Articles

Probing the Agonist Domain of the Nicotinic Acetylcholine Receptor by Cysteine Scanning Mutagenesis Reveals Residues in Proximity to the α-Bungarotoxin Binding Site†

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ABSTRACT: We have constructed a series of cysteine-substitution mutants in order to identify residues in the mouse muscle nicotinic acetylcholine receptor (AChR) that are involved in α-bungarotoxin (α-Bgtx) binding. Following transient expression in HEK 293-derived TSA-201 cells, covalent modification of the introduced cysteines with thiol-specific reagents reveals that α subunit residues W187, V188, F189, Y190, and P194 are solvent accessible and are in a position to contribute to the α-Bgtx binding site in native receptors. These results with the intact receptor are consistent with NMR studies of an α-Bgtx/receptor−dodecapeptide complex [Basus, V., Song., G., and Hawrot, E. (1993) *Biochemistry 32*, 12290−12298]. We pursued a more detailed analysis of the F189C mutant as this site varies substantially between AChRs that bind Bgtx and certain neuronal AChRs that do not. Treatment of intact cells expressing F189C with either bromoacetylcholine (BrACh) or [2-(trimethylammonium)ethyl] methane-thiosulfonate (MTSET), both methylammonium-containing thiol-modifying reagents with agonist properties, results in a marked decrease (∼55−70%) in the number of α-Bgtx binding sites, as measured under saturating conditions. The decrease in sites appears to affect both α/γ and α/δ sites to the same extent, as shown for αW187C and αF189C which were the two mutants examined on this issue. In contrast to the results obtained with MTSET and BrACh, modification with reagents that lack the alkylammonium entity, such as methyl-methanethiosulfonate (MMTS), the negatively charged 2-sulfonatoethyl methane-thiosulfonate (MTSES), or the positively charged aminoethyl methylthiosulfonate (MTSEA), has little or no effect on the maximal binding of α-Bgtx to the αW187C, αV188C, or αF189C mutant receptors. The striking alkylammonium dependency suggests that an interaction of the tethered modifying group with the negative subsite within the agonist binding domain is primarily responsible for the observed blockade of toxin binding.

The nicotinic acetylcholine receptor (AChR)[1] at the neuromuscular junction is a pentameric protein composed of four subunit types in a molar ratio 2α:*β*:*γ*:*δ* and has long been the major prototype for ligand-gated ion channels functioning as neurotransmitter receptors. Major determinants for the binding of agonists, such as ACh or carbamylcholine, and competitive antagonists, e.g., α-bungarotoxin (α-Bgtx) and d-tubocurarine, are located on the α subunit, although the *γ*/*δ* subunits also contribute to the binding sites [see ref 1 for review (*2*, *3*)]. Characterization of the agonist binding site has been facilitated by the use of curaremimetic neurotoxins, such as α-Bgtx. This competitive antagonist has been widely employed as a probe for the receptor as it binds specifically and with high affinity.

Several approaches have been undertaken to identify the molecular determinants of the α-Bgtx binding site. Most of these approaches have followed a reductionist strategy and have studied Bgtx binding either to the isolated α subunit (*4*, *5*), proteolytic fragments of the α subunit (*6*), synthetic peptides (*6*−*8*), or recombinant fusion proteins containing receptor sequences (e.g., refs *9*−*11*). A discussion of these

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[1] Abbreviations: ACh, acetylcholine; AChR, nicotinic acetylcholine receptor; BrACh, bromoacetylcholine; α-CTX, α-conotoxin MI; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; MBTA, 4-(*N*-maleimido)-α-benzyltrimethylammonium iodide, MMTS, methyl methane thiosulfonate; MTSACE, [2-(aminocarbonyl)ethyl] methanethiosulfonate; MTS, methanethiosulfonate; MTSEA, aminoethyl methanethiosulfonate; MTSEA-biotin, *N*-biotinylaminoethyl methanethiosulfonate; MTSES, (2-sulfonatoethyl) methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate; NmmI, Naja mossambica mossambica I, SCAM, substituted cysteine accessibility method.

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various approaches is reviewed by Basus et al. (*12*). In general, all these studies argue that the major determinant for the Bgtx binding site is likely to be located between residues 173 and 204 of the α subunit. Furthermore, the NMR-derived structure of a complex formed between Bgtx and a dodecapeptide corresponding to residues 185−196 from the α subunit indicates that peptide residues 186−190 are in close association with Bgtx (*12*). Recently, Ackermann et al. (*13, 14*) have generated a series of receptor mutants and expressed them in HEK 293 cells to study interaction with a short-chain α-neurotoxin NmmI. Most notably, mutants V188K, Y190K, and P197K greatly perturbed toxin binding, indicating a possible role for these sites in the formation of the toxin binding site. No evidence was obtained for a role of the neighboring residues at positions 187 and 189.

We have now applied a cysteine-substitution mutagenesis approach to the examination of the α-Bgtx binding site. In this strategy, individual cysteines are introduced into presumed extracellular regions of potential structural or functional significance. These engineered cysteines, by virtue of their free sulfhydryl group, can subsequently be modified covalently with specific reagents (*15*). In general, these reagents react much faster with Cys at the water-accessible surface of the receptor than with Cys in the interior of the protein or at the lipid-accessible surface (*16, 17*). Previous studies of oocyte-expressed Cys-substitution mutations of α subunit residues 181−197 (*18*) indicate that the majority of these substitutions are well tolerated and lead to minimal perturbations in receptor function. In this study, we have turned to an HEK-293-derived expression system using TSA-201 cells where cell surface binding studies are facilitated. Probing residues 183−198, we describe the chemical modification of the cell surface localized mutant receptors with various thiol-specific reagents and we characterize the effect of these covalent modifications on Bgtx binding, as measured under conditions that saturate the wild-type receptor. The results suggest that α subunit residues W187, V188, F189, Y190, and P194 all lie near or contribute to the α-Bgtx binding site in the native receptor. In addition, our findings support the hypothesis that an electrostatic interaction involving positively charged residues on Bgtx is important for receptor recognition and that Bgtx directly interacts with the negative subsite of the agonist binding domain.

MATERIALS AND METHODS

Mutagenesis. We used a cytomegalovirus-based expression vector (GWI, British Biotechnology, Oxford, U.K.) to express the cDNAs for the α-, *β*-, *γ*-, and *δ* subunits of the mouse muscle nicotinic AChR. Mutations were introduced using the Quikchange Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's specifications. Mutations were confirmed by diagnostic restriction enzyme digests and bidirectional sequencing of the entire insert following a DyeDeoxy terminator protocol (Perkin-Elmer).

