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Glycosylation within the Cysteine Loop and Six Residues Near Conserved Cys192/Cys193 Are Determinants of Neuronal Bungarotoxin Sensitivity on the Neuronal Nicotinic Receptor α3 Subunit

CHARLES W. LUETJE, FLOYD N. MADDOX, and SCOTT C. HARVEY[1]

Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, Florida 33101

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ABSTRACT

Neuronal bungarotoxin (NBT) is a highly selective, slowly reversible, competitive antagonist of the $\alpha3\beta2$ neuronal nicotinic receptor. Contributions to NBT sensitivity are made by both the $\alpha3$ and $\beta2$ subunits. We used a chimeric α subunit to demonstrate that the entire $\alpha3$ contribution lies within sequence segment 84–215. Construction and analysis of a series of mutant $\alpha3$ subunits identified seven amino acid residues (Thr143, Tyr184, Lys185, His186, Ile188, Gln198, Ser203) within this region that contribute to NBT sensitivity. Changing Thr143 to lysine, as in $\alpha2$, resulted in a ~1000-fold loss of NBT sensitivity. The effect on NBT sensitivity of changing each of the other six residues ranged from 1.8- to 40.5-fold. More extensive mutagenesis demonstrated that Thr143 serves as part of the consensus sequence for glycosylation at N141, and it is this glycosylation that is the determinant of NBT sensitivity. Only serine could substitute for threonine to maintain full NBT sensitivity, and changing Asn141 to alanine resulted in a ~300-fold loss of NBT sensitivity. The chimera $\alpha2$–181-$\alpha3$, containing all identified determinants except the glycosylation site, formed receptors insensitive to 300 nM NBT. Installation of threonine to complete the glycosylation consensus site in this chimera conferred NBT sensitivity only 10-fold less than that of wild-type $\alpha3\beta2$. These seven determinants of NBT sensitivity are located in close proximity to a series of conserved residues that are common features of all nicotinic receptor binding sites.

nAChRs are found throughout the central and peripheral nervous systems. Although nAChRs are responsible for rapid neurotransmission in the periphery, these receptors seem to play a modulatory role in the central nervous system (Gray *et al*., 1996; Role and Berg, 1996). Neuronal nAChRs form in a manner similar to muscle nAChRs, as a pentameric assembly of subunits (Anand *et al*., 1991; Cooper *et al*., 1991). These receptors assemble from various combinations of ≥11 distinct subunits, $\alpha2$–9 and $\beta2$–4 (Sargent, 1993; Elgoyhen *et al*., 1994). The resulting nAChRs differ pharmacologically and biophysically, depending on subunit composition (Role, 1992).

The ligand binding sites of muscle type nAChRs are formed at the interface between α and non-α (γ and δ) subunits (Karlin and Akabas, 1995). The ligand binding sites of neuronal nAChRs seem to be formed in a similar fashion; both α and β subunits contribute to the pharmacological properties of these receptors (Duvoisin *et al*., 1989; Luetje and Patrick, 1991). Affinity labeling techniques have identified amino acid residues on the α, γ, and δ subunits of muscle-type nAChRs that contribute to the structure of the ligand binding sites (Karlin and Akabas, 1995). These residues are highly conserved among neuronal nAChR subunits and thus are features of nicotinic binding sites common to all nAChRs. These conserved residues cannot account for the pharmacological diversity that exists among neuronal nAChRs. These differences must be due to residues that differ among neuronal nAChR subunits. As an approach to the identification of residues responsible for conferring pharmacological differences, we constructed and analyzed chimeras of pharmacologically distinct subunits and then used site-directed mutagenesis to identify residues that confer specific pharmacological features on neuronal nAChRs (Luetje *et al*., 1993; Harvey *et al*., 1996, 1997; Harvey and Luetje, 1996).

NBT, a dimeric protein toxin formed by 66 residue monomers, is isolated from the venom of *Bungarus multicinctus*.

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[1] Current affiliation: Eli Lilly and Co., Lilly Corporate Center, Indianapolis, IN 46285.

ABBREVIATIONS: ACh, acetylcholine; DHβE, dihydro-β-erythroidine; nAChR, nicotinic acetylcholine receptor; NBT, neuronal bungarotoxin; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

NBT is a highly selective, slowly reversible, competitive antagonist of the $\alpha3\beta2$ neuronal nAChR subunit combination. Changing either the α or β subunit results in a dramatic loss in NBT sensitivity (Duvoisin *et al*., 1989; Luetje *et al*., 1990). This high degree of specificity makes NBT an ideal probe for studying the ligand binding sites of neuronal nAChRs. We previously used NBT to identify Thr59 of the $\beta2$ subunit as a critical determinant of competitive antagonist sensitivity (Harvey and Luetje, 1996). We also used NBT to identify three distinct sequence segments on the $\alpha3$ subunit (84–121, 121–181, 195–215) that contain determinants of competitive antagonist sensitivity (Luetje *et al*., 1993). Each of these regions was found to be necessary for full NBT sensitivity. The 121–181 and 195–215 regions were particularly critical because chimeric subunits lacking either of these regions formed receptors that were insensitive to high concentrations of NBT. We now show that residues lying within segment 84–215 of $\alpha3$ are entirely sufficient to confer full NBT sensitivity on the $\alpha2$ subunit. We also use site-directed mutagenesis to identify seven residues within this region that contribute to NBT sensitivity. One of these residues, Thr143, fulfills its role as a determinant by serving as part of the consensus sequence for glycosylation at Asn141.

