Nonidentity of the α-Neurotoxin Binding Sites on the Nicotinic Acetylcholine Receptor Revealed by Modification in α-Neurotoxin and Receptor Structures†

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ABSTRACT: α-Neurotoxins constitute a large family of polypeptides that bind with high affinity to the nicotinic acetylcholine receptor (nAChR). Using a recombinant DNA-derived α-neurotoxin (*Naja mossambica mossambica*, NmmI) and mouse muscle nAChR expressed transiently on the surface of HEK 293 cells, we have delineated residues involved in the binding interaction on both the α-neurotoxin and the receptor interface. Several of the studied NmmI mutations, including two residues conserved throughout the α-neurotoxin family (K27 and R33), resulted in substantial decreases in the binding affinity. We have also examined 23 mutations located on the receptor α subunit and have identified 4 positions that appear to be important to NmmI recognition. These determinants represent a conserved aromatic residue (Y190), two positions where neuronal and muscle receptors differ (V188 and P197), and a negatively charged residue (D200). Unlike many of the nAChR agonists and antagonists which bind to the αδ and αγ binding sites on the receptor with different affinities, the wild-type NmmI−wild-type nAChR interaction showed a single affinity. However, by mutating critical toxin or receptor residues, we were able to produce site-selectivity between the αγ and αδ interfaces. These results suggest a nonequivalence in the binding interaction at the two sites, sensitive to discrete structural changes at key contact points on either the toxin or the receptor protein, and underscore the importance of δ and γ receptor subunits in governing binding affinity.

The nicotinic acetylcholine receptor (nAChR)[1] from vertebrate skeletal muscle is a pentameric ligand-gated ion channel composed of four homologous subunits in the stoichiometric ratio of $\alpha_2\beta\delta\gamma$ [reviewed in (*1*−*3*)]. The subunits are arranged in the circular order of αγα$\delta\beta$ (*4*) where the two functional agonist and antagonist binding sites are found at the interfaces between the αγ and αδ subunits (*5*−*7*). As a consequence of the nonidentity of the δ and γ subunits, many ligands bind to these sites with different affinities (*8*−*10*). Despite the absence of a three-dimensional structure, the nAChR binding sites have been partially characterized through site-directed labeling (*11*−*16*), site-specific mutagenesis (*8*, *10*, *17*−*25*), electron microscopy reconstruction analysis (*26*, *27*), and homology modeling (*28*). For the α subunit, three distinct regions in the linear sequence are thought to form part of the agonist and antagonist binding sites (*29*). These domains, some of which may form loops (*28*), are centered around residue 93, residues 149−152, and residues 180−200. Similarly, four potential loops in the δ and γ subunits also contribute to ligand binding specificity.

α-Neurotoxins constitute a family of polypeptides found in snake venoms that are antagonists to the nAChR, have molecular masses of ∼7 kDa, contain 4−5 conserved disulfide bonds, and share a structural motif characterized by 3 extended loops [reviewed in (*30*, *31*)]. Sequence comparisons between the ∼80 known α-neurotoxins have identified 12 residues that are highly conserved throughout the family, 3 of which are positively charged. X-ray crystal structures (*32*−*34*), NMR solution structures (*35*−*37*), and labeling experiments (*38*) have placed these residues on the concave face of these flat, disk-like molecules, suggesting that this face comprises the binding site for receptor recognition.

Even though α-neurotoxins have been used for over 25 years as probes for studying the nAChRs, rather little is known about the molecular mechanism of their interaction. Peptide fragments of the receptor have been employed to gain insight into the structural determinants necessary for toxin recognition (*39*−*43*). Mutagenesis studies carried out on intact, assembled receptors have identified specific amino acids that contribute to the binding of several agonist and antagonists; however, data for the large polypeptidic α-neurotoxins are lacking. Such data may provide a larger framework in which to view the receptor binding sites. For the opposing binding surface located on the toxin, determinants have been identified for at least one short-chain α-neurotoxin (erabutoxin a) through extensive mutagenesis studies (*44*−*46*). These results have shown that the toxin is interacting with the receptor through residues located on loop II and the tips of loops I and III; both conserved and nonconserved amino acids appear to be involved.

The goal of this study was to analyze the complementary binding sites by which an α-neurotoxin and the mouse muscle nAChR interact. For our studies, we have selected a short-chain α-neurotoxin from *Naja mossambica mossambica* (NmmI) and have expressed it by recombinant DNA methods. Previous experiments carried out with native NmmI toxin showed a modest preference for binding to the

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; NmmI, *Naja mossambica mossmbica*; CNBr, cyanogen bromide; SpA, staphylococcal protein A; NH_4OAc, ammonium acetate; HOAc, acetic acid; α-BGTX, α-bungarotoxin.

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αδ over the αγ sites on *Torpedo marmorata* AChR (*47*). Site-selectivity was thought to add another dimension to locating determinants on the δ and γ subunits (*8*−*10*). Examination of recombinantly derived NmmI (rNmmI) toxin, however, showed a single class of sites for the mouse nAChR. Yet, through mutagenesis studies in which important structural determinants on both the toxin and receptor were altered, nonidentity between the two binding sites is revealed.

EXPERIMENTAL PROCEDURES

Materials. The pEZZ18 expression vector and IgG−Sepharose 6 were purchased from Pharmacia Biotech Inc. [^{125}I]-α-Bungarotoxin (specific activity ∼16 μCi/μg) was obtained from DuPont NEN and Na^{125}I (∼1.0 mCi, specific activity 15.5 mCi/μg) from Amersham. α-Conotoxin M1 was purchased from American Peptide Co. HB101 *E. coli* were from Promega, 18% Tris−glycine gels were from Novex Electrophoresis, CNBr was from Sigma, and Econo-Pac High S cartridges were purchased from Bio-Rad. The native NmmI was a generous gift from Dr. Pascale Marchot, CNRS, Marseille, France.

