Although previous results indicate that α-subunit residues Trp<sup>187</sup>, Val<sup>188</sup>, Phe<sup>189</sup>, Tyr<sup>190</sup>, and Pro<sup>194</sup> of the mouse nicotinic acetylcholine receptor are solvent-accessible and are in a position to contribute to the α-bungarotoxin (α-Bgtx) binding site (Spura, A.; Russin, T. S.; Freedman, N. D.; Grant, M.; McLaughlin, J. T., and Hawrot, E. (1989) Biochemistry 28, 4912–4921), little is known about the accessibility of other residues within this region. By determining second-order rate constants for the reaction of cysteine mutants at α184–α197 with the thiol-specific biotin derivative (+)-biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctanediamine, we now show that only very subtle differences in reactivity (−10-fold) are detectable, arguing that the entire region is solvent-exposed. Importantly, biotinylation in the presence of saturating concentrations of the long neurotoxin α-Bgtx is significantly retarded for positions αW187C, αF189C, and reduced wild-type receptors (αCyS<sup>192</sup> and αCyS<sup>193</sup>), further emphasizing their major contribution to the α-Bgtx binding site. Interestingly, although biotinylation of position αV188C is not affected by the presence of α-Bgtx, erabutoxin a, which is a member of the short neurotoxin family, inhibits biotinylation at position αV188C, but not at αW187C or αF189C. Taken together, these results indicate that short and long neurotoxins establish interactions with distinct amino acids on the nicotinic acetylcholine receptor.

The nicotinic acetylcholine receptor (AChR) is the major prototype for neurotransmitter-gated ion channels and is found at high concentrations in the postsynaptic membranes of muscle cells, where it mediates the rapid propagation of electrical signals at the neuromuscular junction. It is a pentameric protein composed of four subunit types in a molar ratio 2α:β:γ:δ (see Ref. 1 for review).

An important first step in assessing the structure and function of such ion channels at a molecular level is to determine their transmembrane topology. To address this issue, a variety of techniques have been developed (see Ref. 2 for review). The most commonly used methods are the epitope protection assay (3–5), in which an epitope that is recognized by a specific antibody is fused to the protein of interest, and N-linked glycosylation tagging, wherein N-linked glycosylation sites can be engineered into the protein under investigation and glycosylation can then be evaluated (6–8).

The above methods, however, are only useful to assess overall topology of membrane proteins. For the nAChR, there is general consensus on the overall topology, although final proof will have to await high resolution structural data; each subunit possesses a large, extracellular amino-terminal domain, which is followed by four transmembrane-spanning regions and a short extracellular carboxyl terminus (9). In contrast, the key structural issue of whether individual residues are solvent-exposed has not been resolved. To address this issue, Gallivan et al. (10) have recently employed the in vivo nonsense suppression technique to incorporate derivatives of the unnatural amino acid biocytin into the nAChR heterologously expressed in Xenopus oocytes. By evaluating the binding of <sup>125</sup>I-streptavidin to biotinylated receptors, they studied the surface exposure of individual residues comprising the main immunogenic region (spanning positions 67–76; Ref. 11) and showed that position α70 was highly exposed.

In the current study, we have used the substituted cysteine accessibility method (12, 13) to systematically map the accessibility of individual residues between positions 184 and 197 of the α-subunit, the main determinant for agonist and competitive antagonist binding to the nAChR (14–16). To achieve this, we have introduced cysteine residues into the nAChR and labeled them with thiol-reactive, water-soluble biotin derivatives. Subsequently, we precipitated biotinylated receptors with immobilized streptavidin and probed the immunoprecipitates by Western blotting. Previous studies of oocyte-expressed Cys substitution mutations of α-subunit residues 181–197 (17) indicate that the majority of these substitutions are well tolerated and lead to minimal perturbations in receptor function. Here, we show that positions 184–197 are all amenable to biotinylation, suggesting that the entire region is surface-exposed. In addition, modifications with the uncharged biotin derivative occur with similar rates for all of these residues. Moreover, we demonstrate that preincubation with the competitive antagonist α-Bgtx, a long-chain α-neurotoxin, selectively

* This work was supported by Research Grant GM32629 (to E. H.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ This work was done in partial fulfillment of the requirements for a Ph.D. degree from Brown University.

§ To whom correspondence should be addressed. Tel.: 401-863-1034; Fax: 401-863-1595; E-mail: edward_hawrot@brown.edu.

† The abbreviations used are: AChR, acetylcholine receptor; αChR, acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; BrACh, bromoacetylcholine; Bgtx, bungarotoxin; DTT, dithiothreitol; Ea, erabutoxin a; MTSEA-biotin, N-biotinylaminomethyl methanethiosulfonate; MTSET-biotin, N-methanethiosulfonylethyl N'-biotinyl-2-2'(ethylenedioxy)bis (ethyamine), MTSET, [2-trimethylammonium]ethyl]methanethiosulfonate; Nnax1, Naja mossambica mossambica 1; PEO-biotin; (+)-biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctanediamine; DMF, dimethylformamide; mAb, monoclonal antibody; Boc, butoxy-carbonyl; RIPA, radioimmune precipitation buffer.

Armin Spura‡§, Ryan U. Riel‡, Neal D. Freedman‡, Shantanu Agrawal‡, Christopher Seto§, and Edward Hawrot‡

From the ‡Department of Molecular Pharmacology, Physiology, and Biotechnology, Division of Biology and Medicine, and the §Department of Chemistry, Brown University, Providence, Rhode Island 02912

This paper is available on line at http://www.jbc.org

Biotinylation of Substituted Cysteines in the Nicotinic Acetylcholine Receptor Reveals Distinct Binding Modes for α-Bungarotoxin and Erabutoxin a*

Received for publication, February 15, 2000, and in revised form, April 27, 2000
Published, JBC Papers in Press, April 28, 2000, DOI 10.1074/jbc.M001283200

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 29, Issue of July 21, pp. 22452–22460, 2000 Printed in U.S.A.
blocks biotinylation of positions 187, 189, and cysteines 192 and 193 in reduced wild-type receptors, demonstrating the importance of these residues in the binding of α-Bgtx and further supporting results we obtained previously (16). In contrast, preincubation with Ea, a short-chain α-neurotoxin, yields a different footprint, preventing only position 188 and reduced wild-type receptors from biotinylation. Thus, our findings strongly argue that long- and short-chain neurotoxins interact selectively with different positions when blocking agonist binding on the nAChR.

