Identification of Sequence Segments Forming the α-Bungarotoxin Binding Sites on Two Nicotinic Acetylcholine Receptor α Subunits from the Avian Brain*

(Received for publication, December 21, 1990)

Kathryn E. Mclanet§, Xiaodong Wu‡, Ralf Schoepfer¶, Jon M. Lindstrom**, and Bianca M. Conti-Tronconi‡‡

From the 1Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55108, the 2Institute of Neurological Sciences, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104-6140, and the 3Zentrum fur Molekulaire Biologie, Universitat Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg 1, Federal Republic of Germany

The relationship between neuronal α-bungarotoxin binding proteins (αBGTBP) and nicotinic acetylcholine receptor function in the brain of higher vertebrates has remained controversial for over a decade. Recently, the cDNAs for two homologous putative ligand binding subunits, designated αBGTBP α1 and αBGTBP α2, have been isolated on the basis of their homology to the N terminus of an αBGTBP purified from chick brain. In the present study, a panel of overlapping synthetic peptides corresponding to the complete chick brain αBGTBP α1 subunit and residues 166–215 of the αBGTBP α2 subunits were tested for their ability to bind 125I-αBGT. The sequence segments corresponding to αBGTBP α1-(181–200) and αBGTBP α2-(181–200) were found to consistently and specifically bind 125I-αBGT. The ability of these peptides to bind αBGT was significantly decreased by reduction and alkylation of the Cys residues at positions 190/191, whereas oxidation had little effect on αBGT binding activity. The relative affinities for αBGT of the peptide sequences αBGTBP α1-(181–200) and αBGTBP α2-(181–200) were compared with those of peptides corresponding to the sequence segments Torpedo α1-(181–200) and chick muscle α1-(179–198). In competition assays, the IC50 for αBGTBP α1-(181–200) and αBGTBP α2-(181–200) were found to be 20-fold higher than that obtained for the other peptides (±2 versus 40 μM). These results indicate that αBGTBP α1 and αBGTBP α2 are ligand binding subunits able to bind αBGT at sites homologous with nAChR α subunits and that these subunits may confer differential ligand binding properties on the two αBGTBP subtypes of which they are components.

* This work was supported by United States Research Grant BNS-8607289 from the National Science Foundation, United States National Institute on Drug Abuse Program Project Grant 5P01-DA06695, Army Research Office Contract DAMD 17-88-C-8120 (to B. M. C.-T.), and by research grants from the Muscular Dystrophy Association, the California Chapter of the Muscular Dystrophy Association, the California Chapter of the Myasthenia Gravis Foundation, and National Institutes of Health Grant NS11323 (to J. M. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a predoctoral fellowship as part of the United States Public Health Service Training Grant 5-T32-GM07323 ("Chemical Basis of Cell and Molecular Biology").

¶ Recipient of a fellowship by Boehringer Ingelheim Fonds.

** To whom correspondence should be addressed.

The α-neurotoxins, isolated from elapid snake venoms, such as α-bungarotoxin (αBGT) from Bungarus multicinctus, bind to the muscle-type nicotinic acetylcholine receptor (nAChR) with subnanomolar affinity. The α-neurotoxins were used to purify nAChRs from Torpedo electric organ and vertebrate muscle, making possible the determination of the complete amino acid sequence of their four subunits (α1, β1, γ, and δ) (Raftery et al., 1980; Conti-Tronconi et al., 1982; Noda et al., 1982, 1983a, 1983b). In addition to the nAChR of skeletal muscle, receptors that bind αBGT have also been found in numerous regions of the mammalian and avian brain and autonomic ganglia (reviewed by Quik and Geertsen, 1988). αBGT binding to these sites displays typical nicotinic pharmacology, i.e. d-tubocurare, nicotine, and carbamylcholine compete with 125I-αBGT binding (Patrick and Stallcup, 1977; Clarke et al., 1985; Marks et al., 1986; Lukas et al., 1986a, 1986b). Heterogeneity of αBGT binding receptors is suggested by reports of high and low affinity binding components in the rat brain (Lukas, 1984; Meeker et al., 1986).

A few nicotinic cholinergic responses in the mammalian brain are inhibited by αBGT, including cholinergic responses in the carotid sinus (Dinger et al., 1985) and cerebellum (de la Garza et al., 1987) and the effects of light on circadian rhythms (Zatz and Brownstein, 1981; Russak and Bina, 1990). The majority of responses elicited by nicotinic agonists in the brain, however, are insensitive to αBGT (Lipton et al., 1987; Loring and Zigmond, 1988; Vidal and Changeux, 1989; Calabresi et al., 1989; Schulz and Zigmond, 1989; de la Garza et al., 1989; Wong and Gallagher, 1989; Mulle and Changeux, 1990). A functional distinction has also been made between a neuronal αBGT binding protein (αBGTBP) and an αBGT-insensitive nAChR, which are both present on neurons of autonomic ganglia (Bursztajn and Gershon, 1977; Patrick and Stallcup, 1977; Carbonetto et al., 1978; Kemp and Edge, 1987; Whiting et al., 1987; Mclanet et al., 1990a). The nAChRs of the brain and autonomic ganglia are predominantly inhibited by α-bungarotoxin (αBGT) (Chiappinelli, 1983, 1985), also referred to as Toxin F (Loring et al., 1984), bungarotoxin 3.1 (Halvorsen and Berg, 1986), and neuronal bungarotoxin (Lindstrom et al., 1987). Neuronal αBGT-binding receptors, comprising two to four subunits, have been purified from chick optic lobe and brain.

