Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 2785–2789, April 1990
Biochemistry

d-Tubocurarine binding sites are located at α–γ and α–δ subunit interfaces of the nicotinic acetylcholine receptor

(*Torpedo*/photoaffinity)

STEEN E. PEDERSEN AND JONATHAN B. COHEN*

Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 South Euclid Avenue, Saint Louis, MO 63110

Communicated by Gerald Fischbach, February 5, 1990

ABSTRACT **The competitive nicotinic antagonist *d*-[^{3}H]tubocurarine was used as a photoaffinity label for the acetylcholine binding sites on the nicotinic acetylcholine receptor (AcChoR) from *Torpedo*. Irradiation with 254-nm UV light of AcChoR-rich membranes equilibrated with *d*-[^{3}H]tubocurarine resulted in covalent incorporation into the α, γ, and δ subunits that could be blocked by α-bungarotoxin or by carbamoylcholine. The concentrations of *d*-[^{3}H]tubocurarine required for half-maximal specific incorporation into the γ and δ subunits were 40 nM and 0.9 μM, respectively, consistent with the dissociation constants for the high- and low-affinity binding sites (K_d = 35 nM and 1.2 μM). The concentration dependence of incorporation into α subunit was biphasic and consistent with labeling of both the high- and low-affinity *d*-tubocurarine binding sites. The specific photolabeling of each AcChoR subunit was inhibited by carbamoylcholine with appropriate dose dependence. These results establish that, in addition to the α subunits, the γ and δ subunits also contribute directly to the acetylcholine binding sites and that each binding site is at an interface of subunits. Because the AcChoR subunits are homologous and are arranged pseudosymmetrically about a central axis, the photolabeling results are inconsistent with an arrangement of subunits in the AcChoR rosette of $\alpha\beta\alpha\gamma\delta$ and indicate that either the γ or δ subunit resides between the α subunits.**

The nicotinic acetylcholine receptor (AcChoR) is a ligand-gated cation channel composed of four homologous subunits. These subunits are present in the stoichiometry $\alpha_2\beta\gamma\delta$ (1, 2) and are arranged pseudosymmetrically about a central axis (3). The opening of the channel requires binding of two acetylcholine (AcCho) molecules to the AcChoR (4, 5). The AcCho binding sites have been localized to the α subunits by affinity labels (6–9) and α-bungarotoxin (α-BTX) binding (10). In addition, the AcChoR contains a binding site for noncompetitive antagonists, which is thought to reside in the ion channel structure (11).

The two binding sites for AcCho are nonidentical and can be distinguished by differential binding of some competitive antagonists. In particular, *d*-tubocurarine (TC) binds with dissociation constants that differ by ≈100-fold (12), leading to the designation of these two sites as the high- and low-affinity TC binding sites. The difference in the binding sites has been attributed to differences in the extent of glycosylation of the α subunits (13) or to differences in influence of nonequivalent neighboring subunits on the conformation of the binding site at the α subunit (12). The hypothesis that differential glycosylation can account for the difference in the binding sites has been rejected (9, 14, 15). Several lines of evidence indicate that neighboring subunits affect ligand binding. α-BTX binds to isolated α subunit and not the other subunits (10, 16–18), while covalent incorporation of α-BTX by chemical (19) or photochemical (20) crosslinking indicates proximity of other subunits to the α-BTX binding site. Recent studies from the coexpression of pairs of subunits in quail fibroblasts indicate that coexpression of α and γ subunits is required for high-affinity binding of TC, whereas low-affinity binding requires the coexpression of α and δ subunits (21). These observations support the notion of nearest-neighbor influence on the binding site properties.

TC has been shown to be a photoaffinity label of the AcCho binding site on the α subunit (9). Here we extend the previous observations to demonstrate that photoaffinity labeling of the AcCho binding sites also results in reaction of [^{3}H]TC with the γ and δ subunits. Furthermore, reaction with the γ subunit is associated with binding to the high-affinity site and labeling of δ subunit is associated with binding to the low-affinity site. The photoincorporation occurs in the presence of aqueous scavengers added to quench reaction of ligand outside the binding site. These results establish that the source of the difference in binding sites is the direct contribution of the γ and δ subunits to the structure of the high- and low-affinity sites, respectively.