Experimental Procedures

Materials. *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI). The care and use of *X. laevis* frogs in this study were approved by the University of Miami Animal Research Committee and meet the guidelines of the National Institutes of Health. RNA transcription kits were from Ambion (Austin, TX). ACh, atropine, and 3-aminobenzoic acid ethyl ester were from Sigma Chemical (St. Louis, MO). Collagenase B was from Boehringer-Mannheim (Indianapolis, IN). Sequenase 2.0 kits were from Amersham Life Sciences (Cleveland, OH). NBT was from Biotoxins (St. Cloud, FL). CloneAmp kits were from GIBCO BRL (Gaithersburg, MD).

Mutagenesis and construction of chimeric receptors. Chimeric and mutant subunits were constructed using PCR (Higuchi, 1990). Our notation for mutant subunits is to list the naturally occurring residue followed by the position of that residue and then followed by the change that has been made. For example, the mutant subunit $\alpha3$, I188K is an $\alpha3$ subunit in which Ile188 has been changed to a lysine. PCR products were subcloned into the pAMP1 vector using a CloneAmp kit. To minimize the amount of PCR product in the final construct that would have to be sequenced, as much PCR product as possible was replaced with appropriate wild-type sequence using existing restriction sites. Remaining sequence derived from PCR product was confirmed by sequencing using Sequenase 2.0.

Injection of *in vitro* synthesized RNA into *X. laevis* oocytes. $m^7G(5')ppp(5')G$ capped cRNA was synthesized *in vitro* from linearized template DNA encoding the $\alpha2$, $\alpha3$, $\alpha4$, and $\beta2$ subunits, as well as the various chimeric and mutant subunits, using an Ambion mMessage mMachine kit. Mature *X. laevis* frogs were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester, and oocytes were surgically removed. Follicle cells were removed by treatment with collagenase B for 2 hr at room temperature. Each oocyte was injected with 10–30 ng of cRNA in 50 nl of water and incubated at 19° in modified Barth's saline (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 100 μg/ml gentamicin, 15 mM HEPES, pH 7.6) for 2–7 days. RNA transcripts encoding each subunit were injected into oocytes at a molar ratio of 1:1.

Electrophysiological recordings. Oocytes were perfused at room temperature (20–25°) in a 300-μl chamber with perfusion solution (115 mM NaCl, 1.8 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES, pH 7.2, 1.0 μM atropine). Perfusion was continuous at a rate of ~20 ml/min. ACh was diluted in perfusion solution, and the oocytes were exposed to ACh for ~10 sec, using a solenoid valve. NBT sensitivity was tested by comparing ACh-induced current responses before and after the oocytes were incubated for 30 min in perfusion solution containing various concentrations of NBT and 100 μg/ml bovine serum albumin. We have shown previously that bovine serum albumin alone has no effect (Luetje *et al*., 1990). A 10-sec wash was included before the postincubation ACh exposure. The postincubation ACh response is presented as a percentage of the preincubation ACh response. ACh concentrations were below the EC_{50} value for each receptor to avoid extensive desensitization. Preincubation with NBT results in a slowly reversible competitive blockade of $\alpha3\beta2$ but not $\alpha2\beta2$ (Luetje *et al*., 1990). Coapplication of NBT with agonist reveals a rapidly reversible blockade of subunit combinations other than $\alpha3\beta2$. Our experimental protocol incorporates a wash period after NBT incubation and before measurement of the postincubation ACh response. This wash step eliminates the rapidly reversible blockade of subunit combinations other than $\alpha3\beta2$. Thus, our protocol detects only slowly reversible blockade by NBT. The slowly reversible nature of NBT blockade allows the postincubation ACh response to be measured without coapplication of toxin. Because toxin and ACh were not in direct competition, the degree of observed block was not dependent on the concentration of ACh, and the ACh concentration used for each receptor did not have to be equipotent with the ACh concentrations used for other receptors.

Current responses to agonist application were measured under two-electrode voltage-clamp, at a holding potential of −70 mV, using a TEV-200 voltage-clamp unit (Dagan, Minneapolis, MN). Micropipettes were filled with 3 M KCl and had resistances of 0.5–1.0 MΩ. Agonist-induced responses were captured, stored, and analyzed on a Macintosh IIci computer using a data acquisition program written with LabVIEW (National Instruments, Austin, TX) and LIBI (University of Arizona, Tuscon, AZ) software (Luetje and Patrick, 1991).

Fold differences between NBT dose-inhibition data for various receptors were estimated through visual inspection of Figs. 2, 5, and 6. Comparisons were made at 50% inhibition unless otherwise noted. Statistical significance was determined by using a two-sample *t* test.

Results

Residues 84–215 of the $\alpha3$ subunit are sufficient to confer NBT sensitivity on the $\alpha2$ subunit. We previously demonstrated that three-sequence segments of the $\alpha3$ subunit are important for NBT sensitivity (Luetje *et al*., 1993). In that study, a series of chimeric α subunits were constructed that consisted of portions of the $\alpha3$ and $\alpha2$ subunits. Each of the three regions identified (84–121, 121–181, and 195–215) was found to be necessary, but not sufficient, to achieve the full NBT sensitivity of the $\alpha3\beta2$ receptor. No data were obtained regarding the potential involvement of the 181–195 region. Residues 1–83 and residues from 216 to the carboxyl terminus were found to be unnecessary for NBT sensitivity.