Transfections and Cell Lines. A human embryonic kidney cell line containing a neomycin resistance gene and expressing T-antigen (TSA-201 cells) was generously provided by Dr. William Green (University of Chicago, Chicago, IL). In our hands, the TSA-201 cell line yielded 50% greater levels of cell surface Bgtx binding than the parental HEK 293 cells following transfection with wild-type AChR subunit-encoding plasmids. Cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (DMEM + FBS) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. TSA-201 cells were transiently transfected with mutant or wild-type AChR subunit cDNAs using Fugene-6 lipofection reagent (Boehringer Mannheim). In all experiments, cDNAs were cotransfected in the following amounts per 10 cm plate of cells: α (3 *μ*g) and *β*, *γ*, and *δ* (1 *μ*g each). Cells were maintained for 3 days at 37 °C in DMEM + FBS prior to binding assay determinations.

Covalent Cysteine Modification and [^{125}I]α-Bgtx Binding Assay. Three days after transfection, cells were harvested by gentle agitation in phosphate-buffered saline containing 5 mM Na_2-EDTA. After a brief centrifugation at ∼600*g*, the cells were resuspended in high-K Ringer's solution (*19*) and divided into two equal aliquots, one of which was exposed to 1.5 mM of the modifying reagent (except where otherwise noted) for 20 min at room temperature. The unbound reagent was removed by pelleting the cells (2 min at room temperature) and resuspending the pellet in 0.6 mL of high-K Ringer's. This wash was repeated three times in total. Reagents were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada) or from RBI (Natick, MA). For incubations with α-CTX (Sigma), cells were incubated for 20 min at room temperature with 100 nM α-CTX following wash-out of BrACh. α-CTX was not removed prior to incubation with Bgtx. α-Bgtx binding levels were determined in a modified saturation binding assay essentially as previously described (*20*). Mechanically harvested cells [$\sim(0.5-5) \times 10^7$ cells obtained from one 75 cm^2 tissue culture flask] were resuspended in 0.6 mL of high-K Ringer's solution and incubated with 5 nM [^{125}I]Bgtx (DuPont) for 2 h at room temperature to allow for saturation of Bgtx binding sites. It has been previously demonstrated that these assay conditions allow measurement of the total number of Bgtx binding sites (*2*). Cell-attached radioactivity was determined with a *γ*-counter following collection of the cells on Whatman GF/C (25 mm) glass fiber filters and 4 washes with 5 mL of high-K Ringer's. Nonspecific binding was determined in the presence of a 500-fold excess of unlabeled Bgtx (RBI). Typically, TSA-201 cells transiently transfected with wild-type AChR subunits yielded 50−100 fmol of surface toxin binding/cm^2 of confluent cells.

Covalent Cysteine Modification and [^{125}I]Bgtx Solid-Phase Binding Assay of Torpedo Membranes. *Torpedo* membranes were purified as described (*6*). For the modifications, 100 *μ*L membranes (at a protein concentration of 10 *μ*g/mL in high-K Ringer's) were plated in flat-bottomed 96-well microtiter plates (Nunc, Immunosorb) and centrifuged at 1000*g* for 30 min at 4 °C. Wells were washed three times with Ringer's and quenched with 0.2 mL of 2% bovine serum albumin in Ringer's for 1 h at room temperature, followed by two washes with 0.2% bovine serum albumin in Ringer's. Modifying reagent (final concentration of 1.5 mM for all reagents with the exception of DTT, which was used at 1 mM) was added for 20 min at room temperature. Bound [^{125}I]Bgtx was determined with a *γ*-counter following three washes with 0.2% BSA in Ringer's (*4*).

RESULTS

Previous work has shown that a cysteine can be substituted for many of the individual amino acids between positions 183 and 197 of the mouse muscle-type α subunit without dramatic effect (<6-fold changes in the EC_{50}) on receptor functionality as measured by ACh-responsiveness in *Xenopus* oocytes (*18*). To explore further the role of the Cys-substituted residues in the binding of Bgtx, we have now expressed these mutations in a cell line (TSA-201) permitting a more detailed analysis of the effects of thiol modification on Bgtx binding. Two of the mutants showed greatly reduced Bgtx binding levels when expressed in the HEK-derived cell lines. In the first case, the αY190C mutant yields very low but detectable levels of binding. In other studies, we have shown that the αY190C mutation leads to a decrease in affinity for Bgtx binding without affecting expression levels (Spura, Freedman and Hawrot, manuscript in preparation). In the second case, the αH186C mutant receptor does not appear to assemble efficiently in the human TSA-201 cell line (manuscript in preparation), despite good expression in oocytes, as no surface [^{125}I]Bgtx binding was observed following numerous transfection attempts.

BrACh and MTSET Modification of the αF189C Site Blocks Binding. Our major goal was to use the Cys-substitution mutants to determine whether modification of the introduced thiols interferes with [^{125}I]α-Bgtx binding. The alkyl methane thiosulfonates have been widely used for such accessibility-directed analyses (*15*). Another thiol modifying agent that has been extensively used in earlier work with the nicotinic AChR is bromoacetylcholine, an alkylammonium compound containing an α-haloacyl ester moiety. BrACh reacts covalently with the AChR to block [^{125}I]Bgtx binding following selective reduction of the disulfide between Cys192 and Cys193 (*21*−*23*). Using BrACh as the thiol modifying reagent on intact cells, we first wanted to determine the optimal concentration of the reagent for the chemical modification of the cysteine mutants. Our major focus was on the αF189C mutant, since no prior indications for a surface accessibility of this residue had been obtained. As shown in Figure 1, when cells expressing the αF189C mutant were treated with various concentrations of BrACh and then tested for binding following washout of the unreacted BrACh, a large decrease in the number of available [^{125}I]Bgtx binding sites was observed. The maximal effect corresponded to a decrease of 55−60%. The concentration that resulted in half-maximal inhibition of Bgtx binding was determined to be 3.5 μM with the maximal effect being obtained with a concentration of 1.5 mM BrACh. This result compares well with the value obtained for half-complete labeling of reduced *Torpedo* membranes with BrACh (5 μM; ref *24*). The blockade of Bgtx binding shown in Figure 1 follows a 20 min incubation with BrACh; we have since shown that the same level of blockade is achieved after a 5 min incubation with BrACh. In similar dose−response studies using the positively charged alkylthiosulfonate reagent, MTSET, to modify the αF189C mutant, we found that maximal blockade of Bgtx binding was likewise achieved with a concentration of 1.5 mM MTSET (data not shown). As inhibition was maximal with BrACh or MTSET concentrations greater than 1 mM, we used 1.5 mM of the modifying reagent for all subsequent modifications of the cysteine mutants.