MATERIALS AND METHODS

Reagents

MTSEA-biotin was from Toronto Research. PEO-biotin and streptavidin-agarose beads were from Pierce, mAb 35 from Research Biochemicals International, and Protein G-agarose beads from Santa Cruz Biotechnology.

Mutagenesis

We used a cytomegalovirus-based expression vector (GWI, British Biotechnology, Oxford, United Kingdom) 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

These have been described previously (16).

Synthesis of N-Methanethiosulfonyle-N’-biotinyl-2,2’-(ethylenedioxy)bis(ethylamine) (MTSEDE-biotin)

MTSEDE-biotin belongs to a class of compound generally known as alkyl alkanethiolsulfonates. It was synthesized as follows (Fig. 1).

Step 1: N-Boc-2,2’-(ethylenedioxy)bis(ethylamine) (MTSEDE-biotin)—The mono-Boc-protected diamine (0.5 g, 0.1 mmol) was dissolved in 10 ml of dimethylformamide (DMF). To this solution was added sodium methanethiosulfonate (0.37 g, 0.003 mmol), and the reaction was allowed to proceed for 1 h, after which the flask was rotary evaporated to remove the tetrahydrofuran and placed under vacuum. Flash chromatography was performed using 10% MeOH/CH2Cl2 as the eluent, giving an 88% yield of an off-white solid (0.06 g, 0.01 mmol).

Step 2: N-Boc-N’-biotinyl-2,2’-(ethylenedioxy)bis(ethylamine) Derivative (18)—The mono-Boc-protected diamine (0.38 g, 0.15 mmol) was dissolved in 8 ml of methanol. To this solution was added diisopropylamine (6 g, 2.4 mmol) of a yellowish oil. The organic layers were removed by rotary evaporation, and the product was flash chromatographed using a 15% MeOH/CH2Cl2 solvent system. An 86% yield (0.64 g) of a yellowish oil was isolated.

Step 3: N’-Biotinyl-2,2’-(ethylenedioxy)bis(ethylamine) Derivative (19)—The Boc-protected biotin diamine (0.5 g, 0.1 mmol) was dissolved in 8 ml of methanol. To this solution was added diisopropylamine (6 g, 2.4 mmol) of a yellowish oil. The organic layers were removed by rotary evaporation, and the product was flash chromatographed using a 15% MeOH/CH2Cl2 solvent system. An 86% yield (0.64 g) of a yellowish oil was isolated.

Step 4: N-Iodoacetyl-N’-biotinyl-2,2’-(ethylenedioxy)bis(ethylamine) Derivative (20)—The biotin diamine (0.06 g, 0.02 mmol) was dissolved in 2 ml of dimethylformamide (DMF). To this solution was added diisopropylamine (0.37 g, 0.005 mmol) and iodoacetic anhydride (0.17 g, 0.05 mmol) and diisopropylethylamine (0.12 g, 0.1 mmol) were added. The reaction was allowed to proceed for 1 h, after which the flask was rotary evaporated to remove the tetrahydrofuran and placed under vacuum. Flash chromatography was performed using 10% MeOH/CH2Cl2 as the eluent, giving an 88% yield of an off-white solid (0.06 g, 0.01 mmol).

Step 5: N-Methanethiosulfonyle-N’-biotinyl-2,2’-(ethylenedioxy)bis(ethylamine)—The iodobiotin diamine (0.12 g, 0.1 mmol) was added to the reaction flask, and the reaction was allowed to stir at room temperature for 2 h. The flask was placed under vacuum to remove the DMF, and the product was flash chromatographed using a 15% MeOH/CH2Cl2 solvent system. An 88% yield (0.64 g) of a yellowish oil was isolated. NMR spectra for MTSEDE-biotin and its intermediates were determined to confirm the purity of the product (data not shown) and are available upon request.

Step 6: Biotin N-Hydroxysuccinimide Ester (21)—Biotin (5.5 g, 22.6

FIG. 1. Synthesis scheme for MTSEDE-biotin.
Distinct Binding Modes for α-Bungarotoxin and Erabutoxin α

mmol) was dissolved in 70 ml of DMF. To this solution was added N-hydroxysuccinimide (3.12 g, 27.1 mmol) and disopropyl carbodiimide (3.42 g, 27 mmol). The reaction was stirred at 90 °C overnight, after which rotary evaporation was used to remove DMF, giving a yellowish solid. Ethyl ether (150 ml) was added to the crude product to dissolve impurities, after which the crude solid was suction filtered. This off-white solid had a melting point range of 177–182 °C. The crude product was recrystallized in isopropanol and suction-filtered to give an 84.5% yield of a white solid (6.5 g, 19 mmol). The melting point of this solid was 200–202 °C.

**Step 7: Sodium Methanesulfonate (21)—Sodium hydrosulfide was dried over P2O5 for 3 days. This dried sodium hydrosulfide (11 g, 20 mmol) was then dissolved in 150 ml of absolute ethanol. Methanesulfonic acid (11.4 g, 9.9 mmol) was added dropwise to this solution as it stirred at room temperature and under a nitrogen atmosphere. After all of the methanesulfonic acid had been added, the reaction was allowed to stir for another 2 h, under nitrogen. As nitrogen gas was bubbled through the reaction, it was forced through a drying tube and then bubbled through 2000 ml of bleach in order to neutralize the developing hydrogen sulfide gas. After 2 h, the reaction flask was heated to 65–70 °C for 1 h. The flask was allowed to cool, and then 100 ml of absolute ethanol were added before leaving the flask under nitrogen overnight. After this time, the solution was gravity-filtered to remove NaCl and then rotary-evaporated to remove ethanol. Recrystallization was performed with warm ethanol to yield a white solid with a melting point of 271.5 °C. The yield was 3.8 g (2.86 mmol).