To provide definitive proof that residues 84–215 of $\alpha3$ are sufficient to confer complete NBT sensitivity, we constructed the chimeric α subunit $\alpha2$–84-$\alpha3$–215-$\alpha2$. This chimera consists of the $\alpha2$ subunit with residues 84–215 replaced by $\alpha3$ sequence. On expression in *X. laevis* oocytes, in combination with the $\beta2$ subunit, this chimera forms receptors that are sensitive to blockade by NBT. In Fig. 1A, we show ACh induced current responses obtained from oocytes expressing $\alpha3\beta2$, $\alpha2\beta2$, or $\alpha2$–84-$\alpha3$–215-$\alpha2$ $\beta2$ before and after a 30-min incubation with 10 nM NBT. The $\alpha3\beta2$ receptors are antagonized by 10 nM NBT. In contrast, the $\alpha2\beta2$ receptors are

insensitive to this concentration of toxin. In fact, concentrations of NBT as high as 1 μM do not cause blockade of the α2β2 receptor (Luetje *et al.*, 1993). The α2–84-α3–215-α2 β2 receptors display a sensitivity to 10 nM NBT similar to that of α3β2. In Fig. 1B, we compared the sensitivity of α2–84-α3–215-α2 β2 and α3β2 to a range of NBT concentrations and found the NBT sensitivity of these two receptors to be indistinguishable. This result confirms the importance of residues within the 84–215 segment and rules out any requirement for residues between the amino terminus and position 83 or for residues between position 216 and the carboxyl terminus.

Although there is evidence that NBT interacts competitively with neuronal nAChRs expressed by neurons (Halvorsen and Berg, 1987; Wolf *et al.*, 1988; Loring *et al.*, 1989), we wanted to confirm that NBT blockade of the rat α3β2 subunit combination expressed in oocytes also was competitive. We previously demonstrated that DHβE is a competitive antagonist of the α3β2 subunit combination (Harvey and Luetje, 1996). We took advantage of the difference in the rate of recovery of α3β2 receptors from DHβE and NBT blockade to demonstrate that DHβE competes with NBT for binding to α3β2 receptors (Fig. 2). A 2.5-min wash period was included after the standard 30-min incubation with NBT. Under these conditions, NBT alone antagonized α3β2 with an IC_{50} value of 2.9 nM. In contrast, 30-min incubation with 100 nM DHβE followed by a 2.5-min wash period resulted in no blockade of α3β2. This lack of blockade is due to the rapid off-rate of DHβE from the receptor. When 100 nM DHβE is included in NBT incubations, the IC_{50} value for receptor blockade was shifted 6.6-fold to 19 nM. This rightward shift of the NBT concentration-inhibition relationship in the presence of DHβE demonstrates direct competition between NBT and DHβE.

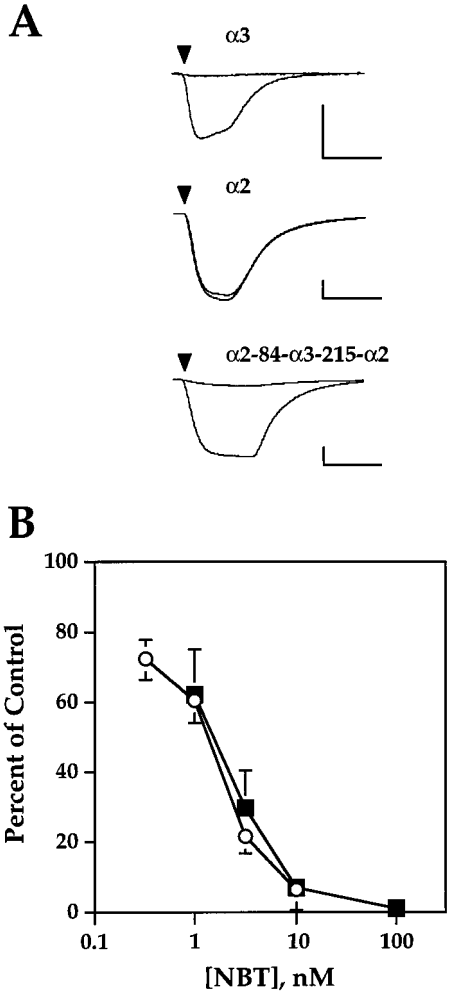

Fig. 1. Residues 84–215 contain the entire contribution of α3 to NBT sensitivity. A: *Top traces*, current responses of an α3β2-expressing oocyte to 3 μM ACh before and after 30-min incubation with 10 nM NBT. *Middle traces*, current responses of an α2β2-expressing oocyte to 1 μM ACh before and after 30-min incubation with 10 nM NBT. *Bottom traces*, current responses of an α2–84-α3–215-α2 β2-expressing oocyte to 100 nM ACh before and after 30-min incubation with 10 nM NBT. *Scale bars*, 100 nA, 10 sec. B, NBT sensitivity of α3β2 (○) and α2–84-α3–215-α2 β2 (■). Current in response to ACh after 30-min incubation with various concentrations of NBT is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three to seven separate oocytes). Some error bars are obscured by symbols.

Identification of seven residues (Thr143, Tyr184, Lys185, His186, Ile188, Gln198, and Ser203) on α3 that contribute to NBT sensitivity. The α3 and α2 subunits differ at 41 of the 132 residues in segment 84–215 (Fig. 3). To identify the amino acid residues in this region that are responsible for NBT sensitivity, we constructed a series of mutant α3 subunits. Residues in α3 were changed to what occurs at the analogous position in α2. In a few cases, we changed two or three residues simultaneously. Each mutant subunit then was coexpressed with the β2 subunit and tested for sensitivity to blockade of ACh-induced current responses by 10 nM NBT (Fig. 4). We found that mutations at seven positions resulted in a significant loss in NBT sensitivity.

