Identification of regions involved in the binding of α-bungarotoxin to the human α7 neuronal nicotinic acetylcholine receptor using synthetic peptides

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The neuronal α7 nicotinic acetylcholine receptor (AChR) binds the neurotoxin α-bungarotoxin (α-Bgt). Fine mapping of the α-Bgt-binding site on the human α7 AChR was performed using synthetic peptides covering the entire extracellular domain of the human α7 subunit (residues 1–206). Screening of these peptides for ^{125}I-α-Bgt binding resulted in the identification of at least two toxin-binding sites, one at residues 186–197, which exhibited the best ^{125}I-α-Bgt binding, and one at residues 159–165, with weak toxin-binding capacity; these correspond, respectively, to loops C and IV of the agonist-binding site. Toxin binding to the α7(186–197) peptide was almost completely inhibited by unlabelled α-Bgt or *d*-tubocurarine. Alanine substitutions within the sequence 186–198 revealed a predominant contribution of aromatic and negatively charged residues to the binding site. This sequence is homologous to the α-Bgt binding site of the α1 subunit (residues 188–200 in *Torpedo* AChR). In competition experiments, the soluble peptides α7(186–197) and *Torpedo* α1(184–200) inhibited the binding of ^{125}I-α-Bgt to the immobilized α7(186–197) peptide, to native *Torpedo* AChR, and to the extracellular domain of the human α1 subunit. These results suggest that the toxin-binding sites of the neuronal α7 and muscle-type AChRs bind to identical or overlapping sites on the α-Bgt molecule. In support of this, when synthetic α-Bgt peptides were tested for binding to the recombinant extracellular domains of the human α7 and α1 subunits, and to native *Torpedo* and α7 AChR, the results indicated that α-Bgt interacts with both neuronal and muscle-type AChRs through its central loop II and C-terminal tail.

Key words: ligand-binding site, multipin peptide synthesis, muscle-type AChR, α-neurotoxin,

INTRODUCTION

Nicotinic acetylcholine receptors (AChRs) are members of the superfamily of ligand-gated ion channels and are found in the central and peripheral nervous system, in vertebrate skeletal muscles, and in the electric organ of electric fish such as *Torpedo*. AChRs form pentameric complexes from different types of subunits, α1–α10, which are responsible for ligand recognition, and β1–β4, γ, δ and ε known as structural subunits [1–3]. Muscle-type AChRs have the subunit composition $(\alpha1)_2\beta1\gamma(\varepsilon)\delta$, while neuronal AChRs form either homomers of five identical α7, α8 or α9 subunits, or heteropentamers formed by various combinations of α and β subunits. The most prevalent subtypes in the central nervous system are α7 and α4β2 (or α4β2α5), while in peripheral ganglia, α7 and α3β4 (or α3β4α5) are more prevalent [3,4].

The α subunit contains major determinants for the binding of agonists, such as the neurotransmitter acetylcholine (ACh), and antagonists, including *d*-tubocurarine and α-neurotoxins. However, site-directed mutagenesis and affinity-labelling studies of the AChR have shown that the ACh binding site must reside at the interface between two adjacent subunits, suggesting that residues from both subunits contribute to the ligand-binding site [5]. In the muscle-type AChR there are two non-equivalent binding sites, located at the interface between the α1 and δ or γ subunits, whereas the five binding sites of the α7 neuronal AChR are situated at the interfaces between the five identical α7 subunits. The ligand-binding site is thought to be formed by the principal component, or (+) face, made up of three segments or loops (A, B, and C) of the α subunit, and the complementary component, or (−) face, formed by three, or possibly four, non-α loops (referred to as D, E and F or I, II, III and IV) [6–9]. X-ray crystallographic analysis of molluscan acetylcholine-binding protein (AChBP), a molecule from snail glial cells that resembles the N-terminal domain of the AChR α subunit, confirmed the existence of loops A, B, C, D, E and F, and suggested that they contribute to the formation of the binding pocket [10]. In the neuronal α7 AChR, both the principal and complementary components of the agonist binding site are present in each α7 subunit. Site-directed mutagenesis experiments revealed the significance of aromatic residues, as well as other residues, in each loop involved in the agonist binding [11,12]. Thus, according to the human α7 numbering, loop A consists of residues Trp-86–Tyr-93, loop B of residues Trp-149–Gly-152, and loop C of residues Arg-186–Asp-197 (containing the conserved residues Tyr-188, Tyr-195 and Cys-190/191). The complementary component includes Ser-34 (loop I), Trp-55, or the entire sequence 55–77 (loop II), Asn-111—Pro-121 (loop III), and Asp-157–Ile-165 (loop IV), which includes Asp-164, a key residue in agonist binding [7,13,14].

α-Neurotoxins compete with typical AChR agonists and antagonists (nicotine, carbamylcholine and *d*-tubocurarine) for binding to the receptor, and their binding is considered to be restricted to α subunits. α-Bungarotoxin (α-Bgt) is a snake-venom-derived long α-neurotoxin containing 74 amino acids which has been extensively studied [15]. Its X-ray crystallographic structure has been solved [16,17] and additional structural information has been obtained in NMR studies [18,19]. It has the characteristic structure of α-neurotoxins, containing three large main loops, five disulphide bonds and a C-terminal

Abbreviations used: ACh, acetylcholine; AChR, nicotinic acetylcholine receptor; AChBP; acetylcholine binding protein, α-Bgt, α-bungarotoxin; Fmoc; 9-fluorenylmethoxycarbonyl group; K_d, dissociation constant; Y188A etc., indicates substitution of Tyr-188 with Ala, etc.

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tail. Its fifth disulphide bond, formed by Cys-29 and Cys-33, is located near the tip of loop II.

The contact regions involved in the interaction between α-Bgt and α subunits have been studied by several research groups [20–22]. Synthetic peptides were used to localize the α-Bgt binding site on the α1 subunit of the *Torpedo* AChR [21–26]. Early studies had shown that the major determinants for α-Bgt binding lay between residues 173–204 of the α1 subunit [23]. A more systematic study of the *Torpedo* α-Bgt binding site, using synthetic peptides, showed that the minimum binding segment of the *Torpedo* AChR was the heptapeptide α189–195 [22]. However, synthetic human α1 peptides had significantly lower affinities for α-Bgt [24,25].

α-Bgt binding to the neuronal α7 subunit has been less well studied. It was previously shown that a bacterial fusion protein, corresponding to residues 124–239 of α7, was able to bind ^{125}I-α-Bgt [27] and that peptide α181–200 of a chick-brain α-Bgt binding protein, homologous to the chick α7 subunit, demonstrated significant α-Bgt binding activity [28].

α-Bgt-derived peptides have been synthesized in order to map the binding sites for *Torpedo* AChR on the α-Bgt molecule [29,30]. One study [30] showed that a loop II peptide corresponding to residues 27–42 of α-Bgt was able to inhibit the binding of α-Bgt to AChR, while another study [29] showed that α-Bgt has three main AChR-binding regions, one in each of the three loops, represented by peptides 1–16, 26–41, and 45–59. Additionally, the NMR solution-structure of a complex between α-Bgt and the *Torpedo* peptide α185–196 showed that 15 residues of α-Bgt within loops I and II (Thr-6–Thr-8, Pro-10, Ile-11, Trp-28–Phe-32, Gly-37–Glu-41) and His-68 in the C-terminal tail are present in the contact zone between the *Torpedo* peptide and α-Bgt [31].

The aim of our study was to identify the regions involved in the binding of α-Bgt to the human α7 subunit, using a systematic solid-phase peptide synthesis system. The α-Bgt binding site within the human α7 subunit was precisely determined and compared with the respective binding site on the α1 subunit. Thus, more than 70 different peptides covering the entire extracellular domain of the human α7 subunit (residues 1–206) and, in some cases, carrying alanine substitutions were synthesized and tested for their ability to bind ^{125}I-α-Bgt in solid-phase assays. This led to the identification of at least two α-Bgt binding subsites on the α7 subunit, and of the α7 residues involved in this interaction. Moreover, the binding of other nicotinic ligands to the α7 peptides was examined by inhibition of ^{125}I-α-Bgt binding. A comparative study of the α-Bgt binding sites on the α1 and α7 subunits was performed by inhibition assays in which soluble α7 and α1 peptides were tested for their ability to inhibit ^{125}I-α-Bgt binding to immobilized α7 peptides, native *Torpedo* AChR, a recombinant fragment of the human α1 subunit, or peptide analogues intermediate between the human α7 and *Torpedo* α1 sequences. Finally, in order to define the binding region on the α-Bgt molecule for each AChR type, we tested the ability of immobilized synthetic α-Bgt peptides to bind to the recombinant extracellular domains of the human α7 and α1 subunits, or to *Torpedo* AChR, and the ability of soluble α-Bgt peptides to bind to neuronal α7 and *Torpedo* AChR.