Cloning and Expression Vector. A cDNA encoding NmmI (*48*) was synthesized as four separate oligonucleotides that were annealed, ligated together, and cloned into pBluescript II. The resulting 195 bp gene, flanked by *Xho*1 and *Bam*H1 restriction sites, contained the following sequence (sense strand): TC GAG TGC CAC AAC CAG CAG TCC TCT GAG CCG CCG ACC ACT ACC CGC TGC AGT GGG GGC GAG ACC AAC TGC TAC AAG AAG CGC TGG CGT GAC CAC CGC GGG TAC CGC ACT GAG CGG GGC TGT GGC TGC CCG ACG GTG AAG AAG GGC ATT GAG CTC AAC TGC TGC ACC ACG GAC CGC TGC AAC AAC TAA GGA TCC.

For expression and secretion of rNmmI into the growth medium of *E. coli*, the fusion expression vector pEZZ18 (*45*, *49*) was utilized. This vector encodes a signal sequence and two IgG binding domains derived from staphylococcal protein A (SpA). The NmmI cDNA was subcloned into pEZZ18 using a linker (AAT TCT TCC CTG GTG CAT ATG C) which encoded a methionine residue just N-terminal to the toxin gene, and ensured the α-neurotoxin was in-frame with the encoded IgG binding domains that reside 5′ to the NmmI sequence.

NmmI Expression and Purification. A small culture of HB101 *E. coli* expressing the SpA−NmmI fusion protein was grown overnight at 37 °C in TB (terrific broth: 12 g of bacto-tryptone, 24 g of bacto-yeast extract, 4 mL of glycerol, 900 mL of H_2O) supplemented with phosphate salts and 250 μg/mL ampicillin. Five milliliter aliquots of the small culture were used to seed 2 × 500 mL cultures (500 mL of TB, phosphate salts, 250 μg/mL ampicillin) that were grown ∼20 h at 37 °C. The cells were sedimented (7000 rpm, 20 min); the supernatant was filtered (0.22 μm) and loaded onto a 10 mL IgG−Sepharose 6 column [flow rate 1.6 mL/(cm^2·min)]. The column was washed with ∼150 mL of equilibration buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20) followed by 50 mL of wash buffer (5 mM NH_4-OAc, pH 5). The fusion protein was eluted from the affinity column with 0.5 M HOAc (pH 3.4) at a flow rate of 0.2 mL/(cm^2·min). Active fractions were pooled and lyophilized. The lyophilizate was resuspended with 1.0 mL of 70% formic acid containing 90 mM CNBr, and incubated at room temperature for 5−6 h. The treated sample was diluted to 10 mL with H_20, lyophilized, and resuspended with 50 mM NH_4OAc (pH 7.2). The sample was centrifuged briefly and the pellet discarded. The supernatant was loaded onto a 1.0 mL S-cartridge (0.5 mL/min), previously equilibrated with 50 mM NH_4OAc (pH 7.2). After being washed with 20 mL of equilibration buffer, the toxins were eluted in a gradient of 50 mM−1.0 M NH_4OAc (pH 7.2) in a volume of 50−70 mL. The peak corresponding to the free toxin was pooled, lyophilized, and resuspended in 1.0 mL of H_2O and the concentration was determined from $E_{280} = 9.8 \times 10^{-3}$ M^{-1} cm^{-1} (*50*). The purity of wild-type and mutant rNmmI was assessed by SDS−PAGE and silver staining.

Mutagenesis. To generate the NmmI mutations, mutagenic oligonucleotides were used with single-stranded pBluescript templates according to Kunkel et al. (*51*). The sequence of the entire gene was verified and then subcloned into the pEZZ18 expression vector. The receptor mutations W187S and W184R were made as previously described (*23*). The remaining receptor mutations were described previously (*9*, *23*, *25*; N. Sugiyama, manuscript in preparation).

Amino Acid and Amino-Terminal Sequence Analyses. Amino acid analyses of rNmmI and nNmmI (0.1 mg of each) were carried out on a ABS Auto Analyzer after hydrolysis of the samples with 6 M HCl and 1% (w/v) phenol for 20 h *in vacuo*. Automated Edman analysis was done on a ABS sequenator with 200 pmol of rNmmI.

Expression of Wild-Type and Mutant nAChR. Mouse muscle nAChR subunit cDNAs (α, β, γ, and δ), in the CMV-based expression vector pRBG4, were cotransfected in a ratio of 2:1:1:1 into 293 HEK cells (at ∼50% confluency), using $Ca_3(PO_4)_2$ precipitation. After 6 h, the medium containing cDNA was replaced with fresh medium (Dulbecco's modified Eagle's plus 10% fetal calf serum), and expression was measured 3−4 days after transfection (*23*).

NmmI Binding Measurements. Binding assays were carried out on assembled pentameric nAChRs expressed on the surface of intact cells. The cells were harvested by gentle agitation in phosphate-buffered saline plus 5 mM EDTA, centrifuged briefly, and resuspended in high-potassium Ringer's solution (*6*). The cells were divided into aliquots for binding measurements such that a final concentration of 100 pM binding sites/assay was achieved (assay volume 200 μL). In some cases, nontransfected HEK cells were added to the assay to ensure formation of a pellet. Specified concentrations of NmmI were added to each tube containing receptor (wild-type or α subunit mutations) and allowed to bind for 5 h. NmmI dissociation constants were measured by competition against initial rates of [^{125}I]-α-bungarotoxin binding (*52*).

Because α-bungarotoxin (α-BGTX) is also a member of the α-neurotoxin family, a caveat concerning its use in examining receptor mutations should be noted. For our experiments, we utilize a concentration of α-BGTX (10 nM) significantly above its K_d (60 pM), and rely upon a comparison of the initial rates of α-BGTX binding in the presence and absence of rNmmI. Thus, even if α-BGTX association is effected by the mutant receptor under study, it will not affect our K_d determinations for rNmmI, as long as the α-BGTX binding is not severely reduced at the concentrations employed.

