A Novel Pharmatope Tag Inserted into the β4 Subunit Confers Allosteric Modulation to Neuronal Nicotinic Receptors*

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α-Bungarotoxin, the classic nicotinic antagonist, has high specificity for muscle type α1 subunits in nicotinic acetylcholine receptors. In this study, we show that an 11-amino-acid pharmatope sequence, containing residues important for α-bungarotoxin binding to α1, confers functional α-bungarotoxin sensitivity when strategically placed into a neuronal non-α subunit, normally insensitive to this toxin. Remarkably, the mechanism of toxin inhibition is allosteric, not competitive as with neuromuscular nicotinic receptors. Our findings argue that α-bungarotoxin binding to the pharmatope, inserted at a subunit-subunit interface diametrically distinct from the agonist binding site, interferes with subunit interface movements critical for receptor activation. Our results, taken together with the structural similarities between nicotinic and GABA<sub>A</sub> receptors, suggest that this allosteric mechanism is conserved in the Cys-loop ion channel family. Furthermore, as a general strategy, the engineering of allosteric inhibitory sites through pharmatope tagging offers a powerful new tool for the study of membrane proteins.

Nicotinic acetylcholine receptors (nAChRs)<sup>1</sup> are prototypical members of the superfamily of ligand-gated pentameric ion channels. They are important for peripheral and central nervous system function and have long been known to mediate synaptic transmission at the vertebrate neuromuscular junction (1). The biochemical and molecular characterization of muscle nAChRs, the first representatives of the superfamily to be characterized in molecular terms, has been greatly facilitated by the availability of highly specific snake venom-derived α-neurotoxins, such as α-bungarotoxin (Bgtx). These small proteins are high affinity competitive antagonists that interact with a subset of agonist binding determinants to disrupt agonist binding (2). Furthermore, the commercial availability of a large number of diversely labeled conjugates of Bgtx has led to major advances in the understanding of the biogenesis and developmental regulation of muscle nAChRs (3). Unfortunately, neuronal heteromeric nAChRs are not recognized by the α-neurotoxins, and comparatively effective tools for their study are lacking.

Biochemical and structural studies show that the major determinants responsible for Bgtx binding to muscle nAChRs are located in Loop C of the α-subunit. The structure of the molluscan acetylcholine-binding protein (AChBP), a generally accepted model for the extracellular domain of homopentameric nAChRs, indicates that Loop C residues form a hairpin turn at the end of two conserved β strand motifs (β9 and β10). These β strands lie on the outer perimeter of the α subunit surface with the hairpin region in close proximity to the surface of the adjoining subunit. In heteromeric nicotinic receptors, the adjacent subunit is always a non-α (4–6). There is considerable evidence that Bgtx binding to the Loop C region prevents the association of acetylcholine (ACh) to its binding site, also in close proximity to Loop C, and as a consequence, the conformational changes leading to channel gating are precluded (7–10).

In contrast to muscle nAChRs, vertebrate heteromeric neuronal nAChRs are insensitive to Bgtx inhibition and lack high affinity Bgtx binding sites (11). We have shown that as few as 5 amino acids from Loop C of the muscle type α1 subunit, when substituted into the homologous region of the Bgtx-insensitive neuronal α3 subunit, are sufficient to impart functional sensitivity to Bgtx (12). These studies, together with observations that Bgtx binds with high affinity to isolated short peptide fragments derived from α1 Loop C, led us to speculate that short sequences derived from α1 Loop C might be effective in transferring Bgtx sensitivity to a more distantly related target, such as the nicotinic β4 subunit, one of the widely expressed neuronal non-α subunits. If successful, we reasoned that such a sequence, which we term a pharmatope in analogy to epitope tagging, could provide a general tool for introducing pharmacological sensitivity into membrane proteins (2).