We previously localized a determinant of NBT sensitivity to region 84–121 (Luetje *et al.*, 1993). However, mutation analysis in Fig. 4 did not reveal any critical residues. The most likely explanation is that several of the residues in this region each make minor contributions that are too small to be detected individually in our assay. Within region 121–181, the T143K mutation caused a significant loss of NBT sensitivity. This residue is of particular interest because it is located directly adjacent to Cys142, one of two cysteines in

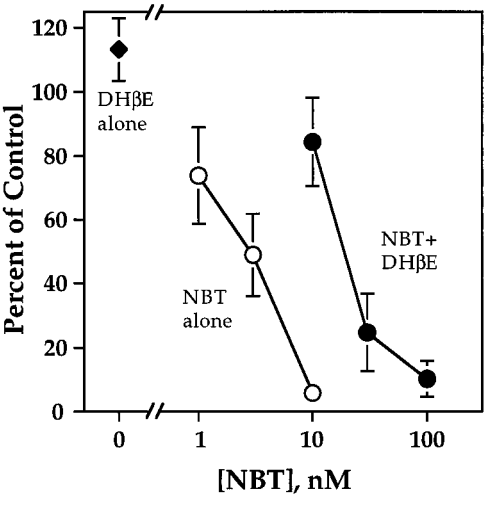

Fig. 2. Effect of DHβE on the concentration dependence of NBT inhibition. Current in response to ACh after a 30-min incubation with 100 nM DHβE (♦), various concentrations of NBT (○), or various concentrations of NBT in the presence of 100 nM DHβE (●), followed by a 2.5-min wash period, is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three separate oocytes). Some error bars are obscured by symbols.

nicotinic receptor subunits that form the conserved cysteine-loop structure. Our analysis of the role of Thr143 in determining NBT sensitivity is presented below.

In previous work (Luetje *et al*., 1993), it was unclear whether amino acid residues between 181 and 195 were important for NBT sensitivity. However, it is clear in Fig. 4 that this region is critical. Four of the seven amino acid residues that we identified on the α3 subunit lie within this region (Tyr184, Lys185, His186, Ile188). In Fig. 5A, we examined the effect of changing each of these residues on sensitivity to a range of NBT concentrations. The change at position 184 from a tyrosine to a threonine, a loss of an aromatic ring, had a modest (1.8-fold) effect on NBT sensitivity. A similarly modest (2.4-fold) effect occurred when His186 was changed to an asparagine. A larger (11.2-fold) effect was achieved by changing Ile188 to lysine, going from a hydrophobic residue to a positively charged residue. Mutation of Lys185 to tyrosine, resulting in a loss of the positive charge and gain of an aromatic ring, resulted in a large shift in NBT sensitivity (40.5-fold).

Within region 195–215, two mutations caused a significant loss of NBT sensitivity (Q198P and S203Y) (Fig. 4). In Fig. 5B, we show that changing Gln198 of α3 to proline, as in α2, causes a 9.5-fold loss in NBT sensitivity. The S203Y mutation, a gain of an aromatic ring, causes a 5.9-fold loss in NBT sensitivity.

The α4 subunit has a threonine at position 143 (and an asparagine at 141; see below), similar to α3, but lacks the other six residues that we identified as determinants of NBT sensitivity in regions 181–195 and 195–215 (see Fig. 3). As might be expected, $\alpha4\beta2$ receptors display little sensitivity to NBT. Even at a concentration of 1 μM, NBT is able to block $\alpha4\beta2$ by only 15.9 $\pm$ 1.4% (five oocytes). We hypothesized that installation of an additional determinant might increase the NBT sensitivity of $\alpha4\beta2$. In previous work, we found that changing P198 of the chimera α3–195-α2 to glutamine increased the NBT sensitivity of the resulting receptors (Luetje *et al*., 1993). Thus, we sought to test the role of glutamine at position 198 by changing Pro198 of α4 to glutamine, as in α3. We found that the mutant subunit (α4, P198Q) formed receptors with a significantly increased NBT sensitivity. At a concentration of 1 μM, NBT was able to block α4, P198Q β2 by 77.4 $\pm$ 6.8% (four oocytes; significantly different from $\alpha4\beta2$, $p < 0.001$).

Thr143 contributes to NBT sensitivity as part of a glycosylation consensus sequence. Receptors formed by α3, T143K are insensitive to both 10 and 100 nM NBT; however, 300 nM NBT is able to cause partial blockade (Fig. 6B). The degree of blockade of α3, T143K β2 receptors by 300 nM NBT is not significantly different from the blockade of $\alpha3\beta2$ receptors achieved by 300 pM NBT. Thus, the loss in NBT sensitivity due to the T143K mutation is ~1000-fold. In contrast to the large effect on NBT sensitivity, the T143K mutation has little effect on ACh sensitivity. The ACh EC_{50} value of α3, T143K β2 is 30.3 $\pm$ 8.5 μM, similar to the EC_{50} value of 70.8 $\pm$ 19.6 μM of $\alpha3\beta2$ (Harvey and Luetje, 1996).

There are two main possibilities why the T143K mutation causes a loss of NBT sensitivity. First, a change in the character of the amino acid side chain at this position may be the important factor. The change from threonine to lysine is nonconservative; the threonine side chain is small in volume and polar, whereas the lysine side chain has a large volume and is positively charged. A change in one or both of these properties could be responsible for the loss of NBT sensitivity. To test the importance of side chain characteristics, we installed a series of residues at this site (Fig. 6A). The installation of alanine, asparagine, glutamine, or tyrosine (representing a variety of side chain volumes) each resulted in a significant loss of NBT sensitivity. If the effect of the T143K mutation was due to the introduction of the positive charge, then introduction of a negative charge might be expected to have an opposite effect. However, installation of aspartate also resulted in a loss of NBT sensitivity. The fact that installation of any of these residues had essentially the same effect suggested that it is the loss of threonine that is important rather than the introduction of any particular side chain