Data were analyzed using least-squares fits to the Hill equation or to two sites of equal population. Nonspecific binding was determined in the presence of either 10 mM carbamylcholine, 300 μM dimethyl *d*-tubocurarine, or 4 μM cobra α-toxin (*N. n. siamensus*). Binding assays conducted in the presence of α-conotoxin MI were carried out in an identical manner. For some K_d determinations, it was necessary to lower the receptor concentration to ∼10 pM (or 20 pM in binding sites) in order to minimize depletion of free ligand. In these cases, the assay volume was 600 μL, and rNmmI was allowed to bind for at least 7 h prior to initiating the initial rate study.

Kinetics of ^{125}I-rNmmI Binding. The association rate of ^{125}I-rNmmI was measured by adding ^{125}I-rNmmI, at final concentrations of 500−900 pM, to suspended HEK cells (expressing wild-type nAChR), and removing aliquots at specified times. The aliquots were immediately diluted into an excess of 10 mM carbamylcholine and washed twice with potassium Ringer's solution followed by centrifugation, and the amount of bound radioactivity was determined. Nonspecific binding was determined in the presence of 10 mM carbamylcholine.

To determine dissociation rate constants, cells were incubated with saturating concentrations of ^{125}I-rNmmI (typically 5 nM) and allowed to equilibrate for over 1 h. To initiate dissociation, the cells were centrifuged briefly to remove excess ^{125}I-rNmmI, and resuspended with potassium Ringer's solution containing either 20 mM carbamylcholine or 1.0 μM unlabeled rNmmI. Aliquots were removed at specified times and washed twice with potassium Ringer's followed by centrifugation, and the amount of bound radioactivity was determined.

Iodination of Recombinant NmmI. Approximately 5 μg (0.7 nmol) of rNmmI was labeled with Na^{125}I (∼1 mCi) using lactoperoxidase as described (*53*). Free iodide was removed by selective adsorption on a Dowex 1-X8 cationic resin. The final specific activity and concentration of ^{125}I-rNmm (typically 950 cpm/fmol, 0.6 mM) were determined by back-titrating with known concentrations of unlabeled α-cobra toxin at various receptor/NmmI ratios. The specific activity is consistent with overall labeling of less than one iodine per toxin molecule.

RESULTS

Cloning, Expression, and Purification of Recombinant Wild-Type and Mutant NmmI. A typical expression−purification scheme of the recombinant α-neurotoxin *Naja mossambica mossambica* started with a 1.0 L culture of HB101 *E. coli* expressing the SpA−NmmI fusion protein. When the bacterial medium was loaded onto a IgG−Sepharose column, the majority of the contaminating proteins passed through the column, while the fusion protein was retained and eluted with 0.5 M acetic acid (Figure 1, lanes 2 and 3), yielding 1000-fold purification in a single step with ∼70% recovery of activity. It is interesting to note that the fusion protein appeared to retain full toxin binding activity despite the conjugation of two IgG binding domains (∼14 kDa).

The toxin was cleaved from the fusion protein by 90 mM CNBr (Figure 1, lane 4) and was purified to homogeneity on a cation exchange column. As shown in Figure 2, rNmmI eluted from the S-cartridge with ∼400 mM NH_4OAc while

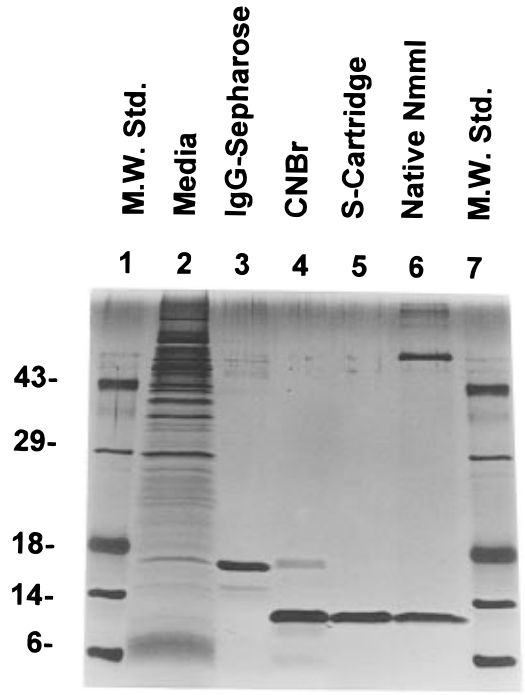

FIGURE 1: SDS−PAGE of fractions obtained from sequential steps of rNmmI purification. Aliquots from each step in the purification were loaded onto 18% SDS−PAGE gels under reducing conditions and visualized by silver stain. Lanes 1 and 7, molecular weight markers; lane 2, SpA−rNmmI fusion protein (∼30 ng) secreted into the culture medium; lane 3, SpA−rNmmI (∼100 ng) eluted from IgG−Sepharose 6; lane 4, rNmmI (∼120 ng) after CNBr cleavage and centrifugation to remove insoluble protein; lane 5, rNmmI (∼120 ng) purified on the S-cartridge; lane 6, native NmmI purified from venom (∼100 ng). Under these conditions, the 21 kDa SpA−NmmI fusion protein runs just below the 18 kDa marker and the 7 kDa rNmmI and nNmmI run slightly below the 14 kDa marker. The high molecular weight band in lane 6 (nNmmI) is added bovine serum albumin.

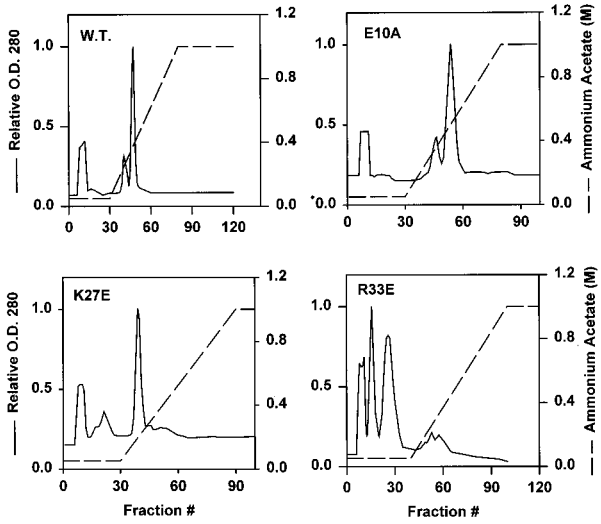

FIGURE 2: Cation-exchange chromatography of rNmmI after CNBr cleavage from the SpA fusion protein. Typical chromatography profiles of rNmmI and rNmmI mutants. O.D. and gradients of NH_4-OAc are shown on the abscissa. Each fraction is ∼1 mL.