In the study reported here, we wanted to determine whether insertion of an α1-derived pharmatope sequence into the neuronal β4 subunit could produce a high affinity Bgtx binding site. We tested this in Xenopus oocytes using functional neuronal heteromeric nAChRs produced by heterologous expression of the chimeric β4 subunit along with the Bgtx-insensitive α3 subunit. Two experimental β4 constructs were prepared: one with the pharmatope inserted en bloc into the L1 loop (4), near the N terminus, and the other with the pharmatope replacing endogenous residues in the homologous Loop C region of β4. We asked whether Bgtx binding to these ectopic sites, distant from the subunit interfaces responsible for ACh binding, could inhibit ACh-evoked activity. We also wanted to determine by independent means whether a reporter Bgtx conjugate could be bound to surface receptors containing either of the two pharmatope-tagged β4 constructs. The observed pharmacological sensitivity introduced through one of the two pharmatope insertions provides novel insights into the conformational changes that couple activation of neuronal nAChRs to channel opening, and we emphasize for the first time an important role

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<sup>1</sup>The abbreviations used are: ACh, acetylcholine; nAChR, nicotinic ACh receptor; AChBP, ACh-binding protein; Bgtx, α-bungarotoxin; GABA, γ-aminobutyric acid.

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for the subunit interfaces distinct from those forming the agonist binding site.

**MATERIALS AND METHODS**

**Chimeric Subunit Constructs**—The α3 cDNA construct used in this study was in pcDNA3.1/Zeo, whereas β2 and β4 were in the pGEMHE vector provided by Charles Luetje. After linearization by restriction enzyme digestion, DNA was purified by ethonal precipitation, and in vitro RNA transcription was performed with the mMESSAGE mMAXICHINE kit from Invitrogen. cRNA was purified by lithium chloride precipitation and stored at −80 °C. Chimeras were generated by QuikChange PCR mutagenesis (Stratagene, La Jolla, CA), and the resulting plasmids were transformed into XL-1 Blue Supercompetent cells. Sequences were determined by PCR cycle sequencing.

**Oocyte Preparation and Injection**—Oocytes were collected from mature Xenopus laevis by survival surgery and were prepared for injection essentially as described previously (12). Oocytes were injected with 46 nL of cRNA and maintained in antibiotic-supplemented buffer for 2–6 days at 15 °C before recording. Equal parts of α to β subunit cRNA (0.125 μg/μl unless otherwise noted) were diluted into OR2 buffer (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, 10 mM HEPES, 0.003 mM atropine sulfate, pH 7.4).

**Electrophysiological Recordings**—ACh-evoked currents were measured using the two-electrode voltage clamp method with a Warner OC-725C. Electrodes of resistance 0.5–2.0 megohms were filled with 3 M KCl, and recordings were performed in a Warner RC-3Z chamber with an OC-725 bath clamp headstage attached. The flow of various drug solutions in OR2 into the chamber was regulated by solenoid valves driven by a Warner BPS-8 controller. The flow rate of the perfusion fluid was adjusted to 5 ml/min by gravity feed. As noted by others, neuronal nAChRs can display rundown with repeated agonist application (35). For each subunit combination, we perform rundown control determinations by measuring currents for the same dose (−EC50) of ACh given 6–10 times over a period similar to that used for the experimental data acquisitions. In cases in which rundown is observed, the results are corrected by linear interpolation. Co-application experiments were used to measure the onset of toxin block. After collecting initial responses evoked by a control dose of ACh, oocytes were incubated in a solution containing the desired Bgtx concentration for 15 min. To measure recovery from toxin block, ACh responses were collected from each oocyte prior to and after exposing the oocyte to Bgtx. Perfusion to wash out the Bgtx was conducted, and cell responsiveness to 100 μM ACh was determined at various times.

