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# Determinants of Specificity for α-Conotoxin MII on α3β2 Neuronal Nicotinic Receptors

SCOTT C. HARVEY, J. MICHAEL MCINTOSH, G. EDWARD CARTIER, FLOYD N. MADDOX, and CHARLES W. LUETJE

*Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, Florida 33101 (S.C.H., F.N.M., C.W.L.), and Departments of Biology (J.M.M., G.E.C.) and Psychiatry (J.M.M.), University of Utah, Salt Lake City, Utah 84112*

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## SUMMARY

The competitive antagonist α-conotoxin-MII (α-CTx-MII) is highly selective for the α3β2 neuronal nicotinic receptor. Other receptor subunit combinations (α2β2, α4β2, α3β4) are >200-fold less sensitive to blockade by this toxin. Using chimeric and mutant subunits, we identified amino acid residues of α3 and β2 that participate in determination of α-CTx-MII sensitivity. Chimeric α subunits, constructed from the α3 and α4 subunits, as well as from the α3 and α2 subunits, were expressed in combination with the β2 subunit in *Xenopus laevis* oocytes. Chimeric β subunits, formed from the β2 and β4 subunits, were expressed in combination with α3. Determinants of α-CTx-MII sensitivity on α3 were found to be within sequence segments 121–181 and 181–195. The 181–195 segment accounted for approximately half the difference in toxin sensitivity between receptors formed by α2 and α3. When this sequence of α2 was replaced with the corresponding α3 sequence, the resulting chimera formed receptors only 26-fold less sensitive to α-CTx-MII than α3β2. Site-directed mutagenesis within segment 181–195 demonstrated that Lys185 and Ile188 are critical in determination of sensitivity to toxin blockade. Determinants of α-CTx-MII sensitivity on β2 were mapped to sequence segments 1–54, 54–63, and 63–80. Site-directed mutagenesis within segment 54–63 of β2 demonstrated that Thr59 is important in determining α-CTx-MII sensitivity.

nAChRs are assembled from a family of ≥11 distinct subunits, α2–9 and β2–4 (1, 2). Similar to what has been shown for muscle-type nAChRs, the ligand binding sites of neuronal nAChRs are complex, with contributions from both the α and non-α (β) subunits. On expression in *Xenopus laevis* oocytes, each functional subunit combination displays unique pharmacological properties (3–7). These pharmacological differences can be exploited as probes with which to explore the structural determinants of receptor subtype specificity.

Affinity labeling techniques have been used to identify a number of amino acid residues on the α, γ, and δ subunits of muscle-type nAChRs (8–16). Many of these residues are conserved among neuronal nAChR subunits and thus may form features of the ligand binding sites common to all nAChRs. Because they are so highly conserved, these residues cannot be responsible for the pharmacological differences that have been observed among different neuronal nAChR subunit combinations. It is the residues that differ among nAChR subunits that must be responsible for this pharmacological diversity.

An approach to identifying residues responsible for pharmacological differences is to construct a series of chimeras of pharmacologically distinct subunits to map critical sequence segments, followed by site-directed mutagenesis to probe the role of individual residues. This technique has been used with success to map residues that determine sensitivity to both agonists and antagonists. Several sequence segments of neuronal nAChR α subunits have been identified that affect sensitivity to agonists and the competitive antagonist neuronal NBT (17). Regions of β2 and β4 have also been identified that determine sensitivity to agonists (18, 19). We used chimeric and mutant β subunits to identify Thr59 of β2 as a major determinant of sensitivity to the competitive antagonists DHβE and NBT (20).

Recently, a novel α-conotoxin (α-CTx-MII) was isolated that is a highly selective antagonist of the α3β2 subunit combination (21). NBT, under certain conditions, is also selective for α3β2 receptors (3, 5, 6; but see Ref. 22). Blockade of α3β2 by α-CTx-MII seems to be competitive because the α3β2 receptor can be protected from α-CTx-MII block by DHβE, a known competitive antagonist (23). In this study, we were interested in identifying residues on the α and β subunits of neuronal nAChRs that determine sensitivity to

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

this toxin. We constructed and screened a series of α subunit chimeras and β subunit chimeras to identify critical sequence segments. We then used site-directed mutagenesis to identify Lys185 and Ile188 of α3 and Thr59 of β2 as residues important in determination of α-CTx-MII sensitivity of the α3β2 subunit combination.

## Experimental Procedures

**Materials.** *X. laevis* frogs were purchased from Nasco (Fort Atkinson, WI). 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 United States Biochemical (Cleveland, OH). CloneAmp kits were from GIBCO BRL (Baltimore, MD). α-CTx-MII was synthesized, and proper disulfide bond formation was achieved as previously described (21).