the toxin mutations differed in their elution profiles in relation to their net overall increase or decrease in positive charge. In all cases, three major 280 nm absorption peaks were found in the chromatogram. The first peak, found in the effluent, was primarily SpA; the second peak, which displayed receptor binding activity, corresponded to uncleaved fusion protein; and the third and largest peak represented the free

Table 1: Amino Acid Composition Analysis of *Naja mossambica mossambica* (NmmI) Toxin

amino acid	from sequence analysis	native NmmI	recombinant NmmI
Asx	7	6.67	6.61
Thr	8	7.44	7.34
Ser	3	2.71	2.70
Glx	7	7.21	7.43
Pro	3	4.05	3.93
Gly	6	6.03	5.98
Val	1	1.03	0.97
Ile	1	0.92	0.93
Leu	2	2.00	2.07
Tyr	2	1.91	1.91
His	2	2.01	2.06
Lys	4	4.10	4.13
Arg	7	6.92	6.92
Ala	0	0	0
Met	0	0	0
Phe	0	0	0
Trp	1	ND[a]	ND[a]
Cys	8	ND[a]	ND[a]

[a] ND, not determined.

toxin. rNmmI eluting from the S-cartridge was found to be purified to apparent homogeneity by SDS−PAGE and silver staining (Figure 1, lane 5). An overall purification of ∼10 000-fold was routinely achieved with ∼40% recoveries and a typical yield of 0.5−1.5 mg of purified α-neurotoxin per 1.0 L of culture.

Characterization of Recombinant and Native NmmI. The amino-terminal residues of rNmmI were found to be LEXH-NQQSSE (where the X denotes a blank step for nonreduced cystine). This sequence matches the sequence of the native toxin (48) and indicates correct processing and cleavage at the methionine residue. Amino acid analysis of the recombinant and native toxins showed identical compositions within experimental error (Table 1) and corresponded to that inferred from the sequence of the synthetic gene. When subjected to reducing SDS−PAGE and silver staining, rNmmI ran as a single sharp band at an identical position as the native toxin (Figure 1, lanes 5 and 6). Equilibrium binding assays for both rNmmI and nNmmI with mouse muscle nAChR expressed transiently on the surface of HEK cells gave K_d's = 140 ± 40 pM (n_H = 1.1) and 72 ± 16 pM (n_H = 1.2), respectively. Thus, it appears that the recombinant toxin is identical to the native toxin by analytical characterization and affinity to the mouse muscle nAChR.

Even though both rNmmI and nNmmI appeared to have a single affinity for the recombinantly expressed mouse muscle nAChR, previous work by Marchot and co-workers (*47*) has shown that nNmmI binds with two affinities to the acetylcholine receptor found in the electric fish, *Torpedo marmorata* (K_{d_1} = 7 pM and K_{d_2} = 51 pM). The two affinities are presumed to be due to inherent differences at the αδ and αγ binding sites on the *Torpedo* receptor, and were shown kinetically to reflect two distinct dissociation rates. Therefore, we also carried out kinetic studies utilizing ^{125}I-labeled rNmmI with the mouse muscle nAChR. Multiple experiments measuring the association and dissociation rates of ^{125}I-rNmm did not show evidence for two-phase kinetics and were found to fit to exponential association or dissociation curves, with typical results shown in Figure 3. The average rate constants obtained from these experiments, k_{on} = 32 × 10^6 M^{-1} min^{-1} and k_{off} = 3.8 × 10^{-3} min^{-1}, give a

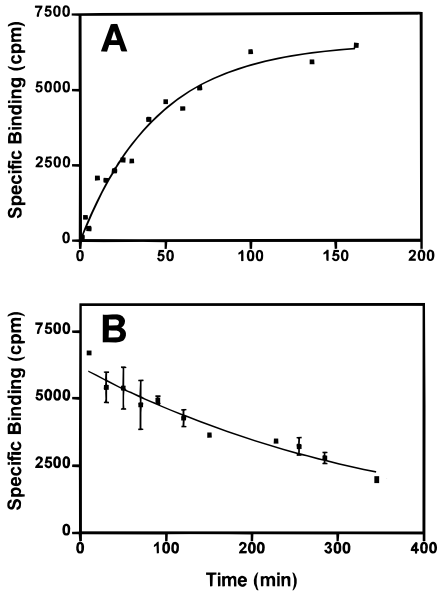

FIGURE 3: Rates of ^{125}I-rNmm I toxin association and dissociation. Kinetics of ^{125}I-rNmm with wild-type nAChR expressed on the surface of HEK cells. (A) Association of 500 pM ^{125}I-rNmmI (850 cpm/fmol) with 20 pM nAChR (k_{on} = 36 × 10^6 M^{-1} min^{-1}). (B) Dissociation of ^{125}I-rNmmI (530 cpm/fmol) after equilibration of the toxin with the cells at a nAChR concentration of 150 pM, centrifugation, and resuspension in a solution containing unlabeled rNmmI (k_{off} = 2.9 × 10^{-3} min^{-1}).

dissociation constant of 120 pM, a value in close agreement with that obtained in the equilibrium binding experiments.

To further distinguish between sites, the equilibrium assays were also carried out in the presence of α-conotoxin MI. α-Conotoxin MI has over a 10 000-fold selectivity for the receptor αδ binding site over the αγ site [$K_{d_{\alpha\delta}}$ = 0.45 nM, $K_{d_{\alpha\gamma}}$ = 20 μM (*9*, *10*)]. At a concentration of 100 nM, α-conotoxin M1 will occupy ∼99% of the αδ sites, but only ∼1% of the αγ sites; thus, the preferential protection enabled us to determine the K_d for the αγ site independently. Both in the presence and in the absence of α-conotoxin MI, Hill coefficients of 1.0 were obtained with similar K_d values (data not shown). Taken together, the observed single phase association and dissociation curves, the equilibrium binding data with Hill coefficients close to unity, and the inability of α-conotoxin MI to affect either affinity or Hill coefficient, all indicate that the binding affinities of the rNmmI to the mouse muscle nAChR are virtually indistinguishable at the αδ and αγ binding interfaces. Thus, the site-selectivity observed in the nNmmI/*Torpedo* AChR but not the rNmmI/mouse nAChR interaction most likely arises from sequence differences between the two receptors.