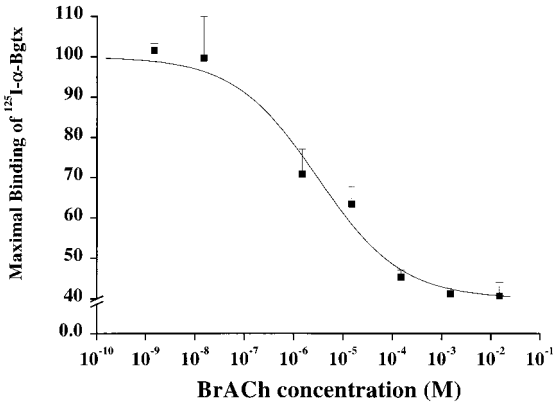

FIGURE 1: Concentration dependence of BrACh modification on Bgtx binding to mutant αF189C-expressing cells. TSA-201 cells were transfected with αF189C cDNA and the three other (β,γ,δ) AChR subunit cDNAs, harvested after 3 days of incubation and treated with the indicated concentrations of BrACh for 20 min at 20 °C. The level of Bgtx binding was determined under saturating conditions as described in the Materials and Methods. Experimental determinations were performed in triplicate for each concentration and were repeated at least three times under comparable conditions. Error bars represent the S. E. M. for all the experiments. A concentration of 3.5 μM was found to be required to achieve 50% of the maximal observed blockade of Bgtx binding sites. At saturation with BrACh, ∼60% of the Bgtx binding sites were blocked. All binding data are compared to the value obtained for cells that were not treated with BrACh (set at 100%).

Cysteine Accessibility Scanning of α Subunit Residues 183−*197 for Effects on α-Bgtx Binding.* As shown in Figure 2, BrACh-mediated modification of mutants αW187C, αV188C, αF189C, αY190C, and αP194C resulted in a 40−65% decrease in Bgtx binding. The most pronounced effect was obtained for mutant αF189C with about 65% inhibition. Importantly, in a wild-type control incubation, where no surface-accessible thiols have been introduced into the AChR, normal Bgtx binding levels were observed following incubation with BrACh (Figure 2). This result clearly indicates that any noncovalently attached BrACh has been effectively removed by the washing procedure. As an additional negative control, we have used mutant αD71C, whose expression was comparable to that of the majority of the Cys-mutants tested in this study. The Asp at position 71 forms part of the main immunogenic region (MIR) spanning residues 67−76 of the AChR (*25*). This position was shown to be important in epitope recognition by anti-MIR antibodies and is therefore likely to be surface-exposed but it is not likely to be near the Bgtx binding site as anti-MIR antibodies have no effect on Bgtx binding to the AChR (*25*). It is clear that no reduction in α-Bgtx binding is observed when this mutant is treated with BrACh (Figure 2). In studies where the positively charged alkyl methane thiosulfonate, MTSET, was used instead of BrACh, results very comparable to those shown in Figure 2 were obtained (Table 1 and Russin et al., manuscript in preparation); the effect on the Cys mutants is therefore not unique to BrACh modification. Taken together, these results suggest that residues αW187, αV188, αF189, αY190, and αP194 are all surface accessible and that they are in a position to contribute to Bgtx binding whereas several other residues tested in this region, αG183C, αW184C, αK185C, αS191C, αT195C, αT196C, and αP197C, appear not to provide essential components to the Bgtx

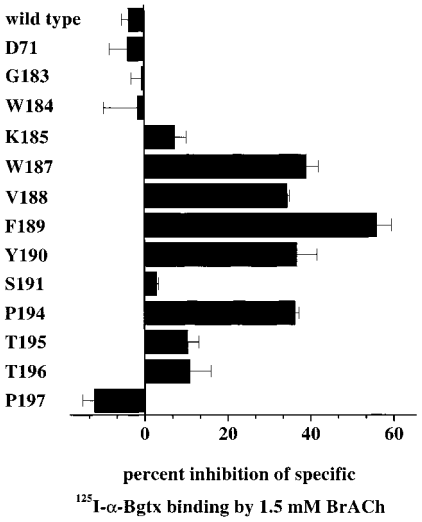

FIGURE 2: Effect of BrACh modification on Bgtx binding to wild-type and various cysteine-substitution mutants. Mutant or wild-type α subunit encoding cDNAs were transfected together with wild-type β, γ, and δ subunits. Bgtx binding was measured as described in the Materials and Methods with half of each cell suspension treated with a concentration of 1.5 mM BrACh. For each mutant, [^{125}I]Bgtx binding was determined on replicate cells not treated with BrACh and the effect of the BrACh treatment on the binding of [^{125}I]Bgtx was plotted relative to this value. Each assay was performed in triplicate and each mutant was tested at least three times under comparable conditions. Error bars represent the S. E. M. for all the experiments. No expression was obtained with the αH186C mutant and αC192 and αC193 are not plotted as these cysteines form a disulfide in the wild-type receptor. Following reduction with DTT, however, modification of these residues does lead to a reduction in Bgtx binding (see Figure 4). Note that as expected the BrACh treatment used under these assay conditions is without effect on Bgtx binding to the native wild-type AChR.

binding site, although affinity reductions of less than ~50-fold cannot be ruled out with these saturation-based studies.

Effect of the Charge of the Modifying Reagent on the Inhibition of Bgtx Binding. To assess the effect that charge and size play in mediating the observed inhibition of Bgtx binding, various cysteine specific methane thiosulfonate (MTS) derivatives were compared on αF189C and the results are shown in Figure 3. Both BrACh and MTSET, which are positively charged, lead to a ~60−70% decrease in Bgtx binding as previously observed (see above), whereas treatment with the negatively charged MTSES did not have any effect on Bgtx binding. Likewise, neither the neutral but similarly sized MTSACE nor the neutral but smaller MMTS have any effect on Bgtx binding (Figure 3). Similarly, the neutral, but bulkier reagent MTSEA-biotin (used at 0.15 mM; the maximum concentration achievable without introduction of significant nonspecific effects) also did not interfere with binding (Figure 3). Interestingly, treatment of αF189C with MTSEA did not result in a reduction of Bgtx binding. MTSEA is predominantly positively charged at neutral pH, but is less bulky than the quaternary amines BrACh and MTSET and lacks the hydrophobic properties of the methylammonium compounds. Furthermore, although MTSET and BrACh have agonist properties, MTSEA does not (*26*). Thus, the covalent attachment of a positive charge to αF189C by itself, while a necessary requirement, is not sufficient to interfere with Bgtx binding. Importantly, very comparable results were also obtained with mutants αW187C and αV188C (Table 1), suggesting that the observed effects are not unique to αF189C.

