α-bungarotoxin residues 28–40 by an order of magnitude. Arg-37 may correspond to the positively charged quaternary ammonium group and Phe-33 to the hydrophobic acetyl methyl group of acetylcholine. These residues may interact with complementary electronegative and hydrophobic sites, respectively, on the receptor α-subunit surface in the vicinity of Cys-192 and Cys-193. The largest decrease in affinity (~20-fold) followed substitution of Gly-38 with a tryptophan residue. The introduction of a bulky indole side chain at this site may sterically interfere with the interaction of Arg-37 with an anionic site on the receptor. Substitution of Asp-31, a potential counterpart of the hydrogen bond acceptor formed by the carbonyl oxygen of acetylcholine, with an alanine had no effect on affinity. The rabies virus glycoprotein synthetic peptides acted in a manner very similar to that of the neurotoxin peptides. Since this region of the glycoprotein contains residues corresponding to all of the functionally invariant neurotoxin residues, it may interact with the acetylcholine receptor through a mechanism similar to that of the neurotoxins.

Neurotoxins from the venoms of certain snakes such as cobras, kraits, mambas, and others bind specifically and with high affinity ($K_D = 10^{-9}$–10^{-10} M) to the nicotinic acetylcholine receptor (AChR)[1] (Lee, 1972; Karlsson, 1979; Dufton & Hider, 1983; Endo & Tamiya, 1987; Lentz & Wilson, 1988). These properties of the neurotoxins enabled the purification and biochemical characterization of this neurotransmitter receptor. The neurotoxins competitively block the binding of acetylcholine and other cholinergic ligands to the AChR and thus are believed to bind at or near the acetylcholine-binding site on the receptor. Thus, the toxins have been used extensively as biological probes to characterize the acetylcholine-binding site on the AChR.

X-ray crystallographic studies of neurotoxins reveal them to be hand-shaped molecules consisting of a disulfide-bonded core from which extend a long central loop and two shorter flanking loops (Low et al., 1976; Tsernoglou & Petsko, 1976; Walkinshaw et al., 1980; Bourne et al., 1985; Love & Stroud, 1986; Low & Corfield, 1986). A large number of neurotoxins and related venom proteins have been sequenced, and these comparative sequence data have been used to deduce structure–function relationships (Karlsson, 1979; Dufton & Hider, 1983; Ménez et al., 1984; Endo & Tamiya, 1987). Some residues are conserved in neurotoxins and nonneurotoxic venom polypeptides. These residues are considered structurally invariant residues and are most likely involved in disulfide pairing and conformation. Other residues are conserved in the neurotoxins but not in the nonneurotoxins and are considered functionally invariant residues. The functionally invariant residues are Trp-29, Asp-31, Phe-, His- or Trp-33, Arg-37, and Gly-38 (Karlsson homology alignment positions). Lys-27 and Lys-53 are highly conserved, and position 42 is Asp or Glu. These residues are located in the three-stranded β-sheet structure of the toxin molecule, and their side chains are oriented in the same direction from the concave surface of the molecule (Love & Stroud, 1986; Endo & Tamiya, 1987). In addition to sequence comparison, chemical modification of toxins has been performed to investigate the role of particular residues in binding (Karlsson, 1979; Dufton & Hider, 1983; Martin et al., 1983; Endo & Tamiya, 1987; Lentz & Wilson, 1988). These studies have shown that modification of certain residues reduces toxicity or binding to the AChR, although no single modification completely abolishes binding.

† This work was supported by National Science Foundation Grant BNS 88-05780 and National Institutes of Health Grant NS 21896.

* Address correspondence to this author at the Department of Cell Biology, Yale University School of Medicine, 333 Cedar St., P.O. Box 3333, New Haven, CT 06510.

[1] Abbreviations: AChR, acetylcholine receptor; α-Btx, α-bungarotoxin; α-Ctx, α-cobratoxin.

0006-2960/91/0430-10949$02.50/0

Most of the functionally invariant residues are located on loop 2 (residues 25–44) of the toxin molecule. The guanidinium group of Arg-37 is the only cationic group common to all of the neurotoxins and may represent the counterpart of the quaternary ammonium group of acetylcholine (Karlsson, 1979). It has been proposed that some of the invariant residues at the tip of loop 2 mimic the structure of acetylcholine or *d*-tubucurarine (Tsernoglou et al., 1978; Low, 1979; Tamiya et al., 1980; Ménez et al., 1984; Low & Corfield, 1986). A hydrogen-bonded ion pair between the guanidinium group of Arg-37 and the side-chain carboxylate of Asp-31 has been proposed to stereochemically resemble acetylcholine (Tsernoglou et al., 1978). Loop 2, therefore, may be important in the recognition and binding of toxin to the acetylcholine-binding site on the AChR. Antibodies against the tip of loop 2 overlap the acetylcholine-binding site (Charpentier et al., 1990). However, a broad area of contact involving multipoint interactions forms between the concave surface of the toxin molecule and the receptor.

Synthetic peptides have been utilized to identify regions of the toxin molecule involved in binding to the AChR. A 33-residue peptide (positions 16–55) of a *Naja naja philippinensis* toxin bound to the receptor with an apparent affinity of $K_D = 2.2 \times 10^{-7}$ M (Juillerat et al., 1982). A synthetic loop peptide corresponding to positions 27–42 of α-bungarotoxin (α-Btx) was shown to bind to the AChR (McDaniel et al., 1987). Peptides corresponding to loop 2 of king cobra (*Ophiophagus hannah*) toxin *b* and the tip of loop 2 of α-Btx bound to the AChR (Lentz et al., 1987). In a functional assay, these peptides behaved as antagonists and inhibited carbachol-induced $^{22}Na^+$ flux into BC3H1 cells (Donnelly-Roberts & Lentz, 1989).

In the present study, synthetic peptides were used to investigate structure–function relationships of the loop 2 interaction with the AChR. Previously, we showed that loop 2 peptides were effective in competing binding of α-Btx to purified AChR (Lentz et al, 1987). Here, a series of N- and C-terminal deletions and substitution of functionally invariant residues in loop 2 peptides were performed in order to determine the role of individual residues in binding. This approach has the advantage that any observed changes in function can be assigned with some degree of certainty to the modification produced. With chemical modification, few reagents are absolutely specific for a particular functional group; it is often difficult to modify a single residue in a protein; and it is not always certain that the desired modification was achieved. In addition to neurotoxin peptides, rabies virus glycoprotein peptides have been investigated in this study. A portion of the glycoprotein of rabies virus, a neurotropic RNA rhabdovirus, bears an amino acid similarity to curaremimetic neurotoxins (Lentz et al., 1984). For example, glycoprotein residues 151–237 show 38% identity with forest cobra (*Naja melanoleuca*) long neurotoxin 2. More significantly, the glycoprotein shows identity with all of the invariant residues of neurotoxin loop 2 (Tyr-25, Trp-29, Asp-31, Arg-37, Gly-38, Gly-44). It has been hypothesized that the AChR might serve as a rabies virus receptor (Lentz et al., 1982), and binding of the virus to the AChR could be mediated by the segment of the glycoprotein that bears a similarity to the neurotoxins. Therefore, it is of interest to compare the interaction with the AChR of glycoprotein peptides and the corresponding neurotoxin peptides.