Characterization of Mutant rNmmI α-Neurotoxin. We selected four site-directed mutations of rNmmI based on previous labeling (*54*−*56*) and mutagenesis studies (*44*, *45*), as well as their position within the structure (*32*, *57*), and have examined their effects on the binding interaction with wild-type nAChR. The three positive residues that are conserved in all α-neurotoxins were mutated to neutral or negative amino acids (K27E, R33E, and K47A). K27 and R33 are located on loop II, while K47 is found on loop III. We also mutated one residue located in loop I (E10A) that is not conserved among the α-neurotoxins.

Figure 4 and Table 2 show the resulting binding curves, K_d's, and changes in affinity of each of the toxin mutations

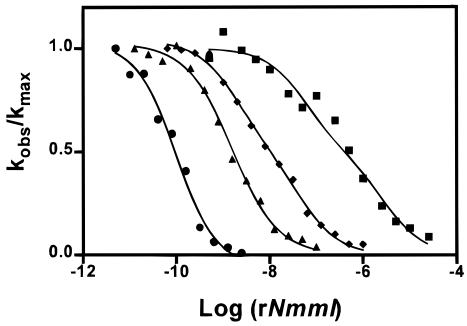

FIGURE 4: Equilibrium binding of wild-type rNmmI and rNmmI mutants to wild-type AChR. Binding of either wild-type rNmmI (●), K47A (▲), K27E (◆), or R33E (■) was measured as the fractional reduction in the initial rates of [^{125}I]-α-bungarotoxin binding in the absence of rNmmI (k_{max}) or the presence of the indicated amounts of rNmmI (k_{obs}). The curves for wild-type and K47A are least-squares fits to the Hill equation with $n_H = 1.0$. The curves for K27E and R33E are least-squares fits to two sites present in equal populations. The concentrations of total binding sites (both αδ and αγ) utilized in each assay were as follows: wild-type rNmmI (15 pM), K47A (140 pM), K27E (120 pM), and R33E (170 pM).

Table 2: Dissociation Constants of Mutant rNmmI Toxin for Nicotinic Acetylcholine Receptor Binding: Site-Selectivity[a]

rNmmI mutation	αδ site K_d	$K_{d(mut)}/K_{d(w.t.)}$	αγ site K_d	$K_{d(mut)}/K_{d(w.t.)}$
wild-type	0.14 ± 0.04	1.0	0.14 ± 0.04	1.0
E10A	0.063 ± 0.036	0.4	0.063 ± 0.036	0.4
K27E	1.8 ± 0.1	13	54 ± 8	390
R33E	95 ± 1	680	2200 ± 300	16000
K47A	1.5 ± 0.1	11	1.5 ± 0.1	11

[a] Binding experiments were carried out as described under Experimental Procedures. Site-Selectivity for K27E and R33E toxins was determined using 100 nM α-conotoxin MI to selectively block toxin binding to the αδ binding site on nAChR.

relative to the wild-type toxin. The K47A and E10A mutations gave binding curves with Hill coefficients ranging from 0.9 to 1.0, indicating similar affinities to the two receptor binding sites. Surprisingly, however, the K27E and R33E mutations resulted in binding curves with Hill coefficients less than unity (0.6), suggesting the presence of two distinct binding sites. When fit to a two-site analysis, the average difference in affinity between the sites was found to be 30-fold for the K27E binding profile, and 23-fold for the R33E profile. To confirm that these results reflected disparate affinities at the αδ and αγ binding interfaces, K_d determinations for both K27E and R33E were carried out in the presence of 100 nM α-conotoxin MI to block selectively the αδ binding site. Figure 5 (panel A) shows the result of K27E assayed under standard conditions in the presence or absence of α-conotoxin MI. In the absence of α-conotoxin MI, the curve gives a Hill coefficient of 0.6 with K_d's = 1.8 nM and 47 nM, when calculated on the basis of an equal population of sites. In the presence of α-conotoxin MI, a single affinity is observed with $K_d = 31$ nM ($n_H = 1.0$). Thus, the mutation K27E appears to influence binding differentially at the two interfaces. Both the K27E and R33E mutations showed the αδ site to have the higher affinity and the αγ site to have the lower affinity (see Table 2). As an alternative method for dissecting the contributions of the αδ and αγ binding sites, the K27E mutation was analyzed using receptor expressed as $\alpha_2\beta\delta_2$ or $\alpha_2\beta\gamma_2$ pentamers. This approach enabled us to confirm high-affinity binding to the