Table 1: Effects of Thiol Modification on Bgtx Binding to Mutant α subunits[a]

mutant	first treatment	second treatment	Bgtx binding (%)
αW187C	MMTS		97 ± 6
	MTSACE		89 ± 3
	MTSEA-biotin (150 μM)		88 ± 4
	BrACh		61 ± 3
	MTSET		54 ± 4
	MTSEA		114 ± 12
	MTSES		91 ± 3
	MTSEA	BrACh	87 ± 3
	MTSES	BrACh	95 ± 4
αV188C	MMTS		92 ± 6
	MTSEA-biotin (150 μM)		85 ± 5
	BrACh		64 ± 1
	MTSET		61 ± 4
	MTSEA		116 ± 3
	MTSES		95 ± 3
	MTSEA	BrACh	97 ± 6
	MTSES	BrACh	97 ± 5
αF189C	MMTS		107 ± 6
	MTSACE		99 ± 3
	MTSEA-biotin (150 μM)		111 ± 8
	BrACh		41 ± 2
		BrACh	43 ± 3
	MTSET		33 ± 6
	MTSEA		99 ± 4
	MTSES		99 ± 3
	MMTS	BrACh	97 ± 3
	MTSACE	BrACh	96 ± 3
	MTSEA-Biotin (150 μM)	BrACh	98 ± 5
	MTSEA	BrACh	97 ± 5
	MTSES	BrACh	92 ± 3

[a] Aliquots of cells expressing $\beta\gamma\delta$ subunits and the α-cysteine mutation indicated were incubated with the indicated first thiol reagent (at 1.5 mM except for MTSEA-biotin, which was applied at 150 μM) or with control buffer (high-K Ringers) for 20 min at room temperature. After a washout period, the cell suspension was either incubated with BrACh (1.5 mM) or with control buffer for 20 min at room temperature. The quantitation of cell surface Bgtx binding sites was determined under saturating conditions, following the second treatment, as described in the Materials and Methods and is expressed as a percentage of that determined for untreated mutant cells. Values are means ± S. E. M. from at least three determinations. For comparison, results obtained for mutant αF189C as shown in Figure 3 are incorporated as well.

Evidence that MTSES and Other Thiol-Specific Reagents Modify αW187C, αV188C, and αF189C without Interfering with Bgtx Binding. It is possible that the lack of binding interference observed with MTS-derivatives lacking an alkylammonium is due to a deficiency in the reactivity of these reagents with the exposed thiols possibly involving unfavorable environmental factors such as electrostatic interactions between the receptor and the modifier. To test this possibility, we devised a sequential incubation protocol to verify that thiol modification had occurred. Mutant cells were first exposed to a modifying reagent (the test reagent) other than BrACh and, after an appropriate wash, were then treated with BrACh under conditions which would normally lead to a decrease in the number of Bgtx binding sites. If the test reagent does indeed react with the mutant thiol, it should prevent the subsequent reaction of that thiol with BrACh, and thus, the second incubation with BrACh should result in no inhibition of Bgtx binding. As shown in Table 1, the effect of a prior incubation of the mutants αW187C, αV188C, and αF189C with MTSES followed by BrACh treatment is identical to the results obtained for the test

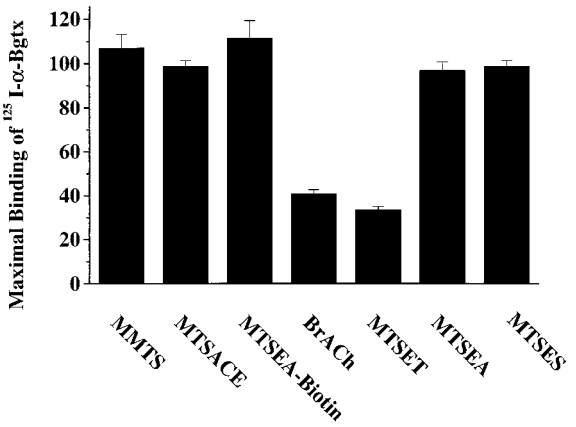

FIGURE 3: Thiol modification with either BrACh or MTSET blocks Bgtx binding to mutant αF189C-expressing cells but other MTS-derivatives have little or no effect. TSA-201 cells were transfected with αF189C, β, γ, and δ subunit cDNAs and incubated with the indicated thiol-modifying reagents for 20 min at room temperature. Concentrations for all reagents were 1.5 mM, except for MTSEA-biotin, which was used at a final concentration of 150 μM. [^{125}I]-Bgtx binding was determined as described in the Materials and Methods. Maximal [^{125}I]Bgtx binding (set at 100%) corresponds to the value obtained for the replicate mutant cells for which the thiol-modification treatment was omitted. Bgtx binding was decreased to 41 ± 2% following treatment with BrACh, and it was decreased to a level of 38 ± 1.5% following a similar treatment with MTSET. All experimental determinations were performed in triplicate and repeated at least three times. Error bars represent the S. E. M.

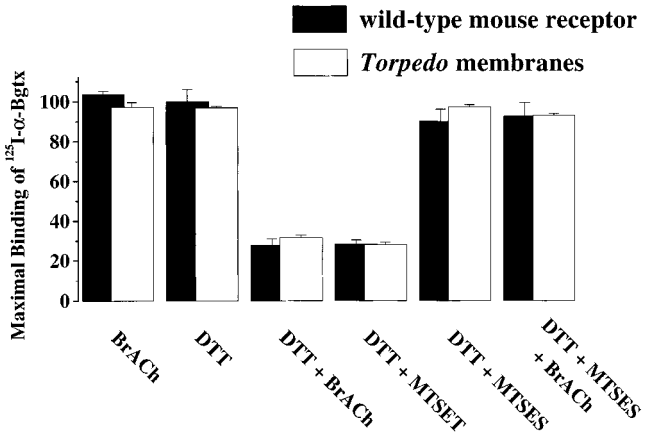

FIGURE 4: Modification of reduced wild-type receptors from mouse and *Torpedo* produces a blockade of Bgtx binding consistent with the results seen with modification of αF189C. For the mouse receptor, all four wild-type subunit-encoding cDNAs were transfected into TSA-201 cells. Prior to modification with thiol-specific reagents (20 min at room temperature), cells were exposed to 1 mM DTT for 20 min at room temperature and washed three times to remove the unreacted DTT. Bgtx binding was determined as described in the Materials and Methods. The preparation and thiol modification of the *Torpedo* electric organ membranes is described in the Materials and Methods. All experiments were performed in triplicate and were repeated at least three times. Error bars correspond to the S. E. M. Maximal binding of [^{125}I]Bgtx is referenced to untreated wild-type cells (set = 100%). Bgtx binding after treatment with DTT and BrACh was decreased to 28 ± 3% (cells) and 32 ± 1% (*Torpedo* membranes) of maximal binding. Similar experiments were performed using MTSET, MMTS, and MTSACE in place of BrACh (see text). Sequential incubation with MTSES followed by BrACh (right column) demonstrates that MTSES is capable of modifying the reduced wild-type AChR, as it provides protection from subsequent BrACh modification. Overall, these results are comparable to those obtained with αF189C mutant cells (Figure 3 and Table 1).

reagent alone (MTSES). This finding indicates that thiol modification of mutants 187−189 does occur with MTSES under the conditions used but that such modification is not sufficient in itself to block Bgtx binding. Results similar to those obtained with MTSES were observed for the four other modifying reagents (MTSEA, MMTS, MTSACE, and MTSEA-Biotin) tested for modification of αF189C (Table 1). Minor changes in affinity are possible and need to be further examined (e.g., MTSEA-biotin treatment of αW187C and αV188C caused a ∼15% inhibition in sites). We therefore conclude that, for mutants αW187C, αV188C, and αF189C, differences in the ability to block Bgtx binding as a result of incubation with different thiol modifiers are unlikely to be due to varying and incomplete thiol modification by these reagents.