EXPERIMENTAL PROCEDURES

Materials. AcChoR-rich postsynaptic membranes from *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) or *Torpedo californica* (Marinus, Westchester, CA) electric organ were isolated by the method of Sobel *et al.* (22) with the modifications described by Pedersen *et al.* (9). Monoclonal antibodies directed against the AcChoR subunits were made as described by Pedersen *et al.* (23). Carbamoylcholine, TC chloride, tetracaine, and oxidized glutathione (GSSG; glutathione disulfide) were from Sigma. α-BTX was from Miami Serpentarium (Miami, FL). HPLC grade acetonitrile was from Baker and trifluoroacetic acid was from Pierce. Acrylamide and bisacrylamide were from Boehringer Mannheim. Proadifen was kindly donated by Smith Kline & French. Histrionicotoxin was donated by Y. Kishi (Harvard University).

[^{3}H]TC was prepared from 13′-iodo-TC by catalytic exchange (New England Nuclear). It was purified by reversed-phase HPLC on a Waters μBondapak C18 column (7.5 × 300 mm) with elution by a gradient of increasing acetonitrile vs. water (with 0.1% trifluoroacetic acid in both solvents). Elution of TC and 13′-iodo-TC were monitored by absorbance at either 225 or 280 nm. Radiochemical and chemical purity were >90% as determined by HPLC. Specific activity of [^{3}H]TC was 30 Ci/mmol (1 Ci = 37 GBq) as determined by analytical HPLC by analyzing a known quantity of radioactivity for mass and comparison to standards. 13′-Iodo-TC

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AcCho, acetylcholine; AcChoR, AcCho receptor; α-BTX, α-bungarotoxin; TC, *d*-tubocurarine; GSSG, oxidized glutathione.

*To whom reprint requests should be addressed.

was synthesized from TC according to Menez *et al.* (24). Other chemicals were from standard sources.

Methods. Irradiation of *Torpedo* postsynaptic membranes was carried out in a 96-well flexible microtiter plate (Falcon) using a Spectroline EF-16 lamp (output, 254-nm UV light; 580 $\mu W/cm^2$ at 15 cm) at a distance of 5 cm. The reaction mixture contained 40–50 μg of protein (50–75 pmol of AcCho binding sites) in a volume between 30 and 200 μl in TPS buffer (250 mM NaCl/5 mM sodium phosphate/3 mM $CaCl_2$/2 mM $MgCl_2$, pH 7.0). All experiments shown were carried out with membranes from *T. nobiliana*; equivalent results were obtained with membranes from *T. californica*. Unless otherwise noted, 0.5 mM GSSG was added to reduce nonspecific incorporation of radioactivity and samples were irradiated for 2 min with 254-nm UV light. To exclude oxygen, reaction mixtures were incubated under Ar for 30 min prior to irradiation. For examining photoincorporation at different wavelengths, 200-μl samples were irradiated in stirred, capped cuvettes in a Spex Fluorolog fluorimeter equipped with a 1000-W xenon lamp and an excitation monochromator with a 9-nm bandwidth. At each wavelength the irradiation time was adjusted for lamp intensity to give equal exposure of photons. After irradiation, membranes were collected by microcentrifugation (Tomy model MTX-150, 18,000 × *g* for 10 min). The supernatant was assayed to determine the concentration of free [^{3}H]TC and the pellets were dissolved in Laemmli sample buffer for electrophoresis.

Incorporation of [^{3}H]TC into the AcChoR subunits was analyzed by $NaDodSO_4$/PAGE using the system of Laemmli (25) as described by Pedersen *et al.* (9). Gels consisting of 8% acrylamide/0.037% bisacrylamide were used. After electrophoresis, gels were stained with Coomassie blue and analyzed by fluorography (9). Radioactive incorporation was quantitated by counting gel slices corresponding to individual subunits as described by Dreyer *et al.* (26).

Specific incorporation of label into subunits with various concentrations of [^{3}H]TC was fit by a nonlinear least-squares algorithm to either single-site [cpm = $A/(1 + K/L)$] or two-site [cpm = $A_1/(1 + K_1/L) + A_2/(1 + K_2/L)$] binding functions, where A represents maximum incorporation of label (cpm), K is the apparent dissociation constant, and L is the free [^{3}H]TC concentration. Inhibition by carbamoylcholine of TC incorporation into AcChoR subunits was fitted to functions representing competition for binding at one [cpm = $A/(1 + L/K)$ + Bcg] or two [cpm = $A_1/(1 + L/K_1) + A_2/(1 + L/K_2)$ + Bcg] sites, where A represents maximum specific labeling (cpm), K is the competitor concentration that yields 50% inhibition, L is the carbamoylcholine concentration, and Bcg is the nonspecific incorporation (cpm).