EXPERIMENTAL

Peptide synthesis

Peptide synthesis was based on the method originally described by Geysen et al. [32]. Briefly, each peptide was synthesized on a polyethylene tip which fits on to a rod activated by radiation grafting. The rods were assembled in a polyethylene holder, with the format and spacing of a microtitre plate, and the peptides were synthesized simultaneously in 96-well microtitre plates. Polyethylene rods, 9-fluorenylmethoxycarbonyl group (Fmoc)-protected amino acids, and all materials needed were provided by Chiron Technologies (Clayton, Victoria, Australia). Solid-phase peptide synthesis consisted of repetitive cycles of Fmoc-deprotection, washing and amino acid coupling. After completion of the final coupling reaction and Fmoc deprotection, the terminal amino group was acetylated and all side-chain protecting groups removed. At least two rods for each peptide were produced in independent synthesis cycles, some peptides being synthesized in multiple copies. In order to measure non-specific binding, peptides containing a reversed α7 sequence, i.e. α7(198–182), α7(198–186) and α7(155–139), were also synthesized.

Selected peptides were also produced in a soluble form. In this case, polyethylene rods with a diketopiperazine linker (supplied by Chiron Technologies) were used. The peptides were synthesized as described above, then, after completion of synthesis, they were cleaved from the polyethylene tip by overnight treatment at 22 °C with 40 % (v/v) acetonitrile in 0.1 M phosphate buffer, pH 7.6 (0.1 M Na_2HPO_4 adjusted to pH 7.6 with 0.1 M NaH_2PO_4). Each of the peptide concentrations was calculated separately based on the absorbance at 280 nm and its aromatic amino acid content. Peptide solubility was tested by comparing the absorbance of the peptides at 280 nm before and after 1 h centrifugation at 50 000 ***g***.

Recombinant α1 and α7 polypeptides

The N-terminal extracellular domain of the α7 subunit of the human neuronal AChR (amino acids 1–208) has been cloned in PET-15b and expressed as inclusion bodies in *Escherichia coli* by Dr Leslie Jacobson in our laboratory (now at Department of Neurology and Neuroscience, Johns Hopkins School of Medicine, Baltimore, MD, U.S.A.). The protein, solubilized in sacrosyl buffer, is capable of specifically binding ^{125}I-α-Bgt (L. Jacobson and S. J. Tzartos, unpublished work). The K_d of the α7 polypeptide to α-Bgt was estimated by Scatchard analysis and was found to be $(3.2 \pm 1.7) \times 10^{-7}$ M, i.e. an affinity two orders of magnitude lower than that of the native α7 AChR but an order of magnitude higher than that of the rat α7 extracellular domain, expressed in *E. coli* as a soluble fusion protein (K_d of 2.5 μM) [33]. The recombinant polypeptide was ^{125}I-labelled by the chloramine-T method [33a] and iodinated fractions were tested with an anti-α7 rabbit polyclonal antibody (produced by immunization of rabbits with the α7 synthetic peptide 176–212) in radioimmunoprecipitation assay. Then the ^{125}I-α7(1–208) polypeptide was tested for binding to immobilized synthetic α-Bgt peptides.

The N-terminal extracellular domain (amino acids 1–210) of the α1 subunit of the human AChR, expressed in the yeast *Pichia pastoris*, is water soluble and has a native-like conformation as reported by Psaridi-Linardaki et al. [34]. The human α1(1–210) was ^{125}I-labelled by the chloramine-T method and the ^{125}I-α1(1–210) was tested for binding to immobilized α-Bgt peptides.

Solid-phase radioassays

Immobilized α7 peptides were tested for binding to ^{125}I-α-Bgt using the protocol described by Tzartos and Remoundos [22]. The rod-bound peptides were blocked by incubation for 1 h at 22 °C in microtitre plates containing 200 μl of PN buffer (10 mM phosphate buffer, pH 7.2, 60 mM NaCl), and 2 % BSA per well. They were then washed for 10 min with PN buffer and incubated

for 3 h at 22 °C with 150 μl of PN buffer containing 0.5 % BSA, (1–2) × 10^{-10} M ^{125}I-α-βgt (15000–26000 c.p.m.) and unlabelled α-Bgt (final concentration of 1 × 10^{-7} M).

In the case of synthetic α-Bgt peptides, 30 000 c.p.m. of ^{125}I-α7 recombinant polypeptide (residues 1–208), ^{125}I-α1 recombinant polypeptide (residues 1–210), or solubilized membrane-vesicle-bound *Torpedo* AChR [35] partially labelled with ^{125}I-α-Bgt, in PN buffer containing 0.5 % BSA, was incubated overnight at 4 °C with rod-immobilized α-Bgt peptides. Following incubation, the rods were washed with PN buffer with agitation, for 4 × 10 min, then the peptide-bearing tips were detached and radioactivity was measured in a γ-counter. The labelled material was then removed from the rods by sonication for 15–20 min in a 60 °C water bath with 0.1 M phosphate buffer, pH 7.2, containing 1 % SDS and 0.1 % 2-mercaptoethanol. The rods were washed twice with water at 60 °C, once with methanol, then air-dried, and the residual radioactivity on the rods was measured and subtracted from the total measured radioactivity in the next assay.

Competitive radioassays

Multiple copies of the same immobilized peptide were used in the competition assays.

The unlabelled ligands, α-Bgt and *d*-tubocurarine (10^{-7}–10^{-3} M) as well as nicotine, carbamylcholine and methyllycaconitine (1 × 10^{-3}–1.5 × 10^{-2} M), were tested for their ability to inhibit α-Bgt binding by mixing them with 1 × 10^{-10} M ^{125}I-α-Bgt/1 × 10^{-7} M unlabelled α-Bgt, in PN buffer containing 0.5 % BSA, and adding the mixture to the rod-anchored peptides. In some assays the unlabelled ligands were pre-incubated for 1 h with the rod-anchored peptides before the α-Bgt mixture was added.

Competition assays in solution were also performed. In one series of experiments, 150 μl of soluble α7 or α1 synthetic peptides (0.01–2 mg/ml) and 1 × 10^{-10} M ^{125}I-α-Bgt (25 000 c.p.m.)/1 × 10^{-7} M unlabelled α-Bgt in PN buffer with 0.5 % BSA (or 0.5 % gelatin) were pre-incubated for 1–16 h at 4 °C. In another series of experiments, a mixture of soluble α-Bgt peptides (0.08–1.6 mg/ml) and 1 × 10^{-10} M ^{125}I-α-Bgt/1 × 10^{-7} M unlabelled α-Bgt was prepared. In both series of experiments, the mixture was then added to the rod-immobilized peptides and incubated for 2–3 h at 22 °C. In negative controls, equal volumes of the soluble peptide dilution buffer [40 % (v/v) acetonitrile in phosphate buffer, pH 7.6] were used instead of soluble peptide.

The soluble α7 and α1 synthetic peptides were also tested as competitive inhibitors of the binding of ^{125}I-α-Bgt to native *Torpedo* AChR and to the recombinant human α1 subunit extracellular domain. Peptides (0.01–0.03 mg) were incubated overnight at 4 °C with 2 pmol of ^{125}I-α-Bgt in a final volume of 100 μl of PBS/2 % BSA. The non-toxin-binding peptide α7(65–81) or a reverse α7 sequence peptide were used as negative controls whereas, in positive controls, 1 μg of unlabelled α-Bgt was added to the membrane-vesicle-bound *Torpedo* AChR, 10 min before the addition of ^{125}I-α-Bgt. One pmol of membrane vesicle- bound *Torpedo* AChR was then added to all the above mixtures and incubation continued for 2–3 h at 4 °C. The samples were then centrifuged at 50 000 ***g*** for 45 min at 4 °C, the pellets were washed with PBS and recentrifuged, and the final pellets of membrane-bound *Torpedo* AChR with bound ^{125}I-α-Bgt were counted for radioactivity in a γ-counter.

Soluble α-Bgt peptides (0.01–0.1 mg) were also tested for their ability to block the binding of labelled α-Bgt to *Torpedo* AChR, by incubating them for 2 h at 4 °C with 100 000 c.p.m. of ^{125}I-α-Bgt and 0.25 pmol of membrane-bound *Torpedo* AChR in a final volume of 120 μl of PBS/0.2 % BSA, and processing the samples as above, using the same negative and positive controls.