1 The abbreviations used are: αBGT, α-bungarotoxin; αBGTBP, α-bungarotoxin binding protein; βGT, β-bungarotoxin; nAChR, nicotinic acetylcholine receptor; IAA, iodoacetamide; DTT, dithiothreitol; 10BA, 2-iodosobenzoic acid; HPLC, high pressure liquid chromatography; CM-Cys, S-(carboxymethyl)cysteine; PBS, phosphate-buffered saline.
Bungarotoxin Binds Brain Nicotinic Receptor Subunit Peptides

(Blanchard et al., 1991; Betz and Pfeiffer, 1984; Conti-Tronconi et al., 1985) and rat brain (Kemp et al., 1985; Wonnacott, 1986; Whiting and Lindstrom, 1987) using α-neurotoxins as affinity ligands. The N-terminal amino acid sequence of a chick brain αBGT binding protein subunit is homologous to the α subunit of the muscle and Torpedo nAChRs (Conti-Tronconi et al., 1985). A distinct class of neuronal nAChRs that do not bind αBGT are composed of two subunits and have been isolated by immunoadfinity chromatography (Whiting and Lindstrom, 1986, 1987; Whiting et al., 1987). This αBGT-insensitive class of nAChRs is now well characterized, as a result of the isolation of cDNAs for several different nAChR subunits from avian and rodent neurons. The deduced amino acid sequences for four neuronal nAChR α subunits (α2, α3, α4, and α5) and three β subunits (β2, β3, and β4, also designated “non-α” or “structural subunits”) have been reported, and other homologous subunits may exist (Wada et al., 1988; Boulter et al., 1986, 1990; Goldman et al., 1987; Nef et al., 1988; Schoepfer et al., 1988; Boyd et al., 1988; Deneris et al., 1988, 1989; Duvoisin et al., 1989). Functional neuronal nAChRs have been formed by some of these subunits in Xenopus oocytes by coexpression of the α2, α3, or α4 subunits with the β2 or β4 subunits (Goldman et al., 1987; Deneris et al., 1988; Wada et al., 1988; Ballivet et al., 1988; Duvoisin et al., 1989; Poppe et al., 1988; Luetje et al., 1990a). The nAChRs formed by the α3 and α4 subunits are sensitive to αBGT, when coexpressed with the β2 subunit, whereas none of these nAChRs are blocked by αBGT (reviewed in Luetje et al., 1990b). Interestingly, the α3β4 complex is insensitive to αBGT (Duvoisin et al., 1989) indicating that the β subunit is also able to affect the ligand binding characteristics of the neuronal nAChR. It is possible that the other α and β subunits that have not been successfully expressed in Xenopus oocytes require other yet unidentified subunits for functional expression and that one of these complexes is an αBGT-sensitive nAChR.

Two additional subunits, designated αBGTBP α1 and αBGTBP α2, have been isolated recently from the avian brain using an oligonucleotide probe corresponding to the N-terminal sequence of the purified chick brain αBGT binding protein (Schoepfer et al., 1990). The αBGTBP α1 subunit is present in >90% of all αBGTBP subunits in chicken brains, whereas the αBGTBP α2 subunit is present in combination with the αBGTBP α1 subunit in a minor subtype comprising ∼15% of the total αBGTBP subunits. A cDNA identical to αBGTBP α1 was subsequently reported by Couturier et al. (1990) and was designated α7 by this group. They observed that when this cDNA was expressed in Xenopus oocytes it produced a cation channel gated by acetylcholine and nicotine and competitively antagonized muscarinic agonists and other homologous subunits. Evidence that a binding site for acetylcholine is located near the homologous residues at positions Cys 192 and 193 of the Torpedo α1 subunit has come from studies showing their selective labeling by cholineergic affinity ligands (Aylward et al., 1984; Dennis et al., 1988). The binding of conventional nicotinic ligands (Blanchard et al., 1979) and cholineergic affinity reagents (Aylward et al., 1984; Wilson et al., 1984) is competitive with αBGT, indicating that this region contains a shared site for neurotransmitter and αBGT binding. The αBGTBPs are affinity labeled by the same reagents that label Cys-192 and -193 of the Torpedo nAChR α1 subunit (Norman et al., 1982; Kemp et al., 1985). For this reason, we were interested in testing the ability of the αBGTBP α1 and α2 subunits to bind αBGT and in comparing their αBGT binding properties with the chick muscle α1 subunit.

In the present study, overlapping synthetic peptides corresponding to the complete αBGTBP α1 subunit and residues 181-200 of the αBGTBP α2 subunit were tested for their ability to directly bind [125I]αBGT in toxoid blot assays and to compete for αBGT binding with native membrane-bound Torpedo nAChR using a competition assay. The results were compared with those obtained using a chick α1 muscle sequence between residues 179-196 and the Torpedo sequence segment 181-200, which both bind αBGT with relatively high affinity, and with a negative control sequence corresponding to residues 181-200 of the a7 subunit (Burden et al., 1975; Conti-Tronconi et al., 1990; McLane et al., 1991). The results indicate that peptides corresponding to the sequence segments αBGTBP α1-(181-200) and αBGTBP α2-(181-200) are both able to bind αBGT, although with somewhat different affinities. These results support the concepts that αBGTBP α1 and α2 subunits form the αBGT binding sites of neuronal αBGTBP subunits and that these binding sites are formed by a region of the subunit homologous to a cholinergic binding site of muscle nAChR α1 subunits.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Characterization—Peptides, 19-21 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 was consistently present, which accounted for 65-85% of the total absorbance at 214 nm. The amino acid composition of all peptides, determined by derivatization of amino acid residues released by acid hydrolysis with phenylisothiocyanate, followed by separation on a reverse phase HPLC column (PICO-TAG) as described by Heinrickson and Mere-dith (1984), yielded a satisfactory correspondence between experimental and theoretical values. The sequence and purity of peptides corresponding to sequences 181-200 of different α subunits, and other randomly selected peptides, were verified by gas-phase sequencing (Applied Biosystems), indicating that contamination by truncated peptides was less than 5-15%. The sequence and codes of the peptides are reported in Fig. 1.