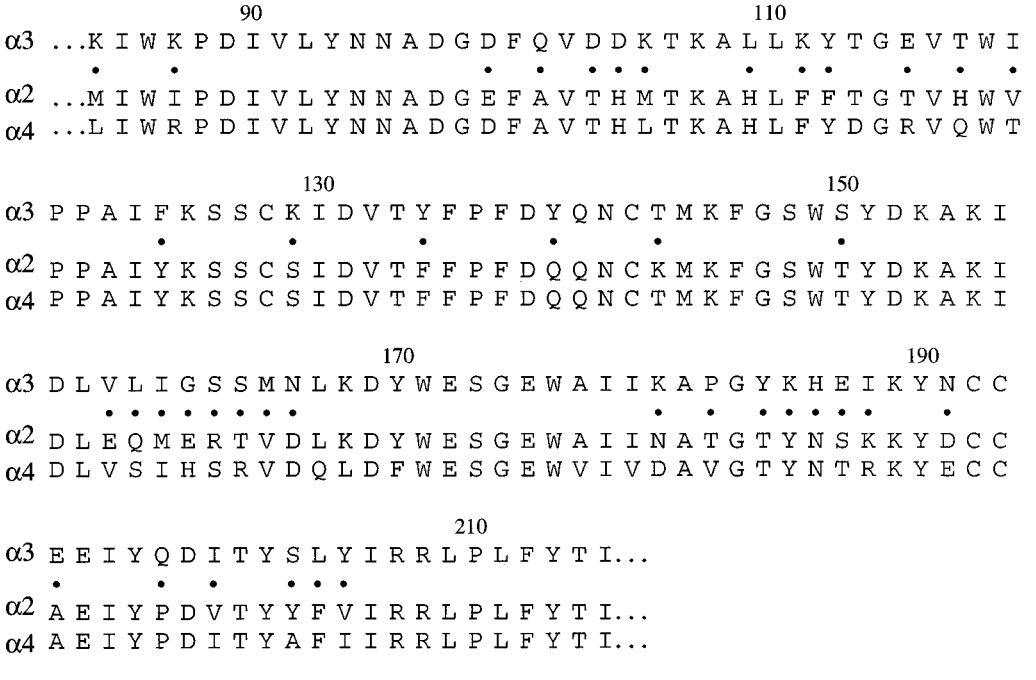

Fig. 3. Amino acid alignment of region 84–215 of α3 with the analogous sequence of α2 and α4. *Dots*, differences between the α2 and α3 sequences.

characteristic. Only the installation of serine allowed conservation of NBT sensitivity.

A second possible explanation for the effect of the T143K mutation concerns the fact that Thr143 serves as part of the consensus sequence for glycosylation (NXT/S) at Asn141. This possibility is supported by the ability of serine to substitute successfully for threonine. The asparagine at position 141 is conserved in both α3 and α2, but only α3 has the appropriate residue at position 143 that would allow glycosylation. To test this possibility, we changed Asn141 to alanine, thus precluding the possibility of glycosylation at position 141. Similar to what we observed for the T143K mutation, the N141A mutation has only a minimal effect on ACh sensitivity (EC_{50} = 18.2 ± 10.9 μM). However, the N141A mutation has a large effect on NBT sensitivity. In Fig. 6B, we show that receptors formed by the N141A mutant are ~300-fold less sensitive to NBT than are α3β2 receptors. This result suggests that glycosylation at position 141 of the α3 subunit is an essential requirement for NBT sensitivity.

A better test of the role of glycosylation at N141 would be to install this glycosylation site in an insensitive subunit. As discussed above, the α2 subunit has an asparagine at position 146 (analogous to Asn141 of α3) but lacks the appropriate threonine or serine at position 148. Thus, installation of a threonine at this site would complete the consensus for glycosylation. However, our previous work with chimeric subunits (Luetje *et al.*, 1993) suggests that simply completing the glycosylation site in α2 would have little effect on NBT sensitivity. The α3–195-α2 chimera, which possesses most of the determinants that we have identified in this study (the glycosylation site, as well as Tyr184, Lys185, His186, and Ile188), was insensitive to 1 μM NBT. Thus, to test the effect of adding the glycosylation site, many of the other critical

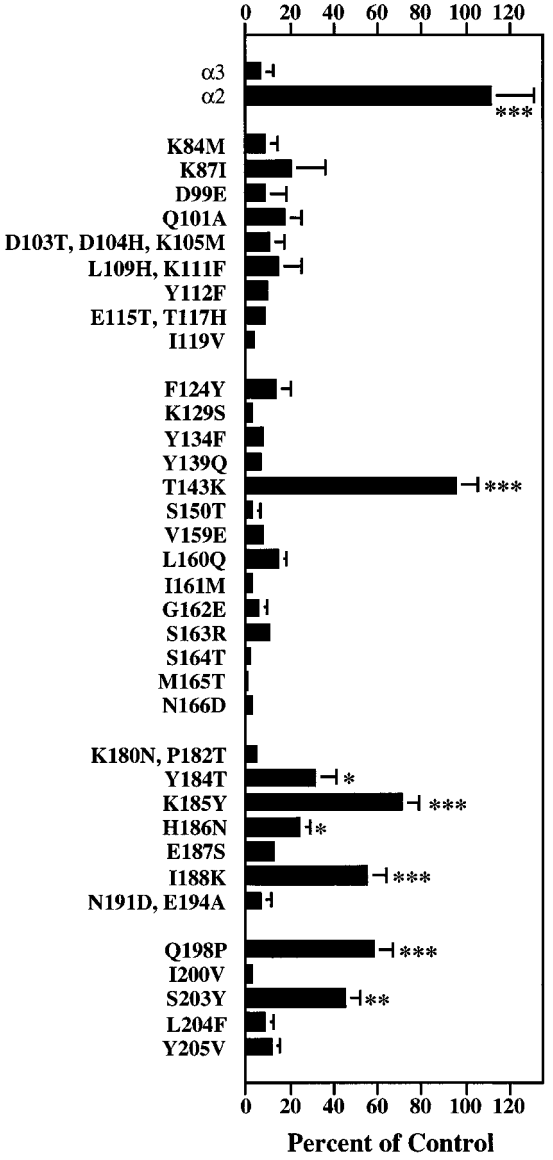

Fig. 4. Mutation analysis of the 84–215 region of α3 identifies seven residues that are important for NBT sensitivity. Sensitivity to 10 nM NBT of each of a series of mutant α3 subunits coexpressed with β2 is shown. Current in response to ACh after 30-min incubation with NBT is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three to seven separate oocytes). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, significant differences from α3. Some error bars are too small to appear.

