A High-Affinity Site for Acetylcholine Occurs Close to the α–γ Subunit Interface of *Torpedo* Nicotinic Acetylcholine Receptor†

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ABSTRACT: Affinity labeling techniques have been used to investigate the location of high-affinity binding sites for cholinergic agonists on the *Torpedo* acetylcholine receptor and the extent of overlap of these sites with those for long α-neurotoxins. Following reduction of the receptor by dithiothreitol, reaction with [^{3}H]bromoacetylcholine leads to covalent incorporation into each of the two α subunits. At high concentrations of [^{3}H]bromoacetylcholine (240 μM) and with prolonged incubation times (1–2 h), this labeling was not inhibited by either α-bungarotoxin or α-najatoxin. Following maximum labeling by [^{3}H]bromoacetylcholine, no residual high binding sites for [^{125}I]-α-bungarotoxin could be detected in the membrane-bound receptor, but 50% of the original sites were recovered by receptor solubilization. Since it has previously been reported that one of the two sites for α-bungarotoxin in the membrane-bound receptor is readily reversible but is converted to a high-affinity state by solubilization [Conti-Tronconi, B. M., Tang, F., Walgrave, S., & Gallagher, W. (1990) *Biochemistry 29*, 1046–1054], these results demonstrate that the covalently bound agonist inhibits the binding of α-bungarotoxin only to its higher affinity site in the membrane. When [^{3}H]bromoacetylcholine labeling was carried out after reduction of the receptor by sodium borohydride rather than dithiothreitol, both α and γ subunits of the receptor were labeled. Labeling of both subunits was completely inhibited if the receptor was first reduced with dithiothreitol and the α subunit sites were previously covalently labeled by unlabeled bromoacetylcholine. These results provide evidence that a high-affinity site for the agonist is located at, or close to, the α–γ subunit interface and that disulfide bonds on each of the α and the γ subunit are located at positions approximately equidistant from the quaternary ammonium binding site.

The nicotinic acetylcholine receptor (nAcChR) is a ligand-gated cation channel formed by four homologous subunits in the ratio $\alpha_2\beta\gamma\delta$ (Raftery et al., 1980; Noda et al., 1983). These subunits are assembled around a central pore (Stroud et al., 1990) that opens rapidly but transiently upon the binding of agonist to the receptor. If agonist remains bound, the receptor undergoes slow conformational transitions, leading to a desensitized state in which the affinity for agonists is high [reviewed by Conti-Tronconi and Raftery (1982), Maelicke (1988), Stroud et al. (1990), and Claudio (1989)]. Antagonists, including α-neurotoxins from the venom of elapid snakes, compete for binding to high-affinity agonist binding sites, and they also block the functional responses of the nAcChR.

The α subunits of the nAcChR have been implicated in forming binding sites for cholinergic ligands since, following reduction of a disulfide bond formed by adjacent cysteines at positions α192 and α193 in *Torpedo* nAcChR (Kao & Karlin, 1986; Mosckovitz & Gershoni, 1988; Kellaris & Ware, 1989), these cysteines may be labeled by the alkylating agonist, [^{3}H]-bromoacetylcholine (Damle et al., 1978; Moore & Raftery, 1979; Wolosin et al., 1980) or by the antagonist, [^{3}H]MBTA[1] (Kao et al., 1984). Several studies of the binding of [^{125}I]-α-bungarotoxin (α-BTX) to proteolytic, synthetic, or biosynthetic peptides have suggested that α-BTX also binds to a short sequence segment, α-181–200, encompassing this region [see Conti-Tronconi et al. (1990) and references therein]. In addition to this segment, other residues in the *Torpedo* nAcChR α subunit that may contribute to the binding site have been identified, and these include Tyr-93 and Tyr-198 identified in affinity labeling studies (Dennis et al., 1988; Galzi et al., 1990) and residues within the segment α55–74, identified by the binding of α-BTX and by antibodies that compete with the binding of nicotinic ligands (Conti-Tronconi et al., 1989, 1990b).