**Binding Assays with Radiolabeled Bgtx**—Seven to eight days after cRNA injection, 100–200 oocytes were disrupted with a Brinkmann Kinematica Polytron homogenizer in 1–2 ml of homogenization buffer (83 mM NaCl, 1 mM MgCl2, 10 mM HEPES, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.0). The solution was diluted to 10 ml before perfusion and ACh-evoked inward currents are corrected by linear interpolation. Co-application experiments were used to measure the onset of toxin block. After collecting initial responses evoked by a control dose of ACh, oocytes were incubated in a solution containing the desired Bgtx concentration for 15 min. To measure recovery from toxin block, ACh responses were collected from each oocyte prior to and after exposing the oocyte to Bgtx. Perfusion to wash out the Bgtx was conducted, and cell responsiveness to 100 μM ACh was determined at various times.

**RESULTS**

**Two Sites in the Extracellular Domain of β4 Targeted for Pharmatope Insertion**—Initially, we compared the Loop C amino acid sequence from an α-bungarotoxin-sensitive α1 subunit found in a skeletal muscle type of nAChR (i.e. from Torpedo californica electric organ) with comparable regions from the toxin-insensitive rat α3 and β4 subunits. As shown in Fig. 1a, the putative Loop C region of β4 is highly variant but anchored by flanking invariant residues. In addition, it contains 4 fewer amino acid residues than Torpedo α1 and 3 less than rat α3. Also, β4 Loop C lacks the adjacent Cys residues (Cys-192–Cys-193; Torpedo α1 numbering) characteristic of all nicotinic α subunits (1). We replaced 7 contiguous residues from the presumed turn 7 of Loop C in β4 (i.e. VNPDQPS) with a sequence that we expected, based on NMR studies of receptor peptide fragments, to be sufficient to generate a high affinity Bgtx binding site (8, 13, 14). Our candidate pharmatope sequence (VVYTTCCPDTP) was derived from the tip of Loop C in the Torpedo α1 subunit. The chimeric β4/α1[11]Loop-C subunit (Fig. 1a) contains a net addition of 4 amino acids in the Loop C region and is identical in length to the Loop C in α1. We produced a second chimeric construct, β4[α1[11]Helix, by inserting the same sequence, VVVVTCCPDTP, into a site distant from Loop C. We chose the L1 region very near the N terminus of the extracellular domain of β4. In the structure of the AChBP, the L1 loop follows a region (residues 1–13) that is α-helical (Fig. 1b). In nAChRs, the homologous L1 loop is most likely situated near the cusp of the receptor vestibule (4). The structures of related ligand-gated receptor subunits, from glycine receptors and GABA receptors, accommodate natural sequence insertions in this region (10, 4).

**The Pharmatope Sequence Is Well Tolerated**—Heterologous expression in Xenopus oocytes was used to prepare functional heteromeric nAChRs containing the β4 pharmatope constructs. As observed previously, the β4 subunit alone is incapable of forming functional ACh-gated ion channels; co-expression of an α subunit such as α3 or α4 is required (11). Therefore, cRNA encoding each β4 chimera was injected into Xenopus oocytes along with cRNA encoding the wild-type, Bgtx-insensitive rat α3 subunit. Neuronal nAChR (α3β4)-mediated currents were measured in the oocytes using the two-electrode voltage clamp recording technique as described previously (12). The β4/α1[11]Loop-C chimera, when co-expressed with α3, generated ACh-evoked currents comparable in size to those of wild-

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**Pharmatope-mediated Allosteric Block of Neuronal nAChRs**

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**Fig. 1. Sequence alignments in the two regions of the β4 subunit targeted for pharmatope insertion.** The peptide sequence, VVVYT TTCCPDTP, was inserted (a) into Loop C of the β4 subunit, replacing residues VNPDQPS, or (b), into the L1 loop immediately following the N-terminal α-helical segment. Asterisks indicate the positions of conserved amino acids among the Torpedo α1, rat α3, and β4 sequences. The Torpedo numbering of the sequence is indicated for the final position in each alignment. The underlined residues in panel b correspond to the α-helical segment observed in the AChBP (4). The locations of Loop C and L1 within the ECD are shown in Fig. 6.

