Chimeric Analysis of a Neuronal Nicotinic Acetylcholine Receptor Reveals Amino Acids Conferring Sensitivity to α-Bungarotoxin*

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† The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; αBgtx, α-bungarotoxin; α3/α1[5], α3 chimeric subunit with the substitutions Y184W, E197W, I188V, K189Y, and N191T; α3/α1[4], α3 chimeric subunit with the substitutions Y184W, E187W, I188V, and N191T; α3/α7[6], α3 chimeric subunit with the substitutions Y184K, K185R, H186N, I188K, K189F, and N191E; α1/α4[2], α1 chimeric subunit with the substitutions V188R and F189K; α3/α1[16], α3 chimeric subunit with residues 177–199 of α3 replaced with the homologous region from Torpedo α1; αBgtx, α-bungarotoxin; NmmI, N. mossambica mossambica toxin I; EC50, the concentration of agonist giving 50% of the maximal response; IC50, the concentration of antagonist that blocks 50% of the control response.

We have investigated the molecular determinants responsible for α-bungarotoxin (αBgtx) binding to nicotinic acetylcholine receptors through chimeric analysis of two homologous α subunits, one highly sensitive to αBgtx block (α1) and the other, αBgtx-insensitive (α3). By replacing rat α3 residues 184–191 with the corresponding region from the Torpedo α1 subunit, we introduced a cluster of five α1 residues (Trp-184, Trp-187, Val-188, Tyr-189, and Thr-191) into the α3 subunit. Functional activity and αBgtx sensitivity were assessed following co-expression in Xenopus oocytes of the chimeric α3 subunit (α3/α1[5]) with either rat β2 or β4 subunits. Agonist-evoked responses of α3/α1[5]-containing receptors were blocked by αBgtx with nanomolar affinity (IC50 values: 41 nM for α3/α1[5]β2 and 19 nM for α3/α1[5]β4). Furthermore, receptors containing the single point mutation α3K189Y acquire significant sensitivity to αBgtx block (IC50 values: 186 nM for α3K189Yβ2 and 179 nM for α3K189Yβ4). Another α3 chimeric subunit, α3a7[6], similar to α3a[5] but incorporating the corresponding residues from the αBgtx-sensitive α7 subunit, also conferred potent αBgtx sensitivity to chimeric receptors when co-expressed with the β4 subunit (IC50 value = 31 nM). Our findings demonstrate that the residues between positions 184 and 191 of the αBgtx-sensitively α3 subunit α1 and α7 play a critical functional role in the interaction of αBgtx with nicotinic acetylcholine receptors sensitive to this toxin.

Nicotinic acetylcholine receptors (nAChRs)† are multimeric ligand-gated ion channels expressed on skeletal muscle cells and on select groups of nerve cells in the peripheral and central nervous systems (1–3). Muscle nAChRs have pentameric structures made up of two α1 subunits and one each β1, γ, and δ (or ε) subunits; they are among the best characterized ion channels and serve as a model for understanding the structure and function of related ligand-gated channels responding to glycine, γ-aminobutyric acid, and 5-hydroxytryptamine (4). Advances in the characterization of muscle nAChRs have been significantly aided by the discovery of a high affinity competitive antagonist, α-bungarotoxin (αBgtx). αBgtx is used extensively in experiments on the molecular properties of nAChRs and for following expression, targeting, and clustering of these receptors on muscle during synapse formation (1–4). Much less is known about nAChRs on neurons, in part because comparable antagonists are in limited quantity or are nonexistent. The purpose of this paper is to define the amino acid residues that are essential for high affinity αBgtx binding through a chimeric subunit approach, by conferring αBgtx sensitivity to a neuronal α subunit that is normally insensitive to αBgtx.