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-iodosobenzoic acid (IOBA) (0.1 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 room temperature. The peptides formed by the α3 and α4 subunits are both able to bind αBGT, although with somewhat different affinities. These results support the concepts that αBGTBP α1 and α2 subunits form the αBGT binding sites of neuronal αBGTBP subunits and that these binding sites are formed by a region of the subunit homologous to a cholinergic binding site of muscle nAChR α1 subunits.
Bungarotoxin Binds Brain Nicotinic Receptor Subunit Peptides

**Preparation and Calibration**

Radiolabeled aBGT-aBGT was prepared (Biotoxins Inc., Lot ABT 88A) and used. The purity of aBGT, as assessed by gas phase sequencing, indicated that contaminating sequences, if present, were below the level of detectability (<3-5%). aBGT was radiolabeled with carrier-free 125I (Lindstrom et al., 1981) and calibrated as described by Blanchard et al. (1979). The specific activity of the 125I-aBGT preparations used in experiments was 4-12 Ci/mmol.

**Toxin Blots and Scatchard Analysis—Nitrocellulose strips (MSI, Inc.) were spotted with 1 μl of each peptide solution (0.25 mg/ml in 10 mM potassium phosphate buffer, pH 7.4 (KP buffer)) and allowed to dry at room temperature. The strips were blocked by incubation with 10 mg/ml cytochrome c (Sigma) in KP buffer for 2 h at room temperature. Cytochrome c (5 mg/ml), which shares similar charge properties to aBGT, was added to solutions of 125I-aBGT to reduce nonspecific binding. The blocked nitrocellulose strips were incubated with 0.1-10 μM 125I-aBGT in PBS (10 mM sodium phosphate, 100 mM NaCl, pH 7.2) for 2-4 h at room temperature. The time course of association of 125I-aBGT to peptides has been reported previously and indicates that equilibrium binding is attained under these conditions (Conti-Tronconi et al., 1990). The toxin blots were washed eight times with 3 ml of PBS containing 0.1% Tween 20 for 2.5 min/wash, mounted for autoradiography using Kodak X-OMat film, and exposed at -70 °C. The concentration dependence of binding was determined by counting triplicate samples (5-mm squares) of nitrocellulose in a Beckman 5500 γ counter. The apparent back-ground binding of 125I-aBGT binding to nitrocellulose as assessed by autoradiography varied between strips. In order to obtain a value for the specific binding to peptides at each concentration of 125I-aBGT, the nonspecific binding of 125I-aBGT was determined by preincubation of replicate strips with 100 μM unlabeled aBGT for 2 h at room temperature prior to addition of 125I-aBGT and was found to be linear with the concentration of 125I-aBGT (5-25% of the specifically bound 125I-aBGT below 3-5 μM). The binding of 125I-aBGT to peptides did not exceed 3% of the total radioactivity added. The relative affinities of peptides bound to nitrocellulose were compared by calculating an apparent Kd using the programs EBDA and LIGAND (Munson and Rodbard, 1980; McPherson, 1983). Given the solid phase nature of these assays and the nonsaturating conditions used, the Kd and Bmax values only approximate the true values.

**Competitive Inhibition of 125I-aBGT Binding by Peptides—Peptides (5-500 μg/ml) were preincubated with 125I-aBGT (0.5-2 pmol) in 10 mM potassium phosphate buffer, pH 7.4, containing 10 mg/ml cytochrome c overnight at 4 °C. To 100 μl of the peptide/ toxin solution, 0.2-1 pmol of membrane-bound Torpedo nAChR (Neubig et al., 1979; Elliot et al., 1980) was added. After incubation for 3 min at room temperature, the assay tubes were centrifuged at 14,000 χ g for 45 min, washed with PBS, and recentrifuged. The pellet was counted in a Beckman 5500 γ counter. Nonspecific binding (<5%) was determined by preincubation of 125I-aBGT and with 20 nM unlabeled aBGT for 10 min prior to addition to the radiolabeled toxin solutions. Binding of 125I-aBGT in the presence of peptides was compared with values obtained with toxin preincubated with only buffer. The IC50 values were determined by logit-log analysis (Rodbard and Frazier, 1975), using the computer program EBDA (McPherson, 1983). The results are presented graphically as a Hofseth plot (Molinoff et al., 1981), in which the IC50 values can be directly compared as the negative slopes of the linear regression lines.

**RESULTS**

**Rationale—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 and Lentz, 1988; Conti-Tronconi et al., 1988, 1989, 1990), and biosynthetic peptides (Barkas et al., 1987; Aronheim et al., 1988; Ohana and Gershoni, 1990) have been used to locate continuous sequence segments of the Torpedo nAChR α subunit able to form independent aBGT binding sites, i.e. "prototopes." We have used synthetic peptides to define an aBGT binding site on the Torpedo nAChR α1 and rat α5 subunits (Conti-Tronconi et al., 1990; McLane et al., 1990b) and a aBGT binding site on the rat α3 nAChR subunit (McLane et al., 1990a).