Evidence has been accumulating to suggest that the binding sites identified in the aforementioned affinity labeling studies involve subunits other than the α subunit. The first indication of this came from photoaffinity labeling with a cholinergic antagonist [^{3}H]bis(3-azidopyridiniumyl)decane perchlorate (DAPA; Witzemann & Raftery, 1977), which was shown to also label specifically the other subunits (β, γ, and δ) in nAcChR-enriched membranes. With the demonstration of the subunit homology of *Torpedo* nAcChR the suggestion was made that all subunits could be involved in forming binding sites (Raftery et al., 1980). Subsequently, other indications of non-α subunit involvement in high-affinity binding sites

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[1] Abbreviations: BrACh, bromoacetylcholine; *o*-BrADMAE, *N*-[2-(acetyloxy)ethyl]-*N*,*N*-dimethylammonium bromide; α-BTX, α-bungarotoxin; DAPA, bis(3-azidopyridiniumyl)decane perchlorate; DDF, *p*-(*N*,*N*-dimethylamino)benzenediazonium fluoroborate; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MBTA, [4-(*N*-maleimido)benzyl]trimethylammonium; MPTA, [4-(*N*-maleimido)phenyl]trimethylammonium; nAcChR, nicotinic acetylcholine receptor; $NaDodSO_4$, sodium dodecyl sulfate; α-NTX, α-najatoxin.

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for cholinergic ligands were obtained. In most equilibrium binding studies, radiolabeled agonists have been found to bind to two high-affinity sites per receptor with identical affinities (reviewed by Conti-Tronconi & Raftery, 1982; McCarthy et al., 1986; Claudio, 1990; Maelicke, 1990). However, the competitive antagonist, *d*-tubocurarine, has been shown to bind to two sites with affinities differing by approximately 100-fold (K_ds of 33 nM and 7.7 μM; Neubig & Cohen, 1979). The results of expression studies have provided evidence that the different association of the α subunit with the γ or δ subunits underlies the nonequivalence of tubocurarine binding sites. When different pairs of mouse nAcChR subunits were stably expressed in fibroblasts, it was found that the α–γ and α–δ pairs displayed high- and low-affinity binding of tubocurarine, respectively (Blount & Merlie, 1989). In photoaffinity labeling experiments, [^{3}H]tubocurarine photoincorporated into not only the α subunits but also into the γ and δ subunits (Pederson & Cohen, 1990). The labeling patterns were consistent with the high-affinity [^{3}H]tubocurarine site being located at the α–γ subunit interface and the low-affinity site at the α–δ subunit interface. Recently, the agonist, [^{3}H]-nicotine, was also used as a photoaffinity ligand and was shown to label α and to a lesser extent γ subunits (Middleton & Cohen, 1991). Inhibition studies suggested that the site of labeling on the γ subunit was also the site that bound tubocurarine with high affinity (Middleton & Cohen, 1991). Another recent study involved the use of a mixed disulfide between glycylcysteinamine and α-Cys-192/193 followed by carbodiimide-induced amide bond formation of the free amino group with nearby carboxylic acid residues, resulting in identification of a region of the δ subunit (residues 164–257) having been cross-linked to the α subunit (Czajkowski & Karlin, 1991). Further indications of the involvement of γ and δ subunits contributing to binding domains have been obtained using the cholinergic antagonist [^{3}H]DDF (Galzi et al., 1991).

Identification of all of the amino acids involved in recognition of agonists and antagonists is central to our understanding of nAcChR structure and function. Although binding studies have shown that α-BTX and other nicotinic ligands compete for binding sites on the nAcChR, it has been suggested from studies using monoclonal antibodies directed against different α subunit sequences that the toxin binding sites may only partially overlap those for other ligands (Watters & Maelicke, 1983; Mihovilovic & Richman, 1987; Chinchetru et al., 1989). A further complexity in locating binding sites is that, in addition to the sites associated with each of the α subunits, the *Torpedo* nAcChR carries additional sites for cholinergic agonists and for neurotoxins (Dunn & Raftery, 1982a,b; Dunn et al., 1983; Conti-Tronconi & Raftery, 1986). It is not, however, known whether these additional sites are located in structural domains homologous to those of the α subunits.

In this report we describe further studies of affinity labeling of *Torpedo* nAcChR by [^{3}H]bromoacetylcholine. It is shown that the binding of α-BTX and labeling by BrACh are not mutually exclusive. Under some experimental conditions, neither α-BTX nor α-najatoxin (α-NTX) inhibited α subunit labeling, and covalently bound [^{3}H]BrACh inhibits the binding of α-BTX to only one of its two sites in the membrane-bound nAcChR. Evidence is also presented to support the notion that a high-affinity site for [^{3}H]BrACh is located at, or close to, the α–γ subunit interface.