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Type β4 (Table I). β4/α1[11]Loop-C alone or when co-expressed with wild-type β4 did not generate functional nAChRs in oocytes (data not shown). The insertion of α1 Loop C residues does not convert the β4 subunit into an α subunit. As with wild-type β4, the β4/α1[11]Loop-C chimera must co-assemble with an agonist binding α-type subunit to generate functional nAChRs (15). Furthermore, the nAChRs comprised of the β4/α1[11]Loop-C chimera and α3 were activated by ACh as in wild-type channels. We obtained an apparent EC50 for ACh of 121 μM, a value comparable with the EC50 of −100 μM reported for wild-type α3β4 nAChRs (12). Our results indicate that insertion of the pharmatope sequence, WYYYTCPPDTTP, within the Loop C region of β4 does not disrupt subunit synthesis or co-assembly with the α3 subunit, critical steps required for generation of functional surface receptors. Pharmatope insertion into the L1 region (β4/α1[11]Helix construct) did not affect cell surface expression of functional receptors upon co-expression with α3 but did decrease the apparent EC50 for ACh −4-fold (Table I). Further investigations would be required to address the mechanisms by which this alteration in the L1 loop, which is distant from the ACh binding pocket, brings about the observed change in the EC50.

The Pharmatope Inserted within Loop C of β4 Confers Bgtx Sensitivity—After confirming that wild-type α3β4 receptors are insensitive to Bgtx (11, 12) (Table I), we tested the toxin sensitivity of ACh-evoked currents in oocytes expressing α3 and the chimera β4/α1[11]Loop-C subunit. Bgtx blocked ACh-evoked responses in oocytes expressing α3 and β4/α1[11]Loop-C subunits with remarkably high affinity. The IC50 for Bgtx block was 34 nM (Table I, Fig. 2a). In addition, we calculated the apparent Hill coefficient for Bgtx block of the β4/α1[11]Loop-C chimera to be −0.7, consistent with occupancy of a single Bgtx binding site per functional receptor being sufficient for functional inhibition.

Next, we tested the reversibility of Bgtx inhibition of β4/α1[11]Loop-C following removal of the toxin from the oocyte perfusion. Bgtx block was long-lived, with an apparent t1/2 for dissociation of −140 min (Fig. 2b). In comparison, Bgtx block of native muscle type nAChRs expressed in oocytes is essentially irreversible over the same time interval (16).

Bgtx Inhibition of nAChRs Containing β4/α1[11]Loop-C Does Not Involve the ACh Binding Site—Bgtx inhibits native muscle nAChRs by competitive antagonism at the agonist binding site; numerous studies have shown that agonists such as ACh inhibit radiolabeled α-neurotoxin binding to muscle-type nAChRs competitively (17). To investigate the mechanism of toxin block in oocytes expressing α3 and chimeric β4/α1[11]Loop-C, we performed competition binding assays on isolated oocyte membranes using 125I-Bgtx (18). In control experiments, we confirmed that 100 μM ACh decreases Bgtx binding to native α1 muscle-type receptors by −90% (Fig. 3). ACh binding to the agonist binding site prevents association of Bgtx with binding determinants that are common to the two ligands (2, 7–9). In contrast, 100 μM ACh has no significant effect on 125I-Bgtx binding to oocyte membranes containing nAChRs composed of α3 and β4/α1[11]Loop-C subunits. These results show that the functional Bgtx binding site in β4/α1[11]Loop-C-bearing receptors is structurally separate from the agonist binding site. Bgtx must therefore inhibit ACh-evoked responses in β4/α1[11]Loop-C-bearing receptors by an allosteric mechanism, not by steric occlusion of the agonist binding site.

