Structural Determinants within Residues 180–199 of the Rodent α5 Nicotinic Acetylcholine Receptor Subunit Involved in α-Bungarotoxin Binding†

Kathyrn E. McLane, Xiadong Wu, and Bianca M. Conti-Tronconi*

Department of Biochemistry, College of Biological Sciences, University of Minnesota, 1479 Gortner Avenue, St. Paul, Minnesota 55108

Received June 6, 1991; Revised Manuscript Received August 15, 1991

ABSTRACT: Synthetic peptides corresponding to sequence segments of the nicotinic acetylcholine receptor (nAChR) α subunits have been used to identify regions that contribute to formation of the binding sites for cholinergic ligands. We have previously defined α-bungarotoxin (α-BTX) binding sequences between residues 180 and 199 of a putative rat neuronal nAChR α subunit, designated α5 [McLane, K. E., Wu, X., & Conti-Tronconi, B. M. (1990) *J. Biol. Chem. 265*, 9816–9824], and between residues 181 and 200 of the chick neuronal α7 and α8 subunits [McLane, K. E., Wu, X., Schoepfer, R., Lindstrom, J., & Conti-Tronconi, B. M. (1991) *J. Biol. Chem.* (in press)]. These sequences are relatively divergent compared with the *Torpedo* and muscle nAChR α1 α-BTX binding sites, which indicates a serious limitation of predicting functional domains of proteins based on homology in general. Given the highly divergent nature of the α5 sequence, we were interested in determining the critical amino acid residues for α-BTX binding. In the present study, the effects of single amino acid substitutions of Gly or Ala for each residue of the rat α5(180–199) sequence were tested, using a competition assay, in which peptides compete for ^{125}I-α-BTX binding with native *Torpedo* nAChR. Four residues appeared to be critical for α-BTX binding to the α5 sequence segment—Lys184, Arg187, Cys191, and Pro195. Peptides containing other amino acid substitutions (Gly185, Asn186, Asp189, Trp193, Tyr194, and Tyr196) retained a measurable level of binding activity, but their affinity for α-BTX was 3–10-fold lower compared to the peptide corresponding to the native sequence. The importance of Cys191 and Cys192 for α-BTX binding was difficult to assess, as peptides substituted at either of these positions tended to form dimers. Oxidation of the peptide corresponding to the native sequence resulted in monomers and dimers, which retained ~60% and ~40% binding activity, respectively, and reduction and alkylation with iodoacetamide slightly decreased its ability to bind α-BTX (~70–80% activity). The same treatment of peptides substituted at Cys191 or Cys192 converted them to monomers and resulted in ~70–80% binding activity for both peptides. These results indicate that a disulfide bridge between the vicinal cysteines at positions 191 and 192 of the α5 sequence is not an absolute requirement for α-BTX binding activity.

Elapid snakes paralyze their prey through postsynaptic curaremimetic antagonists of the muscle nicotinic acetylcholine receptors (nAChRs).[1] α-Neurotoxins from snake venoms, such as α-bungarotoxin (α-BTX) from *Bungarus multicinctus*, bind with subnanomolar affinity to the muscle-type nAChR at sites that reside largely or completely on the α subunits. The use of α-neurotoxins made possible the isolation, purification, and determination of the complete amino acid sequence of the four subunits of the *Torpedo* and mammalian muscle nAChR (α1, β, γ, and δ), which share considerable homology and are likely of common ancestry (Raftery et al., 1980; Conti-Tronconi et al., 1982; Noda et al., 1982, 1983a,b; La Polla et al., 1984; Boulter et al., 1985, 1986a; Isenberg et al., 1986; Buonanno et al., 1986; Yu et al., 1986). Further evidence for an extended supergene family has come from the identification of several different, but homologous, nAChR subunits from rodent, avian, and goldfish neurons, referred to as α2, α3, α4, and α5 (Wada et al., 1988; Boulter et al., 1986b, 1990; Goldman et al., 1987; Nef et al., 1988; Couturier et al., 1990a; Cauley et al., 1990; Hieber et al., 1990a) and β2, β3, and β4 (also designated non-α or structural subunits) (Deneris et al., 1988, 1989; Duvoisin et al., 1989; Nef et al., 1988; Schoepfer et al., 1988; Cauley et al., 1989; Couturier et al., 1990a; Hieber et al., 1990b). The complete sequences of two avian neuronal α-BTX binding proteins, designated α-BGTBP α1 (also referred to as α7) and α-BGTBP α2 (also referred to as α8), have been identified (Schoepfer et al., 1990; Couturier et al., 1990b) using oligonucleotides corresponding to the N-terminal sequence of a putative nAChR isolated from the chick brain by α-BTX affinity chromatography (Conti-Tronconi et al., 1985). In addition, subunits from α-BTX binding proteins of the *Drosophila* and locust nervous systems (Schloss et al., 1988; Sawruk et al., 1990; Marshall et al., 1990) have been reported. Although sequence homology indicates that these different neuronal subunits are related to the muscle nAChR subunits, expression of functional receptors and demonstration of nicotinic pharmacology have only been achieved for some of these subunits. Coexpression of the α2, α3, and α4 subunits with the β2 or β4 subunits in *Xenopus* oocytes results in the formation of functional nAChRs, which are insensitive to α-BTX (Goldman et al., 1987; Deneris et al., 1988, 1989; Ballivet et al., 1988; Wada et al., 1988; Duvoisin et al., 1989; Papke et al., 1989; Luetje et al., 1990),

† This work was supported by NSF Grant BNS-8607289, NIDA Program Project Grant 5P01-DA05695, and ARO Contract DAMD 17-88-C-8120 (to B.M.C.-T.). K.E.M. is a recipient of a predoctoral fellowship as part of the USPHS Training Grant "Chemical Basis of Cell and Molecular Biology" (5-T32-GMO7323).

* To whom correspondence should be addressed.

[1] Abbreviations: α-BTX, α-bungarotoxin; nAChR, nicotinic acetylcholine receptor; IAA, iodoacetamide; DTT, dithiothreitol; IOBA, 2-iodosobenzoic acid; CM-Cys, *S*-(carboxymethyl)cysteine; HPLC, high-pressure liquid chromatography.

0006-2960/91/0430-10730$02.50/0

whereas expression of the α7 subunit as a homoligomer yields an α-BTX-sensitive nAChR (Couturier et al., 1990). Other subunits, such as the α5, α8, and β3 subunits, have not been successfully expressed and may require other yet unidentified subunits to form a functional nAChR.

The neuronal α-BTX binding protein subunits from chick and *Drosophila* (Schoepfer et al., 1990; Couturier et al., 1990b; Sawruk et al., 1990), and the rat α5 subunit (Boulter et al., 1990), have in common with all nAChR α subunits a pair of adjacent cysteine residues at approximately position 190. Cysteines192 and -193 of the *Torpedo* nAChR α1 subunit may form a vicinal disulfide bond (Kao & Karlin, 1986; Mosckovitz & Gershoni, 1988; Kellaris et al., 1989) and are labeled by cholinergic affinity reagents that compete for α-BTX binding (Kao et al., 1984; Kao & Karlin, 1986; Wilson et al., 1984; Middleton & Cohen, 1991). A sequence segment containing cysteines-192 and -193 on the *Torpedo* α1 subunit has also been identified as an important component of the α-BTX binding site by several laboratories, using proteolytic fragments (Wilson et al., 1984, 1985; Pederson et al., 1986; Neumann et al., 1986a), synthetic peptides (Neumann et al., 1986b; Ralston et al., 1987; Wilson et al., 1988; Wilson & Lentz, 1988; Conti-Tronconi et al., 1988, 1989, 1990a), and biosynthetic peptides (Barkas et al., 1987; Aronheim et al., 1988; Ohana & Gershoni, 1990). Our laboratory has used synthetic peptides to define components of α-BTX binding sites on the sequence segments 181–200 from the *Torpedo* α1 subunit (Conti-Tronconi et al., 1990), vertebrate muscle α1 subunits (McLane et al., 1991a), and the avian brain α-BGTBP α1 and α-BGTBP α2 subunits (McLane et al., 1991b), and the sequence segment 199–200 of the rodent α5 subunit (McLane et al., 1990). We have studied the structural requirements for α-BTX binding by comparing the effects of single amino acid substitutions of the *Torpedo* α-BTX binding peptide (Conti-Tronconi et al., 1991), and species-specific substitutions of muscle α1 subunits (McLane et al., 1991a), which are highly homologous sequences. In contrast to the *Torpedo* and vertebrate muscle nAChR α1 subunits, the α-BTX binding sequences of neuronal α subunits (rat brain α5, *Drosophila* SAD, chick brain α-BGTBP α1 and α2) are relatively divergent and offer a unique opportunity to determine what structural features are important to α-BTX binding.