**Mutagenesis and construction of chimeric receptors.** Chimeric and mutant subunits were constructed using the PCR (24). Our notation for chimeric subunits is to list the source of the amino-terminal portion, followed by the residue number in the amino acid sequence where the chimeric joint is made (numbering taken from the mature α3 and β2 subunit sequences), and then followed by the source of the carboxyl-terminal portion. For example, the chimeric subunit α4–216-α3 is composed of α4 sequence from the amino terminus until residue 216, after which it is composed of α3 sequence. Our notation for mutant subunits is to list the naturally occurring residue followed by the position of that residue, followed by the change that has been made. For example, the mutant subunit α3,I188K is an α3 subunit in which Ile188 has been changed to a lysine. PCR products were subcloned into the pAMP1 vector using a CloneAmp kit (GIBCO BRL) or into the pCR-Script $SK^+$ vector (Stratagene, La Jolla, CA). 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 with the use of Sequenase 2.0 (United States Biochemical).

**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 α2, α3, α4, β2, and β4 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 5–50 ng of cRNA in 50 nl of water and was 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. α-CTx-MII sensitivity was tested by comparing ACh-induced current responses before and after the oocytes were incubated for 5 min in perfusion solution containing various concentrations of α-CTx-MII and 100 μg/ml bovine serum albumin. The postincubation ACh response is presented as a percentage of the preincubation ACh response. ACh concentrations were at or below the $EC_{50}$ value for each receptor to avoid extensive desensitization. The slowly reversible nature of α-CTx-MII blockade allowed the postincubation ACh response to be measured without coapplication of toxin. Because toxin and ACh would not be in direct competition, the degree of block is not dependent on the concentration of ACh, and the ACh concentration used for each receptor would not need to be equipotent with the ACh concentrations used for other receptors. The $IC_{50}$ value we obtained for α-CTx-MII block of α3β2 (3.5 nM) was somewhat higher than that obtained previously (0.5 nM) (21). A possible reason for this difference is that in the current study, perfusion was stopped during the α-CTx-MII incubations to conserve toxin, whereas in the previous study, toxin was perfused continuously. Static application of toxin results in an apparent decrease in toxin potency at all receptor subtypes.[1] We are unsure why this difference occurs; however, nonspecific adsorption of toxin to the chamber is a possible reason. This difference was not a problem in the current study because all subunit combinations are affected similarly (compare Fig. 1 with Ref. 21, Fig. 5) and because all experiments in the current study involved the same static toxin application protocol.

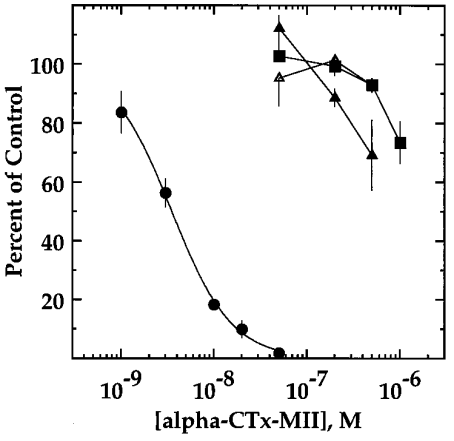


**Fig. 1.** α-CTx-MII is selective for α3β2. α-CTx-MII inhibition of (●) α3β2 (▲), α4β2 (■), α2β2, and (△) α3β4 neuronal nicotinic receptors expressed in *X. laevis* oocytes is shown. The response to a concentration of ACh at or below the $EC_{50}$ value for each receptor after a 5-min incubation with various concentrations of α-CTx-MII is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three or four oocytes). The α3β2 data are fit to a Hill equation (see Experimental Procedures), yielding $IC_{50}$ = 3.5 nM, $n$ = 1.35. Some error bars are obscured by symbols.

Current responses to agonist application were measured under two-electrode voltage-clamp conditions at a holding potential of −70 mV with voltage-clamp units from Dagan Corporation (Minneapolis, MN) and Knight Industrial Technologies (Miami, FL). 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, Tucson, AZ) software (17).

Dose-inhibition data were fit with Passage II software by the nonlinear least squares method using the equation: Current = maximum current/$[1+([\text{antagonist}]/IC_{50})^n]$, where $n$ and $IC_{50}$ represent the Hill coefficient and the antagonist concentration producing half-maximal inhibition, respectively. $IC_{50}$ values were used to determine differences in toxin sensitivity between α3β2 and receptors formed by selected chimeric and mutant subunits. Given the pseudoirreversible nature of α-CTx-MII block under our experimental conditions (see above), the $IC_{50}$ value might be expected to be a reasonable estimate of the dissociation constant (25). This probably is not the case for neuronal nAChRs, which are thought to be similar to muscle nAChRs in that occupation of two binding sites by agonist is required to activate the receptor, whereas occupation of either site by an antagonist will prevent activation (26). Analysis of toxin association

[1] G. E. Cartier and J. M. McIntosh, unpublished observations.

and dissociation rates supports this model for α-CTx-MII block of α3β2 (23). Model 3 of Sine and Taylor ($I = I_o [1 - y]^2$), where I is postincubation current, $I_o$ is preincubation current, and $y$ is fractional occupancy of binding sites) can be used to determine the fractional occupancy of binding sites needed to achieve a given percentage functional blockade (26). Thus, the concentration of α-CTx-MII that occupies 50% of the binding sites (an estimate of the dissociation constant) would block 75% of the functional response. For α3β2, this concentration of α-CTx-MII is 7.9 nM. This concentration of α-CTx-MII (and the fold difference from the α3β2 value) for receptors formed by α2–181-α3–195-α2 is 232 nM (29-fold), by α3,K185Y, 33 nM (4.2-fold); by α3,I188K, 127 nM (16-fold); and by β2,T59K, 34 nM (4.3-fold). This analysis does not change any of the conclusions made in this study.