Competitive filter assays

The ability of soluble α7 peptides to inhibit ^{125}I-α-Bgt-binding to detergent-solubilized *Torpedo* AChR, or to the recombinant extracellular domain of the human α1 subunit, was tested in two competitive filter assays. In the first assay, the soluble α7 and α1 peptides (0.5–18 μg) were pre-incubated with ^{125}I-α-Bgt (50 000 c.p.m.) overnight at 4 °C. Then, 0.42 pmol of solubilized *Torpedo* AChR (derived from membrane *Torpedo* AChR after treatment with PBS/0.5 % Triton X-100) was added at a final volume of 50 μl PBS, 0.2 % BSA per reaction tube and incubated for 2 h at 4 °C. In the second assay, the soluble α7 peptides or the negative control reverse α7(155–139) peptide (0.04–30 μg) were incubated for 2.5 h at 4 °C with ^{125}I-α-Bgt (50 000 c.p.m.) and then soluble human α1(1–210) polypeptide (20–50 ng) was added at a final volume of 50 μl PBS/0.2 % BSA per reaction tube, and incubated for 2 h at 4 °C. In both assays, after incubation, samples were diluted with 1 ml of 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 % Triton X-100, and immediately filtered through two Whatman DE81 filters (Whatman Biosystems Ltd., Maidstone, Kent, U.K.) prewashed with the above buffer. The filters were then washed twice with 1 ml of the same buffer, dried, and the bound radioactivity measured on a γ-counter. The negative control contained either negative peptides or the same volume of the peptide-cleavage buffer.

The soluble α-Bgt peptides were also tested for their ability to block the binding of ^{125}I-α-Bgt to solubilized α7 AChR prepared from IMR32 cells, by pre-incubating 6.5–19 μg of peptides with 50 fmol of solubilized α7 AChR, for 1 h at 4 °C. ^{125}I-α-Bgt [250 fmol (200 000 c.p.m.)] was then added and the solution was incubated for 1 h at 4 °C in a final volume of 50 μl of PBS/0.2 % BSA. After incubation, samples were filtered through two Whatman DE81 filters, as described above.

RESULTS

Identification of the α-Bgt binding region within the extracellular domain of the human α7 subunit

Initially seventeen overlapping synthetic 17-mer peptides, covering the entire extracellular domain (residues 1–206) of the human α7 subunit, were tested for their ability to bind ^{125}I-α-Bgt by incubating the rod-bound peptides with (1–2) × 10^{-10} M ^{125}I-α-Bgt mixed with 1 × 10^{-7} M unlabelled α-Bgt. It was revealed that only peptide 182–198 exhibited a high binding capacity, while peptides 149–165, 159–175 and 188–204 had a lower binding capacity (Figure 1A). In addition, peptides 16–32, 35–51, 95–111 and 169–185 showed a very weak binding to ^{125}I-α-Bgt, close to background levels.

In order to precisely determine the α-Bgt-binding site close to region 182–198, a more detailed analysis of region 174–206 was performed by synthesizing 17-mer peptides overlapping by fifteen residues. Peptide 154–170, partially overlapping with peptides 149–165 and 159–175, was also synthesized. Figure 1(B) shows the ^{125}I-α-Bgt-binding pattern of these peptides. Peptides 184–200 and 186–202 clearly showed a much higher α-Bgt binding capacity than peptide 182–198, whereas peptide 154–170 demonstrated weak toxin binding, similar to that of peptides 149–165 and 159–175. Therefore, human α7 subunit contains at least two toxin-binding subsites, located between residues 186–200, with high binding ability, and between residues 159–165, with low binding ability.

Simultaneously, synthesis of shorter peptides with lengths varying from 15 to 8 residues, by sequential removal of pairs of amino acids from either the N- or C-terminal end of peptide

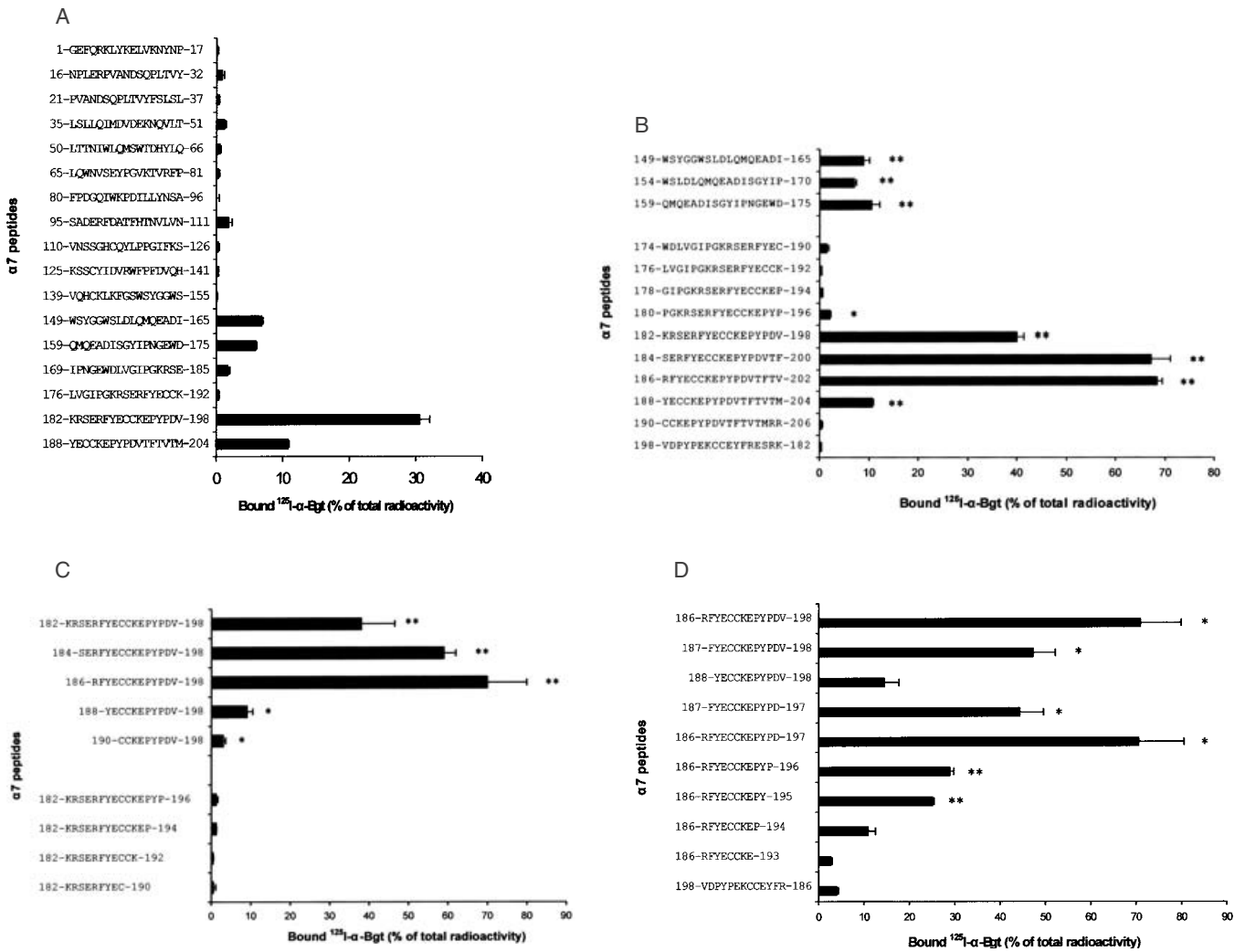

Figure 1 Mapping the α-Bgt binding site on the extracellular domain of the human α7 subunit using continuously overlapping synthetic peptides attached to polyethylene rods

All values are expressed as the percentage of the total ^{125}I-α-Bgt added that bound (% c.p.m. bound/total c.p.m.). Rods were incubated with 14 000–15 000 c.p.m. of ^{125}I-α-Bgt (1×10^{-10} M). Each bar corresponds to the mean ± S.D. for two or more independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the negative control or the low-activity peptides. The values for the reverse sequence negative controls, α7(198–182) and α7(198–186), were approx. 0.5. (**A**) Binding to overlapping 17-mer peptides spanning the entire α7 extracellular domain sequence. (**B**) Binding to continuously overlapping 17-mer peptides within the region α7(149–200). (**C**) Binding to peptides of various lengths within α7(182–198). (**D**) Binding to peptides of various lengths within α7(186–198).

182–198, led to the determination of the minimum length required for α-Bgt binding to the site of high binding capacity. When these peptides were tested for ^{125}I-α-Bgt binding, peptide 186–198 showed the highest activity, similar to that of the longer peptides 184–200 and 186–202, whilst peptides 188–198 and 182–196 showed almost no binding activity (Figure 1C). In addition, the presence of Lys-182 and Arg-183 within the peptide 182–198 decreases the toxin-binding capacity, as substitution of these two positively charged amino acids by two alanine residues increased the α-Bgt binding capacity by about 15 % (results not shown). These results indicate that residues 199–202 are not important for α-Bgt binding, since peptide 186–198 had the same activity as peptide 186–202 and also the increased activity of peptide 184–200 compared with peptide 182–198 was due apparently to the removal of the two charged residues Lys-182 and Arg-183.