Immunoblotting was performed as described by Pedersen *et al.* (23) after electrophoretic transfer of proteins from the gel to Zetabind paper (AMF Cuno). Dissociation constants of TC binding to AcChoR-rich membranes were measured by the centrifugation assay described by Krodel *et al.* (27). Membranes were incubated with various amounts of [^{3}H]TC (diluted isotopically ≈200-fold with nonradioactive TC) in TPS/0.5 mM GSSG at ambient temperature in the absence or presence of 1 mM carbamoylcholine (to define nonspecific binding). Membranes were centrifuged for 15 min in a Tomy MTX-150 microcentrifuge at 18,000 × *g*. Bound and free [^{3}H]TC were determined by assaying the pellets and supernatants, respectively.

RESULTS

Labeling of AcChoR-Rich Membranes by [^{3}H]TC. TC is a photoaffinity label for the AcCho binding site on the *Torpedo* AcChoR (9) and becomes covalently incorporated into the AcChoR α subunit upon irradiation with UV light. To facilitate determination of the residues labeled in the α subunit, we sought to optimize the labeling conditions and by so doing revealed specific labeling of the γ and δ subunits as well. Fig. 1 shows the pattern of [^{3}H]TC incorporation into membrane polypeptides after irradiation for 2 min of a membrane suspension equilibrated with 5 μM [^{3}H]TC in the absence or presence of saturating concentrations of cholinergic ligands. Reactions also included 0.5 mM GSSG as a scavenging reagent. In the absence of competitor, there was predominant incorporation into the AcChoR α subunit with weaker incorporation into the β, γ, and δ subunits as well as some non-AcChoR polypeptides (lane 2). When the reaction was performed in the presence of carbamoylcholine (lane 3) or α-BTX (lane 4), incorporation of label into α, γ, and δ subunits was decreased. The increase in labeling observed for β subunit and other polypeptides in the presence of carbamoylcholine or α-BTX was an increase in nonspecific labeling due to displacement of [^{3}H]TC from the binding site. Parallel experiments were also performed in the presence of high concentrations of the noncompetitive antagonists histrionicotoxin (lanes 5–7) and tetracaine (lanes 8–10). Specific labeling (i.e., incorporation that was inhibited by carbamoylcholine or α-BTX) of the α, γ, and δ subunits was still detected with the noncompetitive antagonist site occupied. As quantitated from gel slices, histrionicotoxin inhibited specific labeling of the α and γ subunits by ≈25%, but tetracaine was without effect. Thus, [^{3}H]TC labeled the α, γ, and δ subunits via the AcCho binding sites and not through interaction at the site of noncompetitive antagonist binding.

Specific labeling was also observed for a band in the gels that migrates slightly slower than the 43 kDa protein (Fig. 1). This band was identified by immunoblotting as a modified form of the α subunit induced by irradiation (Fig. 2). A fluorogram of membranes irradiated in the presence of [^{3}H]TC and in the absence (lane 1) or presence (lane 2) of carbamoylcholine shows the strong specific labeling of the band immediately above the 43-kDa protein (arrow). The Coomassie blue staining pattern of nonirradiated (lane 3) and irradiated (lane 4) membranes shows the appearance of a band that corresponds to the position of the specifically labeled material in the fluorogram. An immunoblot (lanes 5–14) of nonirradiated (odd-numbered lanes) and irradiated

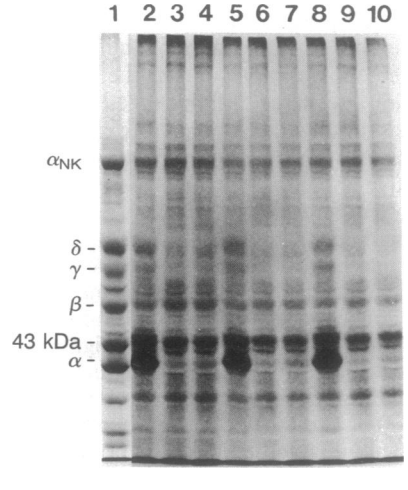

FIG. 1. Photoincorporation of [^{3}H]TC into AcChoR-rich membranes. AcChoR-rich membranes (40 μg) were suspended in 30 μl (1.9 μM AcCho binding sites) of TPS/0.5 mM GSSG/5 μM [^{3}H]TC and irradiated for 2 min with 254-nm UV light. The reaction mixtures contained no other cholinergic ligands (lane 2), 5 mM carbamoylcholine (lanes 3, 6, and 9), 20 μM α-BTX (lanes 4, 7, and 10), 30 μM histrionicotoxin (lanes 5–7), or 30 μM tetracaine (lanes 8–10). Proteins were separated by $NaDodSO_4$/PAGE, stained with Coomassie blue (lane 1), and then processed for fluorography (lanes 2–10).