In the present study, we compared the ability of synthetic peptides corresponding to the homologous sequence segments.
Assays—Peptides (0.25 mg/ml) were spotted onto nitrocellulose under “Experimental Procedures.” Autoradiographs were exposed for 200) of typical qualitative experiments is shown in Fig. 2A (n = 4), where the binding of $^{125}$I-αBGT (2 μM) to the chick muscle α1, and αBGTBP α1 and α2 is compared with control peptides. A strong signal was consistently observed for Torpedo α1-(181–200), chick muscle α1-(179–198), and chick BGTBP α2-(181–200). A weaker but positive signal was also observed for the chick BGTBP α1-(181–200) peptide. Background levels of $^{125}$I-αBGT binding were observed for the negative control peptides, glycine α1-(61–78), rat nAChR β2-(331–350), cow muscle nAChR α1-(181–200), and for other overlapping peptides of the chick brain αBGTBP α1 and α2 subunits, BGTBP α1-(171–189), α1-(185–204), α2-(166–185), and α2-(196–215). In addition, peptides αBGTBP α1-(166–175) and α1-(196–215) did not bind $^{125}$I-αBGT (data not shown). These results indicated that peptides corresponding to residues 181–200 of the αBGTBP α1 and α2 subunits were able to form prototopes that bind $^{125}$I-αBGT.

The specificity of $^{125}$I-αBGT binding to peptides Torpedo α1-(181–200), chick muscle α1-(179–198), chick brain αBGTBP α1-(181–200), and chick brain αBGTBP α2-(181–200) was assessed by blocking the nitrocellulose strips with cytochrome c (10 mg/ml) prior to incubation with $^{125}$I-αBGT (0.5–4 μM). After washing, the strips were dried and mounted, and the ability of a peptide to bind αBGT was determined by autoradiography. The results of typical qualitative experiments is shown in Fig. 2B, where the binding of $^{125}$I-αBGT to the chick muscle α1, and αBGTBP α1 and α2 were used as positive and negative controls, respectively. In addition, we have included two unrelated peptides to control for nonspecific electrostatic effects on αBGT binding, a peptide corresponding to amino acid residues 61–78 of the α subunit of the glycine receptor (Greeningloh et al., 1987), which is negatively charged (pI 2.6), and a positively charged peptide, corresponding to amino acid residues 331–350 of the rat nAChR β2 subunit (pI 12.5). One might predict that αBGT, with a pI of 9.2 (Clark et al., 1972), could bind nonspecifically to negatively charged peptide sequences and/or that a positively charged peptide could mask negatively charged groups, which might contribute to forming an αBGT-binding site on the native Torpedo nAChR. As reported below, however, these effects of nonspecific electrostatic interactions on $^{125}$I-αBGT binding were not detected in this study.

Peptides Corresponding to Chick Brain αBGTBP α1-(181–200) and αBGTBP α2-(181–200) Bind $^{125}$I-αBGT in Toxin Blot Assays—Peptides (0.25 mg/ml) were spotted onto nitrocellulose strips and blocked with cytochrome c (10 mg/ml) prior to incubation with $^{125}$I-αBGT (0.5–4 μM). After washing, the strips were dried and mounted, and the ability of a peptide to bind αBGT was determined by autoradiography. The results of typical qualitative experiments is shown in Fig. 2A (n = 4), where the binding of $^{125}$I-αBGT (2 μM) to the chick muscle α1, and αBGTBP α1 and α2 were used as positive and negative controls, respectively. In addition, we have included two unrelated peptides to control for nonspecific electrostatic effects on αBGT binding, a peptide corresponding to amino acid residues 61–78 of the α subunit of the glycine receptor (Greeningloh et al., 1987), which is negatively charged (pI 2.6), and a positively charged peptide, corresponding to amino acid residues 331–350 of the rat nAChR β2 subunit (pI 12.5). One might predict that αBGT, with a pI of 9.2 (Clark et al., 1972), could bind nonspecifically to negatively charged peptide sequences and/or that a positively charged peptide could mask negatively charged groups, which might contribute to forming an αBGT-binding site on the native Torpedo nAChR. As reported below, however, these effects of nonspecific electrostatic interactions on $^{125}$I-αBGT binding were not detected in this study.

Peptides Corresponding to Chick Brain αBGTBP α1-(181–200) and αBGTBP α2-(181–200) Bind $^{125}$I-αBGT in Toxin Blot Assays—Peptides (0.25 mg/ml) were spotted onto nitrocellulose strips and blocked with cytochrome c (10 mg/ml) prior to incubation with $^{125}$I-αBGT (0.5–4 μM). After washing, the strips were dried and mounted, and the ability of a peptide to bind αBGT was determined by autoradiography. The results of typical qualitative experiments is shown in Fig. 2A (n = 4), where the binding of $^{125}$I-αBGT (2 μM) to the chick muscle α1, and αBGTBP α1 and α2 were compared with control peptides. A strong signal was consistently observed for Torpedo α1-(181–200), chick muscle α1-(179–198), and chick BGTBP α2-(181–200). A weaker but positive signal was also observed for the chick BGTBP α1-(181–200) peptide. Background levels of $^{125}$I-αBGT binding were observed for the negative control peptides, glycine α1-(61–78), rat nAChR β2-(331–350), cow muscle nAChR α1-(181–200), and for other overlapping peptides of the chick brain αBGTBP α1 and α2 subunits, BGTBP α1-(171–189), α1-(185–204), α2-(166–185), and α2-(196–215). In addition, peptides αBGTBP α1-(166–175) and α1-(196–215) did not bind $^{125}$I-αBGT (data not shown). These results indicated that peptides corresponding to residues 181–200 of the αBGTBP α1 and α2 subunits were able to form prototopes that bind $^{125}$I-αBGT.