**Step 8: Iodoacetic Anhydride—Iodoacetic acid (20 g, 107 mmol) was dissolved in 290 ml of ethyl acetate. Diisopropyl carbodiimide (6.8 g, 54 mmol) was added to this solution, and the reaction was stirred at room temperature and under nitrogen. After this time, the solution was gravity-filtered to remove NaCl and then rotary-evaporated to remove ethanol. Recrystallization was performed with warm ethanol to yield a white solid with a melting point of 271.5 °C. The yield was 3.8 g (2.86 mmol).

**Covalent Cysteine Modification with Biotin Reagents and Preincubations with α-Bgtx or Erabutoxin a**

Two days after transfection, cells were harvested by gentle agitation in phosphate-buffered saline containing 5 mM Na2-EDTA (~0.5 × 106 cells obtained from one 75-cm² tissue-culture flask). After a brief centrifugation at ~600 × g, the cells were resuspended in high potassium Ringer’s solution (22), pooled, divided into 300-ml aliquots, and incubated for the specified times with 5–500 μM MTSE-biotin or PEO-biotin. For MTSEA-biotin, the reagent was dissolved in Me6SO instead of water before being added to the cells at 500 μM. For each biotin reagent, we added an excess of BrACh (1.5 mM) to terminate the reaction. The unbound biotin was removed by pelleting the cells at 2 min, 4 °C. The conditions chosen are saturating, 10-fold), and resuspending the pellet in 0.5 ml of high potassium Ringer’s. This wash was repeated three times in total. For preincubations with α-Bgtx or Ea, the cells were incubated for 2 h at room temperature with 10 μM amounts of the respective toxin to allow for a saturation of binding sites. PEO-biotin was then added directly into the tubes for the times indicated. Typically, HEK-293 cells transiently transfected with wild-type α-subunit of the nAChR. Previously, we and others showed that a cysteine can be substituted for all of the individual amino acids between positions 184 and 197 of the mouse muscle-type α-subunit without dramatic effect on receptor functionality (<6-fold changes in the EC50) as measured by ACh responsiveness in Xenopus oocytes (17). Moreover, we showed that within this region, residues Trp187, Val188, Phe189, Tyr190, and Pro194 are solvent-accessible and are in a position to contribute to the α-Bgtx binding site. In order to explore further the topology of the bracketing region extending from 184 to 197, we have now modified the appropriate Cys-substituted mutants with thiol-specific biotin derivatives following their expression in HEK-293 cells, permitting a more detailed analysis of the accessibility of these Cys-substituted residues.

**RESULTS**

In previous studies, we demonstrated that a cysteine can be substituted for all of the individual amino acids between positions 184 and 197 of the mouse muscle-type α-subunit without dramatic effect on receptor functionality (<6-fold changes in the EC50) as measured by ACh responsiveness in Xenopus oocytes (17). Moreover, we showed that within this region, residues Trp187, Val188, Phe189, Tyr190, and Pro194 are solvent-accessible and are in a position to contribute to the α-Bgtx binding site. In order to explore further the topology of the bracketing region extending from 184 to 197, we have now modified the appropriate Cys-substituted mutants with thiol-specific biotin derivatives following their expression in HEK-293 cells, permitting a more detailed analysis of the accessibility of these Cys-substituted residues.

**PEO- and MTSEA-biotin Specifically Modify Cysteines 192/193 in Reduced Wild-type nAChR—**Initially, we wanted to determine whether alkyl thioisulfonate derivatives of biotin could be used to specifically modify surface-exposed cysteines in the nAChR. Previously, we and others showed that smaller alkyl methane thioisulfonate derivatives and bromoacetyltylcholine, an alkylammonium compound containing an α-haloacetyl ester moiety, interact covalently and specifically with Cys192 and Cys193 of the nAChR following their selective reduction with 1 mM DTT (16, 17, 27–29). As shown in Fig. 2, when cells expressing the wild-type nAChR were selectively reduced at positions 192 and 193 with 1 mM DTT and then treated with 500 μM of MTSEA-biotin (Fig. 2A, lane 4), we detected a band corresponding to the α-subunit of the nAChR.
As these studies were under way, a similar biotin derivative (PEO-biotin) became commercially available. When we applied this reagent to reduced wild-type receptors, we obtained results comparable to those for MTSEDE-biotin (Fig. 2B, lane 4). Importantly, this band was not present when unreduced nAChR was exposed to MTSEDE-biotin (Fig. 2A, lane 3) or PEO-biotin (Fig. 2B, lane 3).

Biotinylation was blocked by preincubation with either 1.5 mM BrACh or 1.5 mM MTSET for both MTSEDE- and PEO-biotin, confirming its specificity (Fig. 3B, lane 3; Fig. 5A). In contrast, exposure of cells to the more hydrophobic MTSEA-biotin leads to considerable nonspecific labeling, as we detected α-subunit labeling that was equally pronounced both before and after reduction with DTT. These results suggest that this reagent is capable of penetrating the lipid bilayer to a large degree and may have access to the internal cysteines at position αCys222 (in presumed transmembrane segment M1) and αCys418 (M4) (compare Fig. 2A, lanes 6 and 7). In addition, we observed that MTSEA-mediated biotinylation of reduced wild-type and mutant receptors cannot be blocked by 1.5 mM BrACh (data not shown), further pointing to its reactivity with an internal cysteine. For PEO- and MTSEDE-biotin, we detected nonspecific labeling of native unreduced receptors only when concentrations were raised to 1.5 mM and above (data not shown). The weak signal we obtained in the presence of DTT alone may suggest nonspecific absorption of the receptor to the streptavidin beads that are used for precipitation, although the result presented here was somewhat exceptional, and we generally did not observe a signal in the presence of DTT alone (Fig. 2A, lane 2). Additionally, labeling of surface-expressed α-subunit by incubation of intact cells with the mouse monoclonal nAChR antibody, mAb 35 (11) (directed against region α67–76), followed by immunoprecipitation enabled us to the detect an α-band of an intensity comparable to that for the biotinylated wild-type receptor, indicating that at least a large fraction of the surface population of wild-type nAChRs can be biotinylated (Fig. 2A, lane 5). However, it should be noted that a direct comparison of the signal intensities is not 100% accurate. Although we have optimized conditions such that maximal amounts of both mAb 35-labeled and biotinylated receptor are precipitated, it remains possible that the recovery of receptor eluted from the beads is different for the two methods. Nevertheless, we could use mAb 35 to test surface expression of all the cysteine mutants from position α184C to α197C, and we detected no noticeable difference in cell-surface expression levels among these mutants (Fig. 3A).