Bgtx Binds to Intact Oocytes Expressing nAChRs Containing the β4/α1[11] Helix Subunit—In contrast to β4/α1[11]Loop-C, which renders nAChRs sensitive to Bgtx inhibition, the chi-

| Subunit cRNAs injected | ACh-evoked response | EC50 for ACh | IC50 for Bgtx |
|------------------------|---------------------|--------------|---------------|
|                        | μA                  | μM           | nm            |
| α3 + β4                | 1.0                 | 100 ± 106    | >10,000       |
| α3/α[5]β4              | 4.8                 | 70 ± 106     | 19 ± 3        |
| α3 + β4/α[11]Loop-C    | 3.2                 | 121 ± 4      | 34 ± 3        |
| α3 + β4/α[11]Helix     | 4.4                 | 23 ± 1       | >10,000       |

a Mean value calculated from at least six responses to application of the EC50, concentration of ACh measured in oocytes from at least two different animals.
b Data from Levandoski et al. (12).

Table I: Oocyte expression of pharmatope-tagged nAChRs

![Fig. 2](image-url)
Pharmatope-mediated Allosteric Block of Neuronal nAChRs

FIG. 3. Binding of 125I-Bgtx to membrane preparations and competition with ACh. Bgtx binding was measured using either homogenized oocyte membranes from oocytes injected with α3 and β4/α1 [11] cRNAs or Torpedo electric organ membranes. To determine whether ACh competes with 125I-Bgtx, membranes were incubated with 100 μM ACh prior to the addition of 125I-Bgtx. Non-specific (NS) binding to each set of membranes was determined by the addition of 1 μM unlabeled Bgtx to membranes prior to the addition of 125I-Bgtx. The data shown represent triplicate determinations, and the error bars indicate the standard error of the mean.

We have shown that 11 contiguous amino acids from Loop C of the muscle-type α1 subunit have the capability to generate high affinity binding sites for Bgtx when introduced ectopically into the extracellular region of the non-α nAChR subunit, β4. Although insertion of the pharmatope sequence into the Loop C region produces Bgtx binding and functional block, the same pharmatope produces high affinity Bgtx binding but no detectable pharmacological activity when inserted into the N-terminal L1 loop.

The ability of Bgtx to block ACh-evoked responses in nAChRs formed from co-expressed α3 and β4/α1[11]Loop-C subunits (Fig. 2; Table I) is unexpected based on the conventional view of Bgtx as a competitive inhibitor of ACh. Our results suggest that we have created an allosteric inhibitory site in β4. Furthermore, our findings are entirely consistent with structural considerations based on the crystal structure of the ACh-binding protein (Protein Data Bank accession code 1I9B), a homo-pentamer homologous to the extracellular domain of nAChRs (4). Bgtx, the prototype of the α-neurotoxins, is a competitive inhibitor at the agonist binding site, which is situated at the subunit-subunit interface of the α subunit (1, 2, 7−10, 13). As revealed in the crystal structure of the ACh-binding protein, the agonist binding site consists of three loops, A, B, and C, from the principal (+) face of the α subunit and three loops, D, E, and F, contributed from the complementary (−) face of the adjacent subunit (e.g. γ, ε, or δ in the case of the muscle nAChR or the β4 subunit, as in this study). The introduction of a Bgtx-binding pharmatope into Loop C of the β4 subunit would be expected to direct Bgtx binding to the (+) face of the β4 subunit and diametrically away from the agonist binding site located at least one subunit interface away at the (−) face of the β4 subunit (Fig. 5). The distance from one subunit interface to the next, along the circumference of the extracellular domain at the level of Loop C, is predicted to be ~40−50 Å. In comparison, Bgtx extends ~40 Å in its longest dimension and, therefore, is incapable of interacting simultaneously with the pharmatope-tagged β4 Loop C and with Loop C of the α3 subunit. Consequently, no direct competitive interaction between ACh and Bgtx is expected based on the current structural model. The physical separation of the Bgtx binding site from the agonist binding site in β4/α1[11]Loop-C-bearing nAChRs offers the advantage that the full range of amino acid mutations can now be used to study the contribution of individual amino acids in Loop C to Bgtx binding. This has been technically difficult due to the overlap in determinants for Bgtx and agonist binding (19). In addition, mutational strategies can be used to improve further the affinity of Bgtx for the ectopic binding site.