Effects of Thiol Modifiers on Bgtx Binding to the Reduced Form of the Wild-Type AChR. Residues 192 and 193 form a vicinal disulfide bond in native wild-type receptors and are therefore not amenable to modification with the reagents used above. On the other hand, previous experiments have indicated that Bgtx binding to wild-type *Torpedo* membranes is decreased upon reductive alkylation with BrACh (*21*, *22*). Given the close proximity of these cysteines to the introduced mutations, it is therefore of interest to investigate the precise effects of the same group of thiol modifiers on these residues. We were particularly interested in determining whether modifications with neutral or negatively charged thiosulfonates would interfere with Bgtx binding. Using the same experimental preparation that was used for the analysis of the Cys-substitution mutants, we systematically investigated the effect of either BrACh or various MTS derivatives on Bgtx binding following modification of Cys192/Cys193 of the wild-type AChR. Transfected TSA-201 cells, transiently expressing wild-type AChR, were treated with 1 mM DTT to selectively reduce the vicinal disulfide bond linking αC192 and αC193 (*23*, *27*). As shown in Figure 4, incubation with DTT alone or with 1.5 mM BrACh had no effect. In contrast, in cells treated with 1 mM DTT followed by a brief wash and then incubated with 1.5 mM BrACh, Bgtx binding sites were decreased by 70%. An identical level of blockade was observed when MTSET was substituted for BrACh. Other MTS-derivatives tested at 1.5 mM (MMTS, MTSES, and MTSACE), however, had no effect on Bgtx binding levels to reduced wild-type AChR (see Figure 4 for representative results with MTSES). Just as was shown above with the αW187C, αV188C, and αF189C mutants (Table 1), we could demonstrate that the lack of effect seen with MTSES is not due to a lack of reactivity with the available thiols. MTSES does react with DTT-treated wild-type AChR as demonstrated by the finding that treatment with MTSES completely abolishes the susceptibility of the AChR's Bgtx binding site to blockade by BrACh. These observations indicate that the charge-related effects observed with the Cys-substitution mutants can be extended to positions 192/193. The pattern of reactivity for reduced wild-type mouse AChR expressed in TSA-201 cells was identical to that seen when the native AChR in *Torpedo* electric organ membranes was tested (Figure 4). Treatment of reduced *Torpedo* AChR with either MTSET or BrACh led to a ∼70% decrease in the number of Bgtx binding sites. In contrast, no decrease in the number of Bgtx binding sites was observed following

modification with MTSEA (tested with TSA-201 cells only, data not shown), MMTS, MTSES, or MTSACE (Figure 4).

Evidence that Bgtx Binding Is Not Selectively Impaired on Either the α/γ or α/δ Interface. It was striking that BrACh- or MTSET-evoked inhibition of Bgtx binding hovers around 50−60%, particularly for residues encompassing the 187−190 region. This could imply that inhibition is selective for either the α/γ or α/δ interface, leaving the second site unaffected. We have measured the apparent K_D for toxin binding to BrACh-modified αF189C receptor and find it to be identical to that obtained with the unmodified wild-type receptor. We also have verified that the association and dissociation rates of the residual Bgtx sites following modification are identical to those obtained with the unmodified sites (data not shown). The observed reduction in binding is therefore not due to an incremental change in affinity but rather argues that a large fraction of the sites is essentially no longer accessible. These observations could be taken as consistent with a situation in which only one of the two ligand binding sites per receptor molecule is affected.

To address this question, we employed a sequential incubation protocol; we first incubated with BrACh, followed by an appropriate wash to remove unreacted modifier, followed by an incubation with 100 nM α-CTX. α-CTX exhibits over a 10000-fold selectivity for the receptor α/δ binding site over the α/γ site [$K_{D\alpha/\delta} = 0.5$ nM, $K_{D\alpha/\gamma} = 20$ μM (*28*)]. This approach is justified by the fact that the binding of α-CTX prevents the binding of Bgtx to the same site (*27*). Used at a concentration of 100 nM, α-CTX occupies ∼99% of the α/δ sites, but only ∼1% of the α/γ sites, and therefore allowed us to determine independently the amount of BrACh labeling targeted to the α/γ site. First, we verified that αF189C shows a similar α-CTX selectivity and affinity as wild-type AChR. We obtained K_D values of 0.3 nM (αδ) and 15 μM (αγ), respectively, which are comparable to those of the wild-type receptor. Furthermore, the lack of effect for αF189C on α-CTX binding is in good agreement with results the results of Sugiyama et al. (*28*), where mutation αF189K did not interfere significantly with α-CTX binding. Importantly, incubation with α-CTX following BrACh modification leads to an additional 50% inhibition of Bgtx binding (Figure 5), bringing the final level of Bgtx blockade to a total of 75%. These results suggest that both α/δ or α/γ sites contribute equally to the BrACh-mediated block of Bgtx binding. If only the Bgtx binding sites on the α/γ interface were affected by BrACh, the second incubation with α-CTX would have blocked 100% of Bgtx binding. Likewise, a selective modification of the α/δ site with BrACh would have resulted in no additional inhibition of Bgtx binding following α-CTX addition, i.e., in a final level of Bgtx binding of 50% compared to untreated controls. The intermediate result obtained argues for nonselectivity between the α/γ and α/δ sites. Identical studies with mutant αW187C had a similar outcome. There was no apparent site preference in the blockade introduced by BrACh modification.