Previous work indicates that the main αBgtx binding site is between residues 173 and 204 on the α1 subunit of the muscle nAChR. Specifically, studies of peptides derived from the Torpedo α1 sequence capable of binding αBgtx with sub-micromolar affinity suggest that the major determinants of toxin binding are located in a region adjacent to the vicinal cysteines 192 and 193 (e.g., see Ref. 5). Recent studies of heterologously expressed muscle nAChRs have identified residues in this region of the native receptor that appear to interact with the short α-neurotoxin I from Naja mossambica mossambica (Nmml, 6, 7)) and with αBgtx (8, 9). Residues in this region are also involved in forming the binding sites for agonists and non-α-neurotoxin antagonists (6, 7, 10–12). In such studies, single-site mutations in the muscle type α1 subunit have not been very helpful in fully defining the α-neurotoxin binding site in the native nAChR, as most mutations studied fail to produce large changes in αBgtx affinity (6–9). Therefore, in this study, rather than eliminate αBgtx binding, we have used site-directed mutagenesis of a neuronal nAChR to introduce a toxin binding site. As a consequence, we identified the molecular determinants responsible for αBgtx binding to nAChRs.

Eleven different genes encode neuronal nAChR subunits (1–3): eight α subunits genes (α2–α9) and three β subunits (β2–β4). Sequence homology demonstrates that all muscle and neuronal nAChR subunits share a common structural motif; each has four hydrophobic, putative membrane-spanning domains and a long extracellular amino terminus that contains invariant cysteines at positions 128 and 142 (α1 subunit numbering). In addition, all neuronal α subunits contain the tandem cysteines at residues 192 and 193. Functional expression studies demon-
a1 Subunit Residues Conferring α-Bungarotoxin Sensitivity

175 180 185 190 195 200

| αbungtoxin Sensitivity | α1 | α2 | α3 | α4 |
|------------------------|----|----|----|----|
| + + + +                | + | + | + | + |

**TABLE 1. Summary of αbungtoxin Sensitivity for Various α1 Subunit Constructs.** The table shows the sensitivity of various α1 subunit constructs to αbungtoxin. The constructs are denoted as α1, α2, α3, and α4. The sensitivity levels are indicated as + or ++.

**FIG. 1. Sequence comparison of αbungtoxin-sensitive α1 and αbungtoxin-insensitive α3 nAChR subunits.** The sequence comparison highlights the residues responsible for αbungtoxin binding based on the substituted cysteine accessibility analysis (5). Underlined residues were found to give rise to intermolecular nuclear Overhauser effect signals in a complex formed between αbungtoxin and an α1 subunit-derived dodecapeptide (23).

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Unless otherwise noted, all chemicals were reagent grade from Sigma. αbungtoxin was from Research Biochemicals Inc. (Natick, MA).

**chimeric α3 Subunit Constructs**—Vectors bearing the cDNA genes for the rat α3, β2, and β4 nAChR subunits (in the pcDNA1neo background; Invitrogen, Carlsbad, CA) were gifts of P. Seguela and J. Patrick; pGEM-HE-based plasmids with flanking Xenopus β-globin untranslated sequences were gifts of C. Luetje. The pSPET vectors encoding the Torpedo α1, β1, γ, and δ subunits were gifts of T. Claudio. The pcDNA1neo-α3 plasmid was digested with HindIII and NotI to generate an ~1900-base pair α3 fragment that was subcloned into pcDNA3.1neo + (Invitrogen). All chimeras were generated with the QuickChange (Stratagene, La Jolla, CA) polymerase chain reaction mutagenesis strategy. The α3/α1[15], α3/α7[6], α1/α4[2], and α3K189Y constructs were prepared using pcDNA3.1neo +α3 to template and paired, fully complementary mutagenic primers (synthesized by Life Technologies, Inc.). The α3/α1[16] construct was produced in two steps; the five residues following Cys-193 were first changed using pcDNA3.1neo +α3/α1[15] as a template; a second round of mutagenesis substituted the remaining six residues. The α3/α1[4] construct was made by a Y189K point mutation of the α3/α1[5] subunit. Mutagenic and sequencing primer sequences are available upon request.