In the present study, we identify the critical amino acids for the binding of α-BTX to the peptide sequence 181–200 of the rodent neuronal α5 subunit. We synthesized 20 single-residue-substitution peptide analogues of the sequence segment 180–199 of the α5 subunit, in which 1 of the residues was replaced by a glycine, or if glycine was the amino acid present at that position in the native sequence, it was replaced by alanine. These single-substitution analogues were tested for their ability to bind α-BTX using a competition assay, in which peptides inhibit ^{125}I-α-BTX binding to native *Torpedo* nAChR. Several amino acid substitutions drastically altered the ability of the α5 peptides to inhibit binding of α-BTX to native *Torpedo* nAChR, whereas other substitutions had more subtle effects on the interaction of the α5 peptide with α-BTX, detectable as changes in the IC_{50} values.

Experimental Procedures

Peptide Synthesis and Characterization. Peptides, 20–21 amino acids long, were synthesized by manual parallel synthesis (Houghten, 1985). The purity of the peptides was assessed by reverse-phase HPLC (high-pressure liquid chromatography) using a C18 column (Ultrasphere ODS) and an acetonitrile/water gradient (5–70%) containing 0.1% trifluoroacetic acid. A major peak consistently accounted for 65–85% of the total absorbance at 214 nm. The amino acid composition of the peptides, determined by derivatization of amino acid residues released by acid hydrolysis with phenyl isothiocyanate, followed by separation on a reverse-phase HPLC column (PICO.TAG) as described by Heinrickson and Meredith (1984), gave satisfactory correspondence between experimental and theoretical values for all peptides. The sequence and purity of peptides with altered α-BTX binding activity, and other randomly selected peptides, were verified by gas-phase sequencing (Applied Biosystems, Foster City, CA). Only the expected sequences were found. Contaminating sequences, which would be expected from truncated peptides randomly missing amino acids from incomplete coupling, were below the level of detectability (<3%). The sequence and codes of the peptides are reported in Figure 1.

α5(180-199)$_{Native}$	AMGSKGNRTDSCCWYPYITY
α5(180-199)$_{M181}$	AG**G**SKGNRTDSCCWYPYITY
α5(180-199)$_{G182}$	AM**A**SKGNRTDSCCWYPYITY
α5(180-199)$_{S183}$	AMG**G**KGNRTDSCCWYPYITY
α5(180-199)$_{K184}$	AMGS**G**GNRTDSCCWYPYITY
α5(180-199)$_{G185}$	AMGSK**A**NRTDSCCWYPYITY
α5(180-199)$_{N186}$	AMGSKG**G**RTDSCCWYPYITY
α5(180-199)$_{R187}$	AMGSKGN**G**TDSCCWYPYITY
α5(180-199)$_{T188}$	AMGSKGNR**G**DSCCWYPYITY
α5(180-199)$_{D189}$	AMGSKGNRT**G**SCCWYPYITY
α5(180-199)$_{S190}$	AMGSKGNRTD**G**CCWYPYITY
α5(180-199)$_{C191}$	AMGSKGNRTDS**G**CWYPYITY
α5(180-199)$_{C192}$	AMGSKGNRTDSC**G**WYPYITY
α5(180-199)$_{W193}$	AMGSKGNRTDSCC**G**YPYITY
α5(180-199)$_{Y194}$	AMGSKGNRTDSCCW**G**PYITY
α5(180-199)$_{P195}$	AMGSKGNRTDSCCWY**G**YITY
α5(180-199)$_{Y196}$	AMGSKGNRTDSCCWYP**G**ITY
α5(180-199)$_{I197}$	AMGSKGNRTDSCCWYPY**G**TY
α5(180-199)$_{T198}$	AMGSKGNRTDSCCWYPYI**G**Y
α5(180-199)$_{Y199}$	AMGSKGNRTDSCCWYPYIT**G**

FIGURE 1: Peptide sequences and codes. The rat α5 sequence segment corresponding to residues 180–199 (Boulter et al., 1990) was previously shown to bind ^{125}I-α-BTX (McLane et al., 1990). Each residue of this sequence was sequentially replaced in the synthesis of a panel of peptides corresponding to single amino acid substitutions of Gly for the native residue, or Ala in the case that the native residue was Gly. The sequences and codes of the substituted peptides are shown. For each α5(180–199) amino acid substituted, the final letter corresponds to the amino acid substituted, followed by the residue position in the intact α5 subunit. In the other figures, the peptides are designated by abbrevations including only the amino acid substituted and its position number.

Modification of Cysteine/Cystine Residues. Synthetic peptides (0.5 mg/mL) in 100 mM potassium phosphate buffer, pH 8.5, were treated with either 2-iodobenzoic acid (IOBA) (0.1 or 0.8 mM) or dithiothreitol (DTT) (1.5 mM) for 5 h at room temperature. Samples of untreated, oxidized, and reduced peptides were alkylated with iodoacetamide (IAA) (6 mM) overnight at 4 °C. The reactants and peptides were separated by gel permeation chromatography in 10 mM potassium phosphate buffer, pH 7.0, using a Sephadex G-10 column (0.9 × 4 cm), followed by a P6 column (Bio-Rad) (1.8 × 47 cm), which was calibrated with blue dextran 2000 and the following peptides (obtained from Sigma): bradykinin (1060 daltons), porcine renin substrate tetradecapeptide (1759 daltons), human β-endorphin (3465 daltons), and human growth hormone releasing factor (5041 daltons). Carboxymethylation of free sulfhydryl groups was assessed by amino acid composition analysis, as described above. The *S*-(carboxymethyl)cystein (CM-Cys) derivative was calibrated with a standard (Pierce) and elutes after the Asp and Glu derivatives, and prior to the Ser derivative, using 140 mM sodium acetate buffer (pH 6.4) containing 0.05% triethylamine and 6% acetonitrile (McLane et al., 1991a,b).

Preparation and Calibration of Radiolabeled α-BTX. α-BTX was isolated from *Bungarus multicinctus* venom

(Biotoxins Inc.) as described by Clark et al. (1972). The purity of α-BTX, assessed by gas-phase sequencing, indicated that contaminating sequences, if present, were below the level of detectability (<3–5%). α-BTX was radiolabeled with carrier-free ^{125}I (Lindstrom et al., 1981) and calibrated as described by Blanchard et al. (1979) using membrane-bound nAChR prepared from *Torpedo californica* electric organ (Neubig et al., 1979; Elliot et al., 1980). The specific activity of the ^{125}I-α-BTX was 52–180 Ci/mmol.

Competitive Inhibition of ^{125}I-α-BTX Binding by Peptides. Peptides (1–250 μg/mL) were preincubated overnight at 4 °C with ^{125}I-α-BTX (20 nM) in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mg/mL cytochrome *c*. Membrane-bound *Torpedo* nAChR (0.2–1 pmol) (Neubig et al., 1979; Elliot et al., 1980) was added to 100 μL of peptide/toxin solutions. After 3 min at room temperature, the assay tubes were centrifuged at 14000*g* for 45 min, washed with 10 mM potassium phosphate buffer containing 100 mM NaCl, and recentrifuged. The pellet was counted in a γ counter. Nonspecific binding was determined by preincubation of *Torpedo* nAChR with 20 nM unlabeled α-BTX for 10 min prior to addition to the ^{125}I-α-BTX solutions. Positive controls (100% binding) contained ^{125}I-α-BTX preincubated with buffer in the absence of peptide. The IC_{50} values were determined using the programs EBDA and LIGAND (Munson & Rodbard, 1980; McPherson, 1983). The relative affinities of peptides for α-BTX were also compared by plotting the data in the form of a Hofstee plot (Molinoff et al., 1981), in which the negative slope is equal to the IC_{50}.