Rate of Biotinylation of Reduced Wild-type nAChR and Retardation in the Presence of α-Bxts—In an effort to quantitate the reactivity of reduced wild-type and mutant nAChRs with PEO-biotin, we exposed nAChRs to 5 μM PEO-biotin for various incubation times (Fig. 4A). Second-order reaction rates were
Reduced wild-type p pound was more light-sensitive and degraded rapidly at room SEDE-biotin (data not shown). However, since the MTS com- bond in the wild-type receptor and are not listed separately, since they intesti nes obtained for 10-min incubations with 5, 50, and 500 mimal rates of biotinylation in the presence of PEO-biotin, only estimates of the max- imal of an internal cysteine or other unidentified sites. 

cells, which were harvested after 2 days of incubation, and treated with PEO-biotin. The PEO-biotin concentration for all time points was 5 μM, unless otherwise indicated in the figure. Streptavidin precipitation, electrophoresis, Western blotting, and visualization of the α-subunit were performed as described under "Materials and Methods." A, time course of biotinylation in the absence of α-Bgtx. Biotinylation was terminated by the addition of the thiol-specific BrACh (1.5 mM) at the indicated time points. B, biotinylation in the presence of 10 μM α-Bgtx. Harvested cells were first incubated with 10 μM α-Bgtx for 2 h, and PEO-biotin was then added directly. In lane 1, Torpedo membranes (100 fmol of toxin binding sites) were loaded as a standard reference. For all samples in A and B, transfected cells were pooled prior to modification, and equal amounts of receptor (~200 fmol of toxin binding sites) were modified and loaded onto each lane.

FIG. 5. Reactivity of mutant αV188C with PEO-biotin in the presence or absence of α-Bgtx. The αV188C mutant α-subunit encoding cDNA was transfected together with wild-type β-, γ-, and δ-subunits into HEK-293 cells, which were harvested after 2 days of incubation, and treated with PEO-biotin. The PEO-biotin concentration for all time points was 5 μM, unless otherwise indicated in the figure. Streptavidin precipitation, electrophoresis, Western blotting, and visualization of the α-subunit were performed as described under "Materials and Methods." A, biotinylation in the absence of α-Bgtx. Biotinylation was terminated by the addition of the thiol-specific BrACh (1.5 mM) at the indicated time points. B, biotinylation in the presence of 10 μM α-Bgtx. Harvested cells were first incubated with 10 μM α-Bgtx for 2 h, and PEO-biotin was then added directly. For all samples in A and B, equal amounts of receptor (~200 fmol of toxin binding sites) were modified and loaded onto each lane.

FIG. 4. Reactivity of reduced wild-type receptors with PEO- biotin and inhibition of biotinylation in the presence of α-Bgtx. Wild-type α-subunit encoding cDNAs were transfected together with wild-type β-, γ-, and δ-subunits into HEK-293 cells, which were harvested after 2 days of incubation, and treated with PEO-biotin. The PEO-biotin concentration for all time points was 5 μM, unless otherwise indicated in the figure. Streptavidin precipitation, electrophoresis, Western blotting, and visualization of the α-subunit were performed as described under "Materials and Methods." A, time course of biotinylation in the absence of α-Bgtx. Biotinylation was terminated by the addition of the thiol-specific BrACh (1.5 mM) at the indicated time points. B, biotinylation in the presence of 10 μM α-Bgtx. Harvested cells were first incubated with 10 μM α-Bgtx for 2 h, and PEO-biotin was then added directly. In lane 1, Torpedo membranes (100 fmol of toxin binding sites) were loaded as a standard reference. For all samples in A and B, transfected cells were pooled prior to modification, and equal amounts of receptor (~200 fmol of toxin binding sites) were modified and loaded onto each lane.

| Mutant | Second-order reaction rate k | Ratio k/β<sub>bgtx</sub> |
|--------|-----------------------------|---------------------------|
| αD71C  | 39.5 ± 5.1                  | 0.98 ± 0.03               |
| Reduced wild-type (α192/193) | 103.6 ± 3.4 | >100 |
| αW184C | 41.3 ± 3.9                  | 0.89 ± 0.08               |
| αK185C | 23.5 ± 3.1                  | 1.1 ± 0.05                |
| αH186C | 28.3 ± 3.5                  | 0.91 ± 0.09               |
| αW187C | 10.8 ± 0.9                  | >10                       |
| αV188C | 101.9 ± 12.8                | 1.34 ± 0.07               |
| αF189C | 93.7 ± 9.6                  | >100                      |
| αY190C | 55.6 ± 13.3                 | 0.86 ± 0.07               |
| αS191C | 37.1 ± 1.9                  | 0.88 ± 0.05               |
| αP194C | 21.1 ± 3.2                  | 1.36 ± 0.09               |
| αT195C | 36.8 ± 4.4                  | 0.98 ± 0.05               |
| αT196C | 59.3 ± 3.3                  | 0.96 ± 0.06               |
| αP197C | 47.6 ± 9.4                  | 1.21 ± 0.11               |

Calculated as described under "Materials and Methods." For reduced wild receptors, we obtained a rate constant of 103.6 M<sup>-1</sup> s<sup>-1</sup> (Table I). Comparable results were obtained with MT-SED-biotin (data not shown). However, since the MTS compound was more light-sensitive and degraded rapidly at room temperature, we focused the remainder of our studies exclusively on the effects mediated by PEO-biotin.