**RESULTS AND DISCUSSION**

We prepared four chimeric α3 subunit constructs, focusing on the region between residues 177 and 199. Residues from the αbungtoxin-sensitive Torpedo α1 subunit was substituted for the corresponding residues in the rat α3 subunit and co-expressed with the appropriate non-α subunits (β2 or β4) in Xenopus oocytes. The α3/α1[5] chimera, with the residue changes Y184W, E187W, I188V, K189Y, and N191T, and the parent mutant α3K189Y both expressed well, producing robust ACh-evoked currents with little apparent deleterious effect on ACh sensitivity. As indicated in Table I, the EC50 values for ACh of the various chimeric receptors co-expressed with β2 or β4 subunits, determined from dose-response titrations, are either the same as or less than those for the wild type combinations of α3β2 and α3β4. For α3 chimeric subunits co-expressed...
TABLE I

| Subunit combination | EC<sub>50</sub> for ACh (μM) | IC<sub>50</sub> for αBgtx (nM) | Half-time of recovery (min) |
|---------------------|-----------------------------|-----------------------------|---------------------------|
| α3/α1[5]β2         | 80 ± 40 (n = 4)             | 41 ± 9                      | 24 ± 3                    |
| α3/α1[5]β4         | 70 ± 10 (n = 6)             | 19 ± 3                      | 18 ± 1                    |
| α3K189Yβ2          | 50 ± 20 (n = 7)             | 185 ± 42                    | 1.0 ± 0.2                 |
| α3K189Yβ4          | 230 ± 20 (n = 5)            | 179 ± 59                    | 1.0 ± 0.1                 |
| α3/α1[4]β4         | 450 ± 50 (n = 6)            | 31 ± 2                      | ND                        |
| α3/α1[4]β2         | 40 ± 1 (n = 5)              | >50,000                     | NA                        |
| α3β2               | 70 ± 6 (n = 7)              | >100,000                    | NA                        |
| α3β4               | 100 ± 10 (n = 6)            | >100,000                    | NA                        |

Values reported are ±S.E. as determined by fitting to appropriate equations (described under “Experimental Procedures”) using Origin 5.0 software. IC<sub>50</sub> values for α3/α1[4]β4, α3β2, and α3β4 are limits as determined by control experiments using 1.5 μM (for α3/α1[1]β4) or 10 μM αBgtx (for α3β2 and α3β4). ND, not determined; NA, not applicable.

Properties of chimeric and wild type α3 receptors

with the β2 subunit, the EC<sub>50</sub> values ranged from 50–80 μM, in good agreement with the range 10–150 μM previously reported for wild type α3β2 (17–21); for the chimeras co-expressed with β4, the values ranged from 70–230 μM compared with 100–220 μM reported for wild type α3β4 (15, 17, 18, 20). Increases in agonist sensitivities have also been noted by Luetje and co-workers for several nAChR chimeras (17, 18). Because the EC<sub>50</sub> values we measured for the various chimeric subunits are in the ranges previously reported for the wild type combinations, we conclude that these substitutions cause no major structural perturbations to the receptors.

We also constructed the α3/α1[16] chimeric subunit (see Fig. 1) in which a more extensive region of Torpedo α1 (residues 177–199) was substituted into α3. No expression of this construct was observed following co-injection with either β2 or β4 subunit genes (46 oocytes from 5 frogs). High concentrations of ACh, dimethylphenylpiperazinium, and cytisine failed to evoke currents in these oocytes, suggesting that the additional substitutions in the α3/α1[16] chimera adversely affected subunit folding and/or assembly and membrane targeting. This phenomenon is not well understood but has been observed for other chimeric nAChRs in which residues of the amino-terminal extracellular domain were exchanged (17, 22).