RESULTS

Torpedo nAChR Competition Assay: Correlation with the Physiological Sensitivity to α-BTX of the Corresponding nAChRs. In the competition assay used in the present study, peptides were preincubated with ^{125}I-α-BTX, and their ability to inhibit ^{125}I-α-BTX binding to native *Torpedo* nAChR was determined. This assay adequately reflects the ability of the corresponding α subunits to bind α-BTX as demonstrated in previous studies, where we tested the ability of α-BTX to bind to peptides corresponding to the sequence region 180–200 from α subunits of nAChRs known to differ in their ability to bind α-BTX (McLane et al., 1990, 1991a). The neuronal α2, α3, and α4 rodent subunits, when coexpressed with the β2 subunit in *Xenopus* oocytes, have been shown to form an nAChR insensitive to α-BTX, and peptides corresponding to their sequence segment 181–200 do not compete for α-BTX in the *Torpedo* competition assays, whereas the nAChRs formed by *Torpedo* α1 and rodent α1 coexpressed with β1, γ, and δ subunits are sensitive to α-BTX, and peptides corresponding to these α1 sequences are effective competitors for α-BTX (Mishina et al., 1984; Boulter et al., 1987; Deneris et al., 1988; Wada et al., 1988; McLane et al., 1990). In addition, the cobra muscle nAChR is unique among the vertebrate muscle nAChRs in that it is insensitive to α-BTX (Burden et al., 1975), and synthetic peptides corresponding to the sequence segment 181–200 of the α1 subunit of different species have been shown to bind α-BTX using the *Torpedo* competition assay, with the exception of the cobra sequence, which differs by only a few amino acid residues (McLane et al., 1991a).

Single Amino Acid Substitutions of the Sequence Segment α5(180–199) Alter the Binding of α-BTX. The sequence segment α5(180–199) competitively inhibits ^{125}I-α-BTX binding to native membrane-bound *Torpedo* nAChR (McLane et al., 1990). We examined the effects on α-BTX binding of single amino acid substitutions of this sequence. The single amino acid substitution analogues are depicted in Figure 1.

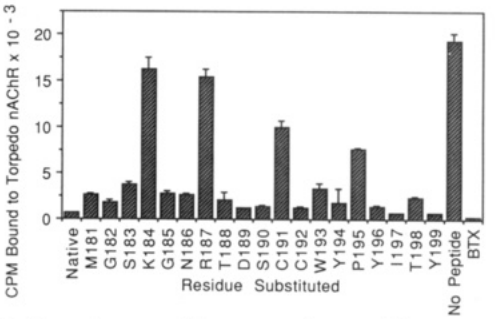

FIGURE 2: *Torpedo* competition assay using peptides corresponding to single amino acid substitutions of the α5(180–199) sequence. Peptides (100 μg/mL) were preincubated with ^{125}I-α-BTX (20 nM, 110 cpm/fmol) prior to addition of 0.2 pmol of membrane-bound *Torpedo* nAChR. Assays were performed as described under Experimental Procedures. "BTX" refers to a negative control with *Torpedo* nAChR that has been preincubated with 20 μM unlabeled α-BTX for 10 min prior to addition to assays, and "No Peptide" corresponds to assays conducted in the absence of peptide. The values given are the mean of triplicate determinations, and the error bars are standard deviations.

Each amino acid from positions 181 to 190 was sequentially replaced by Gly, or by Ala when the native sequence had a glycine residue. This panel of 20 peptides was tested using the *Torpedo* nAChR competition assay to determine the effect of each substitution. The results of a typical experiment (n = 9), where the same concentration of each peptide was used (100 μg/mL, i.e., 40 μM), are shown in Figure 2. At this concentration, peptide α5(180–199)$_{native}$ typically inhibits the binding of ^{125}I-α-BTX to *Torpedo* nAChR by >90% [Figure 2 and McLane et al. (1990)]. The most profound effect on α-BTX binding was found for substitutions of Lys184 (16% inhibition), Arg187 (20% inhibition), Cys191 (48% inhibition), and Pro195 (61% inhibition). The concentration dependence of the inhibition by peptides with these substitutions was compared with the native peptide over a concentration range (3–74 μM) at which >50% inhibition is reached when peptide α5(180–199)$_{native}$ is used. The approximate IC_{50} values for peptide α5(180–199)$_{native}$ and for peptides substituted at Cys191 and Pro195 were 2, 100, and 50 μM, respectively (data not shown). A 50% level of inhibition by the peptides substituted at Lys184 and Arg187 could not be reached within the range of solubility of these peptides.

Other substitutions slightly reduced the ability of the peptide to compete for ^{125}I-α-BTX, but these effects were too small to be assessed using the single concentration of peptide as inhibitor of the experiments depicted in Figure 2. To evaluate subtle effects of amino acid substitutions on the ability of the α5 sequence segment to bind α-BTX, the concentration dependence of inhibition by peptides was determined for peptide concentrations from 1 to 100 μM.[2] The values obtained in three independent experiments were determined by analysis using the program EBDA (Munson & Rodbard, 1980) and are summarized in Table I. The most notable changes in the IC_{50} values were caused by substitutions of Asp189 (~9-fold increase), Asn186 (~5-fold increase), and Trp193 (~4-fold increase) and substitutions of Tyr residues at positions 194 and 196 (4–5-fold increases). Other substitutions had smaller effects on the affinity of α5(180–199) for α-BTX, as indicated in Table I.

Characterization of the Redox State of Cysteine Residues of Peptides α5(180–199)$_{native}$, α5(180–199)$_{C191}$, and α5(180–199)$_{C192}$. In order to investigate if the oxidation state of the

[2] The Hofstee plots of IC_{50} experiments and the data for carboxymethylation analysis are available upon request.

Table I: IC_{50} Values of Single Amino Acid Substitution Peptide Analogues of the Sequence α5(180–199)

amino acid substituted	IC_{50} (μM)[a]	n-fold increase relative to native
native	3.0 (0.9)	1.0
M181	5.3 (2.5)	1.8
G182	6.4 (2.6)	2.1
S183	7.0 (1.6)	2.3
K184	ND	
G185	8.9 (1.8)	3.0
N186	14.0 (0.8)	4.7
R187	ND	
T188	4.5 (3.8)	1.5
D189	26.3 (7.8)	8.8
S190	5.5 (0.7)	1.8
C191	[~100]	>30
C192	4.7 (3.1)	1.6
W193	11.7 (1.1)	3.9
Y194	13.9 (4.3)	4.6
P195	[~50]	>15
Y196	11.3 (3.7)	3.8
I197	5.7 (3.1)	1.9
T198	6.0 (2.8)	2.0
Y199	7.3 (3.0)	2.4

[a] The IC_{50} values are the means of three experiments, and standard deviations are given in parentheses. Values that could not be determined by linear regression and were estimated by inspection are indicated by brackets. ND indicates that IC_{50} values were not determinable.

vicinal cysteine residues of the sequence α5(180–199) affects α-BTX binding, peptides $\alpha5(180–199)_{native}$, $\alpha5(180–199)_{C191}$, and $\alpha5(180–199)_{C192}$ were modified with sulfhydryl reagents. Peptides were either oxidized with iodosobenzoic acid (IOBA) or reduced with dithiothreitol (DTT). Free sulfhydryl groups in untreated peptides, and following reducing or oxidizing treatments, were alkylated with iodoacetamide (IAA) and quantitated by amino acid composition analysis as phenylisothiocarbamyl derivatives of amino acid residues released upon acid hydrolysis. In this assay, cysteinyl residues with free sulfhydryl groups in the peptide preparation are present as their alkylated derivative *S*-(carboxymethyl)cysteine (CM-Cys) due to IAA (McLane et al., 1991a,b). Comparison of the CM-Cys peak obtained for untreated peptides and after full reduction indicated that in the untreated peptide $\alpha5(180–199)_{native}$ 73% of the Cys residues have free sulfhydryl groups. Surprisingly, no CM-Cys peak was present for the untreated peptides $\alpha5(180–199)_{C191}$ and $\alpha5(180–199)_{C192}$, while after reduction the expected molar ratio of CM-Cys was obtained (i.e., ~1). Therefore, the peptide analogues carrying substitutions where one of the vicinal cysteine residues is substituted are fully oxidized in solution, probably in the form of dimers.[2]