When 10 μM α-Bgtx was added to cells and allowed to equilibrate for 2 h, the reactivity of reduced wild-type receptors toward PEO-biotin was substantially slowed (>100-fold). Using 5 and 50 μM PEO-biotin, we could not detect any biotinylation of the α-subunit, and even after incubation with 500 μM PEO-biotin, we obtained only a weak signal (~10% of the maximum intensity; Fig. 4B). A precise determination of the second-order rate constant in the presence of α-Bgtx was not possible, since PEO concentrations of 1.5 mM and above would have to be used, and under these conditions, there was considerable background stemming from the presumed modification of an internal cysteine or other unidentified sites.

Rate of the Biotinylation of Mutant αV188C αChR Is Unaffected by the Presence of α-Bgtx—Previous results showed that mutant αV188K leads to a ~680-fold decrease in affinity for a short α-neurotoxin, Nmnl, although the effect of this mutation was much less pronounced in affecting α-Bgtx binding (15, 30, 31). In addition, modification of αV188C with 1.5 mM αCh or MTSET leads to a ~50% decrease in the number of α-Bgtx binding sites (16). On the other hand, a mutation introducing a negative charge at this position (αV188D) merely produced a ~10-fold decrease in Nmnl binding. To more closely examine the role of this position in α-Bgtx binding, we determined second-order reaction rates for αV188C with 50 μM PEO-biotin in the presence (Fig. 5B) and absence of α-Bgtx (Fig. 5A). In the absence of α-Bgtx, we obtained a rate of 102 M<sup>-1</sup> s<sup>-1</sup>, whereas
Distinct Binding Modes for α-Bungarotoxin and Erabutoxin α

The biotinylation of mutant V188C is less than that for reduced wild-type receptors, despite the fact that their reaction rates are comparable (Table 1). Most likely, two factors contribute to this observation. First, reduced wild-type receptors contain two modifiable free thiol groups (αCys192/193), i.e. although the reaction rate is comparable to that for αV188C, the signal intensity will be twice as high for any given time point. Additionally, it is possible that, for αV188C, only a portion of the receptor population contributes to the signal, whereas the rest is either refractory to biotinylation or to the subsequent sterically constrained reaction with streptavidin beads. This view is strengthened by the fact that preincubation with α-Bgtx enhances the yield of biotinylated receptor approximately 2-fold (Fig. 6C).

Evidence That All Amino Acid Residues within Region α184

to α197 Are Solvent-exposed—Although positions αW187C, αV188C, αF198C, and αP197C were modifiable with BrACh and their modification resulted in a substantial blockade in α-Bgtx binding (16), the surface disposition of the other residues spanning region α184 to α197 is not well understood. Specifically, it was previously impossible to distinguish whether cysteine-substituted residues 184–186, 191, and 195–197 were modified following the application of thiol-specific reagents. By systematically examining the reactivity of these residues with PEO-biotin, we now show that all of these residues are surface-exposed and accessible to biotinylation to a similar degree. Their second-order reaction rate constants fell within the range of ~10 to ~100 m⁻¹ s⁻¹ (αW187C: 10.8 m⁻¹ s⁻¹; αV188C: 101.9 m⁻¹ s⁻¹; Table I).

Table I also lists the second-order reaction rate for positions αD71C and αW184C to αP197C in the presence of saturating concentrations of α-Bgtx. Importantly, the reaction rate for the biotinylation of αD71C is unaffected in the presence of α-Bgtx, confirming its location outside of the α-Bgtx binding site (11). Apart from reduced wild-type receptors, only residues αW187C (at least a 10-fold reduction) and F189C (at least a 100-fold reduction) exhibit a substantial reduction in the reaction rate in the presence of α-Bgtx. It should be noted that an accurate determination of reaction rates for these mutants in the presence of α-Bgtx was not possible. This would have required using concentrations of PEO-biotin in excess of 1.5 mM, a concentration that leads to the modification of other sites in addition to the engineered cysteine. Due to its reduced reactivity with PEO-biotin even in the absence of α-Bgtx, this is especially true for mutant αW187C.

Effects of Erabutoxin α Co-incubation on the Reactivity of

Residues αW184C to αP197C with PEO-biotin—Previous studies suggested contradicting roles for positions αTrp187, αVal188,

\[ \text{PEO-biotin, for lanes } 2-4, \text{ equal amounts of receptor (~200 fmol in toxin binding sites) were reacted and loaded. } \]

\[ \text{B, protection of the biotinylation of mutant αW187C by α-Bgtx and Ea. For all the lanes shown, biotinylations were performed with 500 mM PEO-biotin for 10 min at 20 °C. Lane 1, Torpedo membranes (200 fmol in toxin binding sites); lane 2, PEO-biotin only; lane 3, α-Bgtx (10 μM) and PEO-biotin; lane 4, Ea (10 μM) and PEO-biotin. For lanes 2-4, equal amounts of receptor (~100 fmol in toxin binding sites) were reacted and loaded. C, protection of the biotinylation of mutant αV188C by α-Bgtx and Ea. For all the lanes shown, biotinylations were performed with 50 μM PEO-biotin for 10 min at 20 °C. Lane 1, Torpedo membranes (200 fmol in toxin binding sites); lane 2, PEO-biotin only; lane 3, α-Bgtx (10 μM) and PEO-biotin; lane 4, Ea (10 μM) and PEO-biotin. For lanes 2-4, equal amounts of receptor (~100 fmol in toxin binding sites) were reacted and loaded. D, protection of the biotinylation of mutant αF189C by α-Bgtx and Ea. All biotinylations were performed with 50 μM PEO-biotin for 10 min at 20 °C. Lane 1, PEO-biotin only; lane 2, α-Bgtx (10 μM) and PEO-biotin; lane 3, Ea (10 μM) and PEO-biotin; lane 4, Torpedo membranes (200 fmol in toxin binding sites). For lanes 1–3, equal amounts of receptor (~100 fmol in toxin binding sites) were reacted and loaded. ]
TABLE II

| Position | Trp^{197} | Val^{198} | Phe^{199} | Tyr^{190} | Ser^{191} | Cys^{192} | Cys^{193} |
|----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| α-Bgtx   | +         | +         | +         | +         | +         | +         | +         |
| Erabutoxin a | −         | +         | +         | +         | +         | +         | +         |

