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Photolabeling of Membrane-Bound *Torpedo* Nicotinic Acetylcholine Receptor with the Hydrophobic Probe 3-Trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine†

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ABSTRACT: The hydrophobic, photoactivatable probe 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) was used to label acetylcholine receptor rich membranes purified from *Torpedo californica* electric organ. All four subunits of the acetylcholine receptor (AChR) were found to incorporate label, with the γ-subunit incorporating approximately 4 times as much as each of the other subunits. Carbamylcholine, an agonist, and histrionicotoxin, a noncompetitive antagonist, both strongly inhibited labeling of all AChR subunits in a specific and dose-dependent manner. In contrast, the competitive antagonist α-bungarotoxin and the noncompetitive antagonist phencyclidine had only modest effects on [^{125}I]TID labeling of the AChR. The regions of the AChR α-subunit that incorporate [^{125}I]TID were mapped by *Staphylococcus aureus* V8 protease digestion. The carbamylcholine-sensitive site of labeling was localized to a 20-kDa V8 cleavage fragment that begins at Ser-173 and is of sufficient length to contain the three hydrophobic regions M1, M2, and M3. A 10-kDa fragment beginning at Asn-339 and containing the hydrophobic region M4 also incorporated [^{125}I]TID but in a carbamylcholine-insensitive manner. Two further cleavage fragments, which together span about one-third of the α-subunit amino terminus, incorporated no detectable [^{125}I]TID. The mapping results place constraints on suggested models of AChR subunit topology.

The nicotinic acetylcholine receptor (AChR)[1] is an integral membrane protein with subunit stoichiometry $\alpha_2\beta\gamma\delta$. Each of the AChR subunits is known to span the membrane, and each is thought to contribute structurally to the AChR ion channel which opens in response to the binding of cholinergic agonists [reviewed by Popot and Changeux (1984), Hucho (1986), and McCarthy et al. (1986)]. The binding sites for agonists and competitive antagonists have been localized to the α-subunits by use of affinity labels (Kao et al., 1984; Pedersen et al., 1986; Dennis et al., 1988), while a high-affinity binding site for noncompetitive antagonists is thought to be formed by all subunits and may be located in the channel itself [reviewed by Changeux and Revah (1987)].

The amino acid sequences of all four subunits are known for the AChR of *Torpedo californica* electric tissue (Noda

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; 43K protein, the basic, membrane-bound 43-kDa protein of *Torpedo* postsynaptic membranes; BSA, bovine serum albumin; α-BgTx, α-bungarotoxin; H_{10}-HTX, *d,l*-decahydro(pentenyl)histrionicotoxin; LIS, lithium diiodosalicylate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; [^{125}I]TID, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine; TPS, *Torpedo* physiological saline (250 mM NaCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0); V8 protease, *Staphylococcus aureus* V8 protease.

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et al., 1982, 1983a,b; Claudio et al., 1983). The subunits exhibit considerable sequence homology and share distinct structural features, including four characteristically placed regions of marked hydrophobicity. The length (approximately 20 amino acid residues) of these regions suggests that they are membrane-spanning α-helices in contact with the lipid bilayer. Three of them (M1, M2, and M3) are located centrally in the amino acid sequence of each subunit, and the fourth (M4) is located near the carboxyl terminus. A fifth region, M5, of amphipathic character lying between M3 and M4 has also been proposed to form a membrane-spanning helix on each subunit (Finer-Moore & Stroud, 1984; Guy, 1984).

Experimental work has so far provided only limited, and sometimes conflicting, answers to the question of whether these regions actually span the membrane. Immunological evidence indicates that M4 and M5 are cytoplasmic rather than transmembrane (Ratnam et al., 1986; Kordossi & Tzartos, 1987) and, in addition, implies the existence of two novel membrane-spanning regions (M6 and M7) on the amino-terminal side of M1 (Criado et al., 1985). These conclusions are challenged, in part, by biochemical studies. Work with membrane-impermeable reductants suggests that the carboxyl terminus of the δ-subunit (which flanks M4) is extracellular (McCrea et al., 1984; Dunn et al., 1986) rather than cytoplasmic as indicated by epitope mapping (Young et al., 1985; Ratnam & Lindstrom, 1984). This result implies that M4 is transmembrane, a conclusion also supported by labeling studies with photoreactive phospholipid analogs (Giraudat et al., 1985).

Small, lipophilic photoreagents provide an alternative method of identifying transmembrane regions of the AChR. Several of these, including [^{3}H]pyrenesulfonyl azide (Sator et al., 1979; Clarke et al., 1987), 5-[^{125}I]iodonaphthyl 1-azide (Tarrab-Hazdai et al., 1980; Tarrab-Hazdai & Goldfarb, 1982), and [^{3}H]adamantanediazirine (Middlemas & Raftery, 1983), have been used to demonstrate exposure of the AChR subunits to the membrane, but little work has been done to characterize the labeled regions.

3-Trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]-TID) is another small, hydrophobic photoreagent (Brunner & Semenza, 1981). It differs from azido probes in generating a highly reactive carbene upon photoactivation (instead of the less reactive nitrene), and unlike [^{3}H]adamantanediazirine its photogenerated species does not undergo rearrangement to less lipophilic, reactive intermediates. [^{125}I]TID has been shown to selectively label the integral membrane proteins of erythrocyte ghosts (Brunner & Semenza, 1981) and has been used to identify the membrane-spanning regions of many transmembrane proteins (Brunner & Semenza, 1981; Kahan & Moscarello, 1985; Spiess et al., 1982; Frielle et al., 1982). In several instances the pattern of labeling of amino acid residues has allowed the secondary structure of membrane-spanning regions and their degree of exposure to the lipid bilayer to be inferred (Brunner et al., 1985; Hoppe et al., 1984; Meister et al., 1985).

We characterize in this paper the photoincorporation of [^{125}I]TID into postsynaptic membranes isolated from *Torpedo* electric organ. [^{125}I]TID reacts with the integral membrane proteins of that preparation. All AChR subunits are labeled, though, surprisingly, the extent of labeling is sensitive to the presence of cholinergic agonists and antagonists. Labeled regions of the AChR α-subunit are mapped by the method of *S. aureus* V8 protease digestion (Pedersen et al., 1986). The results presented indicate that [^{125}I]TID is a useful probe not only of the AChR transmembrane regions but also of the conformational changes the AChR undergoes upon ligand binding.