Gel permeation chromatography of these peptides either before or after oxidation, reduction, and alkylation reactions confirmed that peptides $\alpha5(180–199)_{C191}$ and $\alpha5(180–199)_{C192}$ existed in solution predominantly as dimers (4800 ± 500 and 5000 ± 500 Da, respectively) whereas untreated peptide $\alpha5(180–199)_{native}$ existed predominantly as a monomer (2400 ± 200 Da) (Figure 3A). After reduction and alkylation, all peptides eluted as monomers of the expected molecular weight (~2300), indicating that the higher molecular weight forms of the untreated peptides found with the vicinal cysteine substitutions are not merely aggregates, but are disulfide-linked dimers (Figure 3B). Oxidation of peptide $\alpha5(180–199)_{native}$ yielded both monomers and dimers, and amino acid composition analysis showed that no detectable free sulfhydryl groups were present, indicating that both Cys191 and Cys192 of the monomer and dimer forms are either disulfide-linked or oxidized to cysteic acid.

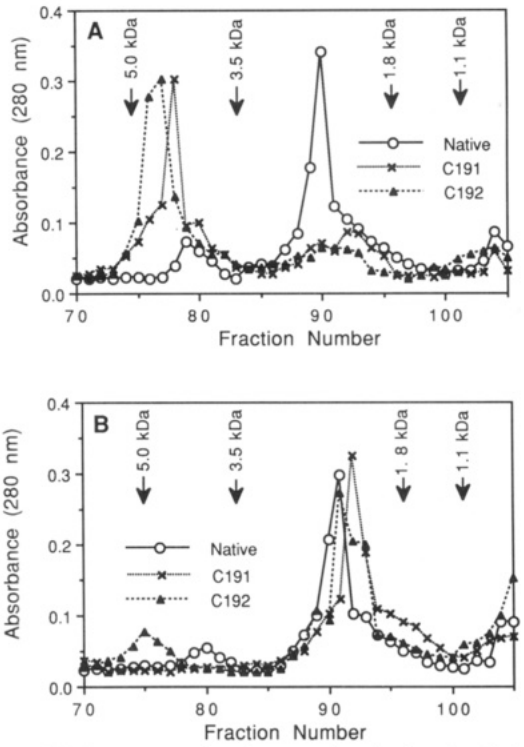

FIGURE 3: P6 chromatography of untreated and reduced and alkylated peptides $\alpha5(180–199)_{native}$, $\alpha5(180–199)_{C191}$, and $\alpha5(180–199)_{C192}$. Untreated peptides (top) and peptides which had been reduced with DTT and alkylated with IAA (bottom) as described under Experimental Procedures were chromatographed on a Biogel P6 column (1.8 × 47 cm) in 10 mM potassium phosphate buffer, pH 7.0. The molecular weight markers are bradykinin (1060), renin substrate tetradecapeptide (1759), β-endorphin (3465), and growth hormone releasing factor (5041).

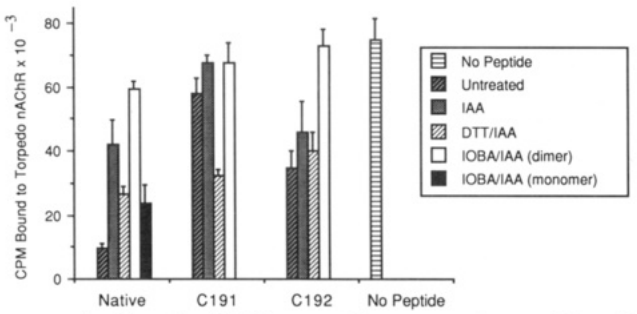

FIGURE 4: *Torpedo* nAChR competition assay using peptides $\alpha5(180–199)_{native}$, $\alpha5(180–199)_{C191}$, and $\alpha5(180–199)_{C192}$ before and after treatment with sulfhydryl reagents. Peptides were modified as described under Experimental Procedures and purified as described in Figure 3. *Torpedo* nAChR competition assays were performed using 20 nM ^{125}I-α-BTX (380 cpm/fmol) and 0.3 pmol of *Torpedo* nAChR per assay, and ~40 μM peptides. The results are expressed as the mean of triplicate determinations, and the error bars are standard deviations.

Effects of Sulfhydryl Modification of Peptides $\alpha5(180–199)_{native}$, $\alpha5(180–199)_{C191}$, and $\alpha5(180–199)_{C192}$ on ^{125}I-α-BTX Binding. The monomeric or dimeric forms of untreated peptides, and those resulting from oxidation and reduction, were tested for their ability to inhibit ^{125}I-α-BTX binding to membrane-bound *Torpedo* nAChR using the competition assay (Figure 4 and Table II). Only the monomeric form of peptide $\alpha5(180–199)_{native}$ could be obtained from untreated peptide, whereas both monomer and dimer forms were obtained in sufficient quantities after oxidation. For peptides $\alpha5(180–199)_{C191}$ and $\alpha5(180–199)_{C192}$, only dimers could be isolated from untreated peptides, or peptides treated with only IAA or IOBA. For all peptides, only the monomeric form

Table II: Inhibition of ^{125}I-α-BTX Binding to *Torpedo* nAChR by Peptides Treated with Sulfhydryl Reagents

peptide	% inhibition relative to native[a]			
	untreated	IAA only	DTT/IAA	IOBA/IAA
$\alpha5(180–199)_{native}$				
monomer	100	76	69	56
dimer				35
$\alpha5(180–199)_{C191}$				
monomer			74	
dimer	44	38		18
$\alpha5(180–199)_{C192}$				
monomer			76	
dimer	80	74		32

[a] The relative level of inhibition of bionding of ^{125}I-α-BTX to *Torpedo* nAChR by different peptides (~40 μM) was averaged for different experiments (n = 6) and divided by the mean level of inhibition observed for untreated peptide $\alpha5(180–199)_{native}$ (77%).

could be isolated and tested after reduction and alkylation. The results of a typical assay using 100 μg/mL peptide solutions (n = 6) are shown in Figure 4. The ability of treated peptides to inhibit ^{125}I-α-BTX binding relative to peptide $\alpha5(180–190)_{native}$, determined in six independent experiments, is summarized in Table II.

Alkylation of peptide $\alpha5(180–199)_{native}$, which contains both Cys191 and -192, reduced its ability to compete for α-BTX by ~30% with or without prior reduction with DTT. Reduction and/or alkylation of peptide $\alpha5(180–199)_{C192}$ did not significantly affect its ability to compete for α-BTX (70–80% inhibition). Peptide $\alpha5(180–199)_{C191}$, on the other hand, was relatively ineffective as a competitor for ^{125}I-α-BTX binding (40–50% inhibition), but upon conversion to the monomer by reduction and alkylation, some activity was restored (~70% inhibition). Although the role of free sulfhydryls in these peptides cannot be tested directly, as conditions necessary to keep the sulfhydryl groups of the peptide in the reduced state would destroy the disulfide loops of α-BTX necessary for activity (Martin et al., 1983), the results of alkylation experiments indicate that a free sulfhydryl is not necessary for α-BTX to bind.

Oxidation of $\alpha5(180–199)_{native}$ with IOBA resulted in the formation of both monomers and dimers. The monomer, which presumably represents the formation of a vicinal disulfide bond, retained 86% α-BTX binding activity, whereas the oxidized dimer was only 50% active. These results confirm that dimerization of the α5(180–199) peptides reduces, but does not eliminate, α-BTX binding activity. In addition, the lack of enhanced α-BTX binding by the completely oxidized monomer of peptide $\alpha5(180–199)_{native}$ indicates that the small proportion of oxidized monomer in untreated preparations of this peptide (~20%) is not responsible for the α-BTX binding observed. Therefore, it appears that α-BTX binding is not affected by the redox state of the adjacent Cys residues at positions 191 and 192.