MATERIALS AND METHODS

Preparation of nAcChR-Enriched Membrane Fragments. nAcChR-enriched membrane fragments were prepared from *Torpedo californica* electric organs as previously described (Elliott et al., 1980) and were subjected to alkali extraction to remove nonreceptor proteins (Neubig et al., 1979; Elliott et al., 1979). The concentration of α-BTX sites was measured by the DEAE–disk assay of Schmidt and Raftery (1973) using [^{125}I]-α-BTX obtained from New England Nuclear and calibrated as described by Blanchard et al. (1979). Unless otherwise stated, all toxin binding assays were carried out in the presence of 0.1% Triton X-100, i.e., conditions in which both of the α-BTX sites on the nAcChR are of high affinity and the complexes formed are pseudo-irreversible (Conti-Tronconi et al., 1990a). Protein was determined by the method of Lowry et al. (1951). Prior to each experiment with bromoacetylcholine, acetylcholinesterase activity was inhibited by the addition of 10^{-5}–10^{-4} M Tetram and only those preparations which were devoid of esterase activity, as measured by the method of Ellman et al. (1961), were used.

Purification of Snake Toxins. α-BTX was purified from *Bungarus multicinctus* venom (Biotoxins, Inc.), α-NTX was purified from *Naja naja siamensis* venom (Biotoxins, Inc.) by the procedures of Ong and Brady (1974), and their purity was assessed as previously described (Conti-Tronconi et al., 1990a,b).

Synthesis of [^{3}H]Bromoacetylcholine. [^{3}H]BrACh perchlorate (60–90 Ci/mmol) was synthesized from [^{3}H]choline (New England Nuclear) and calibrated as previously described (Moore & Raftery, 1979; Dunn et al., 1983). Several different batches of [^{3}H]BrACh were used in the experiments reported here with identical results. *N*-[2-(Acetyloxy)ethyl]-*N*,*N*-dimethylammonium bromide was synthesized from (dimethylamino)ethanol and bromoacetyl bromide using the same procedures as for the synthesis of BrAcCh.

Labeling of nAcChR by [^{3}H]BrACh following Dithiothreitol (DTT) Reduction. Labeling of nAcChR-enriched membrane fragments by [^{3}H]BrACh was carried out using modifications of the procedures of Wolosin et al. (1980) as described by Dunn et al. (1983). Under an argon atmosphere using Aldrich AtmosBags, membrane fragments were diluted to 1 μM in α-BTX sites in degassed 15 mM Tris HCl, pH 8.0, 150 mM NaCl, 4.5 mM NaN_3, and 1.5 mM EDTA. DTT was added to a final concentration of 300 μM, and reduction was allowed to proceed for 45 min at room temperature. Where indicated in the text, membranes were preincubated with 100 μM ACh for 5 min prior to the addition of DTT. After reduction, membranes were pelleted in tightly capped centrifuge tubes in a Sorvall SS34 rotor for 15 min at 18 000 rpm. The tubes were returned to an argon atmosphere, the supernatants were discarded, and the pellets were resuspended in the same buffer but at pH 7.0. Samples were assayed for [^{125}I]-α-BTX binding as described above, appropriate aliquots were taken, and for experiments involving toxin inhibition, membranes were incubated with a 3–60-fold excess of α-BTX or α-NTX for 20 min at room temperature. [^{3}H]BrACh was added, and except where indicated, at the end of the reaction period the reaction was stopped by the addition of 10 mM BrACh. Aliquots of 50 μL were pipetted on to DE-81 discs (Whatman), and unreacted ligand was washed away with three changes of 10 mM sodium phosphate, pH 7.4, containing 50 mM NaCl and 0.1% Triton X-100 (Moore & Raftery, 1979). The remainder of each sample was centrifuged, and the pellets were resuspended in the appropriate buffer for sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis and for measurement of residual sites for [^{125}I]-α-BTX binding.