Materials and Methods

Receptor and α-Btx. Receptor-rich membranes from frozen electric organ tissue of *Torpedo californica* were prepared as described (Gershoni et al., 1983). AChR comprised 5% of the total protein of these preparations. α-Btx was obtained from the Miami Serpentarium, Salt Lake City, UT. α-Btx was iodinated with ^{125}I by the chloramine T method (Wang & Schmidt, 1980). Monoiodinated α-Btx, used in these experiments, was separated from diiodinated α-Btx by ion-exchange chromatography on a CM-25 column (Wang & Schmidt, 1980). The initial specific activity of the labeled toxin was 500–700 cpm/fmol.

Synthetic Peptides. The peptides used in this study are shown in Table I. The peptides were synthesized by the Protein and Nucleic Acid Chemistry Facility, Department of Molecular Biophysics and Biochemistry, Yale University. The peptides exhibited a single major peak after analytical high-pressure liquid chromatography. Peptides were purified by reverse-phase high-pressure liquid chromatography using a Vydac C4 column and a gradient of 0–80% acetonitrile/0.05% trifluoroacetic acid. The eluted peptides were lyophilized and resuspended in distilled water just prior to use. The peptides have the correct composition on the basis of amino acid analysis and correct molecular weight on the basis of mass spectrometry. The protein concentrations of the peptide solutions were determined by amino acid analysis.

Competition Assays. Binding of synthetic peptides to the AChR was determined by measuring their ability to compete binding of ^{125}I-labeled α-Btx to the receptor in a solid-phase assay. Wells of 96-well polystyrene microtiter plates (Nunc, USA Scientific Plastics) were inoculated with 5 μg of *Torpedo* electric organ membrane in 100 μL of coating buffer (0.05 M sodium carbonate/bicarbonate, pH 9.6, containing 0.02% NaN_3). Three replicates were included for each condition. The microtiter plates were then placed in plate carriers and centrifuged for 30 min at 1000 rpm in an International 8R centrifuge. After centrifugation, the plates were washed three times with phosphate-buffered (0.01 M, pH 7.4) saline (0.009%). The wells containing adsorbed membranes were quenched to reduce nonspecific binding by addition of 300 μL of 5% bovine serum albumin to the wells and incubation for 1 h. Competition experiments were performed by adding 50 μL of peptide or ligand over a range of concentrations to the wells. Immediately thereafter, 50 μL of $\sim$1 nM ^{125}I-α-Btx (20 000–40 000 cpm) was added to the wells and incubated for 10 min at room temperature. The wells were then washed five times with phosphate-buffered saline. Bound radioactivity was removed by adding 75 μL of 5% sodium dodecyl sulfate in 0.2 N NaOH. The wells were then swabbed with cotton-tipped applicators and the applicators placed in a vial. The swabbing procedure was repeated once. The samples were then counted in a γ counter. Alternatively, plates with removable wells (Immulon 1 Removawell Strips, Dynatech Laboratories, Alexandria, VA) can be used. After washing, the wells are removed and placed in a vial. This procedure has the disadvantage that it requires handling of wells containing radioactive material. Signals in the absence of competitor were usually about 5000 cpm. Background binding in the absence of membrane ($\sim$150 cpm) was subtracted from experimental values. So that different experiments could be averaged and effects of agents easily compared, the maximum number of counts obtained in a experiment was taken as 100%. Competition experiments were performed with neurotoxin peptides, corresponding rabies glycoprotein peptides, and well-characterized cholinergic ligands. At least 2 separate experiments with 3 replicates each were performed for each agent. Affinities of binding were approximated by determining the concentration of ligand that resulted in a 50% reduction in the

Table I: Inhibition of ^{125}I-α-Btx Binding to *Torpedo* AChR by Neurotoxin and Rabies Virus Glycoprotein Peptides

Agent	Residues[a]	Sequence	IC_{50} (M)[b]	Relative affinity[c]
Cholinergic agents				
Antagonists				
α-Bungarotoxin			$8.4\pm0.7 \times 10^{-9}$	30,952
α-Cobratoxin			$1.7\pm0.2 \times 10^{-7}$	1,529
d-Tubocurarine			$3.4\pm1.2 \times 10^{-6}$	76.5
Agonists				
Suberyldicholine			$2.5\pm1.8 \times 10^{-6}$	104
Nicotine			$1.4\pm0.7 \times 10^{-3}$	0.19
Carbamylcholine			$2.8\pm2.0 \times 10^{-3}$	0.09
Neurotoxin and glycoprotein peptides				
Loop 2 peptides				
CVS 29mer[d]	175-203	YT-IWMPENPRPGTPCDIFTNSRGKRASNG	$2.6\pm0.4 \times 10^{-6}$	100
KC 20mer[e]	25-44	YTKTW----------CDGFCSSRGKRIDLG	$5.7\pm1.9 \times 10^{-6}$	45.6
RV-KC 20mer, reduced		CYTKIW----------SDIFTNSRGKRASNGC	$7.9\pm0.7 \times 10^{-6}$	32.9
RV-KC 20mer, oxidized		CYTKIW----------SDIFTNSRGKRASNGC	$3.0\pm0.3 \times 10^{-5}$	8.7
CVS 19mer	175-203, 179-188 deleted	YT-IW----------CDIFTNSRGKRASNG	$4.1\pm0.8 \times 10^{-5}$	6.3
Truncations				
KC 16mer	25-40	YTKTW----------CDGFCSSRGKR	$5.6\pm1.4 \times 10^{-6}$	46.4
CVS & ERA 15mer	175-199, 179-188 deleted	YT-IW----------CDIFTNSRGKR	$2.9\pm0.7 \times 10^{-5}$	9.0
KC 13mer	28-40	TW----------CDGFCSSRGKR	$2.4\pm0.4 \times 10^{-5}$	10.8
CVS & ERA 13mer	177-199, 179-188 deleted	IW----------CDIFTNSRGKR	$7.7\pm0.8 \times 10^{-5}$	3.4
Btx 13mer	28-40	MW----------CDAFCSSRGKV	$1.3\pm0.2 \times 10^{-5}$	20.0
ERA 13mer	187-199	MSCDIFTNSRGKR	$1.0\pm0.2 \times 10^{-5}$	26.0
CVS 13mer	187-199	TPCDIFTNSRGKR	$6.0\pm1.6 \times 10^{-5}$	4.3
KC 10mer	31-40	DGFCSSRGKR	$1.8\pm0.4 \times 10^{-4}$	1.4
CVS & ERA 10mer	190-199	DIFTNSRGKR	$9.2\pm2.2 \times 10^{-4}$	0.28
Btx 10mer	31-40	DAFCSSRGKV	$1.2\pm0.3 \times 10^{-4}$	2.2
CVS 19mer modifications				
CVS 20mer	179-188P	YT-IW----P-----CDIFTNSRGKRASNG	$7.6\pm0.8 \times 10^{-5}$	3.4
CVS 19mer	I177K	YTK-W----------CDIFTNSRGKRASNG	$1.3\pm0.2 \times 10^{-5}$	20.0
CVS 19mer	W178G	YT-IG----------CDIFTNSRGKRASNG	$1.3\pm0.4 \times 10^{-4}$	2.0
Btx 13mer and ERA 13mer modifications				
Btx 13mer	D31A	MW----------CAAFCSSRGKV	$8.5\pm1.5 \times 10^{-6}$	30.6
Btx 13mer	F33T	MW----------CDATCSSRGKV	$1.1\pm0.3 \times 10^{-4}$	2.4
Btx 13mer	R37D	MW----------CDAFCSSDGKV	$1.3\pm0.4 \times 10^{-4}$	2.0
Btx 13mer	G38W	MW----------CDAFCSSRWKV	$2.1\pm0.5 \times 10^{-4}$	1.2
ERA 13mer	R196D	MSCDIFTNSDGKR	$8.2\pm1.8 \times 10^{-5}$	3.2
Other				
NaCl			$5.5\pm1.5 \times 10^{-2}$	0.005

[a] Residues in mature protein. For neurotoxins, Karlsson homology alignment positions are used. [b] IC_{50} values (±standard error) were determined from logit–log plots of the competition data. [c] The affinity of the CVS 29mer is taken as 100%. [d] Residues identical to neurotoxin residues are underlined. [e] Invariant neurotoxin residues are underlined.

binding of ^{125}I-α-Btx in the absence of competitor (IC_{50} value). The IC_{50} values were calculated from logit–log plots of the competition data (Rodbard & Frazier, 1975). Competition curves are graphically represented by fitted curves derived from a polynomial least-squares fit as performed by the computer program KaleidaGraph (Synergy Software, Reading, PA).