We are unable at present to explain the significantly reduced ability of peptide $\alpha5(180–199)_{C192}$ to bind α-BTX after treatment with IOBA, as this peptide exists predominantly as a dimer in untreated solutions. Iodoso compounds, such as IOBA, are mild oxidants and are known to oxidize cysteine residues both to disulfides and to cysteic acids (Allison, 1976; Torchinsky, 1981; Fontana et al., 1981) and methionine residues to sulfoxides and sulfones (Mahoney & Hermodson, 1979; Fontana et al., 1981). From our amino acid composition analysis (data not shown), it is apparent that the low yield of methionine upon treatment of peptides with IOBA may be due to the formation of the sulfone, which could not be directly quantitated as it coelutes with Asp (Fontana et al., 1981). It

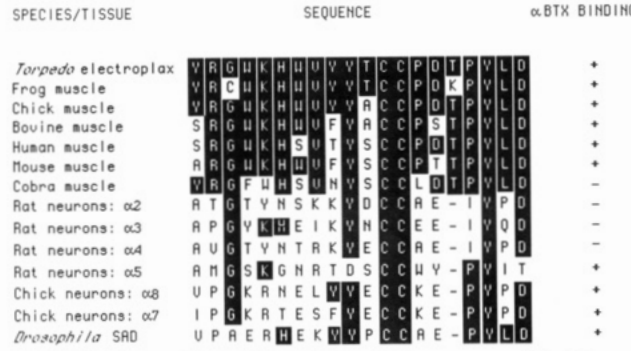

FIGURE 5: Comparison of the α5(180–199) sequence with different α subunits. The sequences corresponding to the deduced amino acid sequences flanking the vicinal cysteine pair, which is the hallmark of a nAChR α subunit, are taken from Conti-Tronconi et al. (1990) and McLane et al. (1990, 1991a,b) and references cited therein. The chick neuron α7 and α8 subunits are also referred to as αBGTBP α1 and αBGTBP α2 in the scientific literature [e.g., see Schoepfer et al. (1990)]. The sequences are aligned with respect to the vicinal cysteine pair at positions 192/193, and amino acid residues that are conserved relative to the *Torpedo* α1 sequence are indicated by a black background.

is possible that the double "mutation" presented as the Met181 sulfone and the substituted Cys residue of oxidized peptides $\alpha5(180–199)_{C191}$ and $\alpha5(180–199)_{C192}$ confers lower α-BTX binding activity than observed for the single amino acid substitutions of either Cys. The results presented for peptides oxidized with IOBA should be interpreted in light of the known side reactions.

Discussion

We have previously identified a sequence segment between amino acid residues 180 and 199 of the rat α5 subunit that contributes to forming the binding site for α-BTX in this putative neuronal nAChR (McLane et al., 1990). Given the divergent nature of the α5 sequence when compared to the highly conserved cholinergic site on the α1 subunits of the muscle nAChR α1 subunits, we were interested in determining what structural features are important for forming the protope for α-BTX. In the present study, a panel of peptides containing single amino acid substitutions of the sequence α5(180–199) were tested for their ability to compete for ^{125}I-α-BTX binding with native membrane-bound *Torpedo* nAChR. Substitutions by glycine of several residues within the sequence α5(180–199) markedly reduced the ability of the corresponding peptides to inhibit ^{125}I-α-BTX binding to *Torpedo* nAChR, when compared to peptide $\alpha5(180–199)_{native}$. These residues were Lys184 (~15% inhibition), Arg187 (~20% inhibition), Cys191 (~50% inhibition), and Pro195 (~60% inhibition). Subtle changes in the affinity for α-BTX occurred when other amino acid residues were substituted, most notably for peptides with substitutions of Asp189 (~10-fold increase in IC_{50} relative to the native sequence) and Tyr194, Tyr196, Asn186, and Trp193 (4–5-fold increase in IC_{50}s).

In order to compare these results with those obtained from previous studies, the sequences of several α subunits corresponding to α5(180–199) and their ability to bind α-BTX are summarized in Figure 5. The sequences are aligned with respect to the vicinal cysteine pair, and the amino acid residues that are identical to the prototypical *Torpedo* electroplax nAChR α1 subunit, which binds α-BTX with high affinity, are indicated by shadowing. (Residue positions specified in the text are with reference to the α5 subunit sequence.) Figure 5 illustrates the high degree of conservation of the amino acid sequences of the vertebrate muscle α-BTX binding site on the α1 subunit, and the relatively divergent nature of the neuronal

nAChR α subunits compared to the *Torpedo* nAChR α1 subunit. On the basis of homology, it would be very difficult to predict which of the neuronal nAChR α subunits might bind α-BTX. On closer scrutiny, however, the results obtained here lend further support to a developing model for the structural requirements for α-BTX binding, as discussed below.

On the basis of the structural analysis of α-BTX, the toxin/nAChR interface is thought to involve primarily hydrophobic and hydrogen-bonding interactions, and only a few charged amino acids (Love & Stroud, 1986). α-BTX is a basic polypeptide (pI ~ 9), and one might expect both specific and nonspecific electrostatic interactions to involve negatively charged residues. We find, however, in addition to substitution of Asp189 with Gly, which results in ~10-fold increase in the IC_{50}, positively charged residues of the α5 subunit, i.e., Lys184 and Arg187, are also critical for α-BTX binding. We interpret these results as indicating that a few negatively charged residues on α-BTX may be involved in specific interactions with the α5 subunit. However, an important caveat to be noted in generalizing these findings to the binding of α-BTX to the native nAChR complex is that the peptide α5(180–199) could conceivably be interacting with α-BTX in a manner different than the native nAChR and the residues identified here as crucial for binding might not be the same as those that interact with α-BTX in the native α5 nAChR. For the sequences of α subunits known to bind α-BTX, Lys184 is conserved, or conservatively replaced by Arg (see Figure 5), and this Lys residue is replaced by Trp in the α-BTX-insensitive cobra α1 sequence, indicating that it may be a common, critical residue for α-BTX binding. Arginine187, which is unique to the α5 sequence, may replace the positive charge of the Arg181 or His186 residues of the *Torpedo* and muscle α1 subunits. The positively charged Lys residue at position 187 of the *Drosophila* SAD subunit may play a similar role. If an insertion is allowed, Pro195 of the α5 sequence aligns with the highly conserved Pro194 residue of the *Torpedo* α1 sequence (Figure 5), which is common to all α-BTX binding sequences, and has been shown to be critical for α-BTX binding to the sequence segment *Torpedo* α1(181–200) (Conti-Tronconi et al., 1991).

Substitution of several aromatic residues (Tyr194, Tyr196, and Trp193) of the α5 sequence decreases the affinity of α-BTX by ~5-fold. Aromatic amino acid residues have been implicated in the high-affinity α-BTX binding of the *Torpedo* sequence by comparison with the α1 subunit sequences of other species that bind α-BTX with lower affinity (Wilson & Lentz, 1988; Ohana & Gershoni, 1990; McLane et al., 1991a), and Trp187 of the *Torpedo* α1 sequence has been demonstrated to be important to α-BTX binding by chemical modification in the synthetic peptide α1(185–196) (Neumann et al., 1986b). 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 through a stabilizing cation–π-electron interaction (Dougherty & Stauffer, 1990). Thus, despite the apparent divergence of the α5 sequence from other α-BTX binding α subunits, certain common features, such as an abundance of aromatic residues and amino acids able to participate in electrostatic and/or hydrogen-bonding interactions, may indicate conserved structural elements that are required for α-BTX binding.