Experimental Procedures

Materials. [^{125}I]TID (~10 Ci/mmol) was purchased from Amersham. Stock solutions in 75% ethanol were stored at 4 °C in the dark. α-Bungarotoxin was from Biotoxins, Inc., and phencyclidine from Alltech Associates. Carbamylcholine and *d*-tubocurarine chloride were from Sigma. *d,l*-Decahydro-(pentenyl)histrionicotoxin (H_{10}-HTX) was the generous gift of Dr. Y. Kishi (Harvard University). *S. aureus* V8 protease was purchased from ICN Biochemicals and endoglycosidase H (Endo H) from Miles Scientific. Carboxypeptidase Y was from Boehringer-Mannheim Biochemicals. Subunit-specific mouse monoclonal antibodies were the gift of Dr. Steen Pedersen and were generated against purified, SDS-solubilized AChR subunits as described by Bridgman et al. (1987).

Preparation of AChR-Rich Membranes, Extraction of Peripheral Proteins, and Isolation of AChR. Membranes were isolated from the electric organ of *T. californica* (Marinus, Inc., Westchester, CA) according to the procedure of Sobel et al. (1977) with the modifications described previously (Pedersen et al., 1986). The final membrane suspensions in 36% sucrose/0.02% NaN_3 were stored at −80 °C under argon and contained 1.3–1.7 nmol of acetylcholine binding sites/mg of protein as measured by a direct [^{3}H]acetylcholine binding assay (Boyd & Cohen, 1980).

For extraction of peripheral proteins, membrane suspensions (1 mg of protein/mL) were incubated for 1 h at 4 °C either at pH 11 (Neubig et al., 1979) or in a solution containing 10 mM lithium diiodosalicylate (LIS), 2 mM EDTA, and 10 mM Tris, pH 8.1 (Porter & Froehner, 1983). The AChR was isolated from detergent extracts of AChR-rich membranes by affinity chromatography on an acetylcholine affinity matrix prepared by the method of Karlin et al. (1976) as modified by Reynolds and Karlin (1978). The purification procedure followed the protocol of Huganir and Racker (1982) with several modifications. For receptor solubilization, 0.5% Lubrol-PX was substituted for 1% cholate, and after adsorption of the extract on the affinity resin, the gel was washed successively with 10 volumes of saline buffers containing 0.5% Lubrol-PX, then 1% Triton X-100, and finally 0.5% Lubrol-PX before elution with 20 mM carbamylcholine in wash buffer. Phospholipids were omitted from the solubilization and wash buffers.

Photolabeling of AChR-Rich Membranes with [^{125}I]TID. Reactions were carried out on a scale appropriate for the analysis of reaction products by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Unless otherwise noted, all labeling experiments were carried out at room temperature with AChR-rich membranes (final concentration, 1 mg of protein/mL) in *Torpedo* physiological saline (TPS, 250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0, and 0.02% NaN_3). All manipulations with [^{125}I]TID were carried out in a fumehood under dim room light. Aliquots of a stock solution of [^{125}I]TID were added to the membranes to the desired final concentration (usually 1–2 μM). After a 15-min incubation, aliquots (30 or 50 μL) of the reaction mixture were placed in the wells of a poly(vinyl chloride) 96-well assay plate (Falcon 3911), incubated under argon for at least 5 min, and then irradiated with a 365-nm lamp (Spectroline EN-16) at a distance of 6.5 cm. Illumination was for either 5 or 10 min. Photolabeling in the presence of cholinergic ligands was accomplished either by diluting the concentrated ligand (in TPS) directly into the membrane/[^{125}I]TID suspension or by diluting the ligand 1:1

into a 2-fold concentrated membrane/[^{125}I]TID suspension.

Samples were prepared for SDS–PAGE in one of two ways. In experiments where the total incorporation of [^{125}I]TID into membranes was monitored, 0.2-mg samples were diluted 1:5 with TPS/1% bovine serum albumin (BSA) and pelleted by centrifugation (Eppendorf 5414) for 15 min. Supernatants were removed and pellets again centrifuged after resuspension in 1.2 mL of TPS/1% BSA. This wash procedure was repeated four times with TPS/1% BSA and twice with TPS alone to remove residual BSA. Samples were then resuspended in sample loading buffer and subjected to SDS–PAGE. In experiments where only protein labeling was monitored, irradiated samples were diluted 1:5 with TPS and pelleted in a Beckman airfuge. Pellets were resuspended in sample loading buffer and subjected to SDS–PAGE.

In some experiments, after labeling with [^{125}I]TID and prior to electrophoresis, membranes suspensions were treated with endoglycosidase H as described by Pedersen et al. (1986). Briefly, 5 μL of 10% SDS and 4.5 munits of endoglycosidase H were added to 0.3-mL aliquots of membranes resuspended at 0.8 mg of protein/mL in 50 mM NaP_i, pH 7.0. After a 12-h incubation at 23 °C, samples were dissolved for electrophoresis.

Partitioning of [^{125}I]TID into AChR-Rich Membranes. [^{125}I]TID was purified by thin-layer chromatography according to the method of Brunner and Semenza (1981) and extracted into absolute ethanol just before the experiment. Equal aliquots of approximately 200 nCi of purified [^{125}I]TID were diluted 1:100 into suspensions of AChR-rich membranes of 0.2, 1, and 5 mg/mL in the presence and absence of 100 μM carbamylcholine. Samples were incubated for 1.5 h in 0.5-mL capped, polypropylene microcentrifuge tubes and then centrifuged (Eppendorf 5414) for 30 min. After centrifugation, supernatants were removed for γ-counting, as were pellets resuspended in 0.2 mL of TPS containing 1% SDS.

Gel Electrophoresis. Proteins were separated by SDS–PAGE essentially by the procedure of Laemmli (1970) except that the electrode buffer contained 50 mM Tris, 380 mM glycine, and 0.15% SDS. Gels of 8% polyacrylamide contained 0.33% bis(acrylamide), while 15% gels contained 0.4% cross-linker. All gels were fixed in 50% methanol/10% acetic acid for at least 30 min before staining with Coomassie Brilliant Blue R in 45% methanol/10% acetic acid.