Statistical significance was determined by using a two-sample $t$ test after an $F$ test to ensure equality of variance. For samples with unequal variance ($p > 0.05$), statistical significance was determined by using a two-sample $t$ test for samples with unequal variance (Cochran's method).

## Results

**Determinants of α-CTx-MII sensitivity on α3 lie within sequence segments 121–181 and 181–195.** α-CTx-MII is highly selective for the α3β2 subunit combination (Fig. 1, ●; $IC_{50}$ = 3.5 nM), which is in agreement with the results of Cartier *et al*. (21). Both the α3 subunit and the β2 subunit are required for high sensitivity to α-CTx-MII. Receptors containing a different α subunit (α4β2 or α2β2) or a different β subunit (α3β4) are ≥200-fold less sensitive to α-CTx-MII than the α3β2 receptor. This high degree of selectivity makes α-CTx-MII a promising probe for investigation of the structure of the antagonist binding sites on neuronal nicotinic receptors. We selected an α-CTx-MII concentration of 50 nM as a test dose for screening chimeric and mutant subunits. This toxin concentration almost completely blocks α3β2 (postincubation response = 1.8 ± 0.5% of control) but has no effect on the α4β2, α2β2, or α3β4 receptors.

To map regions of the α3 sequence responsible for α-CTx-MII sensitivity, we tested a series of chimeric α subunits expressed in combination with β2. We constructed chimeras consisting of portions of α3 and α4. We also used chimeras consisting of portions of α3 and α2, which had been constructed previously (17). Determinants of α-CTx-MII specificity reside entirely within the amino-terminal extracellular domain of α3 (Fig. 2). When this domain of α3 is replaced by α2 or α4 sequence (i.e., α2–215-α3, α4–216-α3), the resulting receptors are completely insensitive to 50 nM α-CTx-MII. Conversely, if this domain of α2 or α4 is replaced by α3 sequence (i.e., α3–215-α2, α3–216-α4), the resulting receptors are as sensitive to α-CTx-MII as wild-type α3β2. This localization of determinants of ligand binding to the amino-terminal extracellular domain has been observed with other antagonists as well as agonists (17).

In Fig. 2A, the chimeras are constructed from α3 and α4. Replacement of the first 175 or 183 residues of α3 with α4 sequence (α4–175-α3, α4–183-α3) had little effect on the α-CTx-MII sensitivity. In contrast, replacement of the first 195 residues of α3 with α4 sequence (α4–195-α3) resulted in a substantial decrease in toxin sensitivity (postincubation response = 81.6 ± 4.1% of control). When the amino-terminal 183 residues of α4 was replaced with α3 sequence (α3–183-α4), the resulting chimera had little sensitivity to α-CTx-MII (postincubation response = 85.3 ± 3.2% of control). Replacement of the amino-terminal 216 residues of α4 with α3 sequence (α3–216-α4) resulted in receptors indistinguishable from α3β2 in terms of α-CTx-MII blockade (postincubation response = 1.9 ± 0.5% of control).