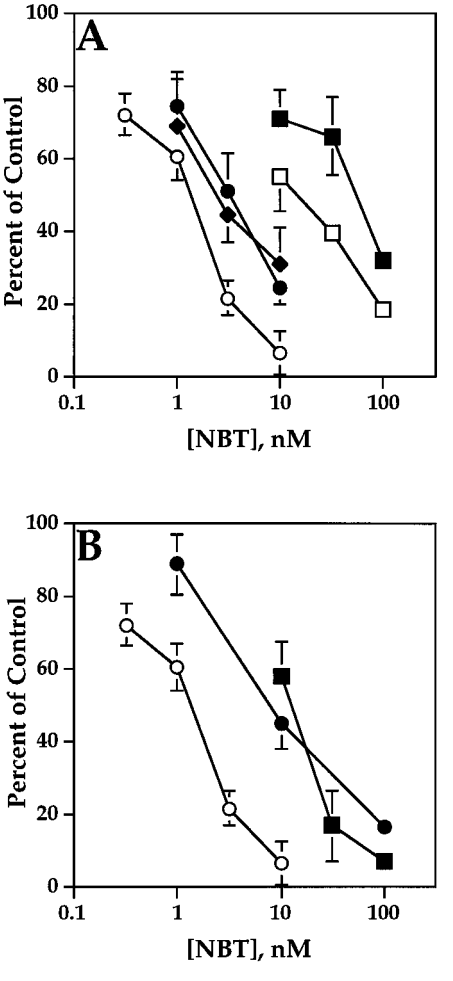

Fig. 5. A, NBT sensitivity of α3β2 (○), α3, Y184T β2 (♦), α3, L185Y β2 (■), α3, H186N β2 (●), and α3, I188K β2 (□). Current in response to ACh after 30-min incubation with various concentrations of NBT is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three to seven separate oocytes). Some error bars are obscured by symbols. B, NBT sensitivity of α3β2 (○), α3, Q198P β2 (■), and α3, S203Y β2 (●). Current in response to ACh after 30-min incubation with various concentrations of NBT is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three to seven separate oocytes). Some error bars are obscured by symbols.

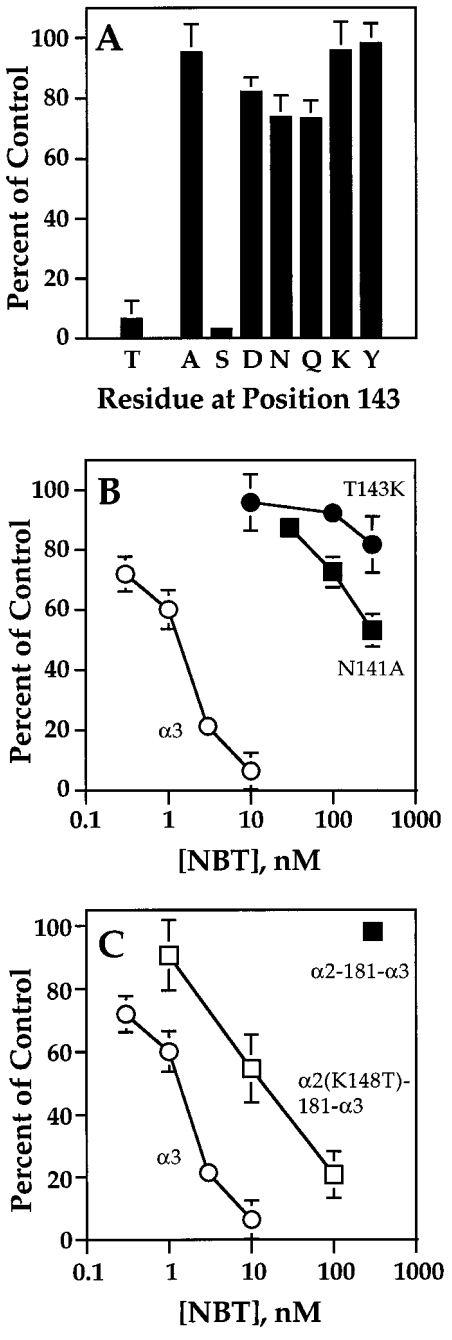

Fig. 6. Thr143 contributes to NBT sensitivity as part of a glycosylation consensus sequence. A, Effect of mutations at position 143 on NBT sensitivity. A series of mutant $\alpha 3$ subunits with changes made at position 143 were coexpressed with $\beta 2$ and tested for sensitivity to 10 nM NBT. Current in response to ACh after 30-min incubation with NBT is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three to seven separate oocytes). The result for each mutant, with the exception of T143S, was significantly different from $\alpha 3$ ($p < 0.001$). *T*, Wild-type $\alpha 3$, which has a threonine at position 143. B, NBT sensitivity of $\alpha 3\beta 2$ (○), $\alpha 3$, T143K $\beta 2$ (●), and $\alpha 3$, N141A $\beta 2$ (■). Current in response to ACh after 30-min incubation with various concentrations of NBT is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three to seven separate oocytes). Some error bars are obscured by symbols. C, NBT sensitivity of $\alpha 3\beta 2$ (○), $\alpha 2$–181-$\alpha 3$ $\beta 2$ (■), and $\alpha 2$(K148T)-181-$\alpha 3$ $\beta 2$ (□). Current in response to ACh after 30-min incubation with various concentrations of NBT is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three to seven separate oocytes). Some error bars are obscured by symbols.