Proteolytic mapping of the α-subunit with *S. aureus* V8 protease was performed according to the procedure of Cleveland et al. (1977) as described by Pedersen et al. (1986). The band containing the α-subunit (25–30 μg/lane), resolved from other proteins on an 8% slab gel and visualized by a brief round of staining and destaining, was excised and soaked for 1 h in overlay buffer (5% sucrose/125 mM Tris-HCl, pH 6.8/0.1% SDS) plus 1 mM DTT. The excised band was then transferred to the well of a mapping gel (15% acrylamide) to which was added 30 μL of overlay buffer containing 3 μg of *S. aureus* V8 protease. Electrophoresis at 120-V constant voltage was carried out until the samples reached the end of the stacking gel. The power was then shut off for 45 min after which time electrophoresis was continued at 250 V until the end of the run.

[^{125}I]TID-labeled proteins and peptides in stained, dried gels were visualized by autoradiography on Kodak XAR-5 film. The film was preflashed as described by Laskey and Mills (1975). The amount of radioactivity incorporated into particular bands was measured by γ-counting of excised bands.

Immunoblotting. Subunits of the purified AChR were resolved on an 8% minigel as described above and electrophoretically transferred to an Immobilon transfer membrane (Millipore). The procedures for transfer and replica processing were essentially those provided by the manufacturer except that the blot buffer contained 10 mM NaP_i, pH 7.0/0.5 M NaCl/0.05% Tween 20. Monoclonal antibodies specific for the AChR subunits were used as primary antibodies on strips of the replica. A 1:1000 dilution of peroxidase-conjugated goat anti-mouse IgG and IgM antisera (Boehringer-Mannheim) was used as the secondary antibody. Labeled bands were visualized by incubating the strips in a solution of 50 mM Tris, pH 7.2, containing 0.01% H_2O_2 and 0.3 mg/mL 3,3′-diaminobenzidine.

Isolation of Proteolytic Fragments of the AChR α-Subunit. Preparative slab gel electrophoresis was used to isolate fragments of the α-subunit produced by *S. aureus* V8 protease. Polypeptides of alkaline-extracted *Torpedo* membranes (~6.5 mg of protein) were resolved on an 8% slab gel, and the AChR α-subunit (~0.6 mg of protein) was excised, soaked in overlay buffer, and placed on top of a 15% mapping gel with 0.2 mL of overlay buffer and 0.35 mL of V8 protease at 0.2 mg/mL. After electrophoresis, the gel was stained and destained, each step taking 1 h, and the bands corresponding to the two higher molecular weight cleavage fragments of the α-subunit (V8-20 and V8-18) were excised and placed in distilled H_2O. The two lower molecular weight cleavage fragments (V8-10 and V8-4) were excised after further destaining (30 min and 5 h, respectively). The excised bands were soaked for 3 h in distilled H_2O with three changes of water and then electroeluted according to the method of Hunkapiller et al. (1983) as described by Pedersen et al. (1986). Excess SDS was removed from the electroeluted peptides by two successive acetone precipitations (4 h at −20 °C) followed by resuspension in digestion buffer (50 mM ammonium acetate, pH 6.5/0.1% SDS/1 mM EDTA/0.001% pepstatin).

Sequencing and Amino Acid Analysis of V8 Protease Cleavage Fragments of the AChR α-Subunit. Amino-terminal sequencing of the four proteolytic fragments of the α-subunit was performed on an Applied Biosystems Model 470A protein sequencer as described by Carr et al. (1987). Carboxyl-terminal sequencing with carboxypeptidase Y (Hayashi, 1977) was accomplished by incubating approximately 20 μg of each peptide with 1 μg of carboxypeptidase Y in 198 μL of digestion buffer. At selected time points a 36-μL aliquot was removed and treated with 4 μL of 10 mM diisopropyl phosphofluoridate to stop the reaction.

Hydrolysates of the four peptide fragments were prepared by the method of Bidlingmeyer et al. (1984). Samples of approximately 1 and 2 μg were dried in 6 × 50 mm Pyrex tubes and placed in a vacuum vial with 200 μL of constant boiling HCl/1% phenol. The vacuum vial was then evacuated, sealed, and incubated at 108 °C for 24 h.

The amino acid composition of hydrolysates and carboxypeptidase digests was determined by the method of Bidlingmeyer et al. (1984). Samples were derivatized with phenyl isothiocyanate [(PITC), Pierce] in 75% methanol/10% H_2O/10% triethylamine, and the phenylthiocarbamyl derivatives of amino acids were separated at 38 °C on an Altex Ultrasphere ODS-PTH column (4.6 × 250 mm). A linear gradient from 90% buffer A (0.15% sodium acetate, pH 6.35/0.05% triethylamine)/10% buffer B (60% acetonitrile/40% H_2O) to 43% buffer A/57% buffer B over 30 min was used to effect the separation.

Results

Initial experiments were designed to characterize the general pattern of photoincorporation of [^{125}I]TID into *Torpedo*

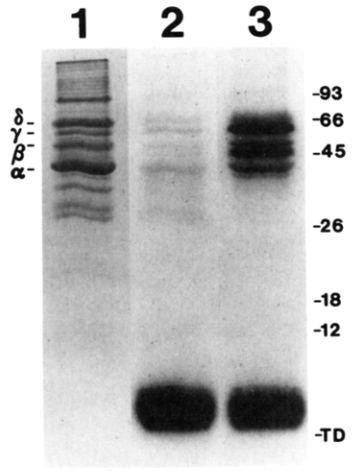

FIGURE 1: Photoincorporation of [^{125}I]TID into AChR-rich membranes in the presence and absence of carbamylcholine. AChR-rich membranes were labeled with [^{125}I]TID in the presence (lanes 1 and 2) or absence (lane 3) of 0.1 mM carbamylcholine. After photolysis, membranes were washed with 1% BSA as described under Experimental Procedures to remove noncovalently bound [^{125}I]TID, and aliquots (containing 20 μg of protein) were subjected to SDS–PAGE on a 12% polyacrylamide gel: (lane 1) Coomassie Blue; (lanes 2 and 3) autoradiography (36-h exposure). The AChR subunits are identified on the left. The α-subunit, unresolved from the nonreceptor 43K protein in this gel system, appears heavily stained. In addition, residual BSA comigrating with the δ-subunit somewhat enhances staining of this band. Migration of the prestained molecular weight markers (Bethesda Research Laboratories) is indicated on the right for phosphorylase *b* (93 000), BSA (66 000), ovalbumin (45 000), α-chymotrypsinogen (26 000), β-lactoglobulin (18 000), and cytochrome *c* (12 000).