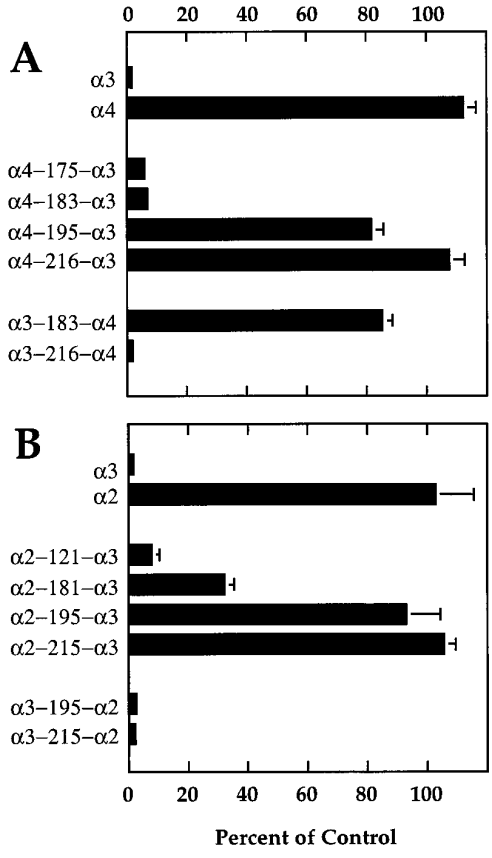


**Fig. 2.** α-CTx-MII sensitivity of receptors formed by chimeric α subunits. A, Chimeras constructed from the α3 and α4 subunits and coexpressed in *X. laevis* oocytes with β2. B, Chimeras constructed from the α3 and α2 subunits and coexpressed in *X. laevis* oocytes with β2. Current in response to an ACh concentration at or below the $EC_{50}$ value for each receptor after a 5-min incubation with 50 nM α-CTx-MII is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three or four separate oocytes). Significantly different from α2 are α2–121-α3, α2–181-α3, α3–195-α2, and α3–215-α2 ($p < 0.001$). Significantly different from α3 are α4–175-α3, α4–183-α3, α4–195-α3, α4–216-α3, α3–183-α4, α2–181-α3, α2–195-α3, and α2–215-α3 ($p < 0.001$) and α2–121-α3 ($p < 0.05$). Significantly different from α4 are α4–175-α3, α4–183-α3, α4–195-α3, α3–183-α4, and α3–216-α4 ($p < 0.001$). Some error bars are too small to appear.

We also tested chimeras of the α3 and α2 subunits (Fig. 2B). Replacement of the first 121 residues of α3 with α2 sequence (α2–121-α3) had little effect on α-CTx-MII sensitivity. Replacement of the amino-terminal 181 residues of α3 with α2 sequence (α2–181-α3) caused some loss in toxin sensitivity (postincubation response = 32.2 ± 3.2% of control), differing from results with the α4–183-α3 chimera (Fig. 2A). This suggests that α3 and α4 possess a determinant of α-CTx-MII sensitivity that α2 lacks and may explain the difference in toxin sensitivity between α4β2 and α2β2 (21) (Fig. 1). When the first 195 residues of α3 were replaced with α2 sequence (α2–195-α3), sensitivity to 50 nM toxin was completely lost (postincubation response = 93.0 ± 11.3% of control). The amino-terminal 195 residues of α3 are sufficient for toxin sensitivity because replacement of the first 195 residues of α2 with α3 sequence (α3–195-α2) confers toxin sen-

sitivity (postincubation response = 2.7 ± 0.6% of control) indistinguishable from that of α3β2.

Our results with α subunit chimeras suggest that sequence segment 181–195 contains determinants of toxin sensitivity. Chimeras containing α3 sequence only carboxyl terminal of 195 or only amino terminal of 181 show little or no sensitivity to 50 nM α-CTx-MII. What all chimeras sensitive to toxin have in common is the 181–195 segment of α3. To directly test the role of this sequence segment, we constructed a chimera consisting almost entirely of α2, with only residues 181–195 replaced with α3 sequence (α2–181-α3–195-α2). Receptors formed by this chimera were blocked by α-CTx-MII with an $IC_{50}$ value of 92 nM (Fig. 3), a toxin sensitivity that is ~26-fold less than that of α3β2. Thus, residues lying within sequence segment 181–195 account for approximately half of the difference in α-CTx-MII sensitivity between α3β2 and α2β2.

**Lys185 and Ile188 of α3 are determinants of α-CTx-MII sensitivity.** Sequence segment 181–195 of α3 is substantially divergent from α2 and α4 sequence, differing at 8 of 15 residues (Fig. 4A). To determine which of these residues are important for toxin sensitivity, we constructed mutants of α3 in which one or two residues in α3 were changed to what occurs in α2. Receptors formed by these mutants were screened for loss of sensitivity to 50 nM α-CTx-MII (Fig. 4B). The mutations K180N, P182T, Y184T, H186N, E187S, N191D, and E194A caused no significant loss in toxin sensitivity. Only the mutants α3,K185Y and α3,I188K formed receptors that were significantly less sensitive to toxin blockade than receptors formed by wild-type α3, identifying Lys185 and Ile188 as determinants of α-CTx-MII sensitivity.

We examined receptors formed by these mutant subunits in more detail by generating dose-inhibition curves (Fig. 5). The α3,K185Y subunit formed receptors that were 3.4-fold less sensitive to α-CTx-MII blockade ($IC_{50}$ = 12 nM) than α3β2. The α3,I188K subunit formed receptors with an $IC_{50}$ value for α-CTx-MII blockade of 39 nM, which is 11.1-fold less sensitive than α3β2.