Several disulfide bonds in the intact *Torpedo* α1 subunit may exist, including a vicinal disulfide between Cys at positions 192 and 193, which appears to be uniquely sensitive to reducing agents (Kao et al., 1984; Kao & Karlin, 1986; Mosckovitz & Gershoni, 1988; Kellaris et al., 1989). A disulfide bridge between flanking cysteine residues requires a cis, nonplanar peptide bond, which is an unusual configuration likely to alter the reactivity of the sensitive cystine (Dayhoff, 1976; Schultz & Schirmer, 1979; Thorton, 1981; Ovchinnikov et al., 1985, 1988). On the other hand, we found here, in agreement with previous determinations of the homologous *Torpedo* and vertebrate muscle peptides (Conti-Tronconi et al., 1991; McLane et al., 1991a), that ~30% of the Cys residues of the untreated peptide $\alpha5(180–199)_{native}$ are oxidized as disulfide bridges and as the amount of dimer present in these preparations is insufficient to account for the total peptide present in oxidized form we must conclude that a disulfide bridge forms spontaneously between the vicinal Cys of the peptide. The role of vicinal disulfide in α-BTX binding to the *Torpedo* α1 sequence, however, remains equivocal. In the intact *Torpedo* nAChR, reduction, or reduction and alkylation, does not change the number of α-BTX binding sites (Moore & Raftery, 1979; Walker et al., 1981). Similarly, Mosckovitz and Gershoni (1988) have shown that alkylation of the vicinal cysteine residues with a large adduct does not interfere with α-BTX binding either to the isolated *Torpedo* α1 subunit or to a proteolytic fragment containing the cholinergic site. The role of the adjacent cysteines in α-BTX binding to the intact *Torpedo* receptor was also studied by mutagenesis of the cloned α1 subunit and expression in *Xenopus* oocytes with the other unmodified subunits (Mishina et al., 1985), and replacement of either Cys192 or Cys193 reduced, but did not eliminate, α-BTX binding (39% and 28% activity, respectively). In the present study, the role of the vicinal cysteine residues at positions 191 and 192 of the α5 sequence could only partially be evaluated due to the tendency for peptides $\alpha5(180–199)_{C191}$ and $\alpha5(180–199)_{C192}$ to form dimers, which retained some activity [$\alpha5(180–199)_{C192}$, ~70–80% inhibition; peptide $\alpha5(180–199)_{C191}$, 40–50% inhibition]. Our results demontrate that both reduced and oxidized monomer forms of the peptide are active and, in addition, ~70% of the α-BTX binding activity is retained after alkylation of the cysteine residues. Thus, although the cysteine residues may assist in the formation of a functional binding surface for α-BTX in the intact receptor, it does not appear that a vicinal disulfide bond is necessary for inducing an active conformation of the α5 subunit.

Given the highly divergent sequences of the rat α5 subunit, the chick α7 and α8 subunits, and the *Drosophila* SAD subunit compared with the muscle type α1 subunits, as depicted in Figure 5, it is surprising that these different sequences accommodate α-BTX binding sites. A comparison of the sequences of different subtypes in Figure 5, and the inability to correlate a particular amino acid sequence with α-BTX binding activity, indicates, in a broader sense, a serious limitation to the use of sequence homology to define functionally related families of proteins. Namely, it is obvious from comparison of the α-BTX binding peptides of the different nAChR subtypes that different primary sequences are able to accommodate three-dimensional structures with comparable hydrophobic, hydrogen-bonding, and charge interactions. This fact is also illustrated by the lack of sequence homology between the nAChR subunits, the muscarinic acetylcholine receptor, and acetylcholinesterase, all which contain acetylcholine binding sites (Schumacher et al., 1986; Hulme et al., 1990). The failure to find a common sequence motif by comparison of the sequence segments of α subunits known to bind α-BTX is also similar to the search for the specific peptide sequences that bind to receptors involved in sorting proteins into cellular

organelles and membrane compartments [reviewed in Verner and Schatz (1988), Hartl et al. (1989), Keegstra (1989), Pfanner and Neupert (1990), Dice (1990), and Jackson et al. (1990)]. In these cases, it has been difficult to identify common recognition sequences, and instead compositional motifs define the targeting sequences of proteins that share a cellular compartment, in which certain amino acids or residues with similar physical characteristics are common, but the exact sequence is unimportant. For example, the signal motif for proteins targeted for lysosomal proteolysis involves amino acid residues of the recognition sequence KFERQ, in which the amino acid residues may occur in any order and can be reversed (Dice et al., 1990). The important structural features of the divergent α-BTX binding proteins of the nAChR superfamily might be presented in a different sequence order. Furthermore, it has been found using site-directed mutagenesis that although a single amino acid substitution can obliterate activity of an enzyme, secondary nonconservative substitutions can restore function (Blacklow & Knowles, 1990). Compensatory, multiple nonconservative substitutions may have occurred during the evolution of α-BTX binding nAChRs, and obscured a "universal" α-BTX binding sequence motif.

In conclusion, we have demonstrated that synthetic peptides with single amino acid substitutions can be used to identify critical residues involved in formation of the functional binding surface of a α-BTX binding site on the α5 subunit. The use of synthetic peptides to study the effects of single amino acid changes has several advantages over mutagenesis and expression of cloned cDNA sequences, such as (i) problems due to difference in the level of expression as a result of mutagenesis do not arise, (ii) the resultant secondary structural changes in peptides are more amenable to analysis than are the intact subunits, and (3) more selective chemical modification is possible with peptides than it is with the membrane-bound or solubilized nAChR. Using synthetic peptides instead of the intact receptor to define the binding sites of ligands, however, requires that enough structural information be contained in a continuous segment of the protein sequence, that the sequence segment is able to fold into the active conformation in the absence of neighboring sequences, and that the affinity of that sequence in the absence of other segments, which may assist in ligand binding in the intact receptor, be high enough to be detectable by the assay method. In the case of the nAChR and α-BTX, we and other investigators have been fortunate.

References

Allison, W. S. (1976) *Acc. Chem. Res. 9*, 293–299.

Aronheim, A., Eshel, Y., Mosckovitz, R., & Gershoni, J. M. (1988) *J. Biol. Chem. 263*, 9933–9937.

Ballivet, M., Nef, P., Couturier, S., Rungger, D., Bader, C. R., Bertrand, D., & Cooper, E. (1988) *Neuron 1*, 847–852.

Barkas, T., Mauron, A., Roth, B., Alliod, C., Tzartos, S. J., & Ballivet, M. (1987) *Science 235*, 77–80.

Blacklow, S. C., & Knowles, J. R. (1990) *Biochemistry 29*, 4099–4108.

Blanchard, S. G., Quast, U., Reed, K., Lee, T., Schimerlik, M. I., Vandlen, R., Claduio, T., Strader, C. D., Moore, H.-P. H., & Raftery, M. A. (1979) *Biochemistry 18*, 1875–1883.

Boulter, J., Luyten, W., Evans, K., Mason, P., Ballivet, M., Goldman, D., Stengelin, S., Martin, G., Heinemann, S., & Patrick, J. (1985) *J. Neurosci. 5*, 2545–2552.

Boulter, J., Evans, K., Martin, G., Mason, P., Stengelin, S., Goldman, D., Heinemann, S., & Patrick, J. (1986a) *J. Neurosci. Res. 16*, 37–49.

Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S., & Patrick, J. (1986b) *Nature 319*, 368–374.

Boulter, J., Connolly, J., Deneris, E., Goldman, D., Heinemann, S., & Patrick, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7763–7767.

Boulter, J., O'Shea-Greenfield, A., Duvoisin, R. M., Connolly, J. G., Wada, E., Jensen, A., Gardner, P. D., Ballivet, M., Deneris, E. S., McKinnon, D., Heinemann, S., & Patrick, J. (1990) *J. Biol. Chem. 265*, 4472–4482.

Buonanno, A., Mudd, J., Shah, V., & Merlie, J. P. (1986) *J. Biol. Chem. 261*, 16451–16458.

Burden, S. Y., Hartzell, M. C., & Yoshikami, D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3245–3249.

Cauley, K., Agranoff, B. W., & Goldman, D. (1989) *J. Cell Biol. 108*, 637–645.

Cauley, K., Agranoff, B. W., & Goldman, D. (1990) *J. Neurosci. 10*, 670–683.