Results

Synthetic Peptides. Sequences and alignments of the peptides used in this study and the modifications produced are shown in Table I. A model for the structure of α-Btx [based on Love and Stroud (1986)] with the sequence for loop 2 of king cobra (*O. hannah*) toxin *b* is shown in Figure 1. The CVS 29mer (CVS strain of rabies virus, glycoprotein residues 175–203) (Yelverton et al., 1983) is the structural equivalent of long neurotoxin loop 2 in the overall alignment between the neurotoxins and glycoprotein (Lentz et al., 1984, 1987). The glycoprotein contains a 10-residue insertion not present in the neurotoxins and predicted to be a random coil loop on the basis of the presence of 4 proline residues (Lentz et al., 1984). The KC 20mer is king cobra toxin *b* loop 2. The CVS 19mer sequence is that of the CVS 29mer with the deletion of the 10-residue segment (residues 179–188). In the CVS 20mer, a single proline residue was substituted for the 10-residue segment. The CVS 19mer was modified by substituting a glycine for Trp-178, the counterpart of invariant neurotoxin residue Trp-29. The CVS 19mer was also modified by substituting a lysine for Ile-177. This modification introduces the equivalent of conserved toxin Lys-27 into the glycoprotein sequence. The CVS & ERA 15mer [same sequence for CVS and ERA (Anilionis et al., 1981) strains] are glycoprotein residues 175–199 with the 10-residue segment deleted. This peptide corresponds to the CVS 19mer with 4 C-terminal residues deleted. Similarly, the KC 16mer is formed by the

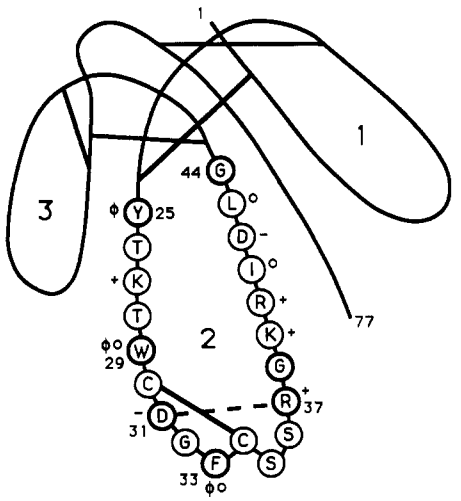

FIGURE 1: Schematic diagram of the structure of α-bungarotoxin viewed from above [based on Love and Stroud (1986)]. The positions of loops 1–3 are shown. Disulfide bonds are shown as straight lines. The proposed hydrogen-bonded ion pair between Asp-31 and Arg-37 is shown as a dashed line (Tsernoglou et al., 1978; Low, 1979). The loop 2 sequence is that of *O. hannah* toxin *b*. The Karlsson (1979) numbering scheme is used. Residues shown in bold circles are invariant among neurotoxins. Lys-27 and Glu/Asp-42 are highly conserved. +, positively charged residues; −, negatively charged residues; o, hydrophobic residues; φ, aromatic residues.

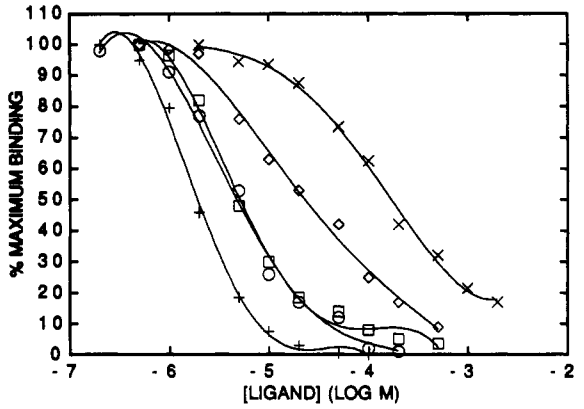

FIGURE 2: Competition curves showing effects of the CVS 29mer, KC 20mer, and truncated forms of the KC 20mer on ^{125}I-α-Btx binding to the AChR. CVS 29mer (+); KC 20mer (circles); KC 16mer (squares); KC 13mer (diamonds); KC 10mer (×). Sequences are shown in Table I. Wells of microtiter plates were coated with 5 μg of electric organ membranes and incubated for 10 min with 1 nM labeled toxin (20 000–40 000 cpm) and increasing amounts of ligand. After washing, bound radioactivity was removed and measured. For each agent, the average of at least 2 experiments with 3 replicates each is shown.

deletion of 4 C-terminal residues of the KC 20mer. The CVS & ERA 13mer was formed by deletion of 2 N-terminal residues of the CVS & ERA 15mer. Similarly, the KC 13mer which corresponds to the CVS & ERA 13mer was formed by deletion of 3 N-terminal residues of the KC 16mer.

The Btx 13mer peptide is residues 28–40 of α-Btx and the equivalent of the KC 13mer and CVS & ERA 13mer. These peptides correspond to the tip of loop 2. The following modifications were made individually in the Btx 13mer: substitution of alanine for Asp-31, substitution of threonine for Phe-33, substitution of aspartate for Arg-37, and substitution of tryptophan for Gly-38. The CVS 13mer and ERA 13mer are residues 187–199 of the glycoproteins of these two strains of rabies virus. These peptides differ in their 2 N-terminal residues. The ERA 13mer was also modified by substitution of aspartate for Arg-196, the equivalent of neurotoxin Arg-37. The CVS & ERA 10mer, KC 10mer, and Btx 10mer were formed by truncation of 3 N-terminal residues of the 13mers. The RV-KC 20mer sequence is that of the CVS 29mer with the deletion of the 10-residue segment, insertion of a lysine residue present in the neurotoxins and not the glycoprotein, and substitution of Cys-189 with a serine. The latter change was made to prevent disulfide bond formation involving this residue. This peptide was synthesized with N- and C-terminal cysteine residues, and some was oxidized with performic acid to form a cyclized peptide that might more closely resemble the conformation of neurotoxin loop 2 in the native protein.