A second set of shorter peptides was synthesized subsequently by subtracting one residue at a time from sequence 186–198 and then testing for ^{125}I-α-Bgt binding. This study showed that the dodecapeptide 186–197 was the smallest peptide to retain high ^{125}I-α-Bgt binding activity (Figure 1D). Loss of Arg-186 reduced considerably the binding capacity (compare results for peptides 186–197 with 187–197), and toxin binding was almost abolished completely when residues 186 and 187 were both eliminated (see peptide 188–198 in Figure 1C). Residues 186 and 187, especially the latter, are therefore needed for toxin binding. Peptides shortened at the C-terminal showed a dramatic decrease in toxin-binding capacity. The specificity of ^{125}I-α-Bgt binding to the synthetic peptides was demonstrated by excluding possible non-specific electrostatic interactions using control peptides. Thus, peptides with a low pI, similar to that of peptide 186–198 (pI 4.7), e.g. peptide α7(16–32) with a pI of 4.4, and the reverse sequence α7 peptides 198–182 and 198–186, consisting of the same amino acids as α7(182–198) and 186–198, but in the reverse order, were both used. ^{125}I-α-Bgt binding to all control peptides was near background levels (see peptide 198–182, Figure 1B and peptide 198–186, Figure 1D).

Table 1 Alignment of the principal α-Bgt-binding region of human α7 with the homologous regions of other AChR α subunits

The underlined sequences correspond to the identified α-Bgt binding sites.

AChR α subunit	Amino acid sequence
Human α7 (186–198)	RFYECCKE-PYPDV
Chick α7 (185–197)	SFYECCKE-PYPDI
Rat α7	KFYECCKE-PYPRC
Torpedo α1 (188–201)	VYYTCCPDTPYLDI
Human α1 (188–201)	VTYSCCPDTPYLDI
Human α3	IKYNCCEE-IYPDI
Chick α3	IKYNCCEE-IYTDI
Rat α3	IKYNCCAE-IYQDI
Chick α4	KKYECCTE-IYPDI
Rat α4	RKYECCAE-IYPDI

Table 2 Alignment of region 149–175 of α7 subunit with the homologous regions of α1 subunits

Underlined sequence corresponds to the identified complementary α-Bgt binding site of α7 of low activity.

Subunit	Amino acid sequence
Human α7 (149–175)	WSYGGWSLDLQMQ-EADISGYIPNGEWD
Chick α7 (148–174)	WTYGGWSLDLQMQ-EADISGYISNGEWD
Torpedo α1(149–176)	WTYDGTKVSISPESDPDLSTFMESGEWV
Human α1 (150–176)	WTYDGSVVAINPESDPDLSNFMESGEWV

Overall, analysis of the entire human α7 extracellular domain revealed that the principal α-Bgt binding site is located in the region 186–197 (Table 1), whereas at least one complementary binding site exists between residues 159–165 (Table 2, underlined sequence), as deduced by the similar weak binding of the three peptides, 149–165, 154–170, and 159–175. However, we cannot exclude the possibility that other regions may also participate in the native toxin-binding site; such regions may correspond to some of the peptides with very weak toxin binding (Figure 1A).

The specificity of ^{125}I-α-Bgt binding to rod-immobilized α7 peptides was confirmed using soluble α7 peptides to block this binding. These peptides were soluble and did not make aggregates as determined by their centrifugation at 50000 ***g*** for 1 h, which did not reduce the peptide concentration of the supernatants. As shown in Figure 2(A), soluble peptides 186–202 and 186–197 totally blocked toxin binding to the rod-immobilized 186–197 peptide with IC_{50} of approx. 0.9×10^{-4} M and 1.2×10^{-4} M, respectively. The soluble reverse-order peptide α7(198–186), used as a negative control, did not show any inhibitory activity.

Binding of various AChR ligands to peptide 186–197 was tested using multiple copies of the rod-anchored peptide in inhibition assays. The ligands tested were unlabelled α-Bgt, *d*-tubocurarine (an antagonist of ACh binding), nicotine and carbamylcholine (AChR agonists), and methyllycaconitine, a specific α7 neuronal receptor ligand. Figure 2(B) shows that *d*-tubocurarine and α-Bgt, inhibited ^{125}I-α-Bgt binding to the immobilized peptide. The IC_{50} for *d*-tubocurarine was 5×10^{-4} M, about 10-fold lower than the reported IC_{50} for the *Torpedo* α1(173–204) peptide (0.86×10^{-4} M [39,40]), while the IC_{50} for α-Bgt was about 8×10^{-5} M, about 2000-fold lower than the reported IC_{50} for the *Torpedo* α1(173–204) peptide (4.2×10^{-8} M [38,39]); however, given the difference in affinity of α-Bgt for the native *Torpedo* and α7 neuronal AChR (about 100-fold), the α7(186–197) peptide would be expected to have a lower affinity for α-Bgt. In contrast,

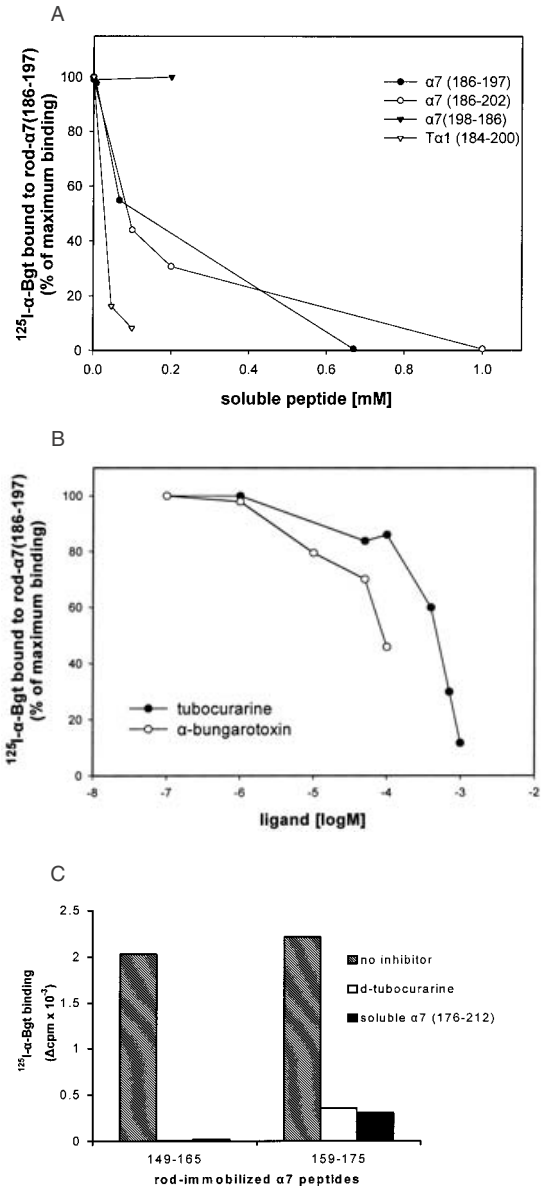

Figure 2 Inhibition of ^{125}I-α-Bgt binding to immobilized peptides α7(186–197), α7(149–165) and α7(159–175) by soluble peptides and unlabelled ligands

(**A**) ^{125}I-α-Bgt binding to rod-immobilized α7(186–197) as a function of the concentration of competing soluble peptides. The values are expressed as the percentage of ^{125}I-α-Bgt bound in the presence of soluble competing peptide compared with that bound in the absence of competing peptide (mean for at least three experiments). The negative control peptide was α7(198–186), a reverse α7 sequence. ^{125}I-α-Bgt (26000 c.p.m.) was added to each rod. ^{125}I-α-Bgt was pre-incubated with the soluble peptides for 1–16 h; the longer preincubation (results shown) resulted in only a slight increase in inhibition. Tα1, *Torpedo* α1 subunit. (**B**) ^{125}I-α-Bgt binding to rod-immobilized α7(186–197) as a function of competing ligand concentration. ^{125}I-α-Bgt (26 000 c.p.m.) was added to each rod. (**C**) ^{125}I-α-Bgt binding to rod-immobilized α7(149–165) and α7(159–175) in the absence of inhibitor (grey bars), in the presence of 1 mM *d*-tubocurarine (open bars), or 1 mg/ml of soluble peptide α7(176–212) (black bars). ^{125}I-α-Bgt (19 000 c.p.m.) was added to each rod.

nicotine and carbamylcholine (at concentrations up to 15 mM) and methyllycaconitine (5 mM) did not inhibit ^{125}I-α-Bgt binding to the synthetic α7 peptides (results not shown).