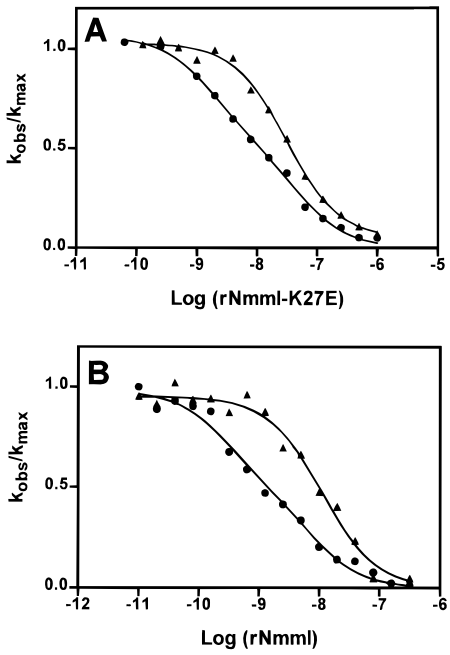

FIGURE 5: Equilibrium binding of rNmmI in the absence and presence of α-conotoxin MI. (Panel A) Binding of rNmmI K27E with wild-type nAChR in the absence (●) or presence (▲) of 100 nM α-conotoxin MI. (Panel B) Binding of wild-type rNmmI with α-subunit mutation D200Q in the absence (●) or presence (▲) of 2.0 μM α-conotoxin MI. Binding was measured by the fractional reduction of the initial rates of [^{125}I]-α-bungarotoxin binding (k_{obs}/k_{max}). K27E: $K_d = 1.8$ nM and 47 nM ($n_H = 0.6$; concentration of binding sites = 120 pM), or in the presence of α-conotoxin MI, $K_d = 31$ nM ($n_H = 1.0$; concentration of binding sites = 220 pM). D200Q: $K_d = 0.31$ nM and 7.5 nM ($n_H = 0.6$; concentration of binding sites = 78 pM), or in the presence of α-conotoxin MI, $K_d = 11$ nM ($n_H = 1.0$; concentration of binding sites = 32 pM).

αδ site and low-affinity binding to the αγ site (data not shown).

Of the four mutations studied, E10A appears to have a similar affinity as the wild-type rNmmI, K47A caused more than a 10-fold increase in the K_d, and K27E and R33E resulted in marked increases in K_d. The R33E mutation affected α-neurotoxin binding to the greatest extent, with over 4 orders of magnitude shift observed at the αγ binding interface and nearly 3 orders at the αδ interface.

Characterization of Receptor Mutations. A series of site-directed mutations of the nAChR α-subunit, located within each of the three domains previously shown to contribute to ligand binding, were examined for their ability to affect rNmmI affinity (*cf.* Table 3). Because of the presumed role of cation−π and Coulombic interactions in the binding energy of the cationic α-neurotoxins (NmmI net charge +5), each of the aromatic residues found in these three domains and many of the charged side chains were mutated. In addition, because α-neurotoxins bind specifically to muscle receptors (α1 subtype), and not to most neuronal receptors (α2−4 subtypes), multiple neuronal-specific mutations were carried out based on sequence differences between muscle and neuronal α subunits.

Due to the sensitivity and practical limitations of the binding assay (low specific activity of ^{125}I-α-bungarotoxin, long incubation times, and assay by centrifugation), it was difficult to screen receptor mutations at concentrations of binding sites less than 100 pM. Therefore, unless indicated, experiments reported in Table 3 were carried out with ~100

Table 3: Residues Located in Three Domains of the Acetylcholine Receptor α-Subunit: Apparent Dissociation Constants (K_d) of Mutant Receptors[a]

	residue no.	wild-type residue	mutated residue	apparent K_d (nM)
		WT		0.40
domain I	93	Y	S	0.25
domain II	149	W	S; F	0.49; 0.13
	150	T	−	−
	151	Y	S; F	0.41; 0.37
	152	D	Q	0.64
domain III	180	E	−	−
	181	A	−	−
	182	R	V	0.46
	183	G	−	−
	184	W	Y; R	0.46; 0.23
	185	K	E	0.51
	186	H	−	−
	187	W	S	0.54
	188	V	D; K	(0.12, 2.7);[b] (2.8, 55)[b]
	189	F	K	0.88
	190	Y	F; T	(3.5, 70);[b] (6.8, 69)[b]
	191	S	−	−
	192	C	−	−
	193	C	−	−
	194	P	L	0.20
	195	T	E	0.33
	196	T	E	0.40
	197	P	H; I	(0.46); (0.47, 9.6)[b]
	198	Y	T	0.51
	199	L	−	−
	200	D	Q	(0.33, 9.2)[b]

[a] Binding experiments were carried out utilizing a concentration of 100 pM binding sites unless indicated. [b] Data shown in Table 4; these measurements were carried out with lower receptor concentration when necessary.

pM binding sites/assay. Because of the high affinity (K_d ~140 pM) of wild-type rNmmI for wild-type nAChR, these conditions result in depletion of free rNmmI in the assay, as seen by the high K_d (400 pM) for the wild-type receptor. Therefore, only apparent K_d's are reported in Table 3; the true values should lie somewhere between 140 and 500 pM.

The majority of nAChR mutations studied gave apparent K_d values close to that of wild-type and therefore were not studied further. However, mutations representing substitutions at four distinct positions in domain III (188, 190, 197, and 200) resulted in substantial decreases in toxin binding affinity, indicating a major role in α-neurotoxin recognition. Interestingly, analysis of the binding curves produced by each of these mutations resulted in Hill coefficients of less than 1. Thus, similar to results obtained with the toxin mutations, substitutions of important residues on the α-subunit appear to reveal a site-selectivity not evident in the interaction of the wild-type rNmmI−nAChR complex.

To establish that these results were actually due to different affinities at the two discrete sites, and to determine which site possessed the higher affinity, binding assays were carried out in the presence of sufficient amounts of α-conotoxin MI to block the αδ binding site. The affinity of α-conotoxin M1 to each of the receptor mutations studied has previously been determined (N. Sugiyama, manuscript in preparation). Figure 5 (panel B) shows the α-subunit mutation D200Q assayed in the presence and absence of α-conotoxin MI. In the presence of α-conotoxin MI, the number of sites decreased by ~50%, and the resulting Hill coefficient sharpens to 1.0 as compared to 0.6 in the absence of