Binding Assay. Binding of the synthetic peptides of the AChR of *Torpedo* electric organ membranes was assessed by testing their ability to inhibit the binding of ^{125}I-α-Btx to the receptor. Increasing amounts of peptide were incubated with ^{125}I-α-Btx, and bound ^{125}I-α-Btx was measured. IC_{50} values were determined and apparent affinities of the peptides for the AChR compared (Table I). The affinities of the peptides were compared with those of other cholinergic ligands. The competition curves for most agents and peptides showing data from which IC_{50} values were determined are presented as Figure S-1 in the supplementary material (see paragraph at end of paper regarding supplementary material). The advantages and disadvantages of the solid-phase assay have been described previously (Wilson et al., 1988). It was noted that affinities as determined with the solid-phase assay are 1–2 orders of magnitude less than those observed with solution assays. This is most likely due to partial denaturation of the receptor as a result of adsorption to plastic. It is likely that individual receptor molecules are denatured to different degrees. Receptor-rich electric organ membranes were used in these studies on the assumption that the receptor in membranes might become less denatured than affinity-purified receptor. However, very little difference between the two preparations was observed (data not shown). Nonetheless, the apparent affinities observed with the solid-phase assay are highly reproducible. Affinities determined in individual experiments generally did not vary by more than a factor of 2. The rank order of affinities of well-characterized cholinergic ligands are preserved (Figure S-1A, supplementary material) so that this assay allows relative affinities of peptides to be determined.

Competition curves were generally steeper for cholinergic agents and long peptides than for the shorter peptides. One explanation for this difference is that the shorter peptides are more flexible and exist in equilibrium with many conformational states which interact differently with the AChR. In the case of α-Btx binding to synthetic receptor peptides, it was suggested that multiple-state rearrangements occur prior to equilibration at the most stable configuration (Pearce & Hawrot, 1990). The cholinergic agents are relatively rigid, and the longer peptides may adopt some native conformation. Circular dichroism studies of the CVS 29mer and the KC 20mer revealed them to be similar in structure and to contain about 2/3 β-sheet and 1/3 random coil (Donnelly-Roberts & Lentz, 1991b). X-ray crystallographic studies of α-Btx have shown that the only secondary structure is triple-stranded, antiparallel β-sheet (Love & Stroud, 1986). A biphasic curve was observed for *d*-tubocurarine consistent with the nonequivalence of the two binding sites on the receptor (Blount & Merlie, 1989).

Binding of Peptides to AChR. Peptides binding to the AChR with the highest apparent affinity were the complete loop 2 counterparts, the CVS 29mer and KC 20mer (Figure 2). The effect of deletion of a 10-residue segment of the CVS

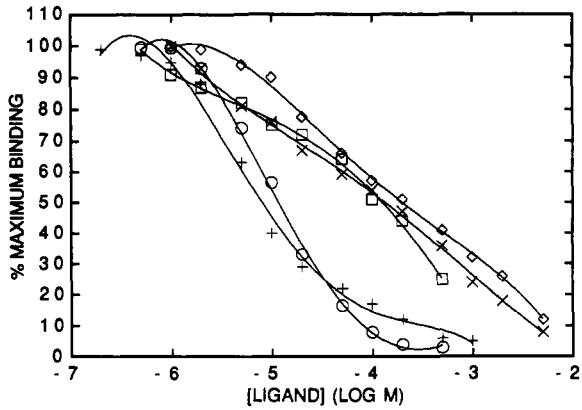

FIGURE 3: Competition curves for Btx 13mer and Btx 13mers in which substitutions were made for functionally invariant residues. Btx 13mer (circles); Btx 13mer R37D (squares); Btx 13mer G38W (diamonds); Btx 13mer F33T (×); Btx 13mer D31A (+). Experiments were performed as described in Figure 2. Sequences are shown in Table I.

29mer not present in the neurotoxins was tested. The CVS 19mer lacking the insertion bound with lower affinity than the CVS 29mer (Figure S-1B, supplementary material). The effects of C- and N-terminal truncation of the KC 20mer (Figure 2) and CVS 19mer (Figure S-1B, supplementary material) were tested. Removal of the 4 C-terminal residues of both peptides producing the KC 16mer and CVS & ERA 15mer resulted in no significant change in affinity. Truncation of 3 N-terminal residues of the KC 16mer to produce the KC 13mer and 2 N-terminal residues of the CVS & ERA 15mer to produce the CVS & ERA 13mer resulted in a significant decrease in affinity. Deletion of 3 additional residues from the KC 13mer and CVS & ERA 13mer producing the KC 10mer and CVS & ERA 10mer resulted in a further significant loss in affinity. The same truncation in the Btx 13mer producing the Btx 10mer similarly decreased affinity.

Modifications were made to the CVS 19mer and effects on binding to the AChR measured (Figure S-1C, supplementary material). Insertion of a proline residue in place of the 10-residue segment (CVS 20mer) had no significant effect. Substitution of a lysine for an isoleucine in the CVS 19mer (CVS 19mer, I177K) increased affinity about 3-fold. On the other hand, substitution of a glycine for tryptophan in the CVS 19mer (CVS 19mer, W178G) reduced affinity about 3-fold. A hybrid glycoprotein–toxin peptide was produced by deleting the 10-residue glycoprotein insertion and introducing a lysine corresponding to toxin Lys-29 (RV-KC 20mer). This peptide in the reduced state bound with 5-fold greater affinity than the CVS 19mer and was comparable to the KC 20mer (Table I). Cyclization of the peptide by oxidation, however, did not increase but reduced the affinity of this peptide.

The 13-residue peptides (Figure S-1D, supplementary material) bound with affinities between 10 and 77 μM. The Btx 13mer bound with an affinity of 13 μM. The functionally invariant residues in the Btx 13mer were substituted (Figure 3). Substitution of positively charged Arg-37 with a negatively charged aspartate reduced affinity about 1 order of magnitude. The same change in the ERA 13mer produced a comparable effect. Similarly, substitution of Phe-33 with a threonine reduced affinity by an order of magnitude. Substitution of Gly-38 with a tryptophan reduced affinity about 20-fold. Substitution of Asp-31 with an alanine, in contrast, had no effect on affinity.

The affinities of the peptides can be compared with those of well-characterized cholinergic ligands (Table I) (Figure S-1A, supplementary material). The loop 2 counterparts (KC 20mer, CVS 29mer) bound with lower affinity than the intact neurotoxins α-Btx and α-cobratoxin (α-Ctx). However, they retained considerable affinity, comparable to those of the competitive antagonist *d*-tubocurarine and the agonist suberyldicholine. The shorter peptides had affinities comparable to nicotine and carbamylcholine. Other unrelated peptides (a vesicular stomatitis virus 13mer corresponding to the CVS 13mer and ERA 13mer, glucagon, microperoxidase 11mer) have previously been shown to not significantly affect binding of ^{125}I-α-Btx to the receptor (Lentz et al., 1987).

Discussion

In the present study, synthetic peptides corresponding to neurotoxin loop 2 and to a structurally similar segment of rabies virus glycoprotein were synthetically modified in order to gain information on structure–function relationships of neurotoxin loop 2 interactions with the AChR. These peptides bound to the AChR as evidenced by their ability to compete ^{125}I-α-Btx binding to the receptor. These findings confirm the importance of loop 2 in the toxin–receptor interaction. The peptides showing the highest apparent affinity for the receptor were the loop 2 counterparts, CVS 29mer and KC 20mer, which bound with affinities (K_D = 2.6 × 10^{-6} M and 5.7 × 10^{-6} M, respectively) comparable to those of *d*-tubocurarine and suberyldicholine. These affinities are also comparable to that of α-Ctx ($K_D \sim 3 \times 10^{-6}$ M) after reduction of all disulfides and carboxyamidomethylation (Martin et al., 1983). The fact that the loop 2 peptides alone have a comparable affinity indicates loop 2 contains the major determinants for binding in the denatured molecule. If a 10-residue segment not aligning with the neurotoxin sequence is omitted from the CVS 29mer, the resultant CVS 19mer shows a decrease in affinity for the receptor, indicating this segment plays some role in binding of the glycoprotein to the receptor.