The specificity of $^{125}$I-αBGT binding to peptides Torpedo α1-(181–200), chick muscle α1-(179–198), chick brain αBGTBP α1-(181–200), and chick brain αBGTBP α2-(181–200) was assessed by blocking the nitrocellulose strips with cytochrome c (10 mg/ml) prior to incubation with $^{125}$I-αBGT (0.5–4 μM). After washing, the strips were dried and mounted, and the ability of a peptide to bind αBGT was determined by autoradiography. The results of typical qualitative experiments is shown in Fig. 2B, where the binding of $^{125}$I-αBGT to the chick muscle α1, and αBGTBP α1 and α2 were compared with control peptides. A strong signal was consistently observed for Torpedo α1-(181–200), chick muscle α1-(179–198), and chick BGTBP α2-(181–200). A weaker but positive signal was also observed for the chick BGTBP α1-(181–200) peptide. Background levels of $^{125}$I-αBGT binding were observed for the negative control peptides, glycine α1-(61–78), rat nAChR β2-(331–350), cow muscle nAChR α1-(181–200), and for other overlapping peptides of the chick brain αBGTBP α1 and α2 subunits, BGTBP α1-(171–189), α1-(185–204), α2-(166–185), and α2-(196–215). In addition, peptides αBGTBP α1-(166–175) and α1-(196–215) did not bind $^{125}$I-αBGT (data not shown). These results indicated that peptides corresponding to residues 181–200 of the αBGTBP α1 and α2 subunits were able to form prototopes that bind $^{125}$I-αBGT.

The concentration dependence of αBGT binding was assessed over a range of 0.1–10 μM $^{125}$I-αBGT as described under “Experimental Procedures” (Table I). The Scatchard analysis

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Apparent $K_a$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torpedo α1-(181–200)</td>
<td>1.9 (0.7)</td>
</tr>
<tr>
<td>Chick muscle α1-(179–198)</td>
<td>0.9 (0.7)</td>
</tr>
<tr>
<td>Chick brain αBGTBP α1-(181–200)</td>
<td>5.3 (0.5)</td>
</tr>
<tr>
<td>Chick brain αBGTBP α2-(181–200)</td>
<td>1.3 (0.5)</td>
</tr>
</tbody>
</table>

$^a$ Standard deviations are given (n = 3 experiments) in parentheses.

Given the solid phase nature of these assays, the $K_a$ values reported here do not represent true dissociation constants.
Bungarotoxin Binds Brain Nicotinic Receptor Subunit Peptides

The concentration dependence of the inhibition of 125I-aBGT binding was compared for peptides Torpedo a1-(181–200), chick muscle a1-(179–198), chick brain aBGTBP a1-(181–200), and aBGTBP a2-(181–200). The results of a typical experiment are shown in Fig. 4, in which the negative slopes of the linear regression lines represent the the IC50 values. The IC50 values determined from different experiments (n = 5) are summarized in Table II. The corresponding Kd values are not given, as such analysis is inappropriate given that the assay is not performed under equilibrium conditions. The IC50 analyses of the relative affinities of peptides for aBGT agree with those results obtained from Scatchard analyses, i.e. the peptides corresponding to sequences Torpedo a1-(181–200), chick muscle a1-(179–198), and chick brain aBGTBP a2-(181–200) are comparable in their potencies (~3–5 μg/ml or ~1–2 μM) as competitors for 125I-aBGT binding with native Torpedo nAChR. These values are similar to those which we obtained previously for the peptides corresponding to the rodent muscle nAChR a1-(182–201) and brain a5-(180–199) sequences (~1 and ~4 μM, respectively). A 10-fold greater concentration of peptide aBGTBP a1-(181–200) is required for equivalent inhibition in the competition assay (~50–100 μg/ml or ~20–40 μM). This level of inhibition is

Fig. 4. Concentration dependence of peptide competition for 125I-aBGT. Torpedo competition assays were performed as described under “Experimental Procedures” and in Fig. 3, except that the concentration of peptide was varied between 5 and 500 μg/ml (~2–200 μM). The assays were performed on different days and contained 20 nM 125I-aBGT (100–150 cpm/fmol) and 2–3 nM membrane-bound Torpedo nAChR. The results are expressed as the mean of triplicate determinations at each concentration of peptide. The “% Displaced” values for peptides, Torpedo a1-(181–200), chick muscle a1-(179–198), chick aBGTBP a1-(181–200), and chick aBGTBP a2-(181–200) are 18,475, 24,652, 27,581, and 24,818 cpm, respectively. The results are presented as a Hofstee plot (Mollhoff et al., 1981), in which the IC50 values can be directly compared as the negative slopes of the linear regression lines. The IC50 values obtained by analysis using the computer analysis program EBDA (McPherson, 1983), and correlation coefficients (in parentheses) from linear regression are: Torpedo a1-(181–200) IC50 0.83 (~0.87), chick muscle a1-(179–198) IC50 1.84 (~0.98), chick aBGTBP a1-(181–200) IC50 1 (0.95), and chick aBGTBP a2-(181–200) IC50 1.20 (~0.96). G. Torpedo a1; x, chick muscle a1; o, chick aBGTBP a1; Δ, chick aBGTBP a2.

Table II

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torpedo a1-(181–200)</td>
<td>1.9 (0.2)</td>
</tr>
<tr>
<td>Chick muscle a1-(179–198)</td>
<td>2.1 (0.7)</td>
</tr>
<tr>
<td>Chick brain aBGTBP a1-(181–200)</td>
<td>37 (10)</td>
</tr>
<tr>
<td>Chick brain aBGTBP a2-(181–200)</td>
<td>1.8 (0.6)</td>
</tr>
</tbody>
</table>

* Standard deviations (n = 5 experiments) are given in parentheses. IC50 values were determined using the computer program EBDA (McPherson, 1983).
similar to the values that we obtained for peptides corresponding to the sequence segments 181–200 of the human and calf muscle \( \alpha_1 \) subunits, which bind \( \alpha BG T \) with relatively low affinity, when compared with peptides corresponding to the Torpedo \( \alpha_1 \) subunit sequence (Wilson and Lentz, 1988; Ohana and Gershoni, 1990; McLane et al., 1991).