TABLE III

| Position | Position | Trp^{197} | Val^{198} | Phe^{199} | Tyr^{190} | Ser^{191} | Cys^{192} | Cys^{193} |
|----------|----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| α-Bgtx   | b        | +         | +         | +         | +         | +         | +         | +         |
| Erabutoxin a | −         | +         | +         | +         | +         | +         | +         | +         |

**DISCUSSION**

Our primary aim was to define in more detail the topology and solvent accessibility of residues 184–197 in the α-subunit of the mouse muscle nicotinic acetylcholine receptor. To achieve this, we engineered individual cysteine substitutions and modified the introduced cysteine side chains with watersoluble, thiol-reactive derivatives of biotin. A similar approach has been used, for example, by Grunewald and co-workers (32) to determine the topology of the astroglial glutamate transporter GLT-1. Likewise, the solvent-accessible structure of the γ-aminobutyric acid A receptor has been investigated with a combined cysteine mutagenesis/biotinylation procedure (33).

**Thiol-reactive Biotin Derivatives That Are Hydrophilic Are More Selective in Modifying External Cysteines**—The success and accuracy of cysteine modifications with thiol-reactive biotin derivatives strongly depends on the hydrophilicity of the reagent. As can be seen in Fig. 1 (A and B), the relatively hydrophobic modifier MTSEA-biotin reacts strongly with wild-type nAChR. In these receptors, there are intrinsic cysteine disulfide pairs at positions 128 and 142 as well as at positions 192 and 193, but no free thiols in the extracellular domain, and thus there should be no reactivity with thiol reagents. The observed labeling is most likely explained by MTSEA-biotin entering the lipid bilayer and reacting with the solvent inaccessible unpaired Cys^{182} and/or Cys^{188}. This is further supported by the fact that preincubation with 1.5 mM BrACh does not protect against biotinylation of reduced wild-type and mutant receptors when MTSEA-biotin is used (data not shown). In sharp contrast, the more hydrophilic, water-soluble derivatives, MTSEDE-biotin and PEO-biotin, do not react with wild-type nAChR unless the disulfide bond at positions 192/193 is selectively reduced with 1 mM DTT (27, 28), indicating that these reagents are not membrane-permeable. With the hydrophilic reagents, biotinylation was blocked completely using either 1.5 mM BrACh or MTSET (33) for MTSEDE-biotin and Fig. 5A for PEO-biotin). As BrACh and MTSET are charged derivatives, their block of biotinylation confirms the specificity of the reaction and supports the conclusion that biotinylation with MTSEDE-biotin or PEO-biotin is restricted to the solvent-accessible surface of the nAChR in our studies using intact cells. Either biotin conjugate would have been useful in the present study, although we decided to concentrate on PEO-biotin, as MTSEDE-biotin was more light-sensitive and degraded more rapidly at room temperature. Nonetheless, MTSEDE-biotin would offer advantages in cases where the introduction of a reversible disulfide bond is desired.

**All Amino Acid Residues between Positions 184 and 197 Are Solvent-exposed**—Table I summarizes the second-order reaction rates for the individual Cys substitutions of amino acid residues between positions 184 and 197. Their second-order reaction rate constants fall within the range of ~10 to ~100 M^{-1} s^{-1} (αW187C: 10.8 M^{-1} s^{-1}; αV188C: 101.9 M^{-1} s^{-1}). As even the least reactive of these positions, αW187C, clearly contributes to the α-Bgtx binding site and is modifiable by the charged reagent BrACh (16), we conclude that second-order reaction rates for modification with PEO-biotin that are as low as 10 M^{-1} s^{-1} are consistent with solvent accessibility. We further conclude, therefore, that all of the residues within the region tested are surface-exposed. Minor perturbations in reactivity occur, in general, over a 10-fold range and are likely to be due to variations in the chemical and steric environment surrounding the individual positions (34). Additionally, favorable electrostatic and steric interactions may enhance biotinylation. As an additional reference point, we included an analysis of mutant αD71C. It is well established that Asp^{71} forms one of the main determinants in the epitope recognized by
antibodies directed against the main immunogenic region (11) and therefore should be surface-accessible. Indeed, the second-order reaction rate (39.5 s⁻¹) is also in the range obtained with the 184–197 series of Cys substitutions. Similarly, a comparable reaction rate constant (∼200 M⁻¹ s⁻¹) has been reported for the reaction of an N-ethylmaleimide derivative with β-mercaptoethanol in phosphate buffer (35).

Further, the second-order reaction rate with PEO-biotin seems to be identical for the two α-subunits of the nAChR, regardless of position of the introduced cysteine mutation. Using a linear regression fitting routine, we calculated a curve with a very good fit to the data (R > 0.98 for all the mutants investigated; data not shown) assuming a single class of binding sites.

Our conclusions on the solvent accessibility of the 184–197 region are in line with a number of recently published studies. Structural predictions derived from the NMR studies of a receptor-peptide fragment bound to α-Bgtx (36) suggest that residues 187–190 are likely to be surface-accessible in the native receptor. The conclusions concerning surface accessibility of α-subunit residues 187–190 are also consistent with studies of the Bgtx-resistant nAChRs found in cobra and mongoose muscle and of HEK-expressed mouse muscle nAChRs containing glycosylation signals found in the cobra and mongoose AChR (23, 37). The surface accessibility of αVal188 was demonstrated by McLaughlin et al. (17) and is also supported by recent studies of Ackermann et al. (30, 31). A recent double mutant cycle analysis concludes that position αPro197 interacts with Nmml residues Arg213 and Lys27 and must therefore be surface-exposed. In addition, our study demonstrates for the first time that positions corresponding to αTrp184, αLys185, αSer191, αThr195, and αThr196 are on the solvent-accessible surface of the receptor.