Labeling of nAcChR by [^{3}H]BrACh following Sodium Borohydride ($NaBH_4$) Reduction. Membranes were washed

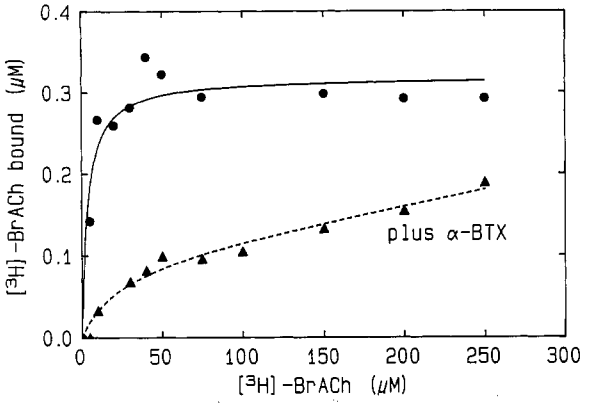

FIGURE 1: Effect of [^{3}H]BrACh concentration on the extent of covalent incorporation in the absence (●) or presence (▲) of 50 μM α-bungarotoxin. Labeling was carried out as described in Materials and Methods, and the time of incubation with [^{3}H]BrACh was 30 min. The data were corrected by subtracting the amount of incorporation in parallel control samples in which the reduction was carried out in the presence of 100 μM ACh (see text for details). Under the control conditions, nonspecific incorporation depended on the concentration of [^{3}H]BrACh and ranged from <25% of total labeling at 10 μM [^{3}H]BrACh to about 50–60% at the highest concentrations used. Data shown are representative of three separate experiments.

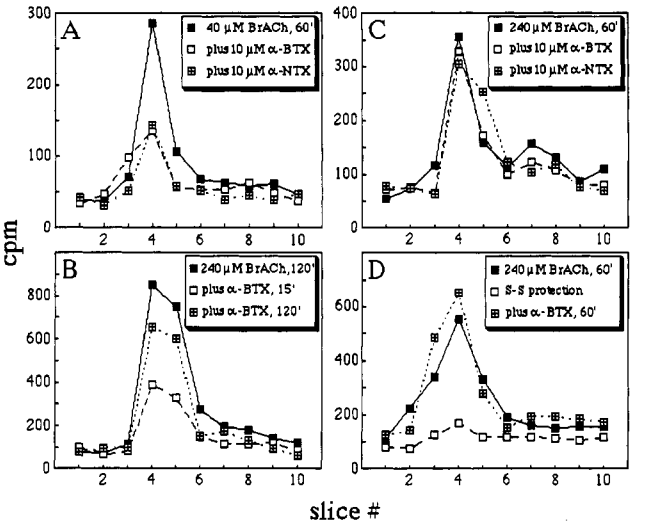

FIGURE 2: Effect of labeling conditions on the extent of inhibition of α subunit labeling by α-neurotoxins. In each experiment, after [^{3}H]BrACh labeling, nAcChR subunits were separated by SDS gel electrophoresis and the gels were cut and counted for [^{3}H]. The migration positions of each subunit indicated were obtained from parallel samples that were stained for protein. In each case the peak of labeling corresponds to the α subunit. In (A) labeling was with 40 μM [^{3}H]BrACh for 60 min in the absence or presence of 10 μM α-bungarotoxin or 10 μM α-najatoxin. The gel profiles in (C) were obtained in a parallel experiment in which the [^{3}H]BrACh concentration was increased to 240 μM. The data in (B) demonstrate that when 240 μM [^{3}H]BrACh is used, the extent of inhibition by 10 μM α-bungarotoxin is time-dependent, with approximately 50% inhibition at 15 min but much less inhibition after 2 h. Panel D shows that the [^{3}H]BrAcCh labeling in the presence of 10 μM α-bungarotoxin is specific for the cysteines generated by reduction of the reactive disulfide near the agonist and antagonist binding sites, since labeling is almost completely inhibited by protection of this disulfide by carrying out the reduction in the presence of 100 μM ACh. All experiments shown have been repeated 2 times (in the case of NTX) and 10 times (in the case of α-BTX) with similar results.

and resuspended at a concentration of 1 μM in sites for [^{125}I]-α-BTX in distilled water containing 0.01% EDTA. After titration to pH 8.5 with 0.1 N NaOH, the membranes were transferred to a round-bottom flask and were cooled on ice while being continually flushed with argon. With continuous stirring on ice, an equal volume of freshly prepared and deaired 0.2 or 0.4 M $NaBH_4$ in water was added dropwise. Reduction was allowed to proceed for 60 min on ice, and the membranes were then centrifuged for 20 min at 18 000 rpm in a Sorvall SS34 rotor. The samples were returned to an argon atmosphere using Aldrich AtmosBags, the supernatants were discarded, and the pellets were resuspended in pH 7.0 buffer (15 mM Tris HCl, 150 mM NaCl, 1.5 mM EDTA, 4.5 mM NaN_3). Labeling by [^{3}H]BrACh was then carried out using the procedures above after DTT reduction.