Five Residues from the α1 Subunit Confer αBgtx Sensitivity to α3—We tested whether the five homologous residues of the Torpedo α1 sequence adjacent to the tandem cysteines 192–193 were sufficient when substituted into the rat α3 sequence to confer sensitivity to αBgtx on these chimeric nAChRs. As shown in Fig. 2A, following an initial co-application of 100 μM ACh and 15 nM αBgtx and further incubation in toxin between successive test co-applications, the evoked currents of an oocyte expressing the α3/α1[5]β4 combination were greatly reduced over a 15-min period. In contrast, the ACh-evoked currents from an oocyte expressing the wild type α3β4 combination showed no block after a 10-min incubation with 1 μM αBgtx (Fig. 2B). In other experiments we confirmed that wild type α3β2 and α3β4 receptors show no block of ACh-evoked currents following incubations for up to 30 min in 10 μM αBgtx (data not shown).

**Fig. 2. ACh-evoked currents in oocytes expressing wild type and chimeric α3 subunits.** A, individual current traces are shown superimposed for an oocyte expressing the α3/α1 chimeric subunit in combination with rat β4. The control responses to a 6-s pulse of 100 μM ACh were recorded with the two-electrode voltage clamp method at a membrane potential of −60 mV. At time 0, a co-application of 100 μM ACh + 15 nM αBgtx was begun, and the response was recorded. Between subsequent co-applications at the indicated times, the cell was maintained in 15 nM αBgtx. B, an oocyte expressing the wild type α3β4 nAChR was challenged with a control 6-s pulse of 100 μM ACh, and the response (left trace) was recorded. After a 10-min incubation in 1 μM αBgtx, the cell was again challenged with 100 μM ACh; the post-toxin response (right trace) is offset on the time axis for clarity, and the arrows indicate onset of ACh application. C, data from co-application experiments as described in A above for chimeric receptors α3/α1[5]β2 (open squares, n = 6 cells) and α3/α1[5]β4 (open circles, n = 3 cells) are shown as plots of 1 − I<sub>co-application/I</sub>control (fractional block) versus time in 15 nM toxin, where I<sub>co-application</sub> is the peak response evoked by the co-application at that time point. Curves represent fits to a bimolecular association with apparent association constants of 1.4 ± 0.1 × 10<sup>5</sup> M<sup>−1</sup>s<sup>−1</sup> for α3/α1[5]β2 and 1.7 ± 0.3 × 10<sup>5</sup> M<sup>−1</sup>s<sup>−1</sup> for α3/α1[5]β4. Those measured for association of αBgtx with muscle type receptors (24, 25). The plateau value of −0.6 for the two data sets shown in Fig. 2C represents the maximal block achievable with 15 nM αBgtx. Operationally, these data also show that for receptors incorporating α3/α1[5] chimeric subunits, an incubation time of 10 min is sufficient to approach full equilibration for αBgtx concentrations in excess of 15 nM.

The concentration dependence of αBgtx block of chimeric α3/α1[5]-containing receptors was determined following a 10-min incubation with αBgtx as shown in Fig. 3. The solid curves represent the best fit to the logistic equation (16), from which...
The IC50 values obtained from these data are given in Table I. Responses to the test dose of ACh were measured in rapid succession under voltage clamp (i.e. block measured in the presence of toxin), the fractional block obtained after 10 min of toxin incubation was identical to that obtained by extrapolating the recovery data. The IC50 values for the Bgtx block of the subunit combinations 3K189Y4 (179 ± 59 nM) and 3K189Yβ2 (186 ± 42 nM) were about an order of magnitude greater than those of the α3/α1[5] receptors and again were not significantly affected by which of the two β subunits was used for co-expression. Considering that the IC50 values of Bgtx block of wild type α3β2 and α3β4 receptors must be greater than 100 μM (see Table I), the sensitivity to block of these α3K189Y chimeric receptors represents an increase in affinity of approximately 3 orders of magnitude or more compared with the wild type α3.