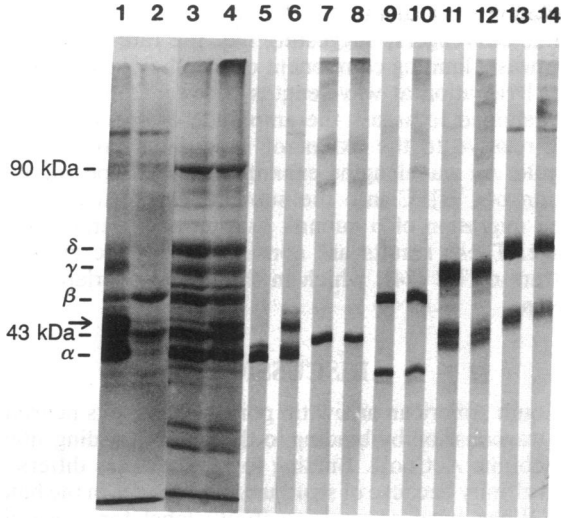

FIG. 2. Irradiation produces α subunit of altered mobility. AcChoR-rich membranes were labeled at a concentration of 330 nM AcCho binding sites in 500 nM [^{3}H]TC/50 μM proadifen in the absence (lanes 1 and 3–14) or presence (lane 2) of 1 mM carbamoylcholine. Samples were irradiated (lane 1 and even-numbered lanes) or not (odd-numbered lanes except for lane 1) with 280-nm UV light for 2 min in a stirred cuvette. Proteins were separated by NaDodSO$_4$/PAGE and either stained with Coomassie blue (lanes 3 and 4) and processed for fluorography (lanes 1 and 2) or transferred to Zetabind paper for immunoblotting (lanes 5–14). After transfer, the replicas were probed with antibodies that bind α subunit (monoclonal antibody A18, lanes 5 and 6), 43-kDa protein (monoclonal antibody 19F4A, lanes 7 and 8), β subunit (monoclonal antibody B9, lanes 9 and 10), γ subunit (monoclonal antibody C8, lanes 11 and 12), or δ subunit (monoclonal antibody D5, lanes 13 and 14).

membranes (even-numbered lanes) using antibodies against each of the AcChoR subunits and the 43-kDa protein illustrates the effects of irradiation on the proteins. The blot of the α subunit clearly shows the appearance of a band upon irradiation that corresponds to the position of the labeled band and to the band evident by Coomassie blue staining. The appearance of this band is independent of the presence of TC.

Effect of Scavenging Reagents on [^{3}H]TC Incorporation. To demonstrate that specific labeling was not due to diffusion of the photoactivated TC from its binding site(s) to a reactive residue distant from the site, scavenging reagents were examined for effects on specific and nonspecific incorporation of the AcChoR subunits. Thiols and disulfides were found to be the most effective agents, but because thiols can affect ligand binding by reducing the disulfide bond in the vicinity of the binding site (28), disulfides were preferred. As shown in Fig. 3, GSSG decreases the nonspecific labeling by >60%, while the specific labeling of the α, γ, and δ subunits was essentially unaffected; there was no specific incorporation of label into the β subunit. These results show that the residues specifically labeled under these conditions must be in close proximity to the TC binding sites.

Labeling of γ and δ Subunits by [^{3}H]TC Is via the High- and Low-Affinity Sites, Respectively. TC binds to the two AcCho binding sites with differing affinities. Under the conditions used here, the dissociation constants (K_d) were measured to be 35 nM and 1.2 μM (data not shown) by a centrifugation binding assay. The difference in K_d of the low-affinity site from published values (1.2 vs. 7 μM; ref. 12) may reflect differences in temperature (4°C vs. 22°C), the presence of GSSG, or changes in membrane preparation (9). It was of interest to determine whether labeling of the AcChoR subunits reflected binding to the low-affinity site, to the high-affinity site, or to both. AcChoR-rich membranes were irradiated in the presence of various concentrations of