α-Bgtx Protects Mutants αW187C, αF189C, and Reduced Wild-type Receptors, but Not Mutant αV188C, from Biotinylation with PEO-biotin.—In the present study, we provide evidence that positions α71, α184, α185, α191, α195, α196, and α197 do not form part of a stable α-Bgtx binding site, as the presence of bound α-Bgtx does not have a significant effect on the second-order reaction rate for PEO-biotin (Table I). One reservation concerning the interpretation of the Cys substitution studies is that the substitution itself may locally distort the structure. Thus, negative results of the protection experiments may not be as conclusive as positive results. Furthermore, it is possible, depending on the local geometry, that an introduced cysteine is both in some proximity to bound Bgtx and in a position where Bgtx does not occlude the site from biotinylation.

Interestingly, the biotinylation of mutants αV188C and αY190C is not affected by the presence of α-Bgtx, even though the tethering of a methylammonium moiety to these residues leads to a blockade of α-Bgtx binding (16). These results allow us to refine our understanding of the interaction between α-Bgtx and positions 188 and 190, as they suggest that these positions, at least when substituted with cysteine side chains, do not interact directly and stably with α-Bgtx (Fig. 3 and Table I). Nevertheless, there is good evidence that these positions are within 8 Å of the toxin binding site. The introduction of an alkylammonium moiety, through the action of either BrACh or MTSET, leads to a significant perturbation of the receptor-toxin interaction (16). With both BrACh and MTSET, a covalently attached adduct of cysteine is formed that would fit into a cylinder 8 Å long and 6 Å in diameter (38). Furthermore, the results presented here are compatible with NMR structural studies of the complex formed between the dodecapeptide α185–196 and α-Bgtx (36). The NMR analysis revealed intermolecular nuclear Overhauser effect cross-peaks between one γ-methyl group of αVal188 and the two γ-methyl groups of Bgtx residue Val199, thus placing these latter methyl protons at a distance of ∼4–5 Å from that of αVal188. Although this distance constraint certainly places Val188 in the proximity of Bgtx residue Val199 in this complex, there was no further indication of a more intimate or extensive contact between these hydrophobic side chains. In contrast, α-Bgtx clearly blocks the biotinylation of residues αW187C and αF189C, and thereby provides independent support that these residues are critical for a stable receptor-toxin interaction (16, 37, 39). Furthermore, the biotinylation of positions 192 and 193 in reduced wild-type receptors is also inhibited by α-Bgtx and underlines the importance of these residues for α-Bgtx binding. Nevertheless, the modification of these cysteines and of αW187C and αF189C with non-methylammonium-containing modifiers does not affect α-Bgtx binding significantly (16), suggesting additional complexity in Bgtx binding. In addition, it should be emphasized that the saturation binding assay used in these modification studies would not have detected decreases of α-Bgtx binding affinity <50-fold (16). Theoretically, it is also possible that the presence of α-Bgtx leads to the complete abrogation of biotinylation on only one of the two α-subunits, whereas the other site remains unaffected. If this were the case, we would expect to see a 2-fold reduction of the maximum intensity of the biotinylation signal. Our results argue against such a scenario, since the maximum intensity of the signal is largely unaltered for all of the investigated mutants.

It is somewhat surprising that the “footprint” of α-Bgtx protection from biotinylation is not larger. Previous studies have suggested that the Bgtx-receptor contact site would involve multiple points and a large portion of the toxin surface (40, 41). Our results suggest that the number of strong contacts may be fewer than expected. In addition, any additional contacts may be flexible enough to allow sufficient structural fluctuation to permit access to the reactive derivatives over the time course of the reaction incubation. Alternatively, some of the residues in this region may interact with Bgtx and the biotinylation reagents via non-overlapping surfaces.

Short and Long Chain Neurotoxins Establish Distinct Contact Points with the nAChR.—Ackermann et al. (31) have argued that the short neurotoxin Nmml interacts with αVal188, whereas their results do not implicate positions αTrp184 and αPhe189 in Nmml binding. The results presented here (Fig. 6, Table II) seem to reconcile the Nmml studies with our previous results, suggesting an important role of αPhe189 in α-Bgtx binding (16, 39). Second-order reaction rates for the biotinylation of positions αW187C and αF189C are inhibited at least 10-fold by bound α-Bgtx, whereas αV188C is not affected. In contrast, Ea, a short neurotoxin very similar to Nmml, leads to a 10-fold protection of position α188C, but does not alter biotinylation at positions αW187C and αF189C. This suggests that α-Bgtx and Ea interact with distinct amino acids on the nAChR.

A Model for the Bgtx-mediated Blockade of Receptor Biotinylation.—The model we propose here for the mechanism of α-Bgtx-mediated blockade of biotinylation is based on the mode of interaction between fasciculin and acetylcholine esterase (42–47). In this model, α-Bgtx, which carries a large net positive charge (+4; see Refs. 48 and 49) could bind to a site near a gorge leading to the agonist binding site and could obstruct PEO-biotin access to substituted cysteines (or ACh access to its binding site), which, in our experiments, would be reflected by a decrease in the second-order reaction rate of biotinylation. It is unlikely, however, that α-Bgtx blocks the putative gorge entirely, as only some of the residues implicated in ACh binding can be protected from biotinylation by concomitant α-Bgtx.
incubation (αW187C, αF189C, and αCys192/193), whereas other residues that are thought to be crucial for the receptor-ACH interaction (e.g. αY190C, Ref. 16), are not affected. Rather, our results argue that the gorge would be only partially blocked by α-Bgtx, and that the remaining cavity is large enough to accommodate the entry of PEO-biotin (29 Å in fully extended length and 5.6 Å in width at the biotin ring). Finally, it is interesting that, between positions α186 and α190, biotinylation of only every other residue is inhibited by α-Bgtx binding. Therefore, our results are consistent with NMR studies arguing that residues α186–190 are in an extended β-sheet orientation (36). In addition, these results suggest that α-Bgtx may establish contact with one of the two faces of the β-sheet, whereas the other face remains accessible to PEO-biotin.