The observation that the single amino acid substitution α3K189Y results in considerable Bgtx affinity demonstrates a central role for position 189 in mediating Bgtx interactions. However, as both the α3/α1[5] and α3K189Y chimeric receptors fall short of the affinity for Bgtx characteristic of wild type Torpedo nAChR (cf. 24, 27), other residues are also likely to contribute to high affinity binding in α1 subunit-containing nAChRs. On the basis of double mutant cycle analysis, Taylor and co-workers (7) suggest that residues Val-188, Tyr-190, and co-workers (7) suggest that residues Val-188, Tyr-190, Phe-189, and Pro-194 (studied in α2 receptors) contribute to high affinity binding in α1 subunit-containing nAChRs. The on/off recovery period using 4–8 peak current measurements out/recovery period using 4–8 peak current measurements out/recovery period using 4–8 peak current measurements. 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**Differing Affinities of α3/α1(5) and α3K189Y nAChRs for αBgtx**

As shown above, the rate constants of association of αBgtx to α3/α1(5)[β2] and α3/α1(5)[β4] chimeric receptors were very similar to that for the association of αBgtx with the muscle type of nAChR (cf. 24, 25). This suggests that the difference in affinities for αBgtx between α3/α1(5) receptors and muscle type nAChRs is due to a difference in dissociation rates. To test this, we carried out measurements of the time course of recovery from toxin block as an indicator of toxin dissociation. After a 10-min incubation with αBgtx, oocytes were continuously perfused with buffer lacking toxin and periodically challenged with the test dose of ACh. Representative results are presented in Fig. 4, where the fraction of maximal block ([I(1) - I] / [I(0) - I]) is plotted as a function of the time of washout. Single exponential fits (solid curves through data points) revealed the following half-times (t1/2) for recovery: 233 min for Torpedo α1βγδ, 18 min for α3/α1(5)[β4], and 1.0 min for α3K189Yβ4. The same ~20-fold difference in t1/2 values for α3/α1(5) and α3K189Y was observed for β2-containing chimeric receptors (Table 1). In all cases, the differences in the IC50 values of αBgtx block demonstrated in Fig. 3 correlate well with the rates of recovery from block obtained for the chimeric receptors.

**Modularity of αBgtx Binding Sequences**

The cysteine residues at positions 192 and 193 are invariant in all nAChR α subunits, and the Gly at position 183 is also highly conserved, occurring in 65% of known sequences. On this basis we postulated that the region 183–193 is structurally conserved in nAChR α subunits and that it may be modular with respect to αBgtx binding. We tested this by preparing another chimeric subunit in which the residues of α3 in the region 183–193 were substituted with those of the αBgtx-sensitive rat α7; this yielded the α3α7[6] construct. Fig. 5 shows a further comparison of some αBgtx-sensitive and αBgtx-insensitive α subunits in this region. Note that the residues that are not invariant or highly conserved among all α subunits in this region, only position 189 is well conserved (either Tyr or Phe) in αBgtx-sensitive subunits.

We found that the α3α7[6] chimera, when co-expressed with the rat β4 subunit, was highly sensitive to block by αBgtx. As shown in Fig. 6, ACh-evoked currents of oocytes expressing the α3α7[6][β4] combination were blocked by αBgtx concentrations in the nanomolar range (IC50 = 31 ± 2 nM) using a 10-min toxin incubation. This is similar to the apparent affinity of αBgtx for the α3α1(5) chimera co-expressed with either the β2 or β4 subunit. The α3α7[6] chimera has an apparent affinity for αBgtx about 1 order of magnitude lower than that of wild type α7 receptors (29, 30). Although the residues 183–193 form a high affinity αBgtx binding unit that is modular in the sense that it can be substituted into the background of insensitive subunits to confer binding, amino acids elsewhere in the sequence must also contribute to give wild type affinity. In comparing α1, α3, and α7 sequences in the region 183–193, position 189 stands out as being most likely to determine the αBgtx sensitivity of α1 and α7 receptors. It is possible and perhaps likely that αBgtx interacts with α1 and α7 nAChRs in subtly different ways, but our results suggest that the core of these interactions is mediated by residues 183–193 and that an aromatic ring at position 189 is an important feature in αBgtx recognition.