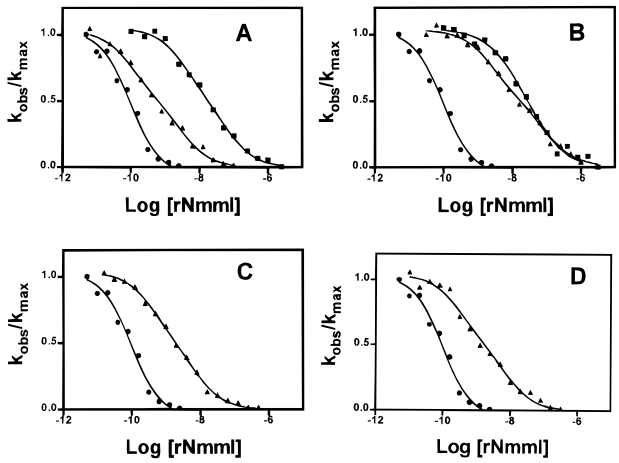

FIGURE 6: Equilibrium binding of wild-type rNmmI with the nAChR containing mutations in the α-subunit. (Panel A) Result of substitutions at position 188 in the α subunit: wild-type nAChR (●), V188D (▲), V188K (■). (Panel B) Result of substitutions at position 190 in the α subunit: wild-type nAChR (●), Y190F (▲), Y190T (■). (Panel C) Result of substitution at position 197 in the α subunit: wild-type nAChR (●), P197I (▲). (Panel D) Result of substitution at position 200: wild-type nAChR (●), D200Q (▲). The concentrations of total binding sites (both αδ and αγ) utilized in each assay were as follows: wild-type nAChR (15 pM), V188D (40 pM), V188K (140 pM), Y190F (91 pM), Y190T (30 pM), P197I (122 pM), and D200Q (78 pM).

Table 4: Dissociation Constants (K_d) for rNmmI Binding to Wild-Type and Selected Mutant Acetylcholine Receptors: Site-Selectivity[a]

nAChR (α mutations)	K_d αδ site	$K_{d(mut)}/K_{d(w.t.)}$	K_d αγ site	$K_{d(mut)}/K_{d(w.t.)}$
wild-type	0.14 ± 0.04	1.0	0.14 ± 0.04	1.0
V188D	0.12 ± 0.01	0.9	2.7 ± 0.2	19
V188K	2.8 ± 1.0	20	55 ± 5	390
Y190F	3.5 ± 1	25	70 ± 16	500
Y190T	6.8 ± 2.5	49	69 ± 6	490
P197I	0.47 ± 0.05	3.4	9.6 ± 2.4	69
D200Q	0.33 ± 0.02	2.3	9.2 ± 1.7	66

[a] Binding experiments were carried out as described under Experimental Procedures. Constants were measured in the absence of α-conotoxin where both sites are available. Site-selectivity for each mutant was determined using α-conotoxin MI to selectively block rNmmI binding to the αδ binding site on nAChR. Concentrations of α-conotoxin MI were based on parallel studies (N. Sugiyama, manuscript in preparation) and were as follows: V188D (300 nM), V188K (200 nM), Y190F (200 nM), Y190T (1.0 μM), P197I (500 nM), and D200Q (2.0 μM).

α-conotoxin MI, consistent with residual α-neurotoxin binding to a single site. K_d determinations were 0.31 and 7.5 nM without α-conotoxin MI and 11 nM in the presence of α-conotoxin MI. Since α-conotoxin MI will protect the αδ site, the residual α-neurotoxin binding of lower affinity is at the αγ site. Accordingly, identical experiments carried out with the V188D, V188K, Y190F, and P197I receptor mutations demonstrated between 20- and 30-fold selectivity for the αδ binding site over the αγ binding site. The Y190T mutation, on the other hand, gave Hill coefficients around 0.8, corresponding to only a 10-fold difference between the two sites (6.8 ± 2.5 nM and 69 ± 6 nM). Affinity differences of less than 10-fold are difficult to discern and cannot be definitively assigned.

The resulting binding profiles and K_d determinations for these mutations are shown in Figure 6 and Table 4. All of these experiments were carried out under conditions of low

receptor concentrations to minimize ligand depletion. Position 190 appeared to be the most sensitive to rNmmI binding, producing the greatest decrease in affinity. Substituting Phe for Tyr at this position (Y190F) produced a 25-fold decrease in affinity at $\alpha\delta$ and a 500-fold decrease at $\alpha\gamma$. Replacing Y190 with threonine produced similar results, with a 50-fold loss observed at $\alpha\delta$ and a 500-fold loss at the $\alpha\gamma$ interface, indicating the importance of an aromatic hydroxyl group at this position. Introducing a positive charge at position 188 (V188K) resulted in 20- and 390-fold decreases in affinity at the $\alpha\delta$ and $\alpha\gamma$ interfaces, respectively. On the other hand, substitution of an anionic side chain at this position (V188D) showed virtually no effect at the $\alpha\delta$ interface, and showed only a 20-fold loss of affinity at the $\alpha\gamma$ interface. At position 197, changing an isoleucine for the proline resulted in a selective 70-fold loss of affinity at the $\alpha\gamma$ site, without significantly affecting affinity at the $\alpha\delta$ site. The mutation D200Q was also selective in destabilizing toxin stability at the $\alpha\gamma$ site without affecting the $\alpha\delta$ site.

DISCUSSION

In this study, we report the expression and purification of a recombinant DNA-derived short-chain α-neurotoxin from *Naja mossambica mossambica* utilizing a bacterial expression system. The purified rNmmI appeared to be identical to the native toxin by multiple analytical assays and by its affinity for mouse muscle nAChR. The toxin affinity was found to be nearly identical at the $\alpha\delta$ and $\alpha\gamma$ binding sites as assessed by: (1) equilibrium binding with Hill coefficients near unity, (2) single phase association and dissociation curves with ^{125}I-labeled rNmmI, and (3) the inability of α-conotoxin M1 to affect the affinity or Hill coefficients of the residual binding site. However, we show that upon dissecting out important structural determinants located on both the toxin and receptor interfaces, site-selectivity is revealed.

Of the four rNmmI mutants analyzed, substitutions at the three conserved positive residues (K27E, R33E, and K47A) resulted in substantial changes in binding affinity. Reductions in affinity would be expected on the basis of previous chemical modification (*54*−*56*) and mutagenesis studies (*44*, *45*) carried out with homologous α-neurotoxins. The largest shifts in affinity were observed with the mutations located in loop II (K27E and R33E). These mutations also differentially influenced binding at the two interfaces, while the K47A and E10A mutations did not exhibit site-selectivity. Hill coefficients of less than unity appear to arise from nonidentity of the $\alpha\gamma$ and $\alpha\delta$ sites rather than negative cooperativity. Binding of α-conotoxin to its high-affinity $\alpha\delta$ site would be expected to destroy the linkage relationship intrinsic to negative cooperativity, and the NmmI dissociation constants at the $\alpha\gamma$ site are identical irrespective of whether α-conotoxin or NmmI is bound to the $\alpha\delta$ site.