Clark, D. G., Macmurchie, D. D., Elliot, E., Wolcott, R. G., Landel, A. M., & Raftery, M. A. (1972) *Biochemistry 11*, 1663–1668.

Conti-Tronconi, B. M., Gotti, C. M., Hunkapillar, M. W., & Raftery, M. A. (1982) *Science 218*, 1227–1229.

Conti-Tronconi, B. M., Dunn, S. M. J., Barnard, E. A., Dolly, J. O., Lai, F. A., Ray, N., & Raftery, M. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5208–5212.

Conti-Tronconi, B. M., Tzartos, S., Spencer, S. R., Kokla, A., Tang, F., & Maelicke, A. (1988) *NATO ASI Ser., Ser. H 25*, 119–136.

Conti-Tronconi, B. M., Fels, G., McLane, K., Tang, F., Bellone, M., Kokla, A., Tzartos, S., Milius, R., & Maelicke, A. (1989) *NATO ASI Ser., Ser. H 32*, 291–310.

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., Schroder, B., Reinhardt-Maelicke, S., & Maelicke, A. (1991) *Biochemistry 30*, 2575–2584.

Couturier, S., Erkman, L., Valera, S., Rungger, D., Bertrand, S., Boulter, J., Ballivet, M., & Bertrand, D. (1990a) *J. Biol. Chem. 265*, 17560–17567.

Couturier, S., Bertrand, D., Matter, J.-M., Herandez, M.-C., Bertrand, S., Millar, N., Valera, S., Barkas, T., & Ballivet, M. (1990b) *Neuron 5*, 847–856.

Dayhoff, M. D. (1976) in *Atlas of Protein Sequence & Structure*, Vol. 5, pp 77–268, National Biomedical Research Foundation, Washington, D.C.

Deneris, E. S., Connolly, J., Boulter, J., Wada, E., Wada, K., Swanson, L. W., Patrick, J., & Heinemann, S. (1988) *Neuron 1*, 45–54.

Deneris, E. S., Boulter, J., Swanson, L. W., Patrick, J., & Heinemann, S. (1989) *J. Biol. Chem. 264*, 6268–6272.

Dice, J. F. (1990) *Trends Biochem. Sci. 15*, 305–309.

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

Duvoisin, R. M., Deneris, E. S., Patrick, J., & Heinemann, S. (1989) *Neuron 3*, 487–496.

Elliot, J., Blanchard, S. G., Wu, W., Miller, J., Strader, C. D., Hartig, P., Moore, H.-P., Racs, J., & Raftery, M. (1980) *Biochem. J. 185*, 667–677.

Fontana, A., Dalzoppo, D., Grandi, C., & Zambonin, M. (1981) *Biochemistry 20*, 6997–7004.

Goldman, D., Deneris, E., Luyten, W., Kochhar, A., Patrick, J., & Heinemann, S. (1987) *Cell 48*, 965–973.

Hartl, F. U., Pfanner, N., Nicholson, D. W., & Neupert, W. (1989) *Biochim. Biophys. Acta 988*, 1–45.

Heinrickson, S., & Meredith, B. (1984) *Anal. Biochem. 136*, 65–74.
Hieber, V., Bouchey, J., Agranoff, B. W., & Goldman, D. (1990a) *Nucleic Acids Res. 18*, 5293.
Hieber, V., Bouchey, J., Agranoff, B. W., & Goldman, D. (1990b) *Nucleic Acids Res. 18*, 5307.
Houghten, R. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5131–5135.
Hulme, E. C., Birdsall, N. J. M., & Buckley, N. J. (1990) *Annu. Rev. Pharmacol. Toxicol. 30*, 633–673.
Isenberg, K. E., Mudd, J., Shah, V., & Merlie, J. P. (1986) *Nucleic Acids Res. 14*, 5111–5118.
Jackson, M. R., Nilsson, T., & Peterson, P. A. (1990) *EMBO J. 9*, 3153–3162.
Kao, P. N., & Karlin, A. (1986) *J. Biol. Chem. 261*, 8085–8088.
Kao, P. N., Dwork, A. J., Kaldany, R. J., Silver, M. L., Wideman, J., Stein, S., & Karlin, A. (1984) *J. Biol. Chem. 259*, 11662–11665.
Keegstra, K. (1989) *Cell 56*, 247–253.
Kellaris, K. V., Ware, D. K., Smith, S., & Kyte, J. (1989) *Biochemistry 28*, 3469–3482.
La Polla, R. J., Mixter-Mayne, K., & Davidson, N. (1984) *Proc. Natl. Acad Sci. U.S.A. 81*, 7970–7974.
Lindstrom, J., Einarson, E., & Tzartos, S. (1981) *Methods Enzymol. 74*, 432–460.
Loring, R. H., & Zigmond, R. E. (1988) *Trends Neurosci. 11*, 73–78.
Love, R. A., & Stroud, R. M. (1986) *Protein Eng. 1*, 37–46.
Luetje, C. W., Wada, K., Rogers, S., Abramson, S. N., Tsuji, K., Heinemann, S., & Patrick, J. (1990) *J. Neurochem. 55*, 632–640.
Mahoney, W. C., & Hermodson, M. A. (1979) *Biochemistry 18*, 3810–3814.
Marshall, J., Buckingham, S. D., Shingai, R., Lunt, G. G., Goosey, M. W., Darlison, M. G., Sattelle, D. B., & Barnard, E. A. (1990) *EMBO J. 9*, 4391–4398.
Martin, B. M., Chibber, B. A., & Maelicke, A. (1983) *J. Biol. Chem. 258*, 8714–8722.
McLane, K. E., Wu, X., & Conti-Tronconi, B. M. (1990) *J. Biol. Chem. 265*, 9816–9824.
McLane, K. E., Wu, X., & Conti-Tronconi, B. M. (1991a) *Biochemistry 30*, 4925–4934.
McLane, K. E., Wu, X., Schoepfer, R., Lindstrom, J. M., & Conti-Tronconi, B. M. (1991b) *J. Biol. Chem.* (in press).
McPherson, G. A. (1983) *Comput. Prog. Biomed. 17*, 107–111.
Middleton, R. E., & Cohen, J. B. (1991) *Biochemistry 30*, 6987–6997.
Mishina, M., Kurosaki, T., Tobimatsu, T., Morimoto, Y., Noda, M., Yamamoto, T., Terao, M., Lindstrom, J., Takahashi, T., Kuno, M., & Numa, S. (1984) *Nature 307*, 604–608.
Mishina, M., Tobimatsu, T., Imoto, K., Tanaka, K., Fujita, Y., Fukuda, K., Kurasaki, M., Takahashi, H., Morimoto, Y., Hirose, T., Inayama, S., Takahashi, T., Kuno, M., & Numa, S. (1985) *Nature 313*, 364–369.
Molinoff, P. B., Wolfe, B. B., & Weiland, G. A. (1981) *Life Sci. 29*, 427–438.
Moore, H.-P., & Raftery, M. A. (1979) *Biochemistry 18*, 1907–1919.
Mosckovitz, R., & Gershoni, J. M. (1988) *J. Biol. Chem. 263*, 1017–1022.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220–239.
Nef, P., Oneyser, C., Alliod, C., Couturier, S., & Ballivet, M. (1988) *EMBO J. 7*, 595–601.
Neubig, R. R., Krodel, E. K., Boyd, N. D., & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 690–694.
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.
Neumann, D., Barchan, D., Horowitz, M., Kochva, E., & Fuchs, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7255–7259.
Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., & Numa, S. (1982) *Nature 299*, 793–797.
Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983a) *Nature 302*, 528–532.
Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kayano, T., Hirose, T., Inayama, S., & Numa, S. (1983b) *Nature 305*, 818–823.
Ohana, B., & Gershoni, J. M. (1990) *Biochemistry 29*, 6409–6415.
Ovchinnikov, Y. A., Lipkin, V. M., Shuvaeva, T. M., Bogachuk, A. P., & Shemyakin, V. V. (1985) *FEBS Lett. 179*, 107–110.
Ovchinnikov, Y. A., Abdulaev, N. G., & Bogachuk, A. P. (1988) *FEBS Lett. 230*, 1–5.
Papke, R. L., Boulter, J., Patrick, J., & Heinemann, S. (1989) *Nature 3*, 589–596.
Pedersen, S. E., Dreyer, E. B., & Cohen, J. B. (1986) *J. Biol. Chem. 261*, 13735–13742.
Pfanner, N., & Neupert, W. (1990) *Annu. Rev. Biochem. 59*, 331–353.
Raftery, M. A., Hunkapillar, M. W., Strader, C. D., & Hood, L. E. (1980) *Science 208*, 1454–1457.
Ralston, S., Sarin, V., Thanh, H. L., Rivier, J., Fox, L., & Lindstrom, J. (1987) *Biochemistry 26*, 3261–3266.
Rodbard, D., & Frazier, G. R. (1975) *Methods Enzymol. 37*, 3–22.
Sawruk, E., Schloss, P., Betz, H., & Schmitt, B. (1990) *EMBO J. 9*, 2671–2677.
Schloss, P., Hermans-Borgmeyer, I., Betz, H., & Gundelfinger, E. D. (1988) *EMBO J. 7*, 2889–2894.
Schoepfer, R., Whiting, P., Esch, F., Blacher, R., Shimasaki, S., & Lindstrom, J. (1988) *Neuron 1*, 241–248.
Schoepfer, R., Conroy, W. G., Whiting, P., Gore, M., & Lindstrom, J. (1990) *Neuron 4*, 35–48.
Schultz, G. E., & Schirmer, R. H. (1979) in *Principles of Protein Structure* (Cantor, C. R., Ed.) p 55, Springer-Verlag, New York.
Schumacher, M., Camp, S., Maulet, Y., Newton, M., MacPhee-Quigley, K., Taylor, S. S., Friedmann, T., & Taylor, P. (1986) *Nature 319*, 407–409.
Thorton, J. M. (1981) *J. Mol. Biol. 151*, 261–287.
Torchinsky, Y. M. (1981) in *Sulfur in Proteins*, p 48, Pergamon Press, New York.
Verner, K., & Schatz, G. (1988) *Annu. Rev. Biochem. 59*, 331–353.
Wada, K., Ballivet, M., Boulter, J., Connolly, J., Wada, E., Deneris, E. S., Swanson, L. W., Heinemann, S., & Patrick, J. (1988) *Science 240*, 330–334.
Walker, J. W., Lukas, R. J., & McNamee, M. G. (1981) *Biochemistry 20*, 2191–2199.