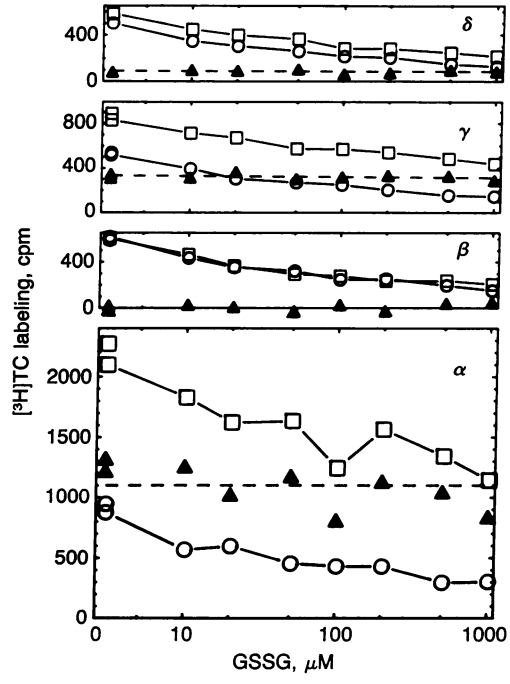

FIG. 3. GSSG reduces nonspecific labeling. AcChoR-rich membranes (40 μg) were incubated in 200 μl (330 nM AcCho binding sites) with 500 nM [^{3}H]TC and various concentrations of GSSG. After separation of membrane proteins by NaDodSO$_4$/PAGE, individual subunits were excised and assayed to quantitate incorporation of [^{3}H]TC. The cpm of [^{3}H]TC incorporated into each subunit (α, β, γ, and δ) is plotted versus GSSG concentration. The specific labeling (▲) is the difference between incorporation in the absence (□) or presence (○) of 1 mM carbamoylcholine.

[^{3}H]TC, 10 μM proadifen to occupy the noncompetitive antagonist binding site, and 0.5 mM GSSG. The total and nonspecific incorporation of [^{3}H]TC into the α, γ, and δ subunits is shown in Fig. 4 (*Left*). The specific labeling (the

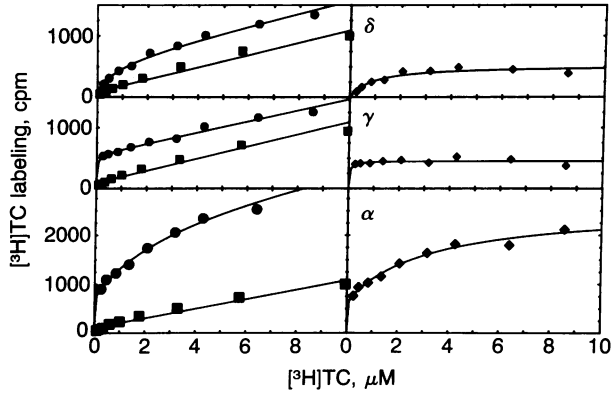

FIG. 4. Labeling of the AcChoR γ and δ subunits via the high- and low-affinity binding sites. AcChoR-rich membranes (50 μg) were incubated with various concentrations of [^{3}H]TC in a vol of 30 μl (2.4 μM AcCho binding sites/0.5 mM GSSG/10 μM proadifen/TPS) in the presence (■) or absence (●) of 1 mM carbamoylcholine. The incorporation of [^{3}H]TC into the AcChoR subunits was quantitated. (*Left*) The total and nonspecific incorporation of the α, γ, δ subunits are plotted as a function of free [^{3}H]TC concentration. (*Right*) The corresponding specific labeling (◆) determined from the difference between total and nonspecific incorporation is shown. Solid lines of nonspecific labeling represent linear fits of the data. Curves of specific labeling represent nonlinear least-squares fits to equations for a single-site (γ and δ subunits) or two-site (α subunits) binding function. The K_{app} values for labeling are given: α, $K_1 < 140$ nM, $K_2 = 3.2 \pm 2.4$ μM; γ, $K < 80$ nM; δ, $K = 0.9 \pm 0.3$ μM.

difference between total and nonspecific incorporation) is shown in Fig. 4 (*Right*). The concentration of [^{3}H]TC required for half-maximal labeling of each subunit (K_{app}) was calculated. Specific labeling of the α subunit was biphasic, consistent with occupancy of two binding sites; a high-affinity site (K_{app} < 100 nM) and a low-affinity site (K_{app} = 3.2 μM). At the concentrations tested, specific labeling of the γ subunit was independent of concentration, consistent with occupancy of a high-affinity binding site. Specific labeling of δ subunit was consistent with occupancy of a single low-affinity binding site (K_{app} = 0.9 μM). When experiments were conducted at a lower concentration of AcChoR and at [^{3}H]TC concentrations between 10 and 250 nM, incorporation into α and γ subunits was characterized by K_{app} values of 46 ± 10 nM for α subunit and 37 ± 8 nM for γ subunit, in good correspondence with the dissociation constant (35 nM) for the high-affinity binding site (data not shown).