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$ gel electrophoresis was performed according to Laemmli (1970) by using 1-mm-thick slab gels containing 8.75% polyacrylamide. For determination of the labeling patterns, gel slices (1 mm) were cut, digested, and counted for [^{3}H] as previously described (Blanchard & Raftery, 1979). Parallel samples were stained with Coomassie Brilliant Blue R-250 in order to locate the position of the nAcChR subunits.

RESULTS AND DISCUSSION

Effect of [^{3}H]BrACh Concentration on Extent of Inhibition of Labeling by α-BTX. In previous studies it was reported that, following reduction of nAcChR with DTT, reaction with [^{3}H]BrACh resulted in covalent labeling of each of the two α subunits and that this incorporation was inhibited by carrying out the alkylation reaction in the presence of excess α-BTX (Wolosin et al., 1980; Dunn et al., 1983). In these early studies, relatively low concentrations of [^{3}H]BrACh (40 μM) and short (3–15-min) incubation times were used. However, in subsequent studies it became apparent that α-BTX becomes a much less effective inhibitor if either higher [^{3}H]BrACh concentrations are used or the alkylation reactions are allowed to proceed for longer times. The results in Figure 1 demonstrate that even when nAcChR was preincubated with excess α-BTX and then incubated with increasing concentrations of [^{3}H]BrACh for 30 min, labeling becomes significant. In this experiment, the extent of [^{3}H]BrACh incorporation was determined by the DEAE–disc assay as described in the Materials and Methods. In order to exclude the possibility that the increased labeling measured in the presence of α-BTX was due to nonspecific incorporation into other than Cys-192 and -193 on the α subunits, the data were corrected by subtracting the measured incorporation in parallel control experiments in which this disulfide was protected from reduction by the inclusion of 100 μM ACh during the reaction with DTT (Bregestovski et al., 1977; Damle & Karlin, 1980). Thus, after correction for nonspecific labeling occurring at high [^{3}H]BrACh concentrations, the progressive increase in labeling seen in the presence of a α-BTX would appear to be specific to the α subunit location.

The specificity of α subunit labeling is confirmed by the gel profile data shown in Figure 2. After 60 min of incubation with 40 μM [^{3}H]BrACh, excess α-BTX or excess α-NTX inhibited only about 50% of the labeling of the α subunits (Figure 2A). When the [^{3}H]BrACh concentration was raised to 240 μM, neither toxin caused any significant inhibition (Figure 2C). Further characterization of this lack of inhibition showed that, at this higher [^{3}H]BrACh concentration, the extent of inhibition by α-BTX was time dependent with about 50% inhibition occurring after 15 min of alkylation and much less inhibition being observed after 120 min (Figure 2B). The profiles in Figure 2D show that the labeling of the α subunits that occurs in the presence of α-BTX is due specifically to labeling of the cysteines generated by reduction of the disulfide

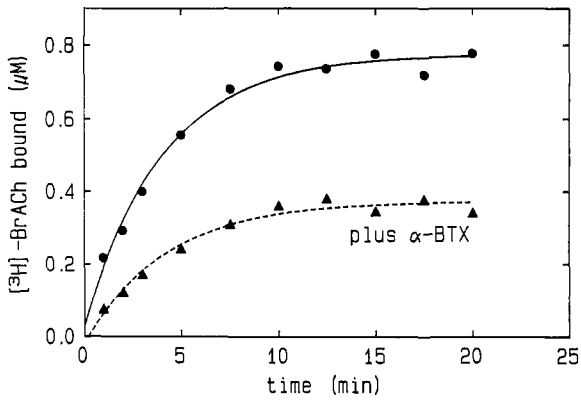

FIGURE 3: Time course of labeling of nAcChR by 40 μM [^{3}H]BrACh. Membranes were reduced with 300 μM DTT and, following removal of excess reducing agent by centrifugation, were incubated for 20 min at room temperature in the absence (●) or presence (▲) of 5 μM α-bungarotoxin. Labeling reactions were initiated by the addition of [^{3}H]BrACh, and at the times indicated, aliquots were removed and the extent of [^{3}H]BrACh incorporation was quantitated as described in Materials and Methods. The data were corrected for nonspecific labeling estimated in parallel controls in which 100 μM ACh was present during DTT reduction. Data shown are representative of four separate experiments.