The proposed structural relationship of the Bgtx binding site relative to the agonist binding site in β4/α1[11]Loop-C-bearing nAChRs (Fig. 5) is strikingly reminiscent of the spatial relationship between the allosteric regulatory site in ligand-gated GABA A receptors and the agonist (GABA) binding site. Nicotinic and GABA A receptors belong to the same superfamily of Cys-loop ligand gated ion channels and share many structural features (1, 4, 20). In GABA A receptors, so-called inverse agonists of the β-carboline drug class bind at a subunit interface that is also responsible for the binding of the classic allosteric regulator, benzodiazepine (4, 20−24). Binding of benzodiazepine to this site potentiates the action of the neurotransmitter GABA, whereas binding of inverse agonists to the same site diminishes responsiveness to GABA, but not in a competitive manner.

Current GABA A receptor models suggest that the inverse agonist binding site is at a subunit interface distinct from the GABA binding site and is formed by the Loop C region at the (+) face of the GABA A α subunit (e.g. α1) and the (−) face of the adjoining γ subunit (e.g. γ2 in the case of α1β2γ2, the most abundant GABA A receptor subtype in rat brain) (20, 21). Meanwhile, the (−) face of the α subunit contributes to the adjoining GABA A binding site in a manner homologous to the predicted contribution of the (−) face of the nicotinic β4 subunit to the ACh binding site in neuronal nAChRs (4, 20−24). The exact mechanism by which binding of inverse agonists to the benzodiazepine site in GABA A receptors produces inhibition of GABA-evoked currents is unknown, but a decrease in the frequency of GABA-evoked channel openings has been observed (25). The structural similarities between nicotinic and GABA A receptors suggest a mechanism common between the action of inverse agonists on GABA A receptors and the inhibition produced by Bgtx on β4/α1[11]Loop-C-bearing nAChRs. Furthermore, although several nicotinic potentiating ligands with allosteric characteristics have been described, their sites of action remain to be fully elucidated (26, 27). Our findings, together with analogies with the benzodiazepine site, would suggest that nicotinic non-α subunit interfaces should receive more attention as potential targets for drug discovery.

What is the molecular mechanism responsible for the allosteric inhibition (Figs. 2 and 3) observed with chimeric β4/α1[11]Loop-C-bearing nAChRs? Based on the types of structural consider-
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FIG. 4. Binding of Alexa Fluor 647®-Bgttx to intact oocytes expressing chimeric nAChRs. Intact oocytes expressing α3 and β4/α1[11]Loop-C subunits (A) or α3 and β4/α1[11]Helix subunits (B and C) were incubated overnight with Alexa Fluor 647®-Bgttx. Unbound Bgttx was removed by five buffer exchanges, and the oocytes were immediately examined by confocal scanning fluorescence microscopy. Nonspecific binding (C) was determined by the addition of 1 μM Bgttx prior to the addition of the fluorescent-Bgttx conjugate. Similar nonspecific binding was observed with oocytes expressing α3 and β4/α1[11]Loop-C subunits and was comparable with the total binding observed with uninjected oocytes.

FIG. 5. Model of subunit arrangement in nAChRs composed of two α3 and three β4/α1[11]Loop-C subunits. The view shown here is looking down onto the extracellular domain of the receptor from the extracellular space. The (+) indicates the principal face of the ACh binding site as demarcated by Loop C, whereas the (−) refers to the opposing complementary subunit interface. For simplification, only one molecule of Bgttx is shown bound to the receptor. The two classic ACh binding sites per pentameric assembly are illustrated at the two subunit-subunit interfaces flanking the introduced Bgttx binding site at the β4(+)α3(−) interface.