membranes and to test the sensitivity of the photoincorporation to cholinergic ligands. Membranes (1 mg of protein/mL) were equilibrated with 1 μM [^{125}I]TID in the presence and absence of 0.1 mM carbamylcholine, an agonist concentration sufficient to produce full receptor occupation and desensitization. After irradiation for 10 min at 365 nm, noncovalently bound radioactivity was removed as described under Experimental Procedures, and the pattern of incorporation was monitored by SDS–PAGE followed by autoradiography. Because most covalently incorporated [^{125}I]TID in other preparations was associated with lipid and electrophoresed with the tracking dye (Brunner & Semenza, 1981; Spiess et al., 1982), the labeled membrane components were resolved on 12% polyacrylamide gels to optimize separation of lipid and protein (Figure 1).

Incorporation of radioactivity into the membranes was dependent upon photolysis and similar in the absence and presence of carbamylcholine, but the distribution of label differed strikingly in the two cases (Table I). Membranes labeled in the absence of agonist showed appreciable incorporation of [^{125}I]TID both into material migrating near the dye front (Figure 1, lane 3) and into polypeptides in the region of the gel containing the AChR subunits. Membranes labeled in the presence of carbamylcholine, however, showed less incorporation into these polypeptides and enhanced labeling of the low molecular weight material (Table I; Figure 1, lane 2). Inclusion during labeling of 50 mM glutathione, an aqueous scavenger, did not alter the differential pattern of protein labeling. In addition, the partitioning of [^{125}I]TID into *Torpedo* membranes did not differ significantly in the absence and presence of agonist. The partition coefficient[2] of the probe

[2] The partition coefficient (P_L) is defined as the amount of [^{125}]TID bound to membranes per milligram of lipid divided by the amount of [^{125}]TID in the aqueous phase per microliter of solution. Lipid content was estimated from the protein concentration by using an average lipid to protein ratio of 0.43 (Schiebler & Hucho, 1978; Popot et al., 1978).

Table I: Incorporation of [^{125}I]TID into *Torpedo* Postsynaptic Membranes[a]

	[^{125}I] radioactivity (10^{-4} cpm)		
	membrane associated	AChR associated[b]	lipid associated[c]
−carbamylcholine	21.2 (25%)[d]	8.2 (37%)[e]	13.1 (58%)[e]
+carbamylcholine	20.2 (24%)[d]	1.2 (6%)[e]	19.9 (89%)[e]
control (no photolysis)	0.2 (0.2%)[d]	ND	ND

[a] *Torpedo* membranes (50 μg of protein) suspended in TPS at 1 mg/mL were incubated with [^{125}I]TID (1 μM, 8.4 × 10^5 cpm) in the absence or presence of 0.1 mM carbamylcholine and then irradiated for 10 min. Irradiated membranes were pelleted and washed as described under Experimental Procedures. Total membrane-associated radioactivity was determined by γ-counting, and the samples were then dissolved in sample loading buffer and electrophoresed on 12% polyacrylamide gels. [b] Total incorporation of ^{125}I into gel slices spanning AChR subunits (see Figure 1). ND indicates none detected. [c] ^{125}I eluting with the tracking dye. [d] Percent total cpm. [e] Percent membrane-associated cpm.

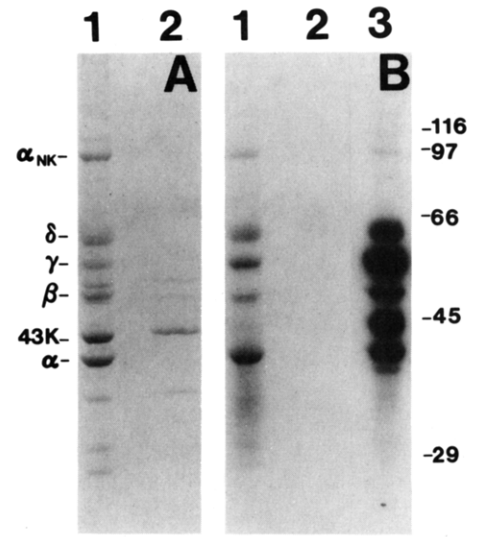

FIGURE 2: Photoincorporation of [^{125}I]TID into peripheral and integral membrane proteins of AChR-rich membranes. AChR-rich membranes were labeled with [^{125}I]TID in the presence (lanes 1 and 2) or absence (lane 3) of 0.1 mM carbamylcholine and the peripheral proteins extracted with 10 mM LIS and 2 mM EDTA as described under Experimental Procedures. Samples containing 15 μg of unextracted membranes (lanes 1 and 3) or the extract from 15 μg of membranes (lane 2) were electrophoresed on an 8% polyacrylamide gel and visualized by Coomassie Blue stain (A) followed by autoradiography (B; 14-day exposure). Labeled lipid and free photolysis products were removed by electrophoresing the tracking dye off the gel. The AChR subunits, the 43K protein, and the Na^+/K^+-ATPase α-subunit (α_{NK}) are indicated on the left. Migration of molecular weight standards is indicated on the right for β-galactosidase (116 000), phosphorylase *b* (97 000), BSA (66 000), ovalbumin (45 000), and carbonic anhydrase (29 000).

at 23 °C, as determined by centrifugation of membrane suspensions containing 0.2–5 mg of protein/mL and constant amounts of [^{125}I]TID, was (1.6 ± 0.5) × 10^5 μL/mg in the absence of carbamylcholine and (1.3 ± 0.3) × 10^5 μL/mg in its presence.

[^{125}I]TID Labeling of Protein in the Presence of Carbamylcholine. To examine the incorporation of [^{125}I]TID into protein in greater detail, polypeptides of labeled AChR-rich membranes were resolved by electrophoresis on 8% SDS–polyacrylamide slab gels (Figure 2, lane 1). Labeled lipid and free photolysis products were removed by electrophoresing the tracking dye off the gels. In addition to the AChR subunits, two other prominent polypeptides are resolved on the 8% gel as visualized by Coomassie Blue stain. One is the AChR associated peripheral protein of M_r 43 000 (43K protein) (Carr

et al., 1987), and the other is the transmembrane α-subunit of the Na^+/K^+-ATPase (M_r ~95000). Positive identification of the latter polypeptide was based upon amino-terminal microsequencing after electroelution of the polypeptide from a preparative SDS–polyacrylamide gel (Sharp, White, and Cohen, unpublished observations). The first 15 cycles corresponded exactly to residues 6–20 of the deduced amino acid sequence of the *T. californica* Na^+/K^+-ATPase α-subunit (Kawakami et al., 1985).