residues will have to be present. To achieve this, we decided to test the effect of completing the glycosylation site on the chimeric subunit $\alpha 2$–181-$\alpha 3$. This subunit possesses all critical residues that we have identified, with the exception of the glycosylation site. As shown in Fig. 6C, receptors formed by this chimera are insensitive to 300 nM NBT. Completion of the glycosylation site in this chimera, yielding $\alpha 2$(K148T)-181-$\alpha 3$, resulted in a dramatic increase in NBT sensitivity. Receptors formed by this mutant chimera were sensitive to NBT with an IC_{50} value of ~13 nM. This is only 10-fold less than the sensitivity of receptors formed by wild-type $\alpha 3$. Taken together, the results presented in Fig. 6 strongly suggest that glycosylation of Asn141 of $\alpha 3$ is critical for achieving full NBT sensitivity.

Discussion

We identified seven amino acid residues on the $\alpha 3$ subunit of neuronal nicotinic receptors that contribute to NBT sensitivity. These residues lie near a series of conserved residues, thought to comprise the common features of all nAChR binding sites (Fig. 7). Originally identified on the *Torpedo californica* electric organ nAChR α subunit using affinity labeling techniques (Karlin and Akabas, 1995), these residues are highly conserved among nAChR α subunits, including the rat neuronal $\alpha 2$ and $\alpha 3$ subunits. The fact that these residues are common to both $\alpha 2$ and $\alpha 3$ excludes them from responsibility for the different pharmacological properties displayed by receptors formed by these subunits. We show that a series of residues positioned near these common residues are responsible for the differing specificity of these receptors. The glycosylation consensus consisting of Asn141 and Thr143 lies close to the conserved Trp149, whereas a cluster of common determinants (Tyr190, Cys192, Cys193, Tyr197) is flanked by Tyr184, Lys185, His186, and Ile188 on the amino-terminal side and by Gln198 and Ser203 on the carboxyl-terminal side. Interestingly, scanning cysteine accessibility mutagenesis has demonstrated the proximity of His186 and Ile188 of the mouse muscle α subunit to the ACh binding site (McLaughlin *et al.*, 1995).

The large size of NBT (a dimer of 66 residue monomers) is a potential problem when using this toxin to study the ligand binding site. Although NBT is a competitive antagonist (Halvorsen and Berg, 1987; Wolf *et al.*, 1988; Loring *et al.*, 1989; current study), some interactions between NBT and the receptor could be quite distant from the binding site. Thus, it is encouraging that we find overlap in our mapping of determinants of moderately sized (α-CTx-MII) and small (DHβE) competitive antagonists on both α and β subunits. Two of the residues on $\alpha 3$ that we have identified as NBT determinants (Lys185 and Ile188) also serve as determinants of α-CTx-MII sensitivity (Harvey *et al.*, 1997). Sequence segment 195–215 of $\alpha 3$, which contains Gln198 and Ser203, also contains a determinant of DHβE sensitivity, although we have not yet identified the individual residue or residues involved (Harvey *et al.*, 1996). The situation is even more striking for the $\beta 2$ subunit. We found that a major determinant of NBT sensitivity, T59, also is a determinant of both DHβE and α-CTx-MII sensitivity (Harvey and Luetje, 1996; Harvey *et al.*, 1997). This overlap in the determinant maps for competitive antagonist sensitivity confirms NBT as a useful tool to

investigate the structure of the ligand binding sites of neuronal nAChRs.

A different approach to the identification of residues responsible for NBT sensitivity has been to test a series of peptides, corresponding to the α3 sequence, for the ability to bind ^{125}I-NBT (McLane *et al*., 1990, 1991, 1993). Peptides representing residues 1–18 and 51–70 of α3 were shown to bind ^{125}I-NBT with low affinity, whereas the α2 peptide analogous to α3:51–70 did not bind NBT. In Fig. 1, we demonstrate that residues 1–83 of α3, which includes both α3 peptides, are unnecessary for NBT blockade of the $\alpha3\beta2$ receptor. McLane *et al*. (1990) also showed that the α3 peptides 180–199 and 183–201, although not able to bind ^{125}I-NBT in a solid-phase assay, were able to partially inhibit the binding of ^{125}I-NBT to PC12 cells. Some of the residues that we identified as determinants of NBT sensitivity (Tyr184, Lys185, His186, Ile188, Gln198) lie within the region covered by these two peptides. Thus, although studies using peptides may identify sequence segments important for ligand binding (i.e., α3:180–199 and α3:183–201), the relative affinities of different peptides for ligands may not be relevant to the role those sequences play in the intact receptor.

Our work strongly suggests that Thr143 fulfills its role as a determinant of NBT sensitivity by serving as part of a glycosylation consensus sequence for glycosylation at Asn141. It is the presence of glycosylation at Asn141 that seems to be required for NBT blockade of α3-containing nAChRs. This surprising result contrasts sharply with the role of glycosylation in conferring α-bungarotoxin resistance on nAChRs of snake and mongoose muscle, where the presence of an additional glycosylation confers resistance, presumably through steric hindrance (Kreienkamp *et al*., 1994; Keller *et al*., 1995).

The presence of the consensus for glycosylation at Asn141 is necessary, but not sufficient, to confer NBT sensitivity. This site is conserved among most nicotinic receptor subunits, even those that form receptors insensitive to NBT. These subunits most likely lack other critical determinants of NBT sensitivity. This dependence on additional residues for achieving NBT sensitivity is demonstrated in our previous work (Luetje *et al*., 1993). We found that the chimera α3–195-α2, which possesses the glycosylation site and lacks only the determinants between 195 and 215, formed receptors that were insensitive to NBT concentrations as high as 1 μM.