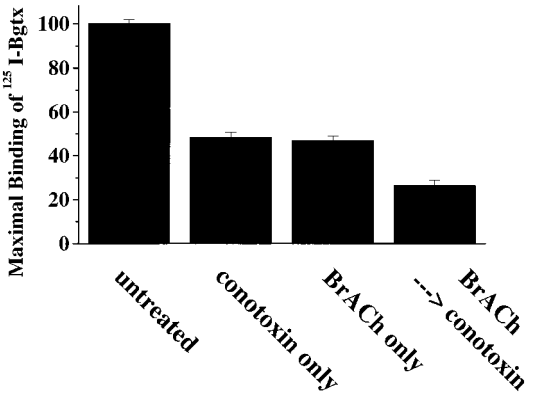

FIGURE 5: Effect of sequential incubations of BrACh and α-CTX on the inhibition of Bgtx binding to mutant αF189C. Cells were transfected with αF189C, along with wild-type β, γ, and δ subunit cDNAs, harvested after 3 days and incubated with either BrACh (1.5 mM) or α-CTX (100 nM) for 20 min at room temperature. For modification with BrACh, unreacted reagent was removed following the incubation and prior to adding [^{125}I]Bgtx. For the α-CTX treatments, the conotoxin was present during the Bgtx binding incubations. [^{125}I]Bgtx binding was determined as described in the Materials and Methods. For the sequential incubation, BrACh (1.5 mM) was first added for 20 min, unreacted reagent was removed, and then α-CTX (100 nM) was added for 20 min, followed by [^{125}I]Bgtx. Bgtx binding was decreased to 25.4 ± 3% upon the combination of BrACh modification followed by α-CTX incubation. All experiments were performed in triplicate and repeated three times; error bars represent the S. E. M. Comparable results were also obtained for αW187C (not shown).

DISCUSSION

Cysteine Mutagenesis Is Well Tolerated in Region 183−197. Most of the mutants in this region appeared to express well as evidenced by cell surface [^{125}I]Bgtx binding levels determined under nominally saturating conditions. These were comparable to wild-type levels, suggesting that the Cys substitutions are well tolerated and do not grossly affect receptor assembly or transport to the cell surface. These results suggest that the substitutions do not produce large structural changes, a conclusion consistent with previous electrophysiological studies (*18*). Cys substitution of residues in the 183−197 region (except for αH186C and αY190C) produces little apparent effect on the observed Bgtx binding levels, although more detailed studies would be needed to assess whether the mutations produce more subtle changes in binding kinetics. A number of similar studies indicate that introduced cysteines themselves produce only very subtle effects on structure and function (*29*).

Methylammonium-Derivative Modification of Cysteines Introduced at Positions α187, α188, α189, α190, and α194 Lead to a Major Reduction in Bgtx Binding Sites. Following MTSET or BrACh-mediated modification of the substituted Cys thiols, there is a substantial (40−65%) drop in Bgtx binding to the cell surface. These effects were observed following the incubation with [^{125}I]Bgtx for 2 h. For mutant αF189C at least, maximal binding was not reached until 4 h after addition of Bgtx, while a 2 h incubation led to about 70% of maximal binding. Nonetheless, prolonged incubation with [^{125}I]Bgtx affected modified and unmodified mutants to the same extent; the observed blockade in Bgtx binding sites following modification for both the 2, 4, or 6 h was in each case 50%. The modification-dependent block of toxin binding provides strong evidence that residues 187, 188, 189, 190, and 194 are surface accessible. Possible reasons why the blockade of Bgtx binding is not complete are discussed below in greater detail.

We decided to focus our attention in these studies on αF189C because there was no prior evidence that this residue

is solvent accessible in the intact receptor. In addition, the residue at 189 varies substantially between those AChRs that bind Bgtx, where it is usually aromatic, and those neuronal AChRs that do not, where it is a positively charged residue. No significant blockade of Bgtx binding is observed when these same reagents are used to treat Cys mutants introduced into positions α71, α183, α184, α185, α191, α195, α196, and α197. These results therefore support the structural predictions derived from the NMR studies (*12*); residues 187−190 (as well as residue 194 which was not identified in the NMR structure) are all clearly surface accessible. They are all in a position to contribute to Bgtx binding to the AChR in that their modification with a suitable reagent drastically reduces Bgtx binding levels. There are two possible explanations for the lack of effect observed with Cys substitutions of αG183, αW184, αK185, αS191, αT195, αT196, or αP197. In the first case, these particular residues may be surface accessible but may lie outside of the α-Bgtx binding site and thus their modification would cause no interference with Bgtx binding. For example, this is the expected situation for the αD71C mutant. In the second possibility, these substituted residues may not be accessible for reaction from the extracellular space. At this point, we have no way of distinguishing between these two possibilities, but in either case we can conclude that these latter substituted residues are unlikely to contribute directly to the Bgtx binding site.

The conclusions concerning surface accessibility of α subunit residues 187−190 are consistent with studies of the Bgtx-resistant AChRs found in cobra and mongoose muscle and of HEK-expressed mouse muscle AChRs containing glycosylation signals found in the cobra and mongoose AChR (*27, 30*). Replacement of αW187 and αF189 with Asn and Thr, respectively, leads to N-linked glycosylation at the introduced αN187 site (*30*), and this glycosylation exerts a major effect in decreasing the affinity for Bgtx binding. Similarly, the introduction of the mutation, αF189N, into the mouse receptor enables the glycosylation of this site, and the glycosylated AChR's affinity for Bgtx is decreased 100-fold (*30*).

The surface accessibility of αV188 was demonstrated by McLaughlin et al. (*18*) and is also supported by recent studies of Ackermann et al. (*13, 14*) where αV188K led to a 20-fold decrease in affinity at the α/δ interface and a 390-fold decrease in affinity at the α/γ interface for a short α-neurotoxin, NmmI. As noted by the authors, however, this mutation did not result in a comparably severe reduction in Bgtx binding (*13*). The lack of a major effect of MTSEA-modification of αV188C on Bgtx binding is therefore most likely due to different interaction of the receptor with short neurotoxins, such as NmmI, versus long neurotoxins, such as Bgtx. Interestingly, a mutation introducing a negative charge at this position (αV188D) only produced a ∼10-fold decrease in toxin binding. The saturation binding assay used in our studies was designed to detect major perturbations of Bgtx binding following thiol modification, and thus relatively minor changes in affinity would escape detection. This may explain the lack of inhibition when αV188C is modified with either the negatively charged MTSES or the positively charged MTSEA. The saturation binding assay does not permit an analysis of more subtle changes leading to reductions of Bgtx binding of ∼50-fold or less. In contrast to our results, Ackermann and Taylor provide no indication of the involvement of αW187 and αF189 in toxin binding. In the case of αW187, introduction of a serine residue had no effect on NmmI binding. At position 189, Ackermann and Taylor observe a very subtle (2-fold) reduction of NmmI binding for mutation αF189K, whereas we detect the elimination of ∼60% of the Bgtx sites at this position following BrACh or MTSET-treatment, but not after MTSEA incubation. The αF189K mutation with the introduction of a positive charge would have more structural resemblance to an MTSEA-modified Cys residue than to the product of BrACh or MTSET modification (*2*). Just as observed for αW187C and αV188C, we only obtain inhibition following modification of αF189C with BrACh/MTSET, but not with MTSEA. The fact that αY190T leads to a >100-fold decrease in the apparent affinity for NmmI toxin (*13*) is consistent with our observation that αY190C leads to a decreased affinity for Bgtx (manuscript in preparation).