Finally, the ability of nicotine, *d*-tubocurarine or the soluble α7(176–212) peptide (kindly provided by Dr Leslie Jacobson) to inhibit ^{125}I-α-Bgt binding to the immobilized low-affinity-site α7

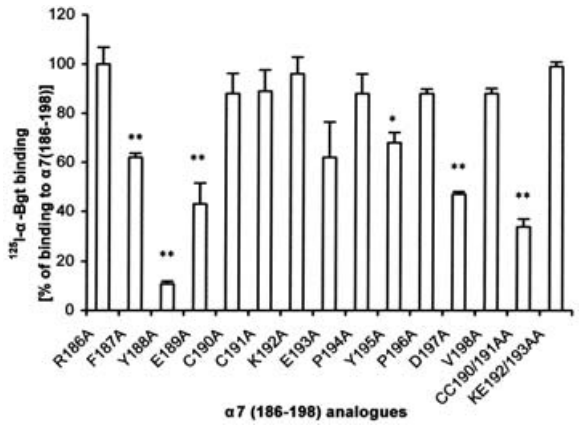

Figure 3 ^{125}I-α-Bgt binding to alanine-substituted α7(186–198) analogues

The values are expressed as the percentage of ^{125}I-α-Bgt binding to analogues compared with ^{125}I-α-Bgt binding to the original α7(186–198) peptide. Results from two experiments (mean ± S.D.) are shown. The residues of the original sequence are indicated to the left of the residue position number and were all substituted by alanine.* $P < 0.05$, ** $P < 0.01$, compared with the values from the original peptide.

peptides, 149–165 and 159–175, was also studied. *d*-Tubocurarine or peptide α7(176–212) totally inhibited toxin binding to the above peptides (Figure 2C), while nicotine had no effect (results not shown).

Determination of the role of individual residues within α7(186–198) in toxin binding

In order to investigate the role of individual residues in the α-Bgt binding site, a series of 15 different analogues of peptide α7(186–198) was synthesized in which one or two residues were substituted with alanine. Figure 3 shows ^{125}I-α-Bgt binding to the α7(186–198) analogues as a percentage of the binding to the original peptide 186–198. Overall, alanine substitutions affected ^{125}I-α-Bgt binding in the order: Y188A ≫ E189A ≈ D197A > F187A ≈ E193A > Y195A. These results demonstrated that residue Tyr-188 is absolutely indispensable for α-Bgt binding, as indicated by the almost total lack of toxin binding to the analogue Y188A, and Glu-189 and Asp-197 seem to play a very important role in α-Bgt binding, whereas the aromatic residue Phe-187 and the Glu-193 seem to play a moderate role. Substitution of either Cys-190 or Cys-191 did not result in reduction of toxin binding capacity, although simultaneous substitution of both cysteines resulted in significant loss of binding activity. Thus, binding of α-Bgt could occur despite the absence of a disulphide bond in the peptide.

Comparative study of the α-Bgt-binding sites on neuronal α7 and muscle-type AChRs

Comparison of the identified principal α-Bgt-binding site on the neuronal human α7 AChR with the α-Bgt-binding site on the α1 subunit of human and *Torpedo* AChR showed that the two prototopes are situated in homologous regions, characterized by the pair of cysteines at positions 192 and 193 and the conserved residues Tyr-190, Tyr-198, Pro-197 and Asp-200, according to the α1 numbering (Table 1). The interactions of α-Bgt with the prototopes of both receptors were simultaneously studied in competition experiments using soluble and immobilized α7 peptides and *Torpedo* α1(184–200) peptide. Soluble *Torpedo* α1(184–200) peptide (4.7×10^{-5} M) strongly inhibited ^{125}I-α-Bgt binding to immobilized α7(186–197) (Figure 2A), its affinity for α-Bgt being 3- to 4-fold higher than that of the soluble α7 peptides (186–202 and 186–197), as shown by the IC_{50} values [3×10^{-5} M for α1(184–200) compared with 0.9×10^{-4} M and 1.2×10^{-4} M for the α7 peptides]. In addition, the soluble α7 peptides 176–212, 186–202 and 186–197, blocked ^{125}I-α-Bgt binding to immobilized α1(184–200) (results not shown).

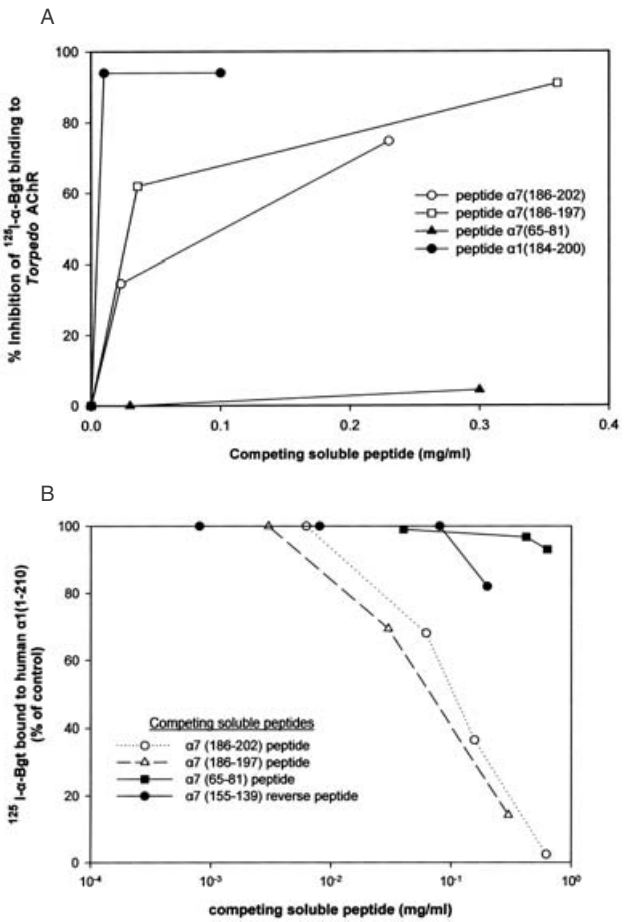

Figure 4 Effect of soluble α7 peptides on ^{125}I-α-Bgt binding to solubilized *Torpedo* AChR (A) and to the soluble human α1(1–210) subunit domain (B)

The reverse peptide α7(155–139) was used as a negative control. Experimental conditions of the filter assays are described in the Experimental section.

Competition for α-Bgt between the principal sites of the two AChR types was also observed when soluble α7 peptides were pre-incubated overnight with ^{125}I-α-Bgt, and muscle-type *Torpedo* AChR was subsequently added. The inhibition experiment was performed using either membrane-bound or Triton X-100 solubilized *Torpedo* AChR; the latter was used to avoid a possible interaction of peptides with the phosholipid membrane through electrostatic bonds or incorporation into the lipid bilayer, which could complicate the results. Figure 4(A) compares the ability of the soluble α7 peptides (186–197 and 186–202) and the *Torpedo* α1(184–200) peptide in inhibiting ^{125}I-α-Bgt binding to detergent-solubilized *Torpedo* AChR. It is shown that both α7 peptides effectively inhibit ^{125}I-α-Bgt binding to *Torpedo* AChR with slight differences in their activity, while *Torpedo* α1(184–200) peptide is a stronger inhibitor. This result was expected considering that *Torpedo* AChR has an affinity for α-Bgt two orders of magnitude higher than that of the neuronal α7 AChR. Inhibitions obtained with membrane-bound AChR were very similar to those

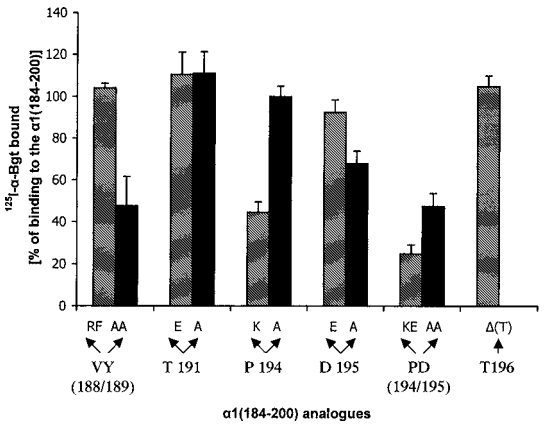

Figure 5 ^{125}I-α-Bgt binding to analogues of the *Torpedo* α1(184–200) peptide

Values are expressed as the percentage of ^{125}I-α-Bgt binding to the analogues compared with the amount of ^{125}I-α-Bgt binding to the original *Torpedo* α1(184–200) peptide. Peptide analogues were synthesized twice in two independent synthesis cycles. The amino acids used for each substitution are shown immediately below the bars with the original α1 amino acids and the respective position numbers beneath. The black bars correspond to substitutions with the homologous human α7 residues and the patterned bars correspond to alanine substitutions. Δ(T) corresponds to the Thr-191 deletion in the original Tα1(184–200) peptide.

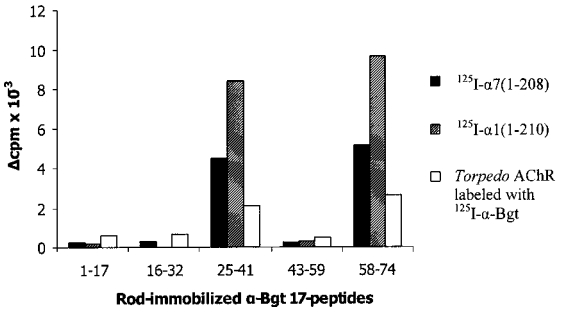

Figure 6 Binding of ^{125}I-α7(1–208), ^{125}I-α1(1–210) and *Torpedo* AChR (partially labelled with ^{125}I-α-Bgt) to synthetic α-Bgt peptides (1–17, 16–32, 25–41,43–59 and 58–74) spanning the entire α-Bgt molecule

Labelled material (30 000 c.p.m.) was incubated overnight at 4 °C with each peptide rod.

obtained with the detergent-solubilized *Torpedo* AChR (results not shown).