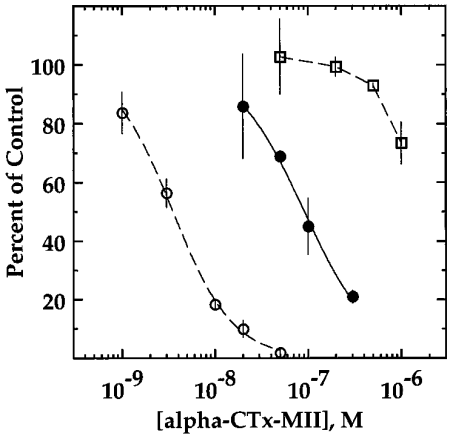


**Fig. 3.** α-CTx-MII sensitivity of receptors formed by α2–181-α3–195-α2. Inhibition by α-CTx-MII of α2–181-α3–195-α2 β2 receptors (●) expressed in *X. laevis* oocytes. The dose-inhibition data for (○) α3β2 and (□) α2β2 from Fig. 1 are shown for reference. The response to a concentration of ACh at or below the $EC_{50}$ value for each receptor after a 5-min incubation with various concentrations of α-CTx-MII is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three or four oocytes). The α2–181-α3–195-α2 β2 data are fit to a Hill equation (see Experimental Procedures), yielding $IC_{50}$ = 91.6 nM, $n$ = 1.19. Some error bars are obscured by symbols.

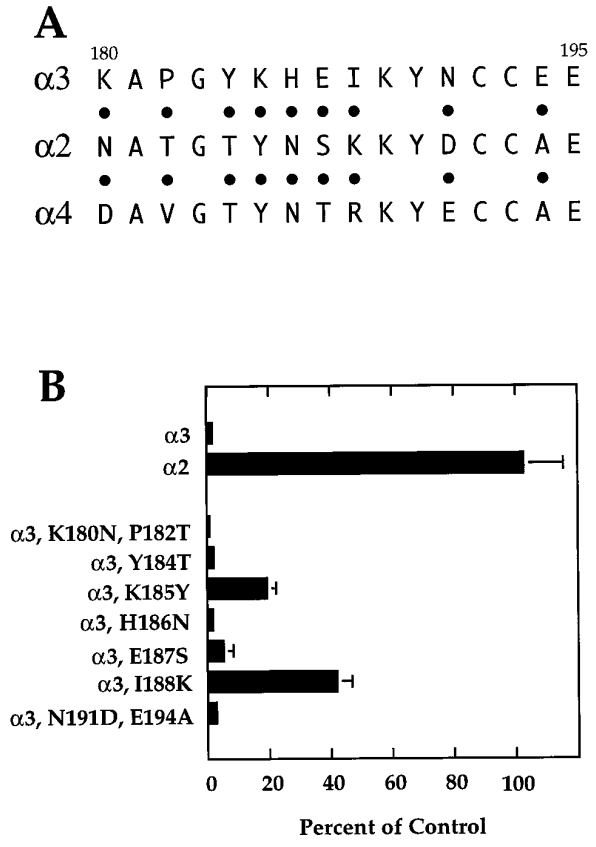


**Fig. 4.** Lys185 and Ile188 of α3 are important for α-CTx-MII sensitivity. A, Amino acid alignment of region 180–195 of α2, α3, and α4. ●, Residues in α2 or α4 that differ from α3. B, α-CTx-MII sensitivity of α3 mutants coexpressed with β2. Current in response to an ACh concentration at or below the $EC_{50}$ value for each receptor after a 5-min incubation with 50 nM α-CTx-MII is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three to four separate oocytes). Significantly different from α3 are α3, K180N, P182T ($p < 0.05$), α3, K185Y ($p < 0.001$), and α3, I188K ($p < 0.001$). Some error bars are too small to appear.

**Determinants of α-CTx-MII sensitivity on β2 lie within sequence segments 1–54, 54–63, and 63–80.** The identity of the β subunit is also critical to determining sensitivity to α-CTx-MII. This is clear in Fig. 1, in which α3β2 is completely blocked by 50 nM α-CTx-MII, whereas α3β4 is blocked only slightly by 500 nM α-CTx-MII (postincubation response = 92.8 ± 2.3% of control). To map residues on β2 that contribute to α-CTx-MII sensitivity, we tested receptors formed by a series of chimeric and mutant β subunits (20) in combination with the α3 subunit. Replacement of the first 54 residues of β2 with β4 sequence (β4–54-β2) had little effect on toxin blockade (Fig. 6), whereas replacement of the first 103 residues of β2 with β4 sequence (β4–103-β2) resulted in a complete loss of sensitivity to 50 nM α-CTx-MII (postincubation response = 99.1 ± 9.7% of control). These results suggest that residues within segment 54–103 are critical to α-CTx-MII sensitivity. To map these residues more closely, we replaced β4 sequence with β2 sequence to determine which sequence segments are required to confer toxin sensitivity. Replacement of the first 54 residues of β4 with β2 sequence conferred some toxin sensitivity, with the β2–54-β4