The aligned sequences of the CVS 29mer and KC 20mer show 12 matches including all of the invariant neurotoxin residues. The effect of C- and N-terminal truncation of these peptides was tested. Omission of 4 C-terminal residues, AlaSerAsnGly in the CVS 29mer and IleAspLeuGly in the KC 20mer, had little effect on affinity, indicating these residues do not play a major role in binding and that neurotoxin residues 25–40 are the most crucial in the interaction with the AChR. Among the residues deleted is the negatively charged residue at neurotoxin position 42. This residue (Asp or Glu) is highly conserved among neurotoxins. There is only a slight reduction in lethality of cobrotoxin when 6 of 7 carboxyl groups, including that at position 42, are modified (Chang et al., 1971). *O. hannah* toxin CM-9 [see Endo and Tamiya (1987)] contains a serine at this position as does the virus glycoprotein.

Deletion of 2 N-terminal residues from the CVS & ERA 15mer (TyrThr) and deletion of 3 N-terminal residues from the KC 16mer (TyrThrLys) produced a further decrease in affinity in the resulting KC 13mer and CVS & ERA 13mer. Tyr-25 is found in most neurotoxins and is considered a structurally invariant residue. In erabutoxin *b* (Low & Corfield, 1986) and other neurotoxins, Tyr-25 is hydrogen bonded to Glu/Asp 42. It has been concluded from a variety of studies that Tyr-25 is part of a hydrophobic core which contributes to maintaining the biologically active conformation of the molecule (Endo & Tamiya, 1987). Since its role appears to be primarily structural, it probably does not play a major role in the binding of the synthetic peptides. If this is the case, the Thr at toxin position 26 may play a role in binding because the glycoprotein lacks a counterpart to Lys-27 and deletion of Tyr and Thr results in a small decrease in affinity.

Lys-27 is conserved in most neurotoxins, although some have a Glu or Met at this position. Chemical modification studies have been performed to investigate the role of lysine residues in toxicity and binding. Modification of all the lysine residues of α-Ctx by reductive methylation, in which the positive charge on the lysine residues is maintained, resulted in a decrease of 21% in toxicity and 75% of the affinity of the unmodified toxin (Martin et al., 1983). Modification by acetylation with acetic anhydride, which abolishes the positive charge, resulted in a 68% decrease in toxicity and 99.95% decrease in affinity (Martin et al., 1983). Dansylation (Chicheportiche et al., 1972), acetylation (Faure et al., 1983), and biotinylation (Lobel et al., 1985) of Lys-27 and Lys-53 resulted in a decrease in affinity of different neurotoxins. The exact function of Lys-27 in binding is uncertain. It appears to be included in the receptor binding site (Faure et al., 1983; Garcia-Borron et al., 1987), but it has been noted that modification could perturb surrounding amino acid side chains including Tyr-25 and Trp-29 (Faure et al., 1983; Endo & Tamiya, 1987). Lys-27, along with other charged residues such as Asp-31, Arg-37, Lys-53, and Glu-42, may be involved in the orientation and steering of the toxin molecule to the receptor surface prior to binding (Low & Corfield, 1986). The present results support a role for Lys-27 in binding. The KC 20mer and the KC 16mer, which contain Lys-27, bind with higher affinity than the corresponding CVS 19mer and CVS & ERA 15mer which lack this residue. When this residue was inserted into the glycoprotein sequence (CVS 19mer, I177K, and RV-KC 20mer), affinity was increased relative to the CVS 19mer.

Further truncation of 3 N-terminal residues of the 13mers leaving toxin residues 31–40 and glycoprotein residues 190–199 in all cases resulted in a significant decrease in affinity (120–920 μM). The deleted residue at toxin position 28 (Thr or Met) is not highly conserved and probably does not play a major role in binding. Also deleted were Cys-30 and functionally invariant Trp-29 and the corresponding residues in the glycoprotein. Cys-30 contributes to the formation of the fifth disulfide bond present at the end of loop 2 of long neurotoxins. The fifth disulfide in long toxins makes the protrusion at the end of the second loop broader than in short toxins (Walkinshaw et al., 1980). Selective reduction of this disulfide of *Naja haje* neurotoxin III and alkylation with iodoacetamide or ethylenimine had little effect on conformational properties and lethality and reduced affinity slightly (Chicheportiche et al., 1975). After reduction of this disulfide in α-Ctx, pyridylethylation and carboxyamidomethylation increased affinity while alkylation with 5-[[2-[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonic acid decreased affinity (Martin et al., 1983). It was concluded from these studies that this disulfide does not play a major role in binding.

Several studies have shown that chemical modification of Trp-29 results in a decrease in toxicity or affinity for the receptor (Chang & Hayashi, 1969; Seto et al., 1970; Faure et al., 1983; Allen & Tu, 1985; Charpentier et al., 1990). Thus, it is most likely that the decrease in affinity from the 13mers to the 10mer is the result of loss of Trp-29 and its equivalent in the glycoprotein. This conclusion is supported by the decreased affinity of the CVS 19mer in which a glycine is substituted for the tryptophan. It has been proposed that Trp-29 lies within a hydrophobic "Trp cleft" on the neurotoxin molecule (Low & Corfield, 1986). It was suggested that, upon the initial interaction of the Asp-31–Arg-37 pair with the AChR, a water-mediated hydrogen bond between Trp-29 and Asp-31 is disrupted, leading to opening up of the cleft and permitting a hydrophobic interaction between neurotoxin cleft residues and a receptor tryptophan.

The ERA 13mer and CVS 13mer are glycoprotein peptides that include the 2 C-terminal residues (MetSer and ThrPro, respectively) of the 10-residue segment that is absent in the CVS & ERA 13mer. When no gap is introduced into the neurotoxins, these residues align with positions 28 and 29. It is surprising that the ERA 13mer bound with at least as high affinity as the other 13mers which contain Trp-29. The CVS 13mer was not as effective. Met or Thr, present in the virus 13mers, is found at position 28 in a number of neurotoxins. It would appear, therefore, that the substitution of serine for proline in the ERA 13mer is responsible for most of the increase in affinity relative to the CVS 13mer and that the serine is able to substitute for the neurotoxin tryptophan, although these are dissimilar amino acids.

The functionally invariant residues in the Btx 13mer were individually substituted. Substitution of an alanine for Asp-31 had no effect on affinity of binding of the 13mer. Asp-31 is conserved in neurotoxins except for two which have weak neurotoxicity (Endo & Tamiya, 1987). Amidation of 6 carboxyl groups including Asp-31 in cobrotoxin decreased lethality only slightly (Chang et al., 1971). The present results indicate the negatively charged Asp-31 does not play a major role in binding of the peptide.

Phe-33 in the Btx 13mer was substituted with a threonine, a hydrophilic residue. This substitution resulted in a decrease in affinity, indicating an important functional role for this residue. Position 33 is invariably occupied by an aromatic amino acid, Phe, His, or Trp. Selective photooxidation of His-33 on cobrotoxin diminished lethal toxicity (Huang et al., 1972). The functional role of this residue is not clear, although it could be involved in hydrophobic interactions with a site on the AChR (Endo & Tamiya, 1987).