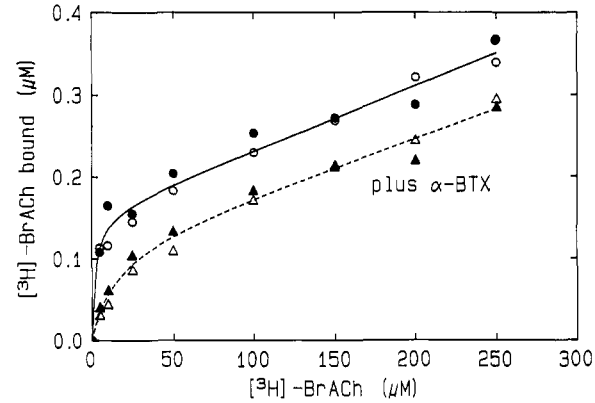

FIGURE 4: Effect of solubilization on the extent of [^{3}H]BrACh labeling and inhibition by α-bungarotoxin. Following DTT reduction, the membrane sample was divided into two aliquots, one of which was solubilized by the addition of Triton X-100 to a final concentration of 1%. Half of each sample was incubated with 5 μM α-bungarotoxin for 20 min at room temperature, prior to reaction with the indicated concentration of [^{3}H]BrACh for 30 min. The data shown are from one of two similar experiments and are for the membrane-bound receptor in the absence (●) or presence (▲) of α-BTX, and for the solubilized receptor in the absence (○) or presence (△) of α-BTX.

bond near the high-affinity binding sites since virtually all labeling was abolished if this disulfide was protected by the inclusion of low (100 μM) concentrations of ACh during the reduction (see above). In these experiments, reduced nAcChR was incubated with a 3–60-fold excess of α-neurotoxin for 20 min prior to the addition of [^{3}H]BrACh; i.e., receptor–toxin complex formation was allowed to reach equilibrium. Since one of the two α-BTX sites in membrane-bound nAcChR is of such high affinity that it is effectively irreversible (see below), the failure of α-BTX to inhibit [^{3}H]BrACh labeling cannot be explained by [^{3}H]BrACh competing for the α-BTX sites. These results show that [^{3}H]BrACh is able to covalently label the α subunits of reduced nAcChR, presumably by reacting with Cys-192/193, even when all of the available toxin binding sites are occupied by α-BTX. Thus α-BTX does not occlude the reduced disulfide, and the sites for α-BTX binding and BrACh labeling are not, therefore, identical or mutually exclusive.

Time Course of [^{3}H]BrACh Labeling. Since at lower [^{3}H]-BrACh concentrations (40 μM), both α-BTX and α-NTX inhibited approximately 50% of the labeling after 60 min (Figure 2A), the possibility arose that one of the two sites for [^{3}H]BrACh labeling was preferentially inhibited by the toxins. In order to investigate possible heterogeneity in [^{3}H]BrACh labeling, the time course of labeling in the presence and absence of α-BTX was investigated. In the absence of α-BTX, no evidence for heterogeneity was observed, and labeling was complete within approximately 11 min (Figure 3). In the presence of excess α-BTX, although the amount of labeling was reduced by more than 50%, the time course of labeling was unchanged. Taken together, the data in Figures 2 and 3 suggest that, in the presence of bound α-BTX, [^{3}H]BrACh labeling of 50% of its sites is unaffected, but labeling of the remaining 50% of the sites occurs more slowly or requires higher [^{3}H]BrACh concentrations.