**Chimeric Mouse α1 with Val-188 and Phe-189 Replaced by Their α4 Counterparts**—Chemical modifications of a substituted cysteine have suggested that Phe-189 of the α1 subunit is in the proximity of the αBgtx binding site in native mouse muscle nAChRs (8). In contrast, mutation of Phe-189 to Lys leads to less than a 3-fold reduction in the apparent dissociation constant for NmmI (6). One interpretation of these results, together with those presented here, is that position 189, although playing an important role in αBgtx binding, may not be as critically involved in the recognition of the short α-neurotoxin NmmI. Because Val-188 has been suggested to contribute to contacts with NmmI (7), we constructed a double mutant of the mouse muscle α1 subunit (α1α4[2], see Fig. 5) in which Val-188 is replaced with the positively charged residue Arg (as in the rat α4 subunit), and Phe-189 is replaced with Lys (as in the rat α4 and α4 subunits). Ackermann and Taylor (6) show that the introduction of a positive charge at position 188 (V188R) of the mouse α1 subunit leads to a 20-fold reduction in affinity for 1 of the 2 neurotoxin binding sites (that associated with the αδ subunit interface) and a 390-fold reduction in affinity for the other (at the αγ interface). NmmI has the unique characteristic among α-neurotoxins of being able to discriminate between the two neurotoxin binding sites in the Torpedo nAChR (31). We reasoned that the effect of a V188R
The fraction of control response after block by α-Bgtx for three chimeric subunits was measured as described in Fig. 3, and the data (I_{post-toxin}/I_{pre-toxin}) are plotted as a function of α-Bgtx concentration. The α1/α4[2] subunit was co-expressed with the mouse β, γ, and δ subunits (upward triangles), and 100 μM AcCh was used to elicit response; the IC_{50} characterizing this block was 7 ± 0.5 nM. In similar studies of α-Bgtx block of oocyte-expressed mouse muscle (α188γδ) receptors, an IC_{50} value of 2.4 nM was observed (27). The α3α7[6] subunit was co-expressed with the rat β4 subunit (downward triangles), and 400 μM AcCh was used to elicit response; the IC_{50} characterizing this block was 31 ± 2 nM. Currents evoked with 100 μM from oocytes expressing the α3/α3[14]β4 combination (×) were not blocked by α-Bgtx at a concentration of 1.5 μM. Data points represent averages (± S.E.) for 3–6 different oocytes at each α-Bgtx concentration.

mutation should be similar to V188K, allowing us to analyze the results in terms of a direct functional comparison between the mouse α1 subunit and the α-Bgtx-insensitive rat α4 subunit. If the two residues Val-188 and Phe-189 are indeed important for α-Bgtx binding, the double mutation V188R and F189K in α1/α4[2] would be expected to produce a very dramatic reduction in α-Bgtx affinity. As shown in Fig. 6, upon co-expression with the mouse β, γ, and δ subunits, the α1/α4[2] chimera gave rise to a receptor that remained very sensitive to α-Bgtx block. With an IC_{50} value of 7 ± 0.5 nM, the sensitivity to α-Bgtx of receptors containing the α1/α4[2] chimera was reduced only about 3-fold compared with wild type mouse α1βγδ receptors (27). This is much less than would be predicted based on the binding studies with Nmml toxin (6) if one assumes that the same residues are recognized by both α-neurotoxins.

First of all, our results suggest a fundamental difference in the role of positions 188 and 189 in α-Bgtx binding and in AcCh binding. The conclusion that α-Bgtx and Nmml differ substantially in their modes of interaction with mouse muscle NmmlRs receives further support from the recent study of Osaka et al. (32). These authors report that Glu-176 of the γ subunit comes into close apposition with Lys-27 of Nmml, and that positions 175 and 176 of the γ and δ subunits contribute to the high affinity of the binding sites at the αγ and αδ interfaces. These conclusions were based on the observation that the homologous residues of the ε subunit, Thr and Ala, conferred 1000-fold lower Nmml affinity to the mouse α1βεδ NmmlR. Of most relevance to the present study, Osaka et al. (32) show that the on-rate of α-Bgtx association to α1βεδ was reduced only about 4-fold compared with mouse α1βγδ receptors (with no significant effect on off-rate), in marked contrast to the dramatic effects observed for the Nmml interaction. Although Lys-27 in Nmml appears to play a very important role in binding to muscle NmmlRs (6, 7, 32), the removal of the positive charge from the corresponding residue in α-Bgtx by the K26A substitu-
tion leads to only a 10-fold reduction in α-Bgtx affinity (27). Furthermore, α-Bgtx blocks homo-oligomeric neuronal α7 receptors with high affinity, whereas short α-neurotoxins show greatly diminished activity on these receptors (33). In combination, these results suggest significant differences in the molecular basis of binding to NmmlRs for the short and long α-neurotoxins (34).