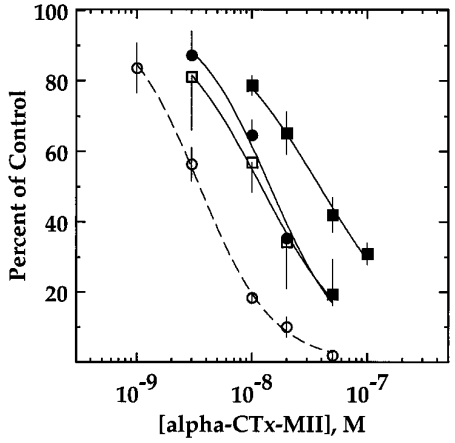


**Fig. 5.** α-CTx-MII sensitivity of receptors formed by α3, K185Y, α3, I188K, and β2, T59K. Inhibition by α-CTx-MII of (□) α3, K185Y β2, (■) α3, I188K β2, and (●) α3 β2, T59K receptors expressed in *X. laevis* oocytes. The dose-inhibition data for (○) α3β2 from Fig. 1 are shown for reference. The response to a concentration of ACh at or below the $EC_{50}$ value for each receptor after a 5-min incubation with various concentrations of α-CTx-MII is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three or four oocytes). The data are fit to a Hill equation (see Experimental Procedures). For α3, K185Y β2, $IC_{50}$ = 12 nM, $n$ = 1.07; for α3, I188K β2, $IC_{50}$ = 39 nM, $n$ = 0.93; and for α3 β2, T59K, $IC_{50}$ = 14 nM, $n$ = 1.29. Some error bars are obscured by symbols.

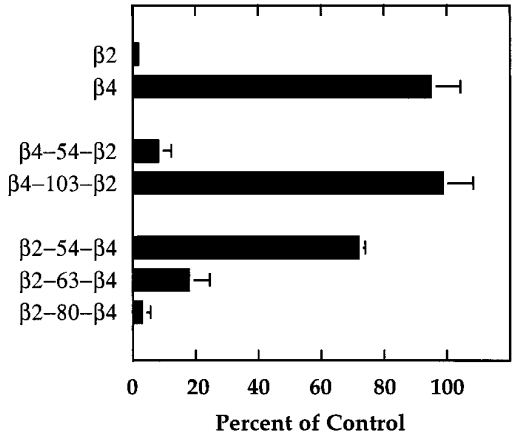


**Fig. 6.** α-CTx-MII sensitivity of receptors formed by chimeric β subunits. Chimeras constructed from the β2 and β4 subunits are coexpressed with the α3 subunit. Current in response to an ACh concentration at or below the $EC_{50}$ value for each receptor after a 5-min incubation with 50 nM α-CTx-MII is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three or four separate oocytes). Significantly different from β2 are β4–103-β2 ($p < 0.001$) and β2–54-β4 and β2–63-β4 ($p < 0.01$). Significantly different form β4 are β4–54-β2, β2–63-β4, and β2–80-β4 ($p < 0.001$) and β2–54-β4 ($p < 0.05$). Some error bars are too small to appear.

subunit forming receptors partially blocked by 50 nM α-CTx-MII (postincubation response = 72.2 ± 2.2% of control). The addition of the first 63 residues of β2 (β2–63-β4) conferred a larger portion of toxin sensitivity (postincubation response = 18.0 ± 6.6% of control). Receptors formed by β2–80-β4 were as sensitive to toxin blockade as wild-type α3β2, suggesting that an additional determinant lies between residues 63 and 80. Thus, β2 contributes several determinants to the α-CTx-MII sensitivity of α3β2, lying within sequence segments 1–54, 54–63, and 63–80.

**Thr59 of β2 is a determinant of α-CTx-MII sensitivity.** Previously, we found a major determinant of NBT and DHβE sensitivity to reside within segment 54–63. We identified this determinant as Thr59 (20). To determine whether this residue also plays a role in determining α-CTx-MII sensitivity, we examined a series of mutant β2 subunits in which each residue that differs between β2 and β4 within segment 54–63 was changed from what occurs in β2 to what occurs in β4 (Fig. 7). The mutations N55S, V56I, and E63T had no effect on toxin sensitivity. Only receptors formed by the mutant β2,T59K were significantly less sensitive to toxin than wild-type α3β2. Thus, similar to our results for NBT and DHβE, Thr59 is involved in determining the α-CTx-MII sensitivity of receptors formed by β2. On testing a range of toxin concentrations (Fig. 5), we found that receptors formed by β2,T59K were 4-fold less sensitive to α-CTx-MII ($IC_{50}$ = 14 nM) than α3β2.