In the presence of 0.1 mM carbamylcholine, label was incorporated primarily into the four AChR subunits (Figure 2B, lane 1). Based upon γ-counting of excised gel bands, the relative incorporation into the AChR subunits was 2:(0.8 ± 0.1):(1.3 ± 0.2):(1.4 ● 0.2) (α:β:γ:δ, n = 8). In addition, the Na^+/K^+-ATPase α-subunit incorporated [^{125}I]TID at a level similar to that of the AChR subunits. The peripheral proteins of the AChR-rich membranes were labeled only slightly or not at all. Peripheral proteins extracted from ~15 μg of [^{125}I]TID-labeled membranes with 10 mM LIS and 2 mM EDTA showed no detectable incorporation of label (Figure 2B, lane 2). [^{125}I]TID incorporation into the 43K protein was, however, detected in concentrated samples of extract (from ~90 μg of labeled membranes) with ~5% as much label incorporated as into the AChR α-subunit.

[^{125}I]TID Labeling of Protein in the Absence of Carbamylcholine. Membranes labeled in the absence of carbamylcholine show a sharply different pattern of protein labeling (Figure 2B, lane 3) from that observed for membranes labeled under identical conditions in the presence of the drug (Figure 2B, lane 1). The α-, β-, and δ-subunits of the AChR each incorporate about 5-fold more label and the γ-subunit incorporates about 15-fold more as estimated by gel slice counting. Preferential labeling of the γ-subunit in the absence of agonist is evident from the stoichiometry of labeling, which was ~2:1:4:1 (α:β:γ:δ). While no difference in labeling of the Na^+/K^+-ATPase α-subunit was observed in the absence of carbamylcholine (Figure 2B, lanes 3 and 1), two bands not obviously labeled in the presence of agonist were labeled heavily in its absence (Figure 2B, lane 3). One had slightly lower mobility (M_r 44000) than the 43K protein, and the second had slightly greater mobility (M_r 39000) than the AChR α-subunit.

The carbamylcholine-sensitive incorporation of [^{125}I]TID into these two bands suggested that they might derive from the AChR. This was confirmed by affinity purification of AChR detergent-extracted from membranes photolabeled in the absence of carbamylcholine (Figure 3). In addition to the label associated with the AChR subunits, there was clearly label associated with two additional bands in the affinity-purified material (Figure 3A, lane 4). These bands, evident by autoradiography, were barely detectable by Coomassie stain (Figure 3A, lane 2). Immunoblotting of the affinity-purified material with monoclonal antibodies specific for each of the AChR subunits demonstrated the presence of β- and γ-subunit immunoreactive material migrating at the positions of the additional labeled bands (Figure 3B, lanes 2 and 3). We therefore concluded that the labeled bands of M_r 39000 and 44000 were degradation products of AChR β- and γ-subunits, respectively.

The affinity purification of labeled AChR allowed an accurate estimate of [^{125}I]TID incorporation under standard conditions of labeling, i.e., 1 mg of protein/mL of membranes, ~1 μM TID, and 10 min photolysis. Under these conditions [^{125}I]TID incorporation into AChR was ~8000 cpm/μg, indicating that on average 20% of the AChR molecules present

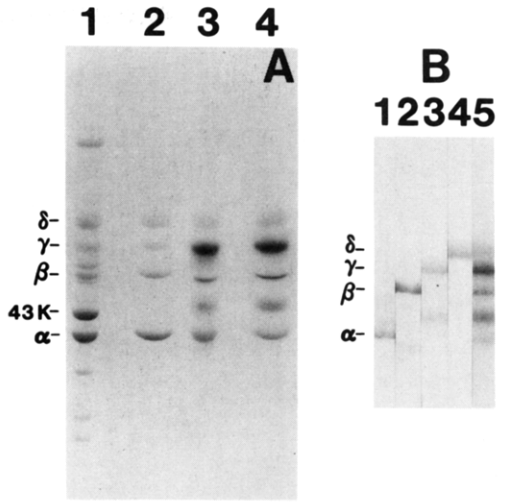

FIGURE 3: Purification by affinity chromatography of AChR labeled with [^{125}I]TID in the absence of carbamylcholine. AChR-rich membranes (2.5 mL) were photolabeled with [^{125}I]TID as described under Experimental Procedures except that labeling was accomplished in three troughs of an Accutran disposable incubation tray (Schleicher and Schuell). Photolabeled membranes, pelleted and resuspended to 1.25 mg of protein/mL in buffer (10 mM NaP_i, pH 8.0, 75 mM NaCl, 85 mM KCl, 1 mM EDTA, 200 μM diisopropyl phosphofluoridate), were solubilized in 0.5% Lubrol-PX for 1 h before pelleting for 30 min in a Beckman airfuge. The supernatant was mixed for 2 h with an equal volume of acetylcholine affinity matrix in buffer supplemented with 0.5% Lubrol-PX and washed as described under Experimental Procedures. Elution of AChR was accomplished with 20 mM carbamylcholine in buffer supplemented with 0.5% Lubrol-PX. (A) Coomassie Blue stain (lanes 1 and 2) and autoradiogram (lanes 3 and 4; 25-h exposure) of labeled starting membranes (15 μg of protein, lanes 1 and 3) and affinity-purified AChR (3.3 μg, lanes 2 and 4) analyzed by SDS–PAGE. (B) Subunits of the affinity-purified AChR, separated by electrophoresis on an 8% polyacrylamide minigel and transferred to an Immobilon transfer membrane, probed with subunit-specific monoclonal antibodies (see Experimental Procedures) to identify the additional [^{125}I]TID labeled bands: (lane 1) anti-α; (lane 2) anti-β; (lane 3) anti-γ; (lane 4) anti-δ. Lane 5 shows the autoradiogram (60-h exposure) of the Immobilon replica.

were labeled. To investigate in greater detail the dependence of [^{125}I]TID incorporation on the labeling conditions, labeling was analyzed as a function of both irradiation time and [^{125}I]TID concentration.