A second conclusion derived from the results with the α1/ α4[2] chimera is that the introduction of a positively charged side chain and the concomitant removal of the aromatic side chain at position 189 cannot alone account for the marked α-Bgtx insensitivity of the α3 and α4 subunits. This contrasts sharply with our demonstration here that the reciprocal mutation at position 189 in the α3 background (i.e. α3K189Y) leads to a dramatic enhancement of α-Bgtx sensitivity of more than 2 orders of magnitude, from an IC_{50} ≥ 100 μM to ~0.2 μM (See Fig. 3 and Table I). In the α1 background, it is possible that multiple alternative contacts with α-Bgtx can accommodate and mitigate the effects of single-site mutations such as F189K, making the α-Bgtx-nmmlR interface effectively over-determined. Differences in the orientations of introduced side chains due to differences in the local environment of the neighboring sequence may also contribute to the apparent nonreciprocal nature of the amino acid substitutions studied at position 189. In any case, the use of the α3 subunit as a background allows for the sensitive detection of residues that contribute to α-Bgtx binding in the α1 subunit. Additional substitution studies will be needed to test whether the major effect of the K189Y mutation in α3 is due to the introduction of an aromatic side chain allowing favorable interactions to occur or due to the removal of a positive charge that interferes with toxin-receptor association. Also, the further application of this approach to investigate the role of other residues divergent in the α1 and α3 sequences should allow a full description of such α residues that directly contribute to α-Bgtx recognition.

**General Implications for α-Bgtx Binding to nAChRs**—Our results with a homologous substitution analysis utilizing an α-Bgtx-insensitive α3 subunit background clearly indicate an important role for residues 184–191 in mediating α-Bgtx recognition for native nAChRs. It is unlikely that the α3 mutations studied here cause gross structural alterations that somehow permit aberrant α-Bgtx binding, given that the chimeric receptors all have EC_{50} values for AcCh activation in the same range as those previously determined for the wild type α3β2 or α3β4 combinations. The studies reported here also provide important support for the physiological relevance of the NMR-based structure of the complex formed between α-Bgtx and a dodecapeptide corresponding to α1 residues 185–196 (23). Tyr-189 was among 5 receptor residues found to be in close contact with α-Bgtx in this slow exchange protein-peptide complex.

The results reported here also demonstrate the benefit and desirability of carrying out reciprocal mutant and chimeric analyses in the study of ligand binding sites. For large ligands with high affinity like α-Bgtx, the intermolecular interactions are probably over-determined, and such redundant interactions may mask the important contributions of individual residues. In addition to mutagenesis aimed at eliminating ligand binding, reciprocal mutations designed to introduce a gain of function such as ligand binding are critical to a complete understanding of ligand-receptor interactions.

The α-Bgtx-sensitive α3α7[5] chimeric subunit may also prove useful in the analysis of endogenous nAChRs whose subunit compositions remain obscure (1, 2). Over-expression of this chimera in appropriate cells should help identify the physiological properties of neuronal nAChRs containing α3 subunits. A similar approach should also be feasible with α4 and
6 subunits. In any event, further studies utilizing the chimeric approach, single-site mutagenesis, and double mutant cycle analysis will be required to fully elucidate the basis of the remarkable affinity and selectivity of αBgtx.

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