Whiting, P. J., Scoepfer, R., Swanson, L. W., Simmons, D. M., & Lindstrom, J. M. (1987) *Nature 327*, 515–518.
Wilson, P. T., & Lentz, T. L. (1988) *Biochemistry 27*, 6667–6674.
Wilson, P. T., Gershoni, J. M., Hawrot, E., & Lentz, T. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2553–2557.
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*, 643–651.
Yu, L., La Polla, R. J., & Davidson, N. (1986) *Nucleic Acids Res. 14*, 2539–2555.

Identification and Characterization of a New Family of High-Affinity Receptors for *Escherichia coli* Heat-Stable Enterotoxin in Rat Intestinal Membranes†

Michel Hugues,‡ Marlys Crane,§ Shereen Hakki,‡ Peter O'Hanley,§ and Scott A. Waldman*,‡

Departments of Medicine and Pharmacology, Division of Clinical Pharmacology, Thomas Jefferson University, MOB-813, 1100 Walnut Street, Philadelphia, Pennsylvania 19107, and Departments of Medicine and Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94043

Received May 21, 1991; Revised Manuscript Received July 24, 1991

ABSTRACT: Novel high-affinity, low-capacity binding sites in intestinal membranes for the heat-stable toxin produced by *Escherichia coli* have been defined. The appearance of these sites is observed in the presence of physiological concentrations of NaCl in binding reactions. Scatchard analyses of equilibrium binding in the absence of NaCl demonstrated a single class of binding sites with $K_D = 1.9 \times 10^{-9}$ M and $B_{max} = 0.75$ pmol/mg of protein. In contrast, similar experiments in the presence of NaCl demonstrated, in addition to the previously described low-affinity site, a high-affinity site with a K_D of 2.1×10^{-11} M and a B_{max} of 73 fmol/mg of protein. Confirmation of the presence of high- and low-affinity sites was obtained in studies of the kinetics of ST binding. These sites exhibited similar dissociation but markedly different association kinetics. Determination of the association and dissociation constants permitted calculation of the K_D's for the high- and low-affinity sites, which were 1.15×10^{-11} M and 1.89×10^{-9} M, respectively. These data agree closely with those obtained in studies of equilibrium binding. Futhermore, similar values for the K_D's of these sites were obtained in experiments of competitive displacement of labeled ST, confirming the presence of two receptors for this toxin. Binding of ST to high-affinity sites is completely reversible and does not appear to be coupled to activation of particulate guanylate cyclase. In contrast, binding of ST to low-affinity sites appears to be partially reversible and may be coupled to activation of guanylate cyclase. The structure, associated second messengers, and pathophysiological role of these different receptors are currently being studied in this laboratory.

Escherichia coli heat-stable enterotoxin (ST) is one of the major etiologic agents responsible for endemic diarrhea in the developing world (Walsh & Warren, 1979; Rhode, 1984; Gold, 1988). This toxin is a low molecular weight peptide of 18 or 19 amino acids whose structure is stabilized by three intrachain disulfide bridges (Alderete & Robertson, 1978; Staples et al., 1980; Chan & Giannella, 1981; Aimoto et al., 1982; Dreyfus et al., 1984a; Okamoto et al., 1987). The integrity of these disulfide bridges is critical for the biological and biochemical activities of the toxin (Dreyfus et al., 1984a; Okamoto et al., 1987; Guarino et al., 1987a). ST induces secretion by binding to receptors in brush borders of intestinal cells (Gianella et al., 1983; Dreyfus et al., 1984a; Frantz et al., 1984; Kuno et al., 1986; Cohen et al., 1987a,b; Guarino et al., 1987b,c). Occupancy of these receptors by ST activates particulate guanylate cyclase, resulting in an increase in intracellular cyclic GMP (Giannella et al., 1983; Dreyfus et al., 1984b; Kuno et al., 1986; Waldman et al., 1986; Guarino et al., 1987b). It has been suggested that this cyclic nucleotide mediates the actions of ST by directly altering intestinal fluid and electrolyte transport (Hughes et al., 1978; Field et al., 1978; Huott et al., 1988). Interestingly, femtomole quantities of ST induce intestinal secretion in animal models whereas guanylate cyclase activation or accumulation of intracellular cyclic GMP in intact cells is observed with 10^{-8}–10^{-6} M toxin (Dreyfus et al., 1983; Giannella et al., 1983; Guarino et al., 1987b; Gariepy et al., 1987; Saeed & Greenberg, 1988; Ivens et al., 1990; Carr et al., 1989). Recent studies suggest that ST induction of intestinal secretion may involve other signal transduction pathways, including the production of inositol polyphosphates and diacylglycerol and the activation of protein kinase C (Banik & Ganguly, 1988, 1989; Weikel et al., 1990). Furthermore, structural studies of the ST receptor demonstrated a complex subunit composition suggesting multiple populations of these receptors (Kuno et al., 1986; Gariepy & Schoolnik, 1986; Ivens et al., 1990; Thompson & Giannella, 1990). These data suggest that ST receptors differing in structure and binding may be coupled to various signal transduction cascades and mediate different physiological responses, a subset of which is intestinal secretion. However, previous studies have demonstrated a single class of functional ST receptor binding sites. Thus, Scatchard analysis of ^{125}I-ST binding to intestinal cells

† This work was supported in part by a PMA Foundation Research Starter Grant. M.H. was supported in part by funds from the CNRS, France, NATO, and the Philippe Foundation.
‡ Thomas Jefferson University.
§ Stanford University School of Medicine.

0006-2960/91/0430-10738$02.50/0