Labeling of the α, γ, and δ subunits by [^{3}H]TC was inhibited by carbamoylcholine in a dose-dependent manner (Fig. 5). The concentrations of carbamoylcholine required for inhibition of half the specific labeling (IC_{50}) of the γ and δ subunits were 7 μM and 60 nM, respectively. These values agree well with IC_{50} values expected for inhibition of binding to the high- and low-affinity sites [4 μM and 200 nM, respectively, assuming a K_d for carbamoylcholine of 100 nM (12)]. The inhibition of labeling of the α subunit was best fit by a two-site model with IC_{50} values of 36 μM and 100 nM. Both the concentration dependence of labeling by [^{3}H]TC and the inhibition of labeling by carbamoylcholine demonstrate that the α subunit was specifically labeled via both the high- and low-affinity TC binding sites, while the γ subunit was labeled only via the high-affinity site and the δ subunit was labeled only via the low-affinity site.

Amplitude of Photoincorporation. For the experiment shown in Fig. 4, at maximal specific incorporation, 0.4% of the AcChoRs was labeled on the α subunit and 0.1% was labeled on each of the γ and δ subunits. Because the extent of incorporation was low, we also examined the incorporation as a function of irradiation time and wavelength. The amount of [^{3}H]TC incorporated into subunits could be increased with irradiation times up to 30 min, but this also resulted in extensive crosslinking of the subunits as shown by Coomassie blue stain (see also Fig. 2). The slow rate of photoincorporation was consistent with the half-time of 7–10 min measured for photolytic degradation of TC. The rate of degradation was unaffected by the presence of thiol or disulfide scavengers, suggesting that the rate of photoactivation was a limiting component of the photolabeling experiment. Irradiation at wavelengths between 250 and 300 nm was examined to assess the amplitude of specific photolabeling relative to the extent of irradiation damage to the AcChoR. All wavelengths examined yielded specific incorporation of [^{3}H]TC into the subunits but also resulted in altered migration of α subunit as well as general radiation damage. These results are consistent with the absorption spectrum of TC (24), which in this range overlaps that of proteins.

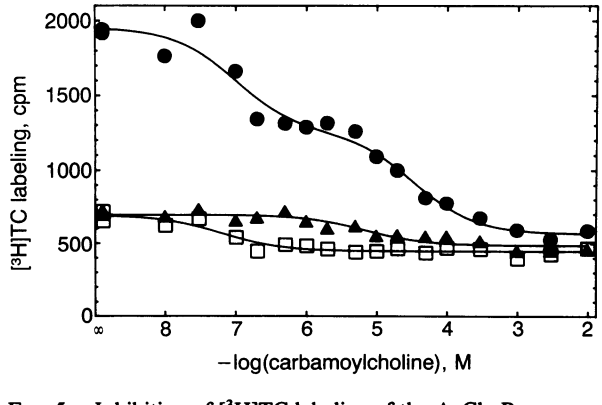

FIG. 5. Inhibition of [^{3}H]TC labeling of the AcChoR α, γ, and δ subunits by carbamoylcholine. AcChoR-rich membranes (40 μg, 570 nM AcCho binding sites) were incubated in 100 μl (2 μM [^{3}H]TC/0.5 mM GSSG/10 μM proadifen/TPS) with various concentrations of carbamoylcholine. Incorporation of [^{3}H]TC into the α (●), γ (▲), and δ (□) subunits was quantitated. Solid lines represent nonlinear least-squares fitting of the data to inhibition at a single site (γ and δ) or two sites (α). K_{app} values for inhibition are as follows: α, K_1 = 100 ± 60 nM, K_2 = 36 ± 18 μM; γ, K = 7 ± 3 μM; δ, K = 60 ± 30 nM.

DISCUSSION

The South American arrow tip poison TC blocks neuromuscular transmission by binding to the AcCho binding sites on the nicotinic AcChoR. Binding to the two sites differs 100-fold in affinity because of structural differences in the binding sites. When used as a photoaffinity label for these sites, labeling of the γ and δ subunits by [^{3}H]TC paralleled the expected occupancy for the high- and low-affinity sites, respectively, as shown by labeling at various concentrations of [^{3}H]TC (Fig. 4) and by inhibition of labeling by carbamoylcholine (Fig. 5). The γ and δ subunits, in addition to the α subunit, therefore appear to be directly involved in the binding of TC. An α–γ subunit pair forms the high-affinity TC binding site and an α–δ subunit pair forms the low-affinity site. The direct contribution of the γ and δ subunits to the high- and low-affinity binding sites further establishes that the TC binding sites are at the interfaces between the α–γ and α–δ subunits. Our conclusion is reinforced by the observations of TC binding to coexpressed pairs of subunits (21), which demonstrates that α–γ subunits form high-affinity binding sites, α–δ subunits form low-affinity sites, and α–β coexpression does not permit TC binding.