In contrast to our results, mutagenesis studies carried out with the α-neurotoxin erabutoxin a, which included the R33E and K27E substitutions, did not show site-selectivity upon binding to the *Torpedo* receptor (*44*, *45*). Quantitative results with the R33E mutation also differed between the two toxins, with a decrease in affinity of 680- and 16 000-fold observed with rNmmI and 318-fold observed with erabutoxin a. In addition, we find that substitution at the nonconserved residue (E10A) did not affect rNmmI binding, while the homologous mutation Q10A in erabutoxin a resulted in a 210-fold decrease in affinity. These different results may be due to sequence differences between *Torpedo* and mouse nAChR, but they may also suggest that the family of α-neurotoxins utilize different side chains for contributing to stabilization of the respective complexes.

Several explanations might be advanced to explain the $\alpha\delta$ versus $\alpha\gamma$ site-selectivity conferred by the loop II mutations. Although both binding sites on the receptor are formed with the same face of the two respective α subunits, they differ by the opposing γ or δ subunits. Thus, upon binding to the receptor, if loop II of the toxin comes in close apposition to the subunit interfaces—or directly binds to the γ and δ subunits—mutations in this loop might produce differing effects between the binding sites. Furthermore, because both K27E and R33E mutations introduce a charge difference of (−2), and both mutations differentially affected binding at the $\alpha\gamma$ site more than the $\alpha\delta$ site, it is possible that the observed selectivity arises from a negative charge and Coulombic repulsion found in γ but not in δ. The proximity of loop II to the δ and γ subunits is in agreement with photolabeling studies (*58*, *59*). These studies showed that an α-neurotoxin containing a photoactivatable group on loop II (position 27 with rNmmI numbering) labeled both δ and γ subunits, while the same labeling reagent located on the third loop (position 47 with rNmmI numbering) labeled the α subunit. Alternative explanations for the observed site-selectivity are considered below.

In order to define the binding interface on the nAChR responsible for α-neurotoxin recognition, we have studied a series of nAChR site-directed mutations. Our initial studies focused on the α subunit because substantial evidence indicates it to be the primary surface for toxin binding. α-Bungarotoxin will bind to the α subunit alone, albeit with lower affinity, in the absence of an interface formed with either δ or γ subunits (*60*, *61*). In addition, the resistance of cobra and mongoose receptors to α-bungarotoxin binding has been shown to be largely due to glycosylation at positions 189 and 187 on the α subunit, respectively (*9*).

The determinants in domain III identified to influence NmmI affinity fall in three categories: (1) a conserved tyrosine (Y190), (2) a negative charge (D200), and (3) modifications found in neuronal but not muscle nAChRs (V188K, P197I). Y190 is one of three conserved tyrosine residues found in all α subunits (Y93, Y198, Y190) and shown to contribute to stabilization of quaternary ammonium groups on agonist and antagonists (*23*, *62*). While mutations in Y93 and Y198 did not affect rNmmI binding, substitutions at position 190 where the hydroxyl aromatic side chain was eliminated caused the most dramatic changes of all the receptor mutations studied. It is intriguing to speculate that similar to other ligands, this aromatic residue is stabilizing positive charges on the toxin. D200, which is also conserved in α-subunits, has been shown to affect receptor channel opening without significantly affecting binding of some agonists and antagonists (*18*, *63*). The D200Q mutation selectively affected rNmmI binding at the $\alpha\gamma$ interface, but not the $\alpha\delta$ interface. Because of the net negative charge on the receptor extracellular domain (*28*) and net positive charge on the α-neurotoxins, this result is consistent with the presumed role of Coulombic interactions in stabilizing the complex. Valine at position 188 is conserved in most α1 subunits but is a positive charge in most neuronal α subunits. Larger changes in affinity found with the V188K mutation,

versus the V188D mutation, suggest that electrostatic repulsion may be engendered by the lysine substitution. Taken together, the decreases in affinity found with receptor α subunit mutations at positions 188, 190, 197, and 200 all suggest that rNmmI, and specifically key cationic side chains, is binding in close proximity to this segment of sequence in the receptor. Alternately, one or more of these residues may exhibit a long-range interaction, therein alter receptor conformation and indirectly reduce NmmI affinity.

Importantly, each of these receptor mutations was found to affect differentially the binding of the toxin to the αδ and αγ interfaces, even though the face presented by the α subunits should be equivalent at the two binding sites. These results suggest that mutations of key contact residues on the receptor force the peptide to adjust its binding position and thus enable the different energetic contributions of δ and γ subunits to become manifest. Alternatively, the toxin may be binding to the two receptor sites with two different orientations, using distinct loci on both the α and non-α subunits. This model is supported by the fact that several of the receptor mutations (V188D, P197I, D200Q) were almost completely selective in destabilizing binding at the αγ versus the αδ sites. The position of the bound α-neurotoxin has yet to be resolved by the higher resolution electron microscopy reconstruction analysis, and this might arise from multiple binding orientations for the α-neurotoxin.

Results of combined α-neurotoxin and receptor mutagenesis yield an initial view of the molecular mechanism of stabilization of the toxin−receptor complex. Early studies of α-neurotoxin−receptor binding have employed isolated peptide fragments from the α subunit (*39*−*43*). However, the demonstration that both rNmmI and α subunit mutations produce site-selectivity underscores the importance of the δ and γ subunits in toxin recognition, and suggests a multipoint attachment where the toxin interacts with both α subunit and δ/γ subunit determinants. Such a view emphasizes the importance of also studying intact, assembled receptors, as the single subunit or peptide fragments likely present only a portion of the binding determinants seen in the intact receptor. Further studies should focus on key residues on the δ and γ subunits; ultimately we hope such studies will yield a comprehensive view of the binding interaction.

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