**Scanning of the Complete Chick Brain \( \alpha BG T BP \ \alpha_1 \) Sequence for Other \( \alpha BG T \) Binding Sequence Segments**—In order to determine if other sequence segments of the chick brain \( \alpha BG T BP \ \alpha_1 \) subunit are involved in forming the \( \alpha BG T \) binding site, a panel of 38 overlapping peptides were synthesized corresponding to the complete subunit, as shown in Fig. 1. These peptides were tested using toxin blot assays and Torpedo nAChR competition assays for their ability to bind \( ^{125}\text{I}-\alpha BG T \). The results obtained in one of several experiments \((n = 4)\) are shown in Fig. 5. Only peptide \( \alpha BG T BP \ \alpha_1-(181–200) \), at a concentration of 200 \( \mu g/ml \), efficiently inhibited the binding of \( ^{125}\text{I}-\alpha BG T \) to Torpedo nAChR (78–88% inhibition of total \( ^{125}\text{I}-\alpha BG T \) binding), i.e. levels of inhibition comparable to those found for inhibition by pretreatment of Torpedo nAChR with 20 nM unlabeled \( \alpha BG T \). Therefore, peptide \( \alpha BG T BP \ \alpha_1-(181–200) \) represents a principal prototipe forming the \( \alpha BG T \) binding site on the chick brain \( \alpha BG T BP \ \alpha_1 \) subunit.

**Effect of Modification of Cysteine/Cystine Residues on \( \alpha BG T \) Binding**—In order to assess the importance for \( \alpha BG T \) binding of the redox state of cysteine residues at positions 190/191 of the \( \alpha BG T BP \ \alpha_2 \) subunits, we modified peptides Torpedo \( \alpha_1-(181–200) \), cobra muscle \( \alpha_1-(181–200) \), chick muscle \( \alpha_1-(179–198) \), and \( \alpha_2-(181–200) \) with sulfhydryl reagents. The peptides were treated in the following ways: 1) oxidation with iodosobenzoic acid and alkylation of free sulfhydryl groups available in untreated peptide (IAA). The latter treatment was used to determine the proportion of peptide in the reduced and oxidized state under normal assay conditions. The products of the reaction were assessed by amino acid composition analysis following acid hydrolysis and derivatization with phenylisothiocyanate, as described under “Experimental Procedures.”

The carbamide derivative of cysteine, the product of IAA treatment, is converted to CM-Cys by acid hydrolysis prior to derivatization, and the yield of CM-Cys for peptides alkylated with or without prior reduction was used to estimate the proportion of peptide in reduced and oxidized states. The estimated percentage of peptide molecules with free sulfhydryl groups was estimated to be 76% for chick muscle \( \alpha_1-(179–198) \) and 45% for chick \( \alpha BG T BP \ \alpha_1-(181–200) \). These results are typical of peptides homologous to Torpedo \( \alpha_1-(181–200) \) from the muscle nAChR \( \alpha_1 \) subunits of different species, which we have found to be 40–80% reduced (McLane et al., 1991). In contrast, the peptide chick \( \alpha BG T BP \ \alpha_2-(181–200) \) appeared to be >90% in the oxidized form, as evidenced by the lack of a CM-Cys peak after IAA treatment without prior reduction, as shown in Fig. 6.

In order to determine if the oxidized forms of the peptides were in the form of monomers or dimers, samples of peptides were analyzed by gel permeation chromatography on a Bio-
Red P6 column. The chromatograms for the untreated peptides are shown in Fig. 7A. The principle peaks for chick muscle \(\alpha_1-(179-198)\), chick \(\alpha\)BGTBP \(\alpha_1-(181-200)\), and \(\alpha\)BGTBP \(\alpha_2-(181-200)\) chromatographed as peptides of molecular masses 2.2 \(\pm\) 0.2 kDa, 2.7 \(\pm\) 0.2 kDa, and 2.7 \(\pm\) 0.2 kDa, respectively. A small peak at \(\approx 5.0\) kDa, corresponding to a dimer, was observed for both chick \(\alpha\)BGTBP peptides and accounted for \(\approx 10-20\%\) of the absorbance at 280 nm. Similar results were obtained with the peptides which had been oxidized, reduced and/or alkylated, and with peptides corresponding to \(\text{Torpedo}\) \(\alpha_1-(181-200)\) and \(\text{cobra muscle}\) \(\alpha_1-(181-200)\) (data not shown). Therefore, the predominant form of the peptides under the conditions of the assays used in this study is the monomer.

Peptides modified by reduction, oxidation, and/or alkylation were tested for their ability to inhibit \(^{125}\text{I}\)-aBGT binding to \(\text{Torpedo nAcHR}\) using the competition assay described in Fig. 3. The results of a typical experiment \((n = 3)\) are shown in Fig. 7B and are compared with control peptides \(\text{Torpedo}\) \(\alpha_1-(181-200)\) and \(\text{cobra muscle}\) \(\alpha_1-(181-200)\). The cobra peptide does not compete for \(^{125}\text{I}\)-aBGT in untreated form or when reduced, oxidized, and/or alkylated. The \(\text{Torpedo}\) \(\alpha_1-(181-200)\) peptide, which is \(\approx 70\%\) reduced under assay conditions, shows reduced ability to bind aBGT when alkylated with or without prior reduction, whereas oxidation either slightly improves aBGT binding or has no effect (Conti-Tronconi et al., 1990; McLane et al. 1991). The results for different treatments of the peptides chick muscle \(\alpha_1-(179-198)\) and \(\text{chick\ muscle}\) \(\alpha_1-(181-200)\) show the same pattern of inhibition as the \(\text{Torpedo}\) peptide. The chick \(\alpha\)BGTBP \(\alpha_2-(181-200)\) peptide has a similar pattern of inhibition for oxidation and reduction/alkylation conditions, but alkylaton with IAA without prior reduction does not alter the ability of the peptide to inhibit \(^{125}\text{I}\)-aBGT binding to \(\text{Torpedo nAcHR}.\)

These results are consistent with the HPLC analysis, which indicate that the peptide is \(\geq 90\%\) oxidized and that few free sulfhydryl residues are available in the untreated peptide for alkylation.