The specificity of [^{3}H]TC photoincorporation into the AcChoR is demonstrated by the fact that carbamoylcholine and α-BTX inhibit labeling of α, γ, and δ subunits. Furthermore, TC labeling of the noncompetitive antagonist site can be ruled out since histrionicotoxin did not alter [^{3}H]TC incorporation in the presence of carbamoylcholine or α-BTX. Histrionicotoxin did, however, reduce specific labeling of the α and γ subunits by ≈25%, an effect also exhibited by proadifen and phencyclidine (data not shown), two desensitizing noncompetitive antagonists (29), but not by tetracaine, a noncompetitive antagonist that interacts preferentially with a nondesensitized conformation of the AcChoR (Fig. 1). These results suggest that binding of noncompetitive antagonists that desensitize the AcChoR causes a conformational change in the AcChoR that affects labeling of the α and γ subunits.

In previous affinity labeling studies of the AcCho binding sites, attention has focused on the contributions from the α subunit. However, it was noted (8) that *p*-(dimethylamino)benzenediazonium fluoroborate was specifically photoincorporated into the γ subunit at a level 10% of α subunit. In addition, chemical crosslinking of ^{3}H-labeled cobra toxin to the AcChoR resulted in reaction with all subunits that could be blocked selectively by TC (19). UV-induced crosslinking of ^{125}I-labeled α-BTX to the AcChoR resulted in covalent attachment to the α, γ, and δ subunits (20). However, because of the large size of the toxin (8 kDa) and the length of the chemical crosslinker (9 Å), it is difficult to evaluate the proximity of the labeled subunits to the AcCho binding sites.

The localization of the AcCho binding sites to the interfaces between the α–γ and α–δ subunits also provides important information about the relative location of the AcChoR subunits. The subunits are arranged in a rosette with rotational pseudosymmetry about the channel pore (3). It is

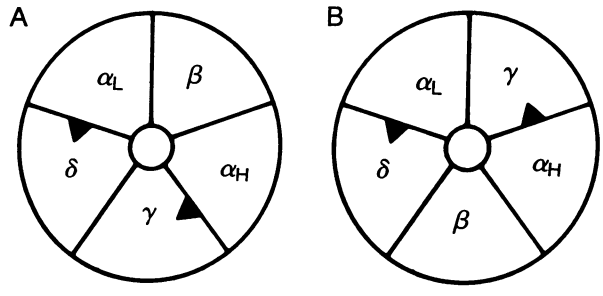

FIG. 6. Possible models for the arrangement of the AcChoR subunits. Two of the possible arrangements of the AcChoR subunits in a rosette are indicated with L and H denoting α subunits associated with the low- and high-affinity binding sites and the triangles symbolizing the ligand binding sites at subunit interfaces. (*A*) An arrangement that places the β subunit between the α subunits. (*B*) An arrangement consistent with rotational symmetry of binding sites located at α–γ and α–δ interfaces. Placement of the δ subunit between α subunits is also consistent with rotational symmetry of the binding sites.

likely that equivalent regions of each α subunit contribute to the binding sites. Therefore, binding sites must also be related by rotational symmetry about the channel pore. If the β subunit is placed between the two α subunits in the rosette and the binding sites are assigned to the α–γ and γ–δ interfaces (Fig. 6*A*), the rotational symmetry is violated. Symmetric arrangement of the binding sites thus requires that either the γ or δ subunit resides between the α subunits (Fig. 6*B*).

This conclusion is in agreement with electron microscopic visualization of α subunits tagged with avidin that places the γ subunit between the α subunits (30), but it differs from the results of a more recent analysis by electron microscopic imaging of crystalline AcChoR that places the β subunit between the α subunits (31). However, in the latter study the α and δ subunits were identified through extracellular ligands, while the β subunit was identified by an antibody directed to a cytoplasmic determinant and yielded only a diffuse localization. Furthermore, the analysis relied on the following untested assumptions: (*i*) the subunits are not tilted with respect to the membrane, and (*ii*) ligand binding does not induce structural changes in the AcChoR. In consideration of these limitations of the localization of the β subunit by electron microscopic image analysis, we conclude that our inferences from [^{3}H]TC labeling are more plausible. Thus, either the γ or δ subunit resides between the α subunits.

Although the efficiency of TC photoincorporation is low, the availability of [^{3}H]TC of high radiochemical specific activity results in specific incorporation into AcChoR subunits of $\approx$50 Ci/mol. Identification of the labeled amino acids within each subunit is thus feasible and will provide information about the residues contributing to the binding sites and the subunit interfaces.