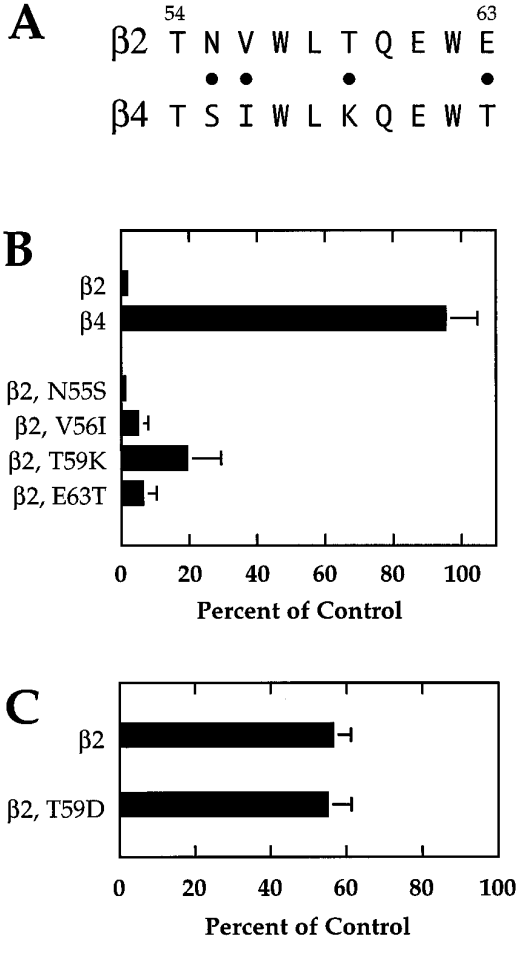


**Fig. 7.** Thr59 of β2 is important for α-CTx-MII sensitivity. A, Alignment of β2 and β4 sequences within segment 54–63. ●, Residues that differ. B, α-CTx-MII sensitivity of receptors formed by each of a series of mutant β2 subunits. Current in response to an ACh concentration at or below the $EC_{50}$ value for each receptor after a 5-min incubation with 50 nM α-CTx-MII is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three to six separate oocytes). β2, T59K is significantly different from β2 ($p < 0.01$). Some error bars are too small to appear. C, Installation of aspartate at position 59 had no effect on α-CTx-MII sensitivity. Current in response to an ACh concentration at or below the $EC_{50}$ value for each receptor after a 5-min incubation with 3 nM α-CTx-MII is presented as a percentage of the preincubation ACh response (mean ± standard deviation of three separate oocytes).

In our previous work, we found that changing Thr59 to aspartate rather than lysine, thus introducing a negative rather than positive charge, resulted in an increase in NBT sensitivity (20). To determine whether the mutation T59D would also have an effect on α-CTx-MII sensitivity, we used a toxin concentration of 3 nM (Fig. 7C). Wild-type α3β2 is partially blocked by 3 nM α-CTx-MII (postincubation response = 56.4 ± 4.8% of control). Receptors formed by β2,T59D were indistinguishable from wild-type in their sensitivity to this concentration of toxin (postincubation response = 55.0 ± 6.4% of control). Thus, unlike NBT block, α-CTx-MII block is unaffected by introducing a negative charge at residue 59 of β2.

## Discussion

We found that determinants of α-CTx-MII sensitivity on the α3 subunit reside within sequence segments 121–181 and 181–195. This contrasts with the distribution of determinants for sensitivity to NBT, a peptide neurotoxin isolated from snake venom that has a selectivity for α3β2 receptors. Major determinants of NBT sensitivity on α3 lie within sequence segments 84–121, 121–181, and 195–215 (17). Only a minor determinant of NBT sensitivity has been found to reside within segment 181–195.[2] NBT is much larger than α-CTx-MII (66 and 16 amino acid residues, respectively), and this may explain why NBT sensitivity requires a larger array of determinants. It is interesting, given that both toxins are competitive antagonists, that residues critical to α-CTx-MII sensitivity (181–195) are of only minor importance to NBT sensitivity. Within this region, residues Y190, C192, and C193 have been identified in affinity labeling experiments as part of the ligand binding site of muscle-type nAChRs isolated from electric organs of various *Torpedo* species (10, 12, 14). These residues are highly conserved in both muscle-type and neuronal-type nAChRs across many species and thus can be thought of as the common features of nAChR binding sites. This conservation rules out these residues in terms of conferring pharmacological differences among nAChR subtypes. It is the residues that differ among subunits that must play this role.

The amino acid residues within sequence segment 181–195 of α3 that determine α-CTx-MII sensitivity are Lys185 and Ile188, positioned close to Y190, C192, and C193, the common features of nicotinic binding sites. The α2 and α4 subunits both have a tyrosine at the position analogous to Lys185 of α3. The 3.4-fold loss in α-CTx-MII sensitivity that we see with the α3,K185Y mutant may be due to the increase in side-chain volume (32.3 Å$^3$), or to the loss of the positive charge. Lys185 of α3 may be interacting with Glu11 of α-CTx-MII, and thus the loss of the positive charge may be a critical factor. At the position analogous to Ile188, α2 has a lysine and α4 has an arginine. The 11-fold loss in α-CTx-MII sensitivity with the α3,I188K mutant is unlikely to be due to the modest change in side-chain volume (2.5 Å$^3$). Another possibility is that the introduction of the positive charge might be responsible for the loss in toxin sensitivity through electrostatic repulsion. This seems unlikely because α-CTx-MII contains no lysines or arginines (21), although the two histidines could be charged. A third possibility is that loss of the isoleucine and the resulting decrease in the hydrophobic character of the α-CTx-MII binding site are responsible for the decrease in sensitivity. These questions can be addressed with more extensive mutational analysis.