Time Course and Concentration Dependence of [^{125}I]TID Labeling. When the labeling reaction was analyzed as a function of irradiation time in both the presence and absence of carbamylcholine, incorporation into the AChR and Na^+/K^+-ATPase α-subunits followed an exponential time course characterized by a half-time of 6 min (Figure 4A). When membranes were incubated with [^{125}I]TID concentrations varying from 0.05 to 4 μM and then irradiated for 5 min, incorporation of label into the AChR and Na^+/K^+-ATPase α-subunits varied linearly with [^{125}I]TID concentration both in the presence and in the absence of carbamylcholine (Figure 4B).

Effects of Cholinergic Antagonists on [^{125}I]TID Labeling of AChR. Two competitive antagonists, *d*-tubocurarine and α-bungarotoxin (α-BgTx), and two noncompetitive antagonists, phencyclidine and decahydrohistrionicotoxin (H_{10}-HTX), were tested for their effects on [^{125}I]TID labeling of AChR-rich membranes. The effects of these ligands were first examined by autoradiography (Figure 5) at ligand concentrations chosen to produce full occupancy of their binding sites on the AChR. In contrast to carbamylcholine neither α-BgTx (10 μM) nor *d*-tubocurarine (100 μM) significantly inhibited AChR labeling by [^{125}I]TID (lanes 2 and 5). However, the presence of α-BgTx did block the effect of carbamylcholine (lane 4 vs

Table II: Effect of Cholinergic Ligands on Photoincorporation of [^{125}I]TID into AChR Subunits[a]

	% inhibition				
	carbamylcholine (100 μM), n = 8	d-tubocurarine (100 μM), n = 6	α-bungarotoxin (10 μM), n = 4	histrionicotoxin (30 μM), n = 6	phencyclidine (50 μM), n = 7
α	74 ± 6	32 ± 9	16 ± 11	66 ± 5	25 ± 8
β	84 ± 4	38 ± 11	27 ± 6	73 ± 4	23 ± 15
γ	93 ± 2	34 ± 11	13 ± 17	82 ± 3	29 ± 9
δ	72 ± 4	35 ± 11	18 ± 11	72 ± 4	−9 ± 14

[a] Membrane suspension (1 mg/mL) equilibrated with 1–2 μM [^{125}I]TID were irradiated in the presence of cholinergic ligands at the indicated concentrations and subjected to SDS–PAGE on 8% polyacrylamide gels. Excised AChR subunits were counted for incorporation of ^{125}I, and the percent incorporation in the presence of ligand (with respect to the incorporation in its absence) was calculated. The percent inhibition was calculated by subtracting this number from 100. Average percentages for n experiments are given with standard deviations.

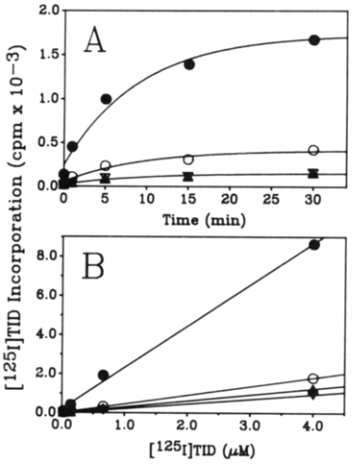

FIGURE 4: Time course and concentration dependence of [^{125}I]TID incorporation into membranes in the presence and absence of carbamylcholine. Membrane suspensions (1 mg of protein/mL) equilibrated with [^{125}I]TID were irradiated at 365 nm in the presence and absence of 0.1 mM carbamylcholine and subjected to SDS–PAGE. The bands corresponding to the α-subunits of the AChR (●, ○) and the Na^+/K^+-ATPase (▲, ▽) were excised and counted for [^{125}I]. Filled and open symbols indicate the absence and presence of carbamylcholine during labeling, respectively. (A) Time course of [^{125}I]TID incorporation. Membrane suspensions with 2 μM [^{125}I]TID were irradiated for the times indicated. The time course were fit to functions of the form $\text{cpm}(t) = A(1 - e^{-t/6}) + B$. (B) Concentration dependence of [^{125}I]TID incorporation. Stock [^{125}I]TID was serially diluted (1:5) into 75% ethanol, and aliquots were further diluted 1:100 into membrane suspensions before irradiation for 5 min. The final [^{125}I]TID concentrations were calculated on the basis of radiochemical specific activity.

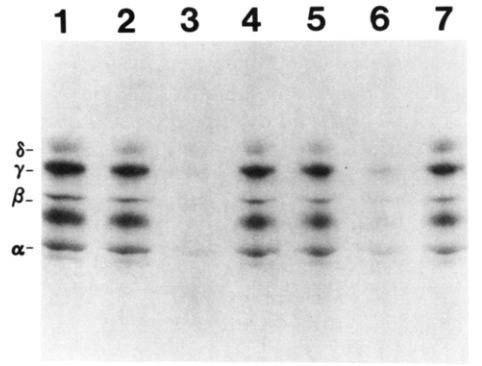

FIGURE 5: Effects of cholinergic antagonists on [^{125}I]TID incorporation into AChR-rich membranes. Suspensions of AChR-rich membranes (1 mg of protein/mL) and [^{125}I]TID (2 μM) were equilibrated for 45 min with the following concentrations of cholinergic ligands: (lane 1) no ligand; (lane 2) 10 μM α-BgTx; (lane 3) 100 μM carbamylcholine; (lane 4) 100 μM carbamylcholine and 10 μM α-BgTx; (lane 5) 100 μM d-tubocurarine; (lane 6) 30 μM H_{10}-HTX; (lane 7) 50 μM phencyclidine. The sample with both α-BgTx and carbamylcholine was equilibrated with α-BgTx alone for 30 min and then further incubated for 15 min after addition of agonist. After equilibration, samples were irradiated for 5 min and then subjected to SDS–PAGE on an 8% polyacrylamide gel. An autoradiogram of the gel exposed for 2 days is shown.

lane 3), indicating that the agonist acts specifically in inhibiting [^{125}I]TID labeling but that its action is not simply to block labeling of the agonist binding site by [^{125}I]TID. H_{10}-HTX (30 μM) profoundly inhibited labeling of AChR subunits by [^{125}I]TID (lane 6), while phencyclidine at 50 μM did not (lane 7). Table II summarizes the extent of inhibition of AChR subunit labeling as determined by γ-counting of subunits excised from stained polyacrylamide gels.