The soluble α7 peptides 186–197 and 186–202 were also tested for inhibiting ^{125}I-α-Bgt binding to the extracellular domain of the human α1 subunit, α1(1–210), expressed in *Pichia pastoris*, in a filter assay. α1(1–210) is water-soluble and has a native-like conformation as reported by Psaridi-Linardaki et al. [34]. It binds α-Bgt with a K_d of 5.1 ± 2.4 nm, i.e. only one order of magnitude lower than that of the native human AChR, and it also binds conformation-dependent monoclonal antibodies that are known to bind exclusively to the native human AChR [34]. As shown in Figure 4(B), both α7 peptides inhibited almost totally toxin binding to this α1 subunit domain. Taken together, these results support the idea that the α-Bgt binding sites of both types of receptors (α1 and α7) must be recognized by the same domain of the α-Bgt molecule.

In order to investigate whether substitution of specific residues within the toxin-binding regions of the α1 and α7 subunits was tolerated and to determine whether differences in primary sequence between these two subunits reflect differences in affinity for α-Bgt, two series of analogues of the *Torpedo* α1(184–200) sequence were synthesized. In the first series, one or two amino acids were substituted by the homologous human α7 residues, while, in the second, the same residues were substituted by alanine. As shown in Figure 5, the double substitution of Val-188/Tyr-189 by the homologous human α7 Arg-186/Phe-187 did not result in any decrease in toxin binding, whereas substitution by two alanine residues reduced toxin binding by about 50 %. Since a significant role of Tyr-189 in the α1 subunit has been demonstrated in previous studies [22,41,42], this decreased toxin binding of the alanine-substituted *Torpedo* peptide should be mainly due to substitution of the tyrosine residue. However, the conservative substitution of Tyr-189 by phenylalanine did not affect α-Bgt binding (Figure 5), suggesting that the presence of an aromatic residue at this position is crucial for toxin binding. Similarly, the analogue in which Asp-195 was conservatively substituted with glutamic acid did not show any significant reduction in toxin binding reactivity, whereas alanine substitution reduced α-Bgt binding. In contrast, substitution of Pro-194 by the homologous α7 subunit Lys residue caused a significant drop in toxin binding activity, while the corresponding Ala substitution had no effect; in addition, the double substitution of Pro-194/Asp-195 by the α7 residues Lys/Glu resulted in a dramatic decrease in toxin binding activity. This effect of P194K substitution may partially explain the difference in α-Bgt binding affinity between intact *Torpedo* and α7 AChRs.

Study of individual regions on the α-Bgt molecule involved in binding to neuronal α7 and muscle-type AChRs

In order to study the regions of the α-Bgt molecule involved in the interaction with α7 and α1 AChRs, we synthesized five 17-mer peptides corresponding to five overlapping regions of the toxin, based on the X-ray structure of α-Bgt [16,17,42]. Three of these corresponded to the three main loops of the toxin, i.e. peptide α-Bgt(1–17) corresponded to loop I, α-Bgt(25–41) to part of loop II, and α-Bgt(43–59) to loop III, while the other two included the C-terminal tail of the toxin (residues 68–74) plus part of the core [peptide α-Bgt(58–74)], or another part of the core plus one side of loop II [peptide α-Bgt(16–32)]. Since most of these peptides correspond with external regions of the molecule, thus probably not heavily affected by neighbouring regions, we expect that they may be able to take a native-like folding, at least in their bound state. Thus, the rod-immobilized peptides were tested for binding to the ^{125}I-labelled recombinant extracellular domains of the human α7 subunit, ^{125}I-α7(1–208), and the human α1 subunit, ^{125}I-α1(1–210), and to native *Torpedo* AChR partially labelled with ^{125}I-α-Bgt. Figure 6 shows that the binding of both recombinant fragments (α1 and α7) and *Torpedo* AChR was mainly restricted to peptides 25–41 and 58–74, suggesting that loop II of the toxin and the C-terminal tail, possibly together with some additional residues from the core, are the main contact sites between α-Bgt and both the muscle-type and neuronal α7 AChRs.

When α-Bgt peptides 25–41, and 58–74 were prepared in a soluble form (centrifugation at 50000 ***g*** showed that peptides are soluble) and tested in inhibition experiments, 0.13 mg/ml and 0.32 mg/ml of the soluble α-Bgt peptides 25–41 and 58–74 were required for inhibiting ^{125}I-α-Bgt binding to the native α7 AChR by 36.8 % and 19.9 %, respectively (Table 3). Recent reports have shown an albumin-induced enhancement of AChR activity (in whole receptors), possibly through removal of inhibitory fatty acids from AChR [36] or through increase of the steady-state channel opening [37]. Based on these reports, we tested the possible effect of BSA on the toxin/peptide binding to α7 AChR, either by removing BSA or adding human serum albumin

Table 3 Inhibition of ^{125}I-α-Bgt binding to solubilized α7 AChR by soluble α-Bgt peptides

Inhibition percentages ($\pm$ S.D.) represent c.p.m. bound in the presence of inhibitor, subtracted from the c.p.m. bound without inhibitor (control) versus the control value. α4(106–122) corresponds to the neuronal α4 AChR that does not bind α-Bgt.

Peptide inhibitor	Concentration used (mg/ml)	Inhibition of ^{125}I-α-Bgt binding to α7 AChR (%)
α-Bgt(25–41)	0.13	36.8 $\pm$ 1.5
α-Bgt(58–74)	0.32	19.9 $\pm$ 3.5
α4(106–122)	0.22	3.8 $\pm$ 0.1

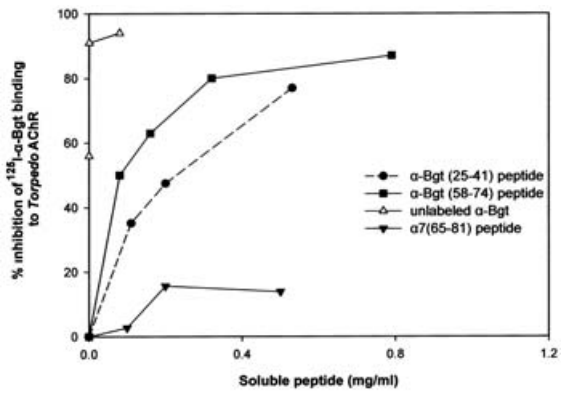

Figure 7 Effect of soluble α-Bgt peptides on ^{125}I-α-Bgt binding to membrane-bound *Torpedo* AChR

The effect on ^{125}I-α-Bgt binding to *Torpedo* AChR of soluble peptides α-Bgt(25–41) (●) and α-Bgt(58–74) (■) was compared with the effect of α7(65–81) (▼), used as negative control with no toxin-binding activity, and unlabelled α-Bgt (△), used as a positive control. Experimental conditions are shown in the Experimental section.

in incubation buffers (according to Conroy et al., human serum albumin does not enhance α7 AChR responses [37]). Results showed that toxin binding to α7 AChR and inhibition percentages with peptides were not affected by the use of BSA.

Similarly, when these soluble α-Bgt peptides were tested for their ability to inhibit ^{125}I-α-Bgt binding to membrane-bound *Torpedo* AChR, they inhibited ^{125}I-α-Bgt binding in a concentration-dependent manner, 50 % inhibition being achieved with 0.07 mg/ml α-Bgt(58–74) and with 0.22 mg/ml α-Bgt(25–41) (Figure 7). These peptides were also tested using solubilized *Torpedo* AChR but no significant differences from results with the membrane-bound AChR were evident (results not shown). Finally, oxidation of toxin peptides with various ratios of reduced/oxidized forms of glutathione (GSH/GSSG; to achieve disulphide bond formation), did not improve the results (results not shown). This could be due possibly to spontaneous formation of the correct disulphide bonds within the peptides. Overall, we can conclude that both AChR types, the neuronal α7 and *Torpedo* α1, interact with the C-terminal tail and loop II of α-Bgt (represented by peptides 58–74 and 25–41, respectively).

DISCUSSION

The α-Bgt binding site on the human α7 subunit

In-depth analysis of α-Bgt binding within the entire extracellular domain of the α7 subunit of the human neuronal AChR, by the systematic synthesis of a large number of peptides, revealed two distinct α-Bgt binding subsites, located in loop C of the principal agonist-binding component (region 186–197) and in loop IV of the complementary agonist-binding component (region 159–165). It is possible that other regions within α7 may also be involved in the toxin-binding site; however, it is not safe to consider the very weak toxin binding of peptides 16–32, 35–51, 95–111 and 169–185 as specific. Furthermore, these regions do not correspond to the known loops of the principal or complementary agonist-binding sites which would render this binding interesting.