It is clear in this and previous studies that neuronal β subunits contribute to the pharmacological properties of neuronal nicotinic receptors. This can be seen clearly by comparing the α-CTx-MII sensitivities of α3β2 and α3β4 in Fig. 1. The α3β4 receptor is also much less sensitive to the antagonists NBT and DHβE than is the α3β2 receptor (20). The β subunits are also involved in determining sensitivity to agonists (4). One of the sequence segments of β2 that determines α-CTx-MII sensitivity (54–63) is also important in determining NBT and DHβE sensitivity (20). Within this region, Thr59 is important for sensitivity to all three competitive antagonists. However, the role that Thr59 plays in determining antagonist sensitivity may differ for these three antagonists. The T59K mutation may reduce NBT sensitivity through electrostatic repulsion because the introduction of a negative charge (T59D) actually results in an increase in NBT sensitivity (20). In contrast, the T59D mutation has no effect on sensitivity to either DHβE or α-CTx-MII.

Despite a confusing nomenclature, the β subunits of neuronal nAChRs are thought to fulfill a role analogous to that of muscle nicotinic γ and δ subunits; they pair with α subunits to form ligand binding sites. It is therefore useful to compare our results with those obtained in the mapping of binding site determinants on muscle γ and δ subunits. Of particular interest are covalent labeling experiments involving the competitive antagonist *d*-tubocurarine, which demonstrated incorporation of label onto a tryptophan residue of the γ and δ subunits (residues 55 and 57, respectively) (8). This residue is conserved in the rat neuronal β2 and β4 subunits (position 57, Fig. 7A), located near residue T/K 59 of β2/β4 that we have identified as important in determination of sensitivity to α-CTx-MII, DHβE, and NBT (current study and Ref. 20). Other important residues have also been identified on γ and δ subunits. Affinity labeling, cross-linking, and mutational analysis identified Asp180 and Glu189 of the δ subunit (9, 11). Both β2 and β4 have a glutamate at a position analogous to E189 of the δ subunit. Tyr117 of the γ subunit (T in δ) has been shown to be critically important in determining curare sensitivity (27, 28). This residue is not conserved in either β2 or β4. Also of interest is another α-conotoxin from *Conus magus* (α-CTx-MI) that shows a 10,000-fold selectivity for the α-δ binding site over the α-γ binding site of mouse muscle nAChRs (29). Chimeric and mutant δ and γ subunits were used to identify the residues Ser36/Lys34, Tyr113/Ser111, and Ile178/Phe172 of the δ/γ subunits as responsible for most of the difference in binding site affinity. There is little conservation of these determinants in neuronal β subunits, which helps explain the failure of α-CTx-MI to antagonize neuronal nAChRs (5, 30).

The fact that both α and β subunits affect the sensitivity of neuronal nAChRs to a wide variety of agonists and antagonists (3–6, 20, 21) suggests that the ligand binding sites of neuronal nAChRs are formed similarly to those of muscle nAChRs, at the interface between α and non-α (β) subunits. For muscle nAChRs, the subunits are thought to be organized within the receptor with a positive face of one subunit associating with a negative face of the next subunit in a rotationally symmetrical fashion (11, 29, 31). In this model,

[2] C. W. Luetje, unpublished observations.

the ligand binding sites are formed by the positive face of an $\alpha$ subunit and the negative face of a $\gamma$ or $\delta$ subunit. In neuronal nAChRs such as $\alpha3\beta2$, the ligand binding sites may also be formed in this fashion: from the positive face of the $\alpha$ subunit and the negative face of the $\beta$ subunit. The $\alpha7$ subunit, which can form homomeric receptors in the *X. laevis* oocyte expression system, seems to be able to provide both faces of the ligand binding site. To provide the positive face, $\alpha7$ has the residues that $\alpha$ subunits contribute to the binding site (Tyr93, Trp149, Tyr190, Cys192, Cys193, Tyr198). In addition, $\alpha7$ has a tryptophan at position 54, analogous to Trp55/57 of $\gamma/\delta$. Mutational analysis of this residue demonstrated involvement in determining sensitivity to both agonists and antagonists, leading to the proposal that $\alpha7$ contributes both an "$\alpha$ component" (i.e., a positive face) and a "non-$\alpha$ component" (i.e., a negative face) when forming homomeric receptors (32). For $\alpha7$ to achieve this, the activity of a prolyl isomerase seems to be required (33). The formation of two binding sites by two pairs of subunits within each receptor suggests that just as for muscle nAChRs, neuronal nAChRs may exist with pharmacologically distinct ligand binding sites within a single receptor. In fact, the existence of neuronal nAChRs containing more than one type of $\alpha$ or $\beta$ subunit seems to be possible (34–38).

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