While Ackermann and Taylor observe a ∼30-fold reduction of NmmI affinity with mutation αP197I (whereas αP197H is without effect), we do not detect any decrease of available Bgtx binding sites with modification of αP197C. Again, because our studies are performed under nominally saturating conditions using 5 nM Bgtx, they would not reliably detect a 30-fold change in affinity. Ackermann et al. (*14*) conclude that αV188, αY190, αP197, and αD200 interact with NmmI residues Arg 33 and Lys 27, based on calculations of interaction energies from double mutant cycle analyses of *mutant* receptor and toxin pairs in which new functionalities have been introduced. While these calculations provide strong evidence for interactions between the mutations introduced into the receptor and into NmmI, they do not necessarily reflect energetic interactions between the *wild-type* receptor and toxin residues. As stated by Ackermann et al. (*14*), an analysis based on mutations to alanine would be required to address this issue. It is also possible that some differences would be expected due to the likely different modes of interaction between the AChR and short neurotoxins, of which NmmI is an example, and long neurotoxins, such as Bgtx.

Importance of the Methylammonium Group in Producing a Substantial Decrease in Bgtx Binding Sites. Whereas BrACh and MTSET each led to a substantial (∼50−65%) decrease in Bgtx binding to the αW187C, αV188C, and αF189C mutants, other MTS derivatives such as MTSEA, MMTS, MTSES, MTSACE, and MTSEA-biotin are essentially without effect (Table 1). Furthermore, we show that prior treatment with MTSES and the other MTS-reagents abolishes the ability of BrACh to affect Bgtx binding (Figure 3 and Table 1). The most likely explanation for this effect is that MTSES and the other reagents react with the thiol, forming the expected disulfide, and thus prevent the subsequent modification of this site with BrACh.

MTSET and BrACh both contain positively charged quaternary amines and they are among the bulkiest of the reagents tested (*29*). Only MTSEA-biotin is bulkier but this reagent was tested at only 0.15 mM due to limited solubility. It is clear that the smaller uncharged (MMTS, MTSACE) or negatively charged MTS derivatives (MTSES) do not effectively block Bgtx binding to either αW187C or αF189C, nor to the reduced wild-type receptor. The less bulky modifications are unlikely to provide much steric hindrance to Bgtx binding. This is consistent with the observation that

most *single* site-directed mutations of residues in the ligand-binding domain appear to have minimal effects on Bgtx binding (*13, 14, 27, 31*−*33*).

MTSET and BrACh may be much more effective than the positively charged MTSEA and the other MTS-derivatives tested because of the presence of the quaternary methylammonium group that confers agonist-like properties to these reagents. We speculate that MTSET and BrACh first react covalently with the engineered cysteines, and, after tethering, the methylammonium moiety interacts with the agonist or Bgtx binding site, if it is in proximity. Occupation of the receptor subsite with the alkylammonium may then prevent Bgtx binding to the same or a nearby site. Further agonist protection experiments may shed light on this issue. For example, preincubation with either 50 μM carbachol or 50 μM ACh does not appear to retard modification of αF189C with 5 μM BrACh (unpublished observations). A similar lack of protection was observed with suberyldicholine and nicotine (Russin et al. *Soc. Neurosc*. Abstr. 24, no. 332.20, p 838). These results suggests that BrACh and MTSET do not require an interaction with the agonist binding site for modification.

An alternative explanation for our results is that the tethered MTSET or BrACh produces a conformational change of the receptor which then induces an obstruction of the Bgtx binding sites. In this case, blockade following modification of a particular residue would not necessarily imply that the Bgtx binding site is nearby. We are currently unable to distinguish experimentally between these two alternative interpretations. The double mutant cycle analysis between receptor and toxin (*14*) provides good evidence, however, that residues substituted into positions α188 and α190 are sufficiently close to bound NmmI to contribute energetically to its binding. Furthermore, studies involving α1/α3 chimeric receptors (Levandoski et al., *Soc. Neurosc.* Abstr. 24, no. 332.12, p 837) indicate that substitution of *Torpedo* α1 residues W184, W187, V188, Y189, and T191 for the corresponding rat α3 residues Y, E, I, K, and N, respectively, confers significant Bgtx sensitivity to the intact chimeric receptors expressed in oocytes. In combination, these results are most consistent with Bgtx binding in close proximity to the region encompassing Cys 192/193 in the α subunit.

Possible Explanations for Incomplete Inhibition of Bgtx Binding Following the Covalent Attachment of BrACh and MTSET to the Cys Mutants. BrACh-mediated modification of four of the Cys-substitution mutants (α187, α188, α190, and α194) led to a maximal effect of ∼40% reduction in Bgtx binding sites. Modification of the αF189C mutant receptor led to significantly lower levels of Bgtx binding (∼55−65% reduction) but the reasons underlying the differences in binding inhibition are unclear. There is evidence with the wild-type receptor that partial reactivity is responsible for the observed incomplete blockade of Bgtx binding upon modification of reduced wild-type receptor with BrACh following reduction of the αC192−C193 disulfide (*34*). According to these studies, one of the agonist binding sites is easily labeled with bromoacetylcholine, while more extreme conditions are required to label the other. A number of other reports indicate that labeling of both agonist sites with BrACh is difficult to accomplish (*24*). In addition, the concentration of BrACh required to achieve full labeling varies considerably, from 100 μM (*24*) to reports that complete labeling of the second site is impossible (*21*, *22*).

One possible explanation for the near 50% reduction in Bgtx binding seen with the Cys-substitution mutants is that only one of the two Bgtx binding sites per receptor is being effectively modified (i.e., half-site reactivity). The two ligand binding sites in each receptor are formed at the α/γ and at the α/δ interface and environmental differences apparently account for the differential affinity of various agonists and antagonists for the two sites (*35*). By examining single channel currents recorded with *Torpedo* AChR stably expressed in mouse fibroblasts, Sine et al. (*36*) demonstrated that ACh affinities are about 100-fold higher for the α/δ interface than for the α/γ site. Similar results were also obtained by Zhang et al. (*37*) for mouse embryonic nicotinic AChR. Therefore, we tested the hypothesis that the ∼50% blockade of Bgtx binding was due to selective BrACh modification at either the α/γ or α/δ interface. Our results with α-CTX (see Figure 5), however, demonstrate that there is no preferential block of toxin binding at either the α/γ or α/δ interface. We conclude that BrACh modification does not show selectivity for one of the two sites and that Bgtx binding to both sites appears to be equally affected.