**DISCUSSION**

The results of these studies demonstrate that peptides corresponding to sequence segments of the \(\alpha\)BGTBP \(\alpha_1\) and \(\alpha_2\) subunits are able to bind aBGT. The relative affinities of peptides \(\alpha\)BGTBP \(\alpha_1-(181-200)\) and \(\alpha\)BGTBP \(\alpha_2-(181-200)\) were compared by IC\(_{50}\) analysis (Fig. 4, Table II). The IC\(_{50}\), determined using a competition assay, also indicated that \(\alpha\)BGTBP \(\alpha_1-(181-200)\) bound aBGT with relatively low affinity (IC\(_{50}\) \(\approx 40\mu\text{M}\)), whereas the peptide \(\alpha\)BGTBP \(\alpha_2-(181-200)\) was able to compete for aBGT as efficiently as \(\text{Torpedo}\) \(\alpha_1-(181-200)\) and \(\text{chick muscle}\) \(\alpha_1-(179-198),\) IC\(_{50}\) of \(\approx 1-2\mu\text{M}\.\) The functional subunit aggregate formed by expression of the \(\alpha\)BGTBP \(\alpha_1-(\text{a7})\) subunit in \(\text{Xenopus}\) oocytes is blocked by aBGT with an IC\(_{50}\) of 0.7 nM (Couturier et al., 1990), indicating a much higher affinity for aBGT than that found for peptide \(\alpha\)BGTBP \(\alpha_1-(181-200)\). The relative differences in affinities reported previously for aBGT binding to synthetic or biosynthetic peptides, when compared with native muscle and \(\text{Torpedo nAcHRs},\) are of the same order of magnitude (Wilson et al., 1985, 1988; Neumann et al., 1986; Gotti et al., 1987; Griesmann et al., 1990; Aronheim et al., 1988; Ohana and Gershoni, 1990; Conti-Tronconi et al., 1990). aBGT binds to synthetic peptides with an affinity that compares with that of the isolated and denatured \(\text{Torpedo}\) \(\alpha_1\) subunits \((K_d \approx 10^{-7}\text{ M})\) (Hagerty and Froehner, 1981; Gershoni et al., 1983), but with much lower affinity compared with the native \(\text{Torpedo nAcHR} \langle 10^{-9}\text{ M}\rangle\), where other sequence segments of the \(\alpha, \gamma, \) and \(\delta\) subunits may contribute to the formation of the ligand binding site (Kurosaki et al., 1987; Sumikawa and Miledi, 1989; Blount and Merlie, 1989; Federson and Cohen, 1990; Conroy et al., 1990; Conti-Tronconi et al., 1990; Suedi et al., 1991).

The adjacent cysteine residues at positions 190/191 of the \(\alpha\)BGTBP \(\alpha_1\) and \(\alpha_2\) subunits are a hallmark of the nAcHR \(\alpha\) subunits and are believed to form a vicinal disulfide bond in the native \(\text{Torpedo}\) \(\alpha_1\) subunit (Kao et al., 1984; Kao and Karlin, 1986; Moscovitz and Gershoni, 1988; Kellaris et al., 1989). The effects of cysteine/cystine modification of the peptides \(\alpha\)BGTBP \(\alpha_1-(181-200)\) and \(\alpha\)BGTBP \(\alpha_2-(181-200)\) on aBGT binding were tested using the competition assay (Fig. 7B), and these experiments indicated that oxidation of the peptides had little effect on aBGT binding, whereas alkylation of free sulfhydryl groups drastically reduced the ability of the peptides to compete for aBGT with native \(\text{Torpedo nAcHR}.\) We have observed previously that reduction and
alkylation of peptides corresponding to the Torpedo a1 subunit sequence a1-(172–205) (Rait et al., 1987), and the Torpedo and muscle nAChR a1 subunit sequences a1-(181–200) of different species (Conti-Tronconi et al., 1990; McLane et al., 1991) reduced their aBGT binding activity. The convergence of these results indicates that although oxidation of Cys-190/191 to form a vicinal disulfide bond may not be critical for aBGT binding, one or both cysteines are involved in forming the interface of this sequence segment with aBGT, as reflected in the reduced ability of the aBGT to bind to the peptides modified by cysteine alkylation.

Recent studies have demonstrated that aBGT competes for affinity labeling of Tyr-93, as well as Tyr-190, Cys-192, and Cys-193 of the Torpedo nAChR a1 subunit (Galzi et al., 1990). In previous studies, we have also found that in addition to the sequence segment containing the vicinal cysteine pair at positions 192/193 relative to the Torpedo a1 subunit sequence, and spanning residues 181–200, other more N-terminal sequence segments contribute to forming the aBGT binding site on the Torpedo nAChR a1 subunit (Conti-Tronconi et al., 1990) and the rat neuronal a5 subunit (McLane et al., 1990b). The corresponding sequences of the aBGTTP a1 subunit, however, share only 20–35% homology with these other aBGT binding sequences on the Torpedo a1 and a5 subunits. In the present study, overlapping synthetic peptides scanning the complete aBGTTP a1 subunit were tested for their ability to bind 125I-aBGT directly and to compete for aBGT with native Torpedo nAChR, but we were only able to detect aBGT binding to the sequence segment aBGTTP a1-(181–200). Therefore, if other sequence segments contribute to the formation of a high affinity aBGT binding site on the aBGTTP a1 subunit, they are either incapable of forming a prototope or have not been properly represented by our panel of synthetic peptides. Given the high degree of homology of the putative extracellular segments of the aBGTTP a1 and a2 subunits, it is likely that a panel of peptides corresponding to the aBGTTP a2 subunit would yield similar results.