Thr143 and Asn141 lie on either side of Cys142, which forms a disulfide bond with Cys128, raising the question of whether glycosylation can occur at Asn141. In fact, the analogous site on *T. californica* electric organ δ subunit has been demonstrated to be glycosylated (Strecker *et al*., 1994). Rickert and Imperiali (1995) have shown, using a peptide encompassing the Cys-loop sequence of the *T. californica* α subunit, that Asn141 can be glycosylated before and after oxidation of Cys128 and Cys142 and that glycosylation of Asn141 actually favors disulfide bond formation. An additional concern is that *X. laevis* oocytes apparently are not capable of complex glycosylation. When *T. californica* nAChRs are expressed in *X. laevis* oocytes, only oligosaccharides of the high mannose type are incorporated (Buller and White, 1990). However, although the other glycosylation sites on the natively expressed *T. californica* δ subunit are of the complex type, glycosylation at the position analogous to Asn141 is of the high mannose type (Strecker *et al*., 1994). Whether this site on α3 receives a high mannose oligosaccharide when this subunit is expressed by a mammalian neuron is not known, but long term block by NBT, similar to what we have observed for rat $\alpha3\beta2$ expressed in oocytes, has been demonstrated with rat sympathetic neurons in culture (Sah *et al*., 1987).

The question of proper glycosylation of nAChRs expressed in oocytes raises the larger issue of whether the pharmacological properties of nAChRs expressed in oocytes are an accurate reflection of the properties these receptors display *in vivo*. We addressed this issue for both muscle and neuronal nAChRs. The agonist pharmacology of mouse muscle $\alpha1\beta1\gamma\delta$ expressed in oocytes and assayed electrophysiologically (Luetje and Patrick, 1991), was found to be quite similar to that of the same receptor natively expressed by BC3H-1 cells (Sine and Steinbach, 1986, 1987). We also measured the affinity of rat $\alpha4\beta2$ expressed in oocytes for a series of agonists and antagonists in a radioligand binding using [^{3}H]cytisine (Parker and Luetje, 1996). Comparison with the properties of the high affinity cytisine binding site in rat brain (Pabreza *et al*., 1991), thought to be $\alpha4\beta2$ (Flores *et al*., 1992), shows that neuronal nAChRs expressed in *X. laevis* oocytes display the same pharmacological features that these receptors display in a native context.

Recently, models were proposed for the extracellular domains of nicotinic receptors (Tsigelny *et al*., 1997) and glycine receptors (Gready *et al*., 1997). Given the homology between nicotinic and glycine receptor subunits, it seems likely that nAChR subunit extracellular domains would be structurally similar to the extracellular domains of glycine receptor subunits. However, these two models are completely different from each other. The nAChR extracellular domain model is based on homology with the copper binding proteins plastocyanin and pseudoazurin, whereas the glycine receptor extracellular domain model is based on homology with the SH2 and SH3 domains of the *Escherichia coli* biotin repressor. The determinants of NBT sensitivity that we have identified fit well with both models. This is not surprising because both

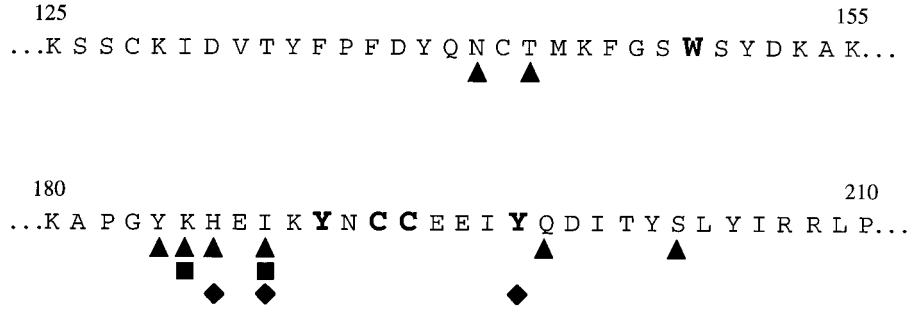

Fig. 7. Determinants of NBT sensitivity on the α3 subunit. Sequence segments 125–155 and 180–210 of α3 are shown. ▲, Residues identified in the current study as determinants of NBT sensitivity. ■, Residues previously identified as determinants of α-CTx-MII sensitivity (Harvey *et al*., 1997). *Boldface*, conserved features of nicotinic binding sites (Karlin and Akabas, 1995). ◆, Residues at positions analogous to those identified by scanning cysteine mutagenesis in the mouse muscle α subunit (McLaughlin *et al*., 1995).

models conform to data from earlier mutagenesis, antibody mapping, and affinity labeling studies. Determination of whether either of these models is useful to the study of ligand-gated channels in general and neuronal nAChRs in particular will require further mutation analysis.

The exact role of each residue we identified currently is unclear. Each residue may be interacting directly with NBT. It also is possible that a residue acts indirectly to change the properties of the binding site without a direct interaction with NBT. Resolution of this issue will require manipulation of the toxin structure. The recent use of a synthetic gene to express recombinant NBT in *E. coli* and *Pichiia pastoris* has allowed structure function analysis to begin on the NBT molecule (Fiordalisi *et al.*, 1994, 1991, 1996). A promising approach to identifying interacting residues on the toxin and the receptor will be the generation and analysis of compensatory mutations on toxin and receptor.

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Send reprint requests to: Dr. Charles W. Luetje, Department of Molecular and Cellular Pharmacology (R-189), University of Miami School of Medicine, P.O. Box 016189, Miami, FL 33101. E-mail: cluetje@chroma.med.miami.edu