Peptide 186–197, which corresponds to loop C of the human α7 AChR, was shown to be the minimal α7 binding segment with the highest ^{125}I-α-Bgt-binding activity. The addition of residues Lys-182 and Arg-183 to the critical sequence 186–197 resulted in decreased toxin-binding ability (as shown by the binding reactivity of peptide 182–198), apparently due to unfavourable conformational states imposed by the two residues. In support of this, substitution of Lys-182 and Arg-183, within the sequence 182–198, by two alanine residues increased the α-Bgt binding (results not shown). ^{125}I-α-Bgt bound to both immobilized and soluble α7 peptide 186–197 with an IC_{50} of about 1.2×10^{-4} M, confirming that the dodecapeptide 186–197 was sufficient for the binding of α-Bgt. Synthetic *Torpedo* α1 subunit peptides (188–197, 189–195, 173–204, 186–196 and 169–181) are also reported to have high α-Bgt binding activity [21,22,39], whereas human α1 peptides have a significantly lower affinity [24,25]. These differences may be explained by the differential role of glycosylation on *Torpedo* α1, human α1 and human α7 subunits; while α-Bgt binding to human α1 is practically abolished after deglycosylation [34], its binding to unglycosylated *Torpedo* α1 and human α7 recombinant polypeptides (expressed in *Escherichia coli*), is very significant (L. Jacobson and S. J. Tzartos, unpublished results [33]). In addition, deglycosylation of the glycosylated human α7(1–208), expressed in yeast, has no effect on ^{125}I-α-Bgt binding (S. Avramopoulou, A. Mamalaki and S. J. Tzartos, unpublished results). Finally, a recent NMR structural analysis of a complex between α-Bgt and the chick α7 peptide 178–196 showed that the contact points in the peptide are the residues Phe-186, Tyr-187, and Glu-188 of loop C [43]. Thus our results are in agreement with the NMR data and support the idea that α7 loop C must be the major contributor to the toxin-binding site.

As regards other AChR ligands, the antagonist, *d*-tubocurarine, almost completely inhibited ^{125}I-α-Bgt binding to the α7(186–197) peptide with an IC_{50} of $\approx 5 \times 10^{-4}$ M (Figure 2B). In an earlier study, α-Bgt binding to the synthetic peptide α181–200 from a chick neuronal ACh binding protein (now known to be the chick α7 subunit) was not blocked by *d*-tubocurarine or other nicotinic ligands (10 mM) [28]. The observed competition in our study between α-Bgt and *d*-tubocurarine suggests that several of the major determinants of *d*-tubocurarine binding are also situated within the α7 region 186–197. However, the agonists nicotine and carbamylcholine, and the α7-selective ligand, methyllycaconitine, did not inhibit binding of α-Bgt to α7(186–197). This is not surprising as, in previous studies, significant competition of ^{125}I-α-Bgt binding to α1 peptides of the *Torpedo* and human AChR was seen using *d*-tubocurarine or unlabelled α-Bgt or α-cobratoxin, but not using nicotine or other agonists [22,34,38,39]. In addition, various α1 subunit peptides displayed differential binding for nicotine and α-Bgt. Thus, nicotine and α-Bgt bound to somewhat different determinants within the α1(173–204) region, despite some overlap of their binding sites [39]. Specifically, regions 181–185 and 194–204 of the α1 subunit were shown to be involved in nicotine binding. Therefore, we could hypothesize that the absence of the homologous α7 residues or the absence of the (−)

face determinants from peptide 186–197 may explain the lack of agonist binding.

A complementary toxin-binding region, exhibiting low ^{125}I-α-Bgt-binding activity, was identified between residues 159–165, as mentioned above. Peptides 149–165, 154–170 and 159–175 of α7 bound weakly to ^{125}I-α-Bgt, suggesting that they contain toxin-binding determinants, but, in the absence of residues 186–197, their binding capacity is quite low. The binding activity of peptide 149–165 could be due partially to the conserved residue Trp-149 (Table 3), a key residue in cholinergic ligand binding and possibly in α-Bgt binding, as shown by electrophysiological studies of chick α7 mutants [11]. Specifically, mutation of chick α7 Trp-148 to phenylalanine, was shown to decrease the apparent affinity for ACh and nicotine. An effect on α-Bgt binding was seen indirectly by reduced α-Bgt inhibition of the ACh response of the mutant. Furthermore, mutations W149T, Y151T and G153S of the human α7 AChR had a weak effect on the binding of α-conotoxin ImI [14]. The direct binding of α-Bgt to α7(149–165) suggests that residues Trp-149, Tyr-151 and Gly-153 (see Table 3) may also be involved in α-Bgt binding. Nevertheless, given the same degree of α-Bgt binding to all three peptides, the overlapping sequence 159–165 must be the most critical region. Interestingly, region 159–165 of the human α7 subunit is included within loop IV of the complementary component of the agonist binding site, indicating that the same key residues within this region are involved in both α-Bgt and agonist binding. Indeed, the conserved Asp-164 of human α7 is involved in agonist binding [7,13] and, recently, the homologous Asp-163 of chick α7 was found to play a moderate role in α-cobratoxin binding by interacting with residue Lys-35 of the toxin [44]. ^{125}I-α-Bgt binding to peptides 149–165 and 159–175 was inhibited by *d*-tubocurarine, showing that *d*-tubocurarine and α-Bgt interact with the same determinants within this region as well. Interestingly, soluble α7(176–212), which contains the principal α-Bgt binding region, competed with peptides 149–165 and 159–175 for ^{125}I-α-Bgt binding (Figure 2C). One might assume that α-Bgt binds to the native receptor through simultaneous interactions with both binding regions, with a possibly additive effect. However, when the peptide 176–212 interacts with the toxin, it could possibly alter the conformation of the toxin and result in the masking of determinants on α-Bgt, critical for its interaction with the complementary α7 binding site. Indeed, conformational changes of α-Bgt on interaction with the chick α7(178–196) peptide have been observed in NMR studies [43]. In conclusion, beside region 186–197, determined as the principal toxin-binding site, a complementary toxin-binding site was identified between residues 159 and 165 of the human α7 subunit, which corresponds to loop IV of the complementary component of the α7 agonist binding site.

According to the crystal structure of the molluscan AChBP [10], which shares a 24 % sequence identity with human α7 AChR, the regions corresponding to the active α7 peptides 186–197, 149–165, 154–170 and 159–175 are external loops with only a minor part of their sequence having a constrained structure of β-strand. Therefore, these regions may not be much involved in intra-molecular interactions in the native α7 AChR and so their structure may not be heavily affected by the presence of other neighbouring residues. Moreover, NMR structural analysis of a complex between α-Bgt and the chick α7 peptide 178–196 showed that, although the free 19-mer peptide lacks a regular secondary structure, it adopts a hairpin-like structure by making contacts with α-Bgt [43]. Therefore, it is likely that the human α7 peptide 186–197 adopts a similar conformation to that taken up by the chick α7 peptide 178–196, when bound to α-Bgt.

The role of each residue within the human α7(186–198) sequence

The use of a series of analogues of α7(186–198) demonstrated the role of individual residues in α-Bgt binding (Figure 3). Overall, alanine substitution of individual residues in peptide 186–198 affected ^{125}I-α-Bgt binding in the order Y188A $\gg$ E189A $\approx$ D197A > F187A $\approx$ E193A > Y195A. These results showed that aromatic and negatively charged residues are involved in the α7 binding site. These findings are in agreement with results using mutants of the chimaeric chick AChR (α7–5HT3), recently reported by Fruchart-Gaillard et al. [44], and furthermore suggest the involvement of additional residues in toxin binding. Fruchart-Gaillard et al. [44] showed that mutations introduced at residues homologous to human α7 residues 164, 166, 186, 187, 188, 195 and 197 decreased the affinity for α-cobratoxin, while our study confirmed the importance of residues 187, 188, 195 and 197 and identified two additional residues, Glu-189 and Glu-193, that contributed to ^{125}I-α-Bgt binding. Taken together, these results provide evidence for the importance of individual residues of loop C in the binding of long toxins.

The negatively charged residues, Glu-189 and Glu-193, may interact electrostatically with positively charged amino acids in α-Bgt (lysine and arginine), thus stabilizing the complex. Glu-193, present in all neuronal α subunits, corresponds to Asp-195 of the muscle-type α1 subunits. Asp-195 of the α1(188–197) peptide of *Torpedo* AChR is absolutely indispensable for α-Bgt binding [22], but the homologous Glu-193 of α7 seems less important (Figure 3). However, the conservative substitution of Asp-195 of *Torpedo* α1(184–200) by the homologous human α7 residue Glu-193 had almost no effect on toxin binding, whereas substitution with alanine decreased toxin binding, suggesting that a negatively charged amino acid is required at this position for full α-Bgt binding.