Effect of [^{3}H]BrACh Labeling on [^{125}I]-α-BTX Binding. We previously reported that maximal incorporation of [^{3}H]-BrACh, i.e., two sites per receptor molecule, inhibited only 50% of residual sites for α-BTX (Dunn et al., 1983). It was later demonstrated that, in the membrane-bound state, only one of the two sites for α-BTX is "irreversible" (Conti-Tronconi et al., 1990a). The other site has lower affinity (K_d 59 ± 35 nM) but is converted to a high-affinity, "irreversible" state by membrane solubilization or by proteolysis of the receptor. In the present study, we have confirmed that 50% of [^{125}I]-α-BTX binding is retained in the [^{3}H]BrACh-labeled receptor, but only when the toxin sites are measured in the presence of Triton X-100, i.e., in solubilized receptor. No binding could be detected when the assay was carried out in *Torpedo* Ringer solution, i.e., in the membrane-bound receptor. Since the DEAE–disc assay used does not detect readily reversible complexes, these data suggest that covalently bound BrACh inhibits the binding of α-BTX to the site which normally has high affinity in the membrane-bound AcChR but does not inhibit binding of α-BTX to its lower affinity site. These findings do not, however, explain the different results reported by Wolosin et al. (1980) who, using a centrifugation assay which would also detect reversible receptor–α-BTX complexes, detected no residual sites for [^{3}H]-α-BTX after BrACh labeling. The present results are thus inconsistent with those of Wolosin et al. (1980), and there does not appear to be any obvious explanation for this discrepancy.

Effect of Solubilization on [^{3}H]BrACh Labeling and Inhibition by α-BTX. As shown above, α-BTX slows down or reduces the apparent affinity for labeling of 50% of the sites for [^{3}H]BrACh. In an attempt to investigate whether this inhibitory effect is due to the occupancy of the higher or lower affinity site for α-BTX, labeling studies have been carried out using both membrane-bound and solubilized receptor. As described above, the α-BTX binding site that is readily reversible in the membrane-bound nAcChR is converted to an effectively irreversible site after receptor solubilization. The two toxin binding sites are thus apparently homogeneous in the solubilized state. The results in Figure 4 demonstrate that the extent of labeling of nAcChR in the presence and absence of α-BTX was unaffected by solubilization. Thus conversion of the lower affinity site for α-BTX to a high-affinity, irreversible state has no effect on the amount of protection from [^{3}H]BrACh labeling afforded by the presence of α-BTX. This suggests, although it does not prove, that it is the high-affinity, irreversible toxin site in the membrane-bound nAcChR that has an inhibitory effect on [^{3}H]BrACh labeling. However, as shown above, this inhibition can readily be surmounted. Since it is also the high-affinity α-BTX site that is inhibited by BrACh labeling, this suggests greater

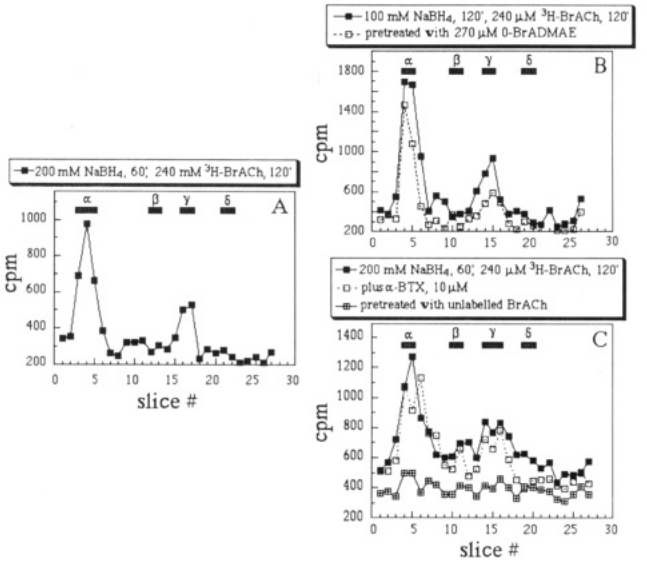

FIGURE 5: [^{3}H]BrACh labeling of the nAcChR following reduction of the membrane-bound receptor by sodium borohydride. (A) Gel profile (representative of six such determinations) showing covalent incorporation of [^{3}H]BrACh into both α and γ subunits. (B) Following reduction by sodium borohydride for 120 min, membranes were incubated for a further 120 min without 270 μM *o*-BrADMAE prior to reaction for 120 min with 240 mM [^{3}H]BrACh. Data shown are from one of two experiments showing similar results. The data shown in (C) are representative of three similar experiments and illustrate that labeling of neither the α nor γ subunits was inhibited by α-bungarotoxin. All labeling was, however, abolished by first reducing nAcChR with DTT and reaction with 240 μM unlabeled BrACh before $NaBH_4$ reduction and reaction with [^{3}H]BrACh.

overlap of this toxin binding site with one of the [^{3}H]BrACh labeling sites.