Substitution of a negatively charged aspartate for positively charged Arg-37 in the Btx 13mer and the comparable residue in the ERA 13mer resulted in 1 order of magnitude decrease in affinity of both peptides, indicating the cationic Arg-37 plays a role in binding. Chemical modification of Arg-37 of α-Ctx with phenylglyoxal caused about a 75% decrease in toxicity (Yang et al., 1974). Modification of all arginine residues in α-Ctx by 1,2-cyclohexanedione caused about an 8-fold decrease in toxicity and a 100-fold decrease in affinity (Martin et al., 1983). The guanidinium group of Arg-37 is the only cationic group common to all of the neurotoxins and may be the counterpart of the quaternary ammonium group of acetylcholine (Karlsson, 1979). Further evidence for the importance of Arg-37 is provided by the ~20-fold decrease in affinity produced by substituting a tryptophan for invariant Gly-38. It is likely that a residue with a side chain cannot be accommodated in the toxin structure at this position and that the bulky indole side chain may sterically interfere with the interaction of Arg-37 with the receptor.

The loop 2 peptides may interact with the acetylcholine-binding site of the receptor because they are able to compete carbachol-induced $^{22}Na^+$ flux into BC3H1 cells (Donnelly-Roberts & Lentz, 1989). It has been proposed that Asp-31 and Arg-37 form an ion pair that stereochemically resembles acetylcholine and interacts with the acetylcholine-binding site on the receptor (Tsernoglou et al., 1978; Low, 1979; Low & Corfield, 1986). In erabutoxin *b* (Low & Corfield, 1986), there is a water-mediated hydrogen bond from the Trp-29 indole nitrogen to the Asp-31 carboxyl oxygen and between the Arg-33 amide nitrogen and the Asp-31 carboxyl oxygen. The ion pairing between Asp-31 and Arg-37 in α-Btx is very similar to that in erabutoxin *b* (Love & Stroud, 1986). On

the other hand, the hydrogen-bonded ion pair between Asp-31 and Arg-37 has not been detected by NMR analyses of neurotoxins in solution (Dufton & Hider, 1983; Endo & Tamiya, 1987).

Acetylcholine is a hydrophobic cation in which the important functional groups are a positively charged quaternary ammonium group, the carbonyl oxygen acting as a hydrogen bond acceptor, and the acetyl methyl group which can participate in hydrophobic interactions (Beers & Reich, 1970). Some organic cations such as tetramethylammonium and dimethylphenylpiperazinium, which do not contain a potential hydrogen bond acceptor, mimic the effects of acetylcholine (Beers & Reich, 1970). The results from modification of the peptides indicate Arg-37 and Phe-33 are important in binding while Asp-31 is not. Arg-37 and Phe-33 may correspond to the positively charged quaternary nitrogen and the hydrophobic methyl group of acetylcholine, respectively. Asp-31, the potential hydrogen bond acceptor, does not appear to contribute to an acetylcholine mimic in the peptides, although such a role in the intact molecule cannot be ruled out.

With the exception of Asp-31, the functionally invariant or conserved residues in loop 2 (Lys-27, Trp-29, Asp-31, Phe-33, Arg-37, and Gly-38) were shown to play a role in binding. As has been noted repeatedly in the case of chemical modification studies (Martin et al., 1983; Endo & Tamiya, 1987; Lentz & Wilson, 1988), no single synthetic modificaton abolished toxicity indicating there are multiple points of interaction between the toxin molecule and the receptor. Although a decrease in affinity following a substitution demonstrates a role for that residue in binding, it does not necessarily indicate the residue is directly interacting with a site on the receptor (Ward et al., 1990). In native proteins, a substitution can alter conformation, interfering with the ability of the protein to bind. Since the synthetic peptides are at least partially denatured, it is more likely that the observed functional change is due to loss of interaction of the original side chain. A substitution, however, could cause a local environmental change affecting the function of adjacent residues, or the new side chain introduced may be capable of a modified interaction with the receptor.

The interaction of neurotoxins with the AChR may involve a local recognition event between structurally and chemically complementary surfaces followed by an induced fit in which hydrophobic interactions are promoted by rearrangements and conformational changes in flexible regions of the toxin and the receptor (Lentz & Wilson, 1988). On the basis of studies using synthetic peptides, the major toxin-binding determinant on the receptor is located in a region of the primary sequence of the α-subunit encompassing Cys-192 and Cys-193 (Wilson et al., 1985; Mulac-Jericevic & Atassi, 1986; Neumann et al., 1986a; Ralston et al., 1987; Wilson et al., 1988; Conti-Tronconi et al., 1990; Donnelly-Roberts & Lentz, 1991a). The sequence of *Torpedo* α-subunit residues 180–201 is DYRGWKHWVYYTCCPDTPYLDI. The antagonist binding site overlaps the agonist binding site because the probes of the agonist binding site, [4-(*N*-maleimido)benzyl]trimethylammonium iodide which labels Cys-192 and Cys-193 (Kao et al., 1984) and *p*-(dimethylamino)benzenediazonium fluoroborate which labels Trp-149, Try-190, Cys-192, and Cys-193 (Dennis et al., 1988), competitively inhibit antagonist binding. Other regions of the α-subunit and the other subunits may contribute to the binding site.

Knowledge of the crucial toxin residues may permit identification of receptor residues participating in binding. On the basis of the present studies, toxin residues Arg-37, Phe-33, Trp-29, and Lys-27 most likely interact with the AChR. The positively charged side chain of Arg-37 may interact with a negative subsite on the receptor. Negatively charged residues are present at positions 195 and 200 of *Torpedo* α-subunit, but these are not conserved or are present in the other subunits. On the other hand, the presence of a residue in β-, γ-, or δ-subunits in addition to the α-subunit does not rule out a role for that residue in toxin binding. Because the toxins interact with the receptor at multiple points, the presence of 1 or even a few binding determinants on other subunits may not be sufficient for high-affinity binding. Glu-180 is conserved in α-subunits and absent in other subunits, but substitution of this residue with glutamine in a calf peptide had no significant effect on toxin binding (Wilson & Lentz, 1988). An electronegative site could be provided by side chains of polar residues occurring in this region (Tyr-181, -189, -190, and -198 and Thr-191 and -196). Finally, it has been pointed out that the quaternary ammonium group of acetylcholine could interact directly with the π electrons of the electron-rich aromatic group of tyrosine (Dougherty & Stauffer, 1990), and it is possible the guanidinium group of Arg-37 interacts in a similar manner with a tyrosine residue in this region.

Trp-29 and Phe-33 could bind to the receptor through hydrophobic or aromatic–aromatic interactions (Burley & Petsko, 1985). Hydrophobic receptor residues in this region are Trp-184 and -187 and Val-188. The tyrosine residues in this region could take part in aromatic–aromatic interactions. As noted above, toxin Trp-29 has been suggested to interact with a receptor tryptophan (Low & Corfield, 1986). It was suggested that Trp-187 is important in toxin binding on the basis of the lower affinity binding of α-Btx to human synthetic peptides in which a serine is substituted for *Torpedo* Trp-187 (Neumann et al., 1986b; Wilson & Lentz, 1988). On the other hand, substitution of a glycine for Trp-187 had little effect on α-Btx binding to a synthetic peptide (residues 181–200) while replacement of Val-188 with glycine reduced binding significantly (Conti-Tronconi et al., 1991). In addition, energy-transfer calculations suggest that the minimum distance between the α-Btx Trp and Trp-187 in a receptor peptide (residues 185–196) is $\sim$12 Å, arguing against Trp–Trp stacking during binding (Pearce & Hawrot, 1990). Because of the distance between Trp-29 and Phe-33, it can be expected that these interact with separate sites on the receptor. Lys-27 also may interact with a negative subsite separate from that interacting with Arg-37.