The highly divergent nature of the avian brain aBGTTP a subunit sequences at positions 181–200 offers a unique opportunity to determine the amino acid residues that are most critical to the interaction with aBGT and can best be appreciated by comparing homologous sequences of a subunits from nAChRs that bind aBGT with those that do not (as shown in Fig. 8). Inspection of these sequences reveals that seven amino acids are characteristic of all a subunits regardless of aBGT binding activity and therefore cannot uniquely confer the ability to bind aBGT: i.e. Gly-183 (or the conservative substitution Ala in the Drosophila neuronal a subunits), Tyr-190, Cys-192, Cys-193, Asp-195 (or the conservative substitution of Gln in a subunits of neuronal origin), Tyr-198, and Asp-200. All a subunits that bind aBGT have Tyr-189 (or the conservative substitution Phe) and Pro-197, whereas Lys-189 and Ile-197 (with the exception of the cobra a1 subunit) are characteristic of all subunits that do not bind aBGT. Therefore, comparison of naturally occurring a sequences suggests that Tyr-189 and, perhaps Pro-197, may be critical for conferring the ability to bind aBGT. Single amino acid residue substitutions by Gly or Ala of the Torpedo a1-(181–200) peptide indicates that both Tyr-189 and Tyr-190 are critical for aBGT binding, whereas Pro-197 is not (Conti-Tronconi et al., 1991). The importance of Tyr-189 to aBGT binding is also indicated by natural substitutions, such as the replacements by Phe-189 in the calf and mouse a1 sequences and Thr-189 in the human a1 sequence, which reduce the affinity for aBGT (Wilson and Lentz, 1988; Ohana and Gershoni, 1990; McLane et al., 1991). Of the five amino acids that differ between the aBGTTP a1 and a2 subunit between residues 181–200, the replacement of Ser/Pro at positions 188/189 of the a1 subunit by Leu/Tyr in the a2 sequence may account for the observed decrease in affinity of the peptide corresponding aBGTTP a1-(181–200). The binding surface of aBGT is believed to involve primarily hydrophobic and hydrogen bonding residues and a few charged amino acids, including Arg and/or Lys side chains (Low, 1979; Karlsson, 1979; Duffton and Hider, 1983; Love and Stroud, 1986; Kosen et al., 1989; chick aBGTTP a1 and a2, Schoepfer et al., 1990; Drosophila ALS, Bossy et al., 1988; and Drosophila ARs, Sawruk et al., 1990; and cobra muscle a1, Neumann et al., 1989).

FIG. 8. Comparison of sequences from nAChRs that bind aBGT with those that do not. Homologous sequences from a subunits are aligned according to the Torpedo nAChR a1 sequence numbering. Amino acids present in all a sequences or at positions where there are only occasional conservative substitutions are indicated by a black background. Similarly, amino acids characteristic only of those sequences that bind aBGT are indicated by a dotted background, whereas those characteristic of only those sequences that cannot bind aBGT are indicated by a hatched background. The sequences of the sources shown are: Torpedo a1, Noda et al., 1982; chick a1, a2, a3, and a4, Nef et al., 1988; rat a2, Wada et al., 1988; rat a3, Boulter et al., 1986; rat a4, Goldman et al., 1987; chick aBGTTP a1 and a2, Schoepfer et al., 1990; Drosophila ALS, Bossy et al., 1988; and Drosophila ARs, Sawruk et al., 1990; and cobra muscle a1, Neumann et al., 1989.
for the ability of αBGT to accommodate small structural differences between nACHRs of different species, as well as highly divergent proteins such as the αBGTBP α1 and α2 subunits. Finally, it has been demonstrated that a single amino acid substitution that destroys the function of a protein molecule, can be compensated by simultaneous nonconservative substitutions of other amino acid residues (Reines et al., 1986; Blacklow and Knowles, 1990). Thus, the few amino acids that are conserved between other αBGT binding α subunits and the αBGTBP α1 and α2 sequences may only adumbrate common structural features.

The αBGTBP α1 subunit was cloned using an oligonucleotide probe based on the N-terminal sequence of a protein isolated by αBGT affinity chromatography (Conti-Tronconi et al., 1985), whereas αBGTBP α2 was isolated on the basis of its homology to αBGTBP α1 (Schoepfer et al., 1990). Monoclonal antibodies against fusion proteins containing unique sequences of the αBGTBP α1 and α2 subunits immunoprecipitate native αBGT binding proteins from the chick brain, indicating that their gene products contribute to the formation of receptor complexes, presumably nACHRs, that bind αBGT. The results reported here indicate that synthetic peptides corresponding to sequence segments of the αBGTBP α1 and α2 subunits, which are homologous to a cholinergic binding site on muscle and neuronal nACHRs, form protomolecules able to bind αBGT. Although it has long been known that αBGTBPs have nicotinic pharmacological properties, and we have now shown that two αBGTBP subunits bind αBGT, the endogenous ligand and functional role of αBGTBPs in the brain remain to be determined.

REFERENCES