Labeling of nAcChR by [^{3}H]BrACh after Sodium Borohydride Reduction. When DTT is used as the reducing agent, the only subunits that are labeled by [^{3}H]BrACh are the α subunits, presumably as a result of incorporation into Cys-192 and/or Cys-193. However, if the membrane-bound nAcChR was reduced by $NaBH_4$ rather than DTT, both α and γ subunits were labeled (Figure 5A). The extent of incorporation of [^{3}H]BrACh into the α subunits was similar to that incorporated after DTT reduction. The extent of labeling of the γ subunit was about 50% of that of the α, an intriguing result since cysteines 192 and 193 are conserved among nAcChR α subunits but are not conserved among the other receptor subunits. Thus, another, as yet unidentified, disulfide bond in the γ subunit must be sensitive to reduction by $NaBH_4$ but not by DTT. Characterization of the labeling of the γ subunit showed that relatively high concentrations of [^{3}H]BrACh were required and labeling occurred more slowly than that of the α subunit, being maximal after about 120 min. Over these long time periods, neither receptor agonists nor excess α-BTX (see Figure 5B) inhibited the labeling of either the α or γ subunits. This lack of inhibition by α-BTX is not surprising since, even when DTT was used as the reducing agent, under the experimental conditions used (240 μM [^{3}H]BrACh, 120 min incubation) α-BTX had no inhibitory effect on labeling (see above). Also the lack of inhibition of labeling by excess ACh can readily be explained since ACh binding is reversible and, over prolonged time periods, there is a finite probability of BrACh binding resulting in covalent incorporation. This explanation is supported by the results of studies of the time course of α subunit labeling by 240 μM [^{3}H]BrACh after $NaBH_4$ reduction, in which it was found that the presence of 10 mM ACh inhibited 60% of labeling after 30 min but only about 10% after 120 min. Similar estimation of the time course of labeling of the γ subunit is more difficult because [^{3}H]BrACh is incorporated more slowly and to a lesser extent. In one experiment, *d*-tubocurarine also did not inhibit labeling over these long incubation times.

In order to determine whether the labeling was specific for cholinergic binding sites or was due only to nonspecific alkylation, the receptor preparation reduced by $NaBH_4$ was incubated with the tertiary derivative of BrACh (*o*-BrADMAE) for 120 min prior to reaction with [^{3}H]BrACh (Figure 5B). These experiments were based on those previously used to demonstrate the specificity of MPTA affinity labeling of nAcChR, in which it was shown that the rate of reaction of the reduced receptor with MPTA was 2–3 orders of magnitude faster than that of the ternary amine analogue (Karlin & Winnik, 1968). After preincubation with *o*-BrADMAE the extent of inhibition of both α and γ subunit labeling was similar, i.e., about 30%. Since some inhibition is to be expected due to the long time of incubation with an alkylating agent, this would support the specificity of the labeling reaction. Further support for specificity of both α and γ subunit labeling is provided by the data illustrated in Figure 5C. If nAcChR was first reduced with DTT and labeled with nonradioactive BrACh under conditions in which both α subunit sites were labeled, subsequent reduction by $NaBH_4$ and reaction with [^{3}H]BrACh resulted in no labeling of either α or γ subunits. We conclude that once [^{3}H]BrACh is covalently attached to Cys (193/193) on the α subunits, binding to its adjacent sites is inhibited and as a consequence no labeling of the γ subunit occurs. However, if free sulfhydryl groups are generated on both α and γ subunits as a result of $NaBH_4$ reduction, the binding of [^{3}H]BrACh can lead to alkylation of either one or the other subunit. These results provide evidence that a high-affinity site for the agonist is located at, or close to, the α–γ subunit interface. This is in agreement with previous results obtained with the bis-quaternary antagonist *d*-tubocurarine (Pederson & Cohen, 1990; Blount & Merlie, 1989) and with the monoquaternary agonist, nicotine (Middleton & Cohen, 1991). We interpret the results presented here to indicate that α Cys-192/193 and an unidentified cysteine on the γ subunit are approximately equidistant (9–10 Å) from the site of interaction of the quaternary nitrogen of BrAcCh with either or both of these subunits.

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