It has been pointed out that the toxin-binding region on the receptor surface will be complementary to the toxin's concave surface which comes into contact with the receptor (Fairclough et al., 1983; Love & Stroud, 1986). Mapping revealed the toxin-binding surface of the receptor to consist of rows of hydrophobic and ionic domains alternating every 5–8 Å (Fairclough et al., 1983). However, the three-dimensional structure of the receptor is not known, making it difficult to predict what regions might be complementary to the toxin surface. The toxin-binding region is predicted to be a β-barrel structure (Finer-Moore & Stroud, 1984; McCarthy & Stroud, 1989; Stroud et al., 1990). Proline residues at positions 194 and 197 may produce a loop with the vicinal cysteines near the tip. Such a loop could contain regions complementary to the alternating toxin domains.

The structurally similar rabies virus glycoprotein peptides behaved in a manner nearly identical to that of the neurotoxin peptides. Modification of the glycoprotein residues corresponding to the functionally invariant toxin residues resulted in changes very similar to those following modification of these

residues in the neurotoxins. These findings support the suggestion that this region of the glycoprotein can interact with the AChR and suggest that the mechanism of binding is the same as for the neurotoxins. This is supported by observations that α-Btx inhibits the infection of cultured chick (Lentz et al., 1982) and rat (Tsiang et al., 1986) myotubes by rabies virus. In addition, monoclonal antibodies were raised against a rabies glycoprotein peptide comprising residues 190–203 (Bracci et al., 1988). The antibodies recognized both the virus glycoprotein and α-Btx and inhibited binding of the glycoprotein and α-Btx to the AChR.

Supplementary Material Available

A figure showing competition curves for cholinergic agonists and antagonists (Figure S-1A), truncated peptides (Figure S-1B), modified CVS 19-mers (Figure S-1C), and 13-residue peptides (Figure S-1D) (2 pages). Ordering information is given on any current masthead page.

References

Allen, M., & Tu, A. T. (1985) *Mol. Pharmacol. 27*, 79–85.

Anilionis, A., Wunner, W. H., & Curtis, P. J. (1981) *Nature 294*, 275–278.

Beers, W. H., & Reich, E. (1970) *Nature 228*, 917–922.

Blount, P., & Merlie, J. P. (1989) *Neuron 3*, 349–357.

Bourne, P. E., Sato, A., Corfield, P. W. R., Rosen, L. S., Birken, S., & Low, B. W. (1985) *Eur. J. Biochem. 153*, 521–527.

Bracci, L., Antoni, G., Cusi, M. G., Lozzi, L., Niccolai, N., Petrini, S., Rustici, M., Santucci, A., Soldani, P., Valensin, P. E., & Neri, P. (1988) *Mol. Immunol. 25*, 881–888.

Burley, S. K., & Petsko, G. A. (1985) *Science 229*, 23–28.

Chang, C. C., & Hayashi, K. (1969) *Biochem. Biophys. Res. Commun. 37*, 841–846.

Chang, C. C., Yang, C. C., Nakai, K., & Hayashi, K. (1971) *Biochim. Biophys. Acta 251*, 334–344.

Charpentier, I., Pillet, L., Karlsson, E., Couderc, J., & Ménez, A. (1990) *J. Mol. Recognit. 3*, 74–81.

Chicheportiche, R., Rochat, C., Sampieri, F., & Lazdunski, M. (1972) *Biochemistry 11*, 1681–1691.

Chicheportiche, R., Vincent, J.-P., Kopeyan, C., Schweitz, H., & Lazdunski, M. (1975) *Biochemistry 14*, 2081–2091.

Conti-Tronconi, B. M., Tang, F., Diethelm, B. M., Spencer, S. R., Reinhardt-Maelicke, S., & Maelicke, A. (1990) *Biochemistry 29*, 6221–6230.

Conti-Tronconi, B. M., Diethelm, B. M., Wu, X., Tang, F., Bertazzon, T., Schröder, B., Reinhardt-Maelicke, S., & Maelicke, A. (1991) *Biochemistry 30*, 2575–2584.

Dennis, M., Giraudat, J., Kotzyba-Hibert, F., Goeldner, M., Hirth, C., Chang, J.-Y., Lazure, C., Chrétien, M., & Changeux, J.-P. (1988) *Biochemistry 27*, 2346–2357.

Donnelly-Roberts, D. L., & Lentz, T. L. (1989) *Peptide Res. 2*, 221–226.

Donnelly-Roberts, D. L., & Lentz, T. L. (1991a) *Biochemistry 30*, 7484–7491.

Donnelly-Roberts, D. L., & Lentz, T. L. (1991b) *Mol. Brain Res. 11*, 107–113.

Dougherty, D. A., & Stauffer, D. A. (1990) *Science 250*, 1558–1560.

Dufton, M. J., & Hider, R. C. (1983) *CRC Crit. Rev. Biochem. 14*, 113–171.

Endo, T., & Tamiya, N. (1987) *Pharmacol. Ther. 34*, 403–451.

Fairclough, R. H., Finer-Moore, J., Love, R. A., Kristofferson, D., Desmeules, P. J., & Stroud, R. M. (1983) *Symp. Quant. Biol. 48*, 9–20.

Faure, G., Boulain, J.-C., Bouet, F., Montenay-Garestier, T., Fromageot, P., & Ménez, A. (1983) *Biochemistry 22*, 2068–2076.

Finer-Moore, J., & Stroud, R. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 155–159.

Garcia-Borron, J.-C., Bieber, A. L., & Martinez-Carrion, M. (1987) *Biochemistry 26*, 4295–4303.

Gershoni, J. M., Hawrot, E., & Lentz, T. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4973–4977.

Huang, J. S., Liu, S. S., Ling, K. H., Chang, C. C., & Yang, C. C. (1972) *J. Formosan Med. Assoc. 71*, 383–388.

Juillerat, M. A., Schwendimann, B., Hauert, J., Fulpius, B. W., & Bargetzi, J. P. (1982) *J. Biol. Chem. 257*, 2901–2907.

Kao, P. N., Dwork, A. J., Kaldany, R.-R. J., Silver, M. L., Wideman, J., Stein, S., & Karlin, A. (1984) *J. Biol. Chem. 259*, 11662–11665.

Karlsson, E. (1979) *Handb. Exp. Pharmacol. 52*, 158–212.

Lee, C. Y. (1972) *Annu. Rev. Pharmacol. 12*, 265–286.

Lentz, T. L., & Wilson, P. T. (1988) *Int. Rev. Neurobiol. 29*, 117–160.

Lentz, T. L., Burrage, T. G., Smith, A. L., Crick, J., & Tignor, G. H. (1982) *Science 215*, 182–184.

Lentz, T. L., Wilson, P. T., Hawrot, E., & Speicher, D. W. (1984) *Science 226*, 847–848.

Lentz, T. L., Hawrot, E., & Wilson, P. T. (1987) *Proteins: Struct., Funct., Genet. 2*, 298–307.

Lobel, P., Kao, P. N., Birken, S., & Karlin, A. (1985) *J. Biol. Chem. 260*, 10605–10612.

Love, R. A., & Stroud, R. M. (1986) *Protein Eng. 1*, 37–46.

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

Low, B. W., & Corfield, P. W. R. (1986) *Eur. J. Biochem. 161*, 579–587.

Low, B. W., Preston, H. S., Sato, A., Rosen, L. S., Searl, J. E., Rudko, A. D., & Richardson, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2991–2994.