FIG. 6. A model of the proposed dimer interface making up the allosteric Bgttx binding site. The x-ray structure of the AChBP forms the framework for this model (4), and the position of bound Bgttx is based on NMR studies using a Bgttx-binding peptide fragment of the rat α7 homomeric receptor (8). The extracellular domain of the β4/α1[11]Loop-C subunit is shown on the left, contributing its (+) face to the Bgttx binding site, where Loop C, with Bgttx in close proximity below, is shown extending toward the α3 subunit on the right. The vertical stripes in Loop C indicate the approximate position of the introduced pharmatope sequence. The rotating arrows at the top of the figure depict the clockwise direction of subunit rotation that has been observed with Torpedo α1 subunits upon agonist activation (30). The exposed loop, L1, immediately following the N-terminal helical region is highlighted in the β4/α1[11]Loop-C subunit.

At this position) found in all α subunits, and their Loop F regions, located at the (−) face of the subunit interface, are identical in length to that of α subunits. This suggests that neuronal β subunits may be more likely to undergo subunit rotations akin to those seen in muscle-type α subunits. If such rotations are essential for gating, then a wrench-in-the-works-like interference due to a stabilization of the resting state subunit interface by bound Bgttx could be the mechanism underlying the functional blockade mediated by the β4/α1[11]-Loop-C subunit (Fig. 6).

Alternatively, it is possible that Bgttx may prevent rotation of the α3 subunit by stabilizing the resting state β(+)+α(−) interface of chimeric β4/α1[11]Loop-C subunit-containing nAChRs. In either case, the functional blockade of β4/α1[11]Loop-C subunit-containing nAChRs by Bgttx supports the view that rigid body-like subunit movements at subunit interfaces play an important role in receptor gating. Whether such movements...
are restricted to α subunits as suggested previously for muscle-type nAChRs or whether neuronal β subunits also undergo conformational movements of their extracellular domains remains to be fully elucidated.

Previous studies have shown that neuronal nAChRs are pentameric with an expected stoichiometry of two α subunits and three β subunits (33, 34). This would suggest that our β4 chimera generate three potential Bgtx binding sites per receptor: two at the β(+)/α(−) interfaces and one at the β(+)/β(−) interface (Fig. 5). The Hill coefficient of −0.7, estimated from our dose-response analysis, argues that Bgtx needs to bind to only a single site on the receptor complex to produce functional inhibition. We do not presently know whether all three potential Bgtx binding sites per β4/α1[11]Loop-C-bearing receptor are capable of binding toxin and producing blockade. We have observed, however, that β4/α1[11]Loop-C-bearing receptors with α4 subunits substituting for α3 are somewhat less sensitive to Bgtx inhibition, suggesting that the (−) face of the α subunit may directly contribute to the introduced Bgtx binding site. This would suggest that it is binding to the β(+)/α(−) interface that is responsible for the observed functional block.

The successful introduction of a Bgtx-binding pharmatope into the extracellular domain of the β4 subunit suggests that similar modifications can be made to the other neuronal nAChR β subunits, and in preliminary studies, we find that insertion of a pharmatope sequence into Loop C of the rat β2 subunit also imparts functional Bgtx sensitivity. In addition, in those cases in which high affinity Bgtx binding is conferred in the absence of pharmacological sensitivity, such constructs would be of considerable utility for studies of receptor localization and metabolism given the many commercially available reporter group derivatives of Bgtx. The relatively small size of Bgtx (∼8 kDa) as compared with antibodies may offer a further advantage in tissue penetration and accessibility. Finally, our findings offer the possibility that similar pharmatope sequences can be similarly inserted into the extracellular domains of other ligand-gated ion channels or, more broadly, into other membrane proteins for which appropriate probes are lacking.

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2 T. Sanders and E. Hawrot, unpublished observations.

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