The dependence of [^{125}I]TID labeling on the concentration of cholinergic ligands is shown in Figure 6 for the AChR γ-subunit. The concentrations of carbamylcholine and HTX that produced half-maximal inhibition (IC_{50}) were ~1 μM in both cases. For phencyclidine, which reduced incorporation by only 30%, the IC_{50} was 7 μM. Dissociation constants (K_D) determined from the inhibition data of Figure 6 (see figure legend) were 0.1 μM for carbamylcholine, 1 μM for H_{10}-HTX, and 7 μM for phencyclidine, values that agree well with the directly measured K_Ds: 0.1 μM for carbamylcholine (Boyd & Cohen, 1980), 0.3 μM for HTX (Heidmann et al., 1983), and 6 μM for phencyclidine (Oswald et al., 1983). The 35% inhibition of [^{125}I]TID labeling by d-tubocurarine appeared in part nonspecific, as judged by its insensitivity to the presence of α-BgTx. More work would be required to quantify the dose dependence of the α-BgTx-sensitive component of the data.

A variety of pharmacological data indicate that phencyclidine competes with HTX for a single high-affinity site on the AChR (Heidmann et al., 1983). Because these two noncompetitive antagonists had markedly different effects on [^{125}I]TID labeling, labeling was also carried out for membranes equilibrated with both HTX and phencyclidine (Figure 7). For each AChR subunit a low concentration of H_{10}-HTX (3 μM) reduced [^{125}I]TID incorporation by ~60%, and that effect was inhibited by the presence of 50 μM phencyclidine.

Mapping of the [^{125}I]TID-Labeled Sites on the AChR α-Subunit by V8 Protease. Digestion of the AChR α-subunit with *S. aureus* V8 protease has been shown to produce four bands that run on a 15% SDS–polyacrylamide gel with apparent molecular weights of 20 000 (V8-20), 18 000 (V8-18), 10 000 (V8-10), and unspecified low molecular weight (Pedersen et al., 1986). Fragment V8-20 was shown to contain the binding sites of agonists and antagonists (both competitive and noncompetitive) in a peptide with amino-terminal sequence beginning at residue Ser-173, while fragment V8-18 was shown to contain the N-linked carbohydrate of the α-subunit in a peptide beginning at Val-46. We used digestion with V8 protease to map the sites of [^{125}I]TID labeling on the α-subunit. Identical sets of membranes were labeled in the presence and absence of 100 μM carbamylcholine. Half of each set was then treated with endoglycosidase H, a glycosidase known to release high mannose and hybrid-type Asn-linked oligosaccharides from glycoproteins (Varki & Kornfeld, 1983), as

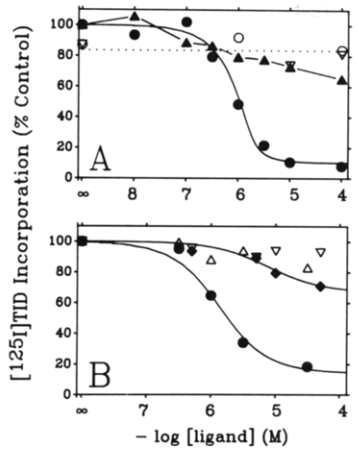

FIGURE 6: Inhibition of [^{125}I]TID labeling by cholinergic ligands. Membrane suspensions (2 mg of protein/mL) equilibrated with 2–4 μM [^{125}I]TID (and 25 μM α-BgTx where indicated) were diluted 1:1 into appropriate concentrations of cholinergic ligands and reequilibrated (30 min for noncompetitive antagonists, 5 min for other ligands) before irradiation for 5 min. Samples were subjected to SDS–PAGE, and the bands corresponding to the γ-subunit and Na^+/K^+-ATPase α-subunit were excised and counted for [^{125}I]. The counts per minute (cpm) incorporated at any given ligand concentration were normalized by dividing by the cpm incorporated in the absence of ligand. (A) Effects on γ-subunit labeling of carbamylcholine (●, ○) and *d*-tubocurarine (▲, ▽). Filled and open symbols indicate labeling in the absence and presence of α-BgTx, respectively. The average [^{125}I]TID incorporation in the presence of α-BgTx (in the presence and absence of other ligands) is indicated by the dotted line. (B) Effects on γ-subunit and Na^+/K^+-ATPase α-subunit labeling of the AChR noncompetitive antagonists H_{10}-HTX (●, ▲) and phencyclidine (◆, ▽). Filled and open symbols indicate labeling of the γ-subunit and Na^+/K^+-ATPase α-subunit, respectively. Carbamylcholine, HTX, and phencyclidine concentration curves for γ-subunit labeling were fit to functions of the form % control = $100 - 50A[(1 + D_0/R_0 + K_D/R_0) - [(1 + D_0/R_0 + K_D/R_0)^2 - 4D_0/R_0]^{1/2}]$, where R_0 is the concentration of binding sites (1.7 μM for carbamylcholine and 0.85 μM for HTX and phencyclidine in these experiments), D_0 is the (varied) total ligand concentration, and A is the inhibitable fraction of the total incorporation. Optimal fits were produced by nonlinear least-squares analysis allowing the parameters A and K_D (the dissociation constant) to vary.