Martin, B. M., Chibber, B. A., & Maelicke, A. (1983) *J. Biol. Chem. 258*, 8714–8722.

McCarthy, M. P., & Stroud, R. M. (1989) *Biochemistry 28*, 40–48.

McDaniel, C. S., Manshouri, T., & Atassi, M. Z. (1987) *J. Protein Chem. 6*, 455–461.

Ménez, A., Boulain, J.-C., Bouet, F., Couderc, J., Faure, G., Rousselet, A., Trémeau, O., Gatineau, É., & Fromageot, P. (1984) *J. Physiol. (Paris) 79*, 196–206.

Mulac-Jericevic, B., & Atassi, M. Z. (1986) *FEBS Lett. 199*, 68–74.

Neumann, D., Barchan, D., Safran, A., Gershoni, J. M., & Fuchs, S. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3008–3011.

Neumann, D., Barchan, D., Fridkin, M., & Fuchs, S. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9250–9253.

Pearce, S. F., & Hawrot, E. (1990) *Biochemistry 29*, 10649–10659.

Ralston, S., Sarin, V., Thanh, H. L., Rivier, J., Fox, J. L., & Lindstrom, J. (1987) *Biochemistry 26*, 3261–3266.

Rodbard, D., & Frazier, G. R. (1975) *Methods Enzymol. 37*, 3–22.

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

Stroud, R. M., McCarthy, M. P., & Shuster, M. (1990) *Biochemistry 29*, 11009–11023.

Tamiya, N., Takasaki, C., Sato, A., Menez, A., Inagaki, F., & Miyazawa, T. (1980) *Biochem. Soc. Trans. 8*, 753–755.

Tsernoglou, D., & Petsko, G. A. (1976) *FEBS Lett. 68*, 1–4.

Tsernoglou, D., Petsko, G. A., & Hudson, R. A. (1978) *Mol. Pharmacol. 14*, 710-716.
Tsiang, H., de la Porte, S., Ambroise, D. J., Derer, M., & Koenig, J. (1986) *J. Neuropathol. Exp. Neurol. 45*, 28-42.
Walkinshaw, M. D., Saenger, W., & Maelicke, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2400-2404.
Wang, G.-K., & Schmidt, J. (1980) *J. Biol. Chem. 255*, 11156-11162.
Ward, W. H. J., Timms, D., & Fersht, A. R. (1990) *Trends Pharmacol. Sci. 11*, 280-284.
Wilson, P. T., & Lentz, T. L. (1988) *Biochemistry 27*, 6667-6674.
Wilson, P. T., Lentz, T. L., & Hawrot, E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8790-8794.
Wilson, P. T., Hawrot, E., & Lentz, T. L. (1988) *Mol. Pharmacol. 34*, 643-650.
Yang, C. C., Chang, C. C., & Liou, I. F. (1974) *Biochim. Biophys. Acta 365*, 1-14.
Yelverton, E., Norton, S., Obijeski, J. F., & Goeddel, D. V. (1983) *Science 219*, 614-620.

Tubulin–G Protein Interactions Involve Microtubule Polymerization Domains†

Nan Wang‡ and Mark M. Rasenick*

Department of Physiology and Biophysics and the Committee on Neuroscience, University of Illinois, College of Medicine, Chicago, Illinois 60680-6998

Received June 19, 1991; Revised Manuscript Received August 29, 1991

ABSTRACT: It has been suggested that elements of the cytoskeleton contribute to the signal transduction process and that they do so in association with one or more members of the signal-transducing G protein family. Relatively high-affinity binding between dimeric tubulin and the α subunits of G_s and G_{i1} has also been reported. Tubulin molecules, which exist in solution as $\alpha\beta$ dimers, have binding domains for microtubule-associated proteins as well as for other tubulin dimers. This study represents an attempt to ascertain whether the association between G proteins and tubulin occurs at one of these sites. Removal of the binding site for MAP2 and tau from tubulin by subtilisin proteolysis did not influence the association of tubulin with G protein, as demonstrated in overlay studies with [^{125}I]tubulin. A functional consequence of that association, the stable inhibition of synaptic membrane adenylyl cyclase, was also unaffected by subtilisin treatment of tubulin. However, ring structures formed from subtilisin-treated tubulin were incapable of effecting such inhibition. Stable G protein–tubulin complexes were formed, and these were separated from free tubulin by Octyl-Sepharose chromatography. Using this methodology, it was demonstrated that assembled microtubules bound G protein quite weakly compared with tubulin dimers. The α subunit of G_{i1} and, to a lesser extent, that of G_o were demonstrated to inhibit microtubule polymerization. In aggregate, these data suggest that dimeric tubulin binds to the α subunits of G protein at the sites where it binds to other tubulin dimers during microtubule polymerization. Interaction with signal-transducing G proteins, thus, might represent a role for tubulin dimers which is independent of microtubule formation.

Guanine nucleotide binding regulatory proteins (G proteins) are linked to a number of surface membrane receptors and they mediate the regulation of a variety of effectors by hormones and neurotransmitters. Functional reconstitution studies with purified hormone receptors, G proteins, and adenylyl cyclase catalytic unit have shown that this signal transduction cascade can be modeled by incorporating the purified components into phospholipid vesicles (Cerione et al., 1986). These studies suggest that coexistence of these three components meet the minimum requirements for hormonal signal transduction. The regulation of adenylyl cyclase in vivo, however, is likely to be much more complicated. This and other laboratories have suggested that the cytoskeleton, particularly the microtubule–tubulin system, may be involved in adenylyl cyclase regulation [see Rasenick et al. (1985, 1989) and Zor (1983) for reviews], although the mechanism remains elusive.

Recent studies suggest that one locus of interactions between cytoskeletal components and the adenylyl cyclase system is between tubulin and G proteins which are involved in the regulation of that enzyme. In those experiments, a brief incubation of the tubulin–Gpp(NH)p[1] with synaptic membranes caused inhibition of adenylyl cyclase which was persistent to

† Supported by USPHS Grant MH 39595. M.M.R. is the recipient of a research scientist development award from the National Institute of Mental Health (MH 00699).

* Address correspondence to M.M.R. at the Department of Physiology and Biophysics, University of Illinois College of Medicine, m/c 901, P.O. Box 6998, Chicago, IL 60680. U20133@UICVM.Bitnet.

‡ Present address: Laboratory of Cell Biology, National Cancer Institute, NIH, Bethesda, MD 20892.

[1] Abbreviations: tubulin-S, tubulin dimer with the carboxyl terminus of both α and β subunit cleaved by subtilisin, and equivalent to $\alpha s\beta s$ tubulin; $\alpha\beta s$ tubulin, tubulin dimer with the carboxyl terminus of β subunit cleaved by subtilisin; PC-tubulin, tubulin deprived of MAPs with phosphocellulose chromatography; tubulin–Gpp(NH)p, tubulin liganded with Gpp(NH)p; tubulin-S–Gpp(NH)p, tubulin-S liganded with Gpp(NH)p; tubulin-S–GTP, tubulin-S liganded with GTP; G_α, the α subunit of G protein; AAGTP, P^3-(4-azidoanilido)-P^1-5′-GTP; Gpp(NH)p, 5′-guanylylimidodiphosphate; G_s, stimulatory GTP-binding regulatory protein of adenylyl cyclase; G_i, inhibitory GTP-binding regulatory protein of adenylyl cyclase; G_o, a G protein abundant in brain with unknown functions; MAPs, high molecular weight microtubule-associated proteins; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid, 1.5 sodium); DTT, dithiothreitol.

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