We do not believe that the incomplete block of Bgtx binding sites in the modified Cys mutants is due to incomplete reactivity of the reagents. Neither longer incubation times nor higher reagent concentrations lead to a further blockade. Complete reaction appears to occur within 5 min following the addition of BrACh, as maximal blockade of Bgtx binding is observed as early as 5 min, at least with mutants αW187C and αF189C (data not shown). In addition, MMTS, MTSEA, and MTSES are all identical in their ability to fully block the effect of the subsequent BrACh addition (Table 1). These results clearly argue that modification with these differently charged reagents is complete and suggest that a full reaction with MTSET and BrACh would also be expected. Most recently we have shown that prior treatment of the αF189C mutant with BrACh results in a nearly full blockade of the reactivity of this site with a thiol-reactive Biotin derivative (manuscript in preparation). This observation further argues against incomplete reactivity of BrACh and MTSET with the introduced cysteines.

The results of the conotoxin experiment (Figure 5) are consistent with a model in which the applied affinity reagent modifies all the available sites on both α/γ or α/δ interface, but that only approximately half of the modified sites are capable of blocking Bgtx binding. The incomplete blockade may be explained by some conformational or state-dependent heterogeneity that could limit the accessibility of the modified thiols to a critical component of the Bgtx binding site. In this model, both sites per receptor are rapidly modified by the reagents. In a stochastic process, one of the two tethered ligands then interacts directly with the agonist binding site, inducing a conformational change that alters the spatial disposition of the second binding site such that the second tethered ligand cannot compete with Bgtx for binding to that site. In theory, it is also possible that partial oxidation of exposed free thiols on the cell surface during cell culture could account for a decrease in the effectiveness of BrACh or MTSET modification. The addition, however, of up to 10 000 units/mL catalase to the medium, in an attempt to maintain extracellular thiol residues on cell surface proteins

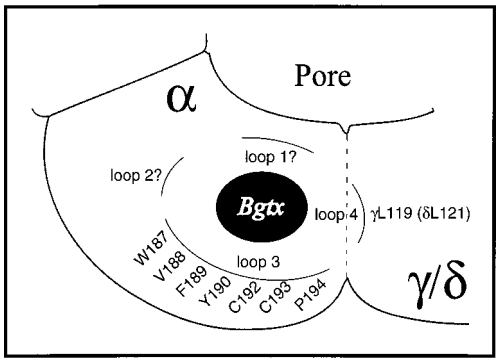

FIGURE 6: Summary model depicting interactions between α-Bgtx and residues on the AChR. This model is adapted from the four-loop ligand binding site model as first proposed by Dennis et al. (55) and shows the binding sites for Bgtx as being located at the interface between α and γ/δ subunits. Evidence for the involvement of residues γL119 and δL121 in the Bgtx binding sites is derived from Sine (*2*). On the basis of the observations reported in this study, residues on the α subunit that are in a position to contribute to Bgtx binding are shown on "loop 3". The orientation of the sequence in loop 3 is arbitrary.

in the reduced state (*38*), did not alter the results obtained (data not shown).

A Model for the Interaction between Bgtx and Its Binding Site on the Receptor. α-Bgtx belongs to a class of long neurotoxins, which consist of 71−74 amino acids folded into three loops held together by four disulfide bonds (for review see ref *39*). Owing to the large net positive charge of Bgtx (+4) and the net negative charge of the receptor, the receptor-toxin complex is likely to be stabilized by multiple electrostatic interactions. Mutation of the highly conserved residue Arg 36 into an alanine reduced the binding affinity of Bgtx to wild-type receptors ~90-fold (Rosenthal et al., manuscript submitted). This is in agreement with the observation that charge reversal of the conserved Arg in short α-neurotoxins reduces toxin binding by >350-fold (*13*, *40*). Our results similarly point at the importance of a negative subsite on the receptor subserving the interaction with Bgtx: covalent attachment of either MTSET and BrACh to α187, α188, α189, α190, and α194 all decrease Bgtx binding to a significant degree. It is indeed tempting to speculate that the side chain of the conserved Arg in the neurotoxins interacts with the same negative subsite on the receptor that mediates the methylammonium dependent blockade seen in our studies (*41*). The data presented here do not allow us to determine the extent to which the individual residues αW187, αV188, αF189, αY190, and αP194 contribute energetically to Bgtx binding. For αV188 and αY190, the work by Ackermann et al. (*14*) suggests a direct interaction between these two residues and NmmI residues although the involvement of an intermediate residue cannot yet be excluded (*14*).

A schematic model that incorporates the currently available data on the structural determinants of Bgtx binding to the native AChR is shown in Figure 6. The postulated role for loops "1" and "2" in Bgtx binding is based on earlier models of the agonist binding domain which suggest an involvement of α subunit residues Y93 (loop 1) and W149 and Y151 (loop 2) (*5*, *42*). At this point there is no direct evidence for loops 1 and 2 being involved in Bgtx binding to the native receptor although peptide-based studies have suggested a role for residues α55−74 (*43*) as well as residues α125−148 (*44*). Recently, Sine has demonstrated that γL119 and the equivalent residues in the δ and ϵ subunit form a localized contact point for Bgtx binding to the native receptor (*2*). Interestingly, this study also reported a difference among MTS-derivatives entirely consistent with our observations. Modification of γL119C with MTSET led to a block in Bgtx binding sites, whereas modification with MTSEA or MTSES had no effect despite the clear demonstration of site modification.

If a negative subsite is involved in Bgtx recognition, it is unlikely to be contributed by δD180, δD189, γD174, γE176, or γE183 (all suggested as possible sites of interaction between the receptor and the methylammonium group on agonists) as mutation of these groups has little or no effect on Bgtx affinity (*45*−*47*). Aromatic residues have also been suggested to contribute to the negative subsite for agonist binding (*32*, *48*−*51*). On the basis of the size of the MTSET modification, which fits into a cylinder 0.8 nm long and 0.6 nm in diameter (*29*), components of any negative subsite important for Bgtx binding would have to be capable of interacting with a positive charge fixed at ~0.8 nm from the Cβ positions of α subunit residues 187, 188, 189, 190, and 194. Studies using the affinity alkylating reagent MBTA to modify the thiols at α192/93 of the reduced wild-type receptor argue that the negative subsite for agonist binding must be within 1 nm of these residues (*52*, *53*).

The proximity of all these residues on the linear sequence as well as the demonstrated surface accessibility of residues α187, α188, α189, α190, and α194 are consistent therefore with an interaction of the modified substituted cysteines with the negative subsite involved in agonist recognition. In this region of the α subunit, only αS191 remains to be localized to the surface. Cys-substitution studies therefore provide firm evidence that at least eight out of the nine residues positioned between H186 and P194 on the α subunit are surface accessible, consistent with models placing this region at the ligand binding site (*54*).

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