This research was supported in part by National Institutes of Health Grants NS 19522 and NS 22828 (Senator Jacob Javits Center for Excellence in Neuroscience).

1. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T. & Numa, S. (1983) *Nature (London)* **302,** 528–532.
2. Raftery, M. A., Hunkapiller, M. W., Strader, C. D. & Hood, L. E. (1980) *Science* **208,** 1454–1457.
3. Brisson, A. & Unwin, P. N. T. (1985) *Nature (London)* **315,** 474–477.
4. Neubig, R. R. & Cohen, J. B. (1980) *Biochemistry* **19,** 2770–2779.
5. Neubig, R. R., Boyd, N. D. & Cohen, J. B. (1982) *Biochemistry* **21,** 3460–3467.
6. Abramson, S. N., Li, Y., Culver, P. & Taylor, P. (1989) *J. Biol. Chem.* **264,** 12666–12672.
7. Kao, P. N., Dwork, A. J., Kaldany, R.-R. J., Silver, M. L., Wideman, J., Stein, S. & Karlin, A. (1984) *J. Biol. Chem.* **254,** 11662–11665.
8. Langenbuch-Cachat, J., Bon, C., Mulle, C., Goeldner, M., Hirth, C. & Changeux, J.-P. (1988) *Biochemistry* **27,** 2337–2345.
9. Pedersen, S. E., Dreyer, E. B. & Cohen, J. B. (1986) *J. Biol. Chem.* **261,** 13735–13743.
10. Tzartos, S. J. & Changeux, J.-P. (1983) *EMBO J.* **2,** 381–387.
11. Giraudat, J., Dennis, M., Heidmann, T., Haumont, P.-Y., Lederer, F. & Changeux, J.-P. (1987) *Biochemistry* **26,** 2410–2418.
12. Neubig, R. R. & Cohen, J. B. (1979) *Biochemistry* **18,** 5464–5475.
13. Conti-Tronconi, B. M., Hunkapiller, M. W. & Raftery, M. A. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2631–2634.
14. Smith, M. M., Schlesinger, S., Lindstrom, J. & Merlie, J. P. (1986) *J. Biol. Chem.* **261,** 14825–14832.
15. Nomoto, H., Takahashi, N., Nagaki, Y., Endo, S., Arata, Y., & Hayashi, K. (1986) *Eur. J. Biochem.* **157,** 233–242.
16. Tzartos, S. J. & Changeux, J.-P. (1984) *J. Biol. Chem.* **259,** 11512–11519.
17. Gershoni, J. M., Hawrot, E. & Lentz, T. L. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 4973–4977.
18. Carlin, B. E., Lawrence, J. C., Jr., Lindstrom, J. M. & Merlie, J. P. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 498–502.
19. Hamilton, S. L., Pratt, D. R. & Eaton, D. C. (1985) *Biochemistry* **24,** 2210–2219.
20. Oswald, R. E. & Changeux, J.-P. (1982) *FEBS Lett.* **139,** 225–229.
21. Blount, P. & Merlie, J. P. (1989) *Neuron* **3,** 5349–5357.
22. Sobel, A., Weber, M. & Changeux, J.-P. (1977) *Eur. J. Biochem.* **80,** 215–224.
23. Pedersen, S. E., Bridgman, P. C., Sharp, S. D. & Cohen, J. B., (1990) *J. Biol. Chem.* **265,** 569–581.
24. Menez, A., Bouet, F., Morgat, J.-L., Ronsseray, A.-M., Boquet, P., Changeux, J.-P. & Fromageot, P. (1973) *Biochimie* **55,** 919–924.
25. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
26. Dreyer, E. B., Hasan, F., Cohen, S. G. & Cohen, J. B. (1986) *J. Biol. Chem.* **261,** 13727–13734.
27. Krodel, E. K., Beckman, R. A. & Cohen, J. B. (1979) *Mol. Pharmacol.* **15,** 294–312.
28. Damle, V. N., & Karlin, A. (1980) *Biochemistry* **19,** 3924–3932.
29. Cohen, J. B., Medynski, D. C. & Strnad, N. P. (1985) in *Effects in Anesthesia,* ed. Fozzard, H. A. (Am. Physiol. Soc., Bethesda, MD), pp. 53–64.
30. Karlin, A., Holtzman, E., Yodh, N., Lobel, P., Wall, J. & Hainfeld, J. (1983) *J. Biol. Chem.* **258,** 6678–6681.
31. Kubalek, E., Ralston, S., Lindstrom, J. & Unwin, N. (1987) *J. Cell Biol.* **105,** 9–18.