REFERENCES

1. Karlin, A., and Akabas, M. H. (1995) Neuron 15, 1231–1244
2. White, S. H. (ed.) (1994) Membrane Structure: Methods in Physiology, Oxford University Press Inc., New York
3. Bennett, J. A., and Dingledine, R. (1995) Neuron 15, 373–384
4. Anand, R., Bason, L., Saedi, M. S., Gerzanich, V., Peng, X., and Lindstrom, J. (1990) J. Cell Biol. 119, 385–393
5. Chavez, R. A., and Hall, Z. W. (1992) J. Cell Biol. 119, 310, 765–769
6. Pedemonte, C. H., Sachs, G., and Kaplan, J. H. (1990) J. Biol. Chem. 265, 272, 6840–6849
7. Olivares, L., Aragon, C., Gimenez, C., and Zafra, F. (1997) J. Biol. Chem. 272, 3137–3144
8. Bennett, E. R., and Kanner, B. I. (1997) J. Biol. Chem. 272, 3137–3144
9. De Souza-Otero, A., and Hamilton, S. L. (1984) Biochemistry 23, 2321–2328
10. Gallivan, J. P., Lester, H. A., and Dougherty, D. A. (1997) J. Biol. Chem. 272, 739–749
11. Saedi, M., Anand, R., Conroy, W., and Lindstrom, J. (1990) FEBS Lett. 267, 55–59
12. Akabas, M. H., Stauffer, D. A., Xu, M., and Karlin, A. (1992) Science 258, 307–310
13. Stauffer, D. A., and Karlin, A. (1994) Biochemistry 33, 6840–6849
14. Sine, S. M. (1997) J. Biol. Chem. 272, 23521–23527
15. Machold, J., Weise, C., Utkin, Y., Teetlin, V., and Huch, F. (1995) Eur. J. Biochem. 234, 427–430
16. Spura, A., Russin T. S., Freedman N. D., Grant M., McLaughlin J. T., and Hawrot, E. (1999) Biochemistry 38, 4912–4921
17. McLaughlin, J. T., Hawrot, E., and Yellen, G. (1995) Biochem. J. 310, 765–769
18. Sigal, G., Mammen, M., Dahmann, G., and Whitesides, G. (1996) J. Am. Chem. Soc. 118, 3789–3800
19. Becker, J. M., Wilchek, M., and Katchalski, E. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 2604–2607
20. Currier, S. F., and Mautner, H. G. (1977) Biochemistry 16, 1944–1948
21. Bruise, T., and Kenyon, G. (1982) J. Protein Chem. 1, 47–58
22. Sine, S., and Taylor, P. (1981) J. Biol. Chem. 256, 6692–6699
23. Kreienkamp, H.-J., Sine, S. M., Maeda, R. K., and Taylor, P. (1994) J. Biol. Chem. 269, 8108–8114
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. Chase, B. A., Holliday, J., Reese, J. H., Chun, L. L., and Hawrot, E. (1987) Neuroscience 3, 959–976
26. Sine, S., and Taylor, P. (1978) J. Biol. Chem. 254, 3315–3325
27. Damle, V. N., McLaughlin, M., and Karlin, A. (1978) Biochem. Biophys. Res. Commun. 84, 845–851
28. De Souza-Otero, A., and Hamilton, S. L. (1984) Biochemistry 23, 2321–2328
29. Criado, M., Sarin, V., Fox, J. L., and Lindstrom, J. (1990) Biochemistry 29, 2839–2846
30. Ackermann, E. J., and Taylor, P. (1997) Biochemistry 36, 12836–12844
31. Ackermann, E. J., Ang, E. T.-H., Kanzler, J. R., Tsujimura, I., and Taylor, P. (1998) J. Biol. Chem. 273, 10958–10965
32. Grunewald, M., Benda, A., and Kanner, B. I. (1998) Neurosci 21, 623–632
33. Boileau, A. J., Evers, A. R., Davis, A. F., and Czajkowski, C. (1999) J. Neurosci. 19, 4847–4854
34. Pascual, J. M., and Karlin, A. (1998) J. Gen. Physiol. 111, 717–739
35. Schelte, P., Boeckler, C., Frisch, B., and Schuber, F. (2000) Bioconjugate Chem. 11, 118–123
36. Basus, V., Song, G., and Hawrot, E. (1993) Biochemistry 32, 12290–12298
37. Keller, S. H., Kreienkamp, H.-J., Kawanishi, C., and Taylor, P. (1995) J. Biol. Chem. 270, 4165–4171
38. Zhang, H., and Karlin, A. (1997) Biochemistry 36, 15
39. Levandoski, M. M., Lin, Y., Moise, L., McLaughlin, J. T., Cooper, E., and Hawrot, E. (1999) J. Biol. Chem. 274, 26113–26119
40. Tremeau, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Ducancel, F., Boulin, J. C., and Menez, A. (1995) J. Biol. Chem. 270, 9362–9369
41. Love, R. A., and Stroud, R. M. (1986) Protein Eng. 1, 37–46
42. Harel, M., and Sussman, J. L. (1993) Proc. Natl. Acad. Sci. U.S.A 90, 9031–9035
43. Miyazawa, A., Fujiyoshi, Y., Stowell, M., and Unwin, N. (1999) J. Mol. Biol. 298, 765–786
44. Cervejanczyk, C., Enstrom, A., and Karlsson, E. (1994) Biochim. Biophys. Acta 1199, 1–5
45. Sussman, J. L., and Silman, I. (1991) Science 253, 872–879
46. Bourne, Y., Taylor, P., and Marchot, P. (1995) Cell 83, 503–512
47. Harel, M., Kleywegt, G. J., Raveli, R. B. G., Silman, I., and Sussman, J. L. (1995) Structure 3, 1355–1366
48. Lentz, T., and Wilson, P. (1995) Int. J. Biochem. 27, 1–5
49. Rosenthal, J. A., Levandoski, M. M., Chang, B., Potts, J. F., Shibata, Q. L., and Hawrot, E. (1999) Biochemistry 38, 7847–855