Tyr-188 seems indispensable for α-Bgt binding, as almost no binding to the analogue Y188A was observed (Figure 3) and a similar role for the homologous Tyr-190 of the *Torpedo* AChR α1 subunit has been reported [22,40]. Additionally, the homologous Tyr of the chick α7 αChR (Tyr-187) is crucial for the binding of α-cobratoxin and α-Bgt. Specifically, mutation of Tyr-187 to phenylalanine in the intact chick α7 AChR results in a 21-fold decrease in the affinity for α-cobratoxin, while non-conservative mutations result in more dramatic effects on α-Bgt and α-cobratoxin binding [44,45]. These results suggest that the presence of an aromatic residue at this position is crucial for toxin binding.

Substitution of Phe-187 by alanine resulted in a considerable decrease in ^{125}I-α-Bgt-binding activity of the α7(186–198) peptide. Moreover, the double substitution of Val-188/Tyr-189 in the α1(184–200) *Torpedo* peptide by the homologous human α7 residues, arginine/phenylalanine, did not reduce the α-Bgt binding capacity, whereas alanine substitutions resulted in a significant drop in α-Bgt binding (Figure 5). These findings correlate well with those from previous studies on mutant rat α7 AChRs, in which F189Y mutant AChR had a K_d for α-Bgt similar to that of the wild-type receptor [46], as on the *Torpedo* α1 subunit, in which substitutions of the homologous residue Tyr-189 revealed its critical role in α-Bgt binding [22] and, moreover, on α-Bgt-insensitive α3β2/β4 AChR, in which rat α3 carrying a single substitution K189Y showed an increase in affinity for α-Bgt by three orders of magnitude compared to the wild-type α3 [41]. Therefore, in the present study, the decrease in toxin binding of the double-alanine-substituted *Torpedo* peptide should be mainly due to substitution of the tyrosine residue while the conservative substitution of Tyr-189 by phenylalanine did not affect toxin binding. Taken together, we can suggest that an

aromatic residue at position 187, according to the human α7 numbering, may be necessary and sufficient for full α-Bgt binding. This hypothesis is also supported by the reduced α-Bgt binding affinity of human α1 peptides [24,25] and the absence of α-Bgt binding to neuronal α3 and α4 receptors that have a Thr and a Lys residue at the homologous position respectively (Table 1).

The conserved amino acids, Tyr-195 and Asp-197, of the human α7 AChR were found to play a major role in the interaction with α-Bgt (Figure 3) whereas the homologous α1 residues of the muscle-type AChR are not involved in the critical α-Bgt binding site [22].

Substitution of either Cys-190 or Cys-191 by alanine did not result in any reduction in toxin-binding activity, although a dramatic drop was seen on substitution of both cysteines. These results show that no disulphide bond needs to be formed between these two cysteines in the peptide for α-Bgt binding to occur. The observed decrease in ^{125}I-α-Bgt binding in the case of the double-substituted peptide may reflect a change in the conformation of the peptide. The lack of a critical role of either cysteine or the disulphide bond in toxin binding has been previously reported. Specifically, substitution of the homologous cysteines 192 and 193 within the α1(188–197) peptide of the *Torpedo* AChR by various amino acids did not significantly decrease ^{125}I-α-Bgt binding [22]. In addition, McLane et al. [28] showed that oxidation of the adjacent cysteines (positions 190/191 according to human α7 numbering) in peptide α181–200 of the neuronal α-Bgt binding protein had almost no effect on α-Bgt binding.

Comparison of the α-Bgt binding sites of neuronal α7 and muscle-type AChRs

Despite the significant differences in the primary sequence between the α-Bgt binding sites of α1 and α7, if both sites bind to the same α-Bgt domain and α-Bgt interacts with both types of receptors in a similar way, replacement of the amino acids of the binding site in one subunit by the equivalent residues from the other should have no dramatic effect on toxin binding. We tested this hypothesis by replacing residues within the *Torpedo* α1(184–200) sequence by the corresponding human α7 residues. With the exception of substitution P194K, which introduces a positive charge, substitution of α1 residues by the corresponding α7 residues did not affect α-Bgt binding, whereas alanine substitutions decreased α-Bgt binding. The finding that substitutions between α1 and α7 are, in general, well tolerated suggests that α-Bgt interacts similarly with both prototopes, recognizing a specific peptide conformation, common to the two peptides. This was further supported by the observed competition between peptides α1(184–200) and α7(186–197) or α7(186–202) for binding to toxin, as well as by the ability of these soluble peptides to inhibit the binding of ^{125}I-α-Bgt to the soluble native-like human α1 subunit extracellular domain and to native *Torpedo* AChR. The observed cross inhibition excludes the possibility that α-Bgt binding to the two AChR types occurs through different sites of α-Bgt molecule. Thus, the binding sites for both neuronal α7 and muscle-type AChRs on the α-Bgt molecule must be identical or overlapping and therefore capture of α-Bgt by one prototope excludes binding by the other. Since it is unlikely that the same domain of α-Bgt recognizes different conformations, this suggests that the α1 and α7 prototopes possibly have a similar conformation.

Study of individual regions on the α-Bgt molecule involved in binding to neuronal α7 and muscle-type AChRs

In order to directly identify and compare the binding regions for the two types of receptors on the α-Bgt molecule, synthetic α-Bgt peptides were tested for binding to the recombinant ^{125}I-α7(1–208), ^{125}I-α1(1–210), and to native *Torpedo* and neuronal α7 AChR. Overall, the results demonstrated that loop II (peptide 25–41) and the C-terminal tail (peptide 58–74) of α-Bgt are the major regions by which the toxin makes contact with both α7 and muscle-type receptor (Figure 6). No binding to loop I or III was observed, indicating the absence of significant binding determinants at these regions.

Our findings are consistent with data obtained by introducing mutations into the α-Bgt or α-cobratoxin molecule [45,47,48]. Residues Trp-25, Asp-27, Phe-29, Arg-33, Lys-35 and Arg-36 in loop II and Phe-65 (homologous to His-68 of α-Bgt) in the C-terminal tail of α-cobratoxin were found to be critical for binding to both neuronal α7 and *Torpedo* AChRs [45,48,49], whereas no mutations introduced into loop I [45,49] or loop III [49] resulted in decreased binding affinity, suggesting that loop I and loop III are not substantially involved in the binding site, in agreement with our findings. Additionally, NMR studies of a complex between α-Bgt and the *Torpedo* α185–196 peptide suggested that His-68 of α-Bgt is in close proximity to the AChR binding site, while residues Trp-28–Phe-32 and Gly-37-Glu-41 within loop II participate in the binding site; however, residues Thr-6–Thr-8, Pro-10 and Ile-11 within loop I were also found to be involved in the interaction with the peptide [31]. Similarly, a recent NMR analysis of a complex between α-Bgt and the chick α7(178–196) peptide showed that residues Ala-7, Ser-9 and Ile-11 in loop I of the toxin and Arg-36, Lys-38, Val-39 and Val-40 in loop II are in contact with the α7 peptide [43]. In this study, the α7 peptide was found to be positioned between loops I and II (facing the concave surface of the toxin, with loop I on the left and loop II on the right). Such a position would favour interaction of α7 with the left side of loop II and the C-terminal tail, thus explaining our results. On the other hand, a study using synthetic α-Bgt peptides, corresponding to the α-Bgt loops, reached different conclusions; peptides 1–16, 26–41, and 45–59, corresponding to loops I, II and III of α-Bgt, but forming artificial disulphide bonds, all bound to *Torpedo*^{125}I-AChR, whereas the C-terminal region did not [29]. The loop II peptide that demonstrated the highest reactivity in the above study lacked certain key residues, such as Cys-29 and Cys-33, which form the fifth disulphide bridge, and Arg-25 and Lys-42, which were replaced by cysteine residues. However, the significance of the fifth disulphide for the high-affinity interaction of α-cobratoxin with the α7 receptor has been reported [45,50]. It is possible that this fifth disulphide (Cys-29 and Cys-33) is formed within our peptide 25–41, allowing its interaction with α7.

Overall, using synthetic peptides, we have identified two α-Bgt binding sites within the human α7 neuronal AChR subunit; these are located in residues 186–197 and 159–165 and demonstrate, respectively, high and low binding activity. Competition assays suggested that the homologous α-Bgt binding sites of the neuronal α7 and muscle-type AChRs adopt a similar conformation and also implied that these regions bind to identical or overlapping sites on the α-Bgt molecule. Results obtained using synthetic α-Bgt peptides indicated that α-Bgt interacts with both neuronal α7 and muscle-type AChRs through its central loop II and C-terminal tail as shown by the binding activity of the α-Bgt peptides 25–41 and 58–74.

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