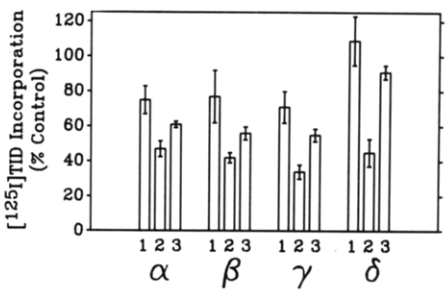

FIGURE 7: Phencyclidine block of H_{10}-HTX-mediated inhibition of AChR labeling by [^{125}I]TID. Membrane suspensions containing 1 μM [^{125}I]TID were irradiated for 5 min after equilibration (40 min) with (bar 1) 50 μM phencyclidine, (bar 2) 3 μM H_{10}-HTX, or (bar 3) 50 μM phencyclidine and 3 μM H_{10}-HTX. The cpm incorporated into each AChR subunit under these conditions were measured from excised gel slices and normalized by dividing by the cpm incorporated in the absence of any ligand. (The error bars indicate the values obtained in two experiments).

described under Experimental Procedures. The other half was treated identically except that no glycosidase was added. AChR α-subunits were then isolated and subjected to cleavage with V8 protease (Figure 8). The four principal cleavage products were evident in the Coomassie Blue stained gel (Figure 8A). We have assigned the name V8-4 to the poorly resolved band with nominal molecular weight of 4000. As previously noted by Pedersen et al. (1986), treatment of

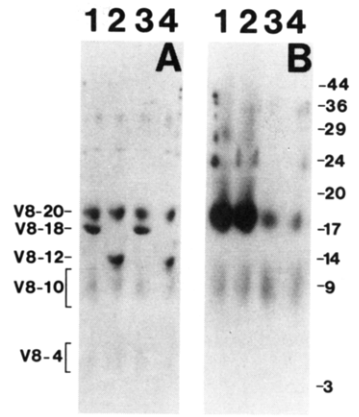

FIGURE 8: Proteolytic mapping of the sites of [^{125}I]TID incorporation on the AChR α-subunit using *S. aureus* V8 protease. AChR-rich membranes were labeled with [^{125}I]TID in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 0.1 mM carbamylcholine and split into 250-μg suspensions for incubation with (lanes 2 and 4) or without (lanes 1 and 3) endoglycosidase H as described under Experimental Procedures. After incubation, membrane samples were subjected to SDS–PAGE on an 8% slab gel and the α-subunit bands excised following identification by brief staining with Coomassie Blue. The excised bands were transferred to the wells of a 15% mapping gel and overlaid with 3 μg of *S. aureus* V8 protease. Following electrophoresis, the mapping gel was stained with Coomassie Blue (A) and subjected to autoradiography for four days (B). The principal proteolytic fragments of the α-subunit are identified on the left according to the nomenclature of Pedersen et al. (1986). In addition, we have assigned the name V8-4 to the poorly resolved band of nominal molecular weight 4000. The molecular weight standards are ovalbumin (44 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), soybean trypsin inhibitor (20 000), α-lactalbumin (14 000), myoglobin (17 000), and myoglobin cyanogen bromide fragments (Sigma) of molecular weight 9000 and 3000.

membranes with endoglycosidase H shifted the electrophoretic mobility of V8-18 to approximately 12 kDa (Figure 8A, lanes 2 and 4) while leaving the mobilities of the other fragments unchanged.

Inspection of the autoradiogram of the V8 protease digests (Figure 8B) revealed that the site of [^{125}I]TID labeling sensitive to carbamylcholine migrated with V8-20 (lanes 1 and 3) and was not sensitive to endoglycosidase H (lanes 2 and 4). That lack of sensitivity confirmed that the site was contained in V8-20 and not in V8-18. Not all of the incorporation of [^{125}I]TID into V8-20 was abolished by 100 μM carbamylcholine (Figure 8B, lanes 3 and 4). Direct counting of the excised gel bands indicated that in the presence of carbamylcholine the labeling of V8-20 was reduced to approximately 15% of its value in the absence of agonist. Incorporation of [^{125}I]TID into V8-10 was the same in the presence and absence of carbamylcholine, and in the presence of agonist this fragment contained over 50% of the total label incorporated into the α-subunit fragments. Neither V8-18 nor V8-4 incorporated a significant amount of label.

Amino-Terminal Sequencing of the α-Subunit Proteolytic Fragments. To identify the positions of the V8-10 and V8-4 fragments in the primary structure of the α-subunit and to confirm the identities of V8-20 and V8-18, these (unlabeled) peptides were isolated as described under Experimental Procedures and subjected to N-terminal Edman degradation. Table III displays the first five cycles of degradation for each peptide together with the yield (in picomoles) of each recovered residue. The major sequences of all four peptides were present in at least 4-fold excess of any minor sequence.

The major sequence of V8-10 began unambiguously at residue Asn-339. Also present was a minor sequence beginning

Table III: Amino-Terminal Sequence Analysis of the AChR α-Subunit Fragments Produced by *S. aureus* V8 Protease Digestion[a]

	V8-4		V8-18		V8-20		V8-10	
cycle	residue	pmol	residue	pmol	residue	pmol	residue	pmol
1	S/ND	103/ND	T/V/S	169/40/36	S/S/T	156/-/NI	N/K	133/NI
2	E/ND	152/ND	N/N/G	194/-/13	G/D/N	84/NI/10	K/Q	NI/26
3	H/I	NI/6	V/Q/E	184/39/22	E/R/V	103/NI/17	I/E	116/10
4	E/F	84/11	R/I/W	NI/36/NI	W/P/R	NI/36/NI	F/N	124/8
5	T/A	71/14	L/V/V	198/22/-	V/D/L	93/10/29	A/K	115/NI
starting residue	S-1/N-339		T-52/V-46/S-173		S-173/S-162/T-52		N-339/K-336	

[a] The four proteolytic fragments (V8-20, V8-18, V8-10, and V8-4) were isolated by preparative slab gel electrophoresis as described under Experimental Procedures. Protein concentrations of the resuspended peptides were estimated by the method of Schaffner and Weissman (1973) using BSA as a standard. Approximately 350 pmol of each sample were subjected to automated Edman degradation. The PTH amino acids released at each cycle of degradation are indicated by the conventional one-letter code along with the corresponding yields (in picomoles). The starting residue of each major and minor sequence and its position in the complete α-subunit sequence are indicated. ND indicates that the residue was not detected, NI, that it was not integrated.

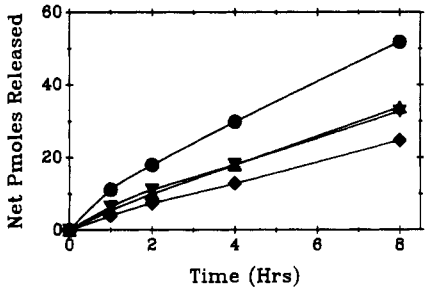

FIGURE 9: Carboxyl-terminal digestion of V8-18 with carboxypeptidase Y. The peptide V8-18, generated by *S. aureus* V8 protease cleavage of the AChR α-subunit, was isolated and subjected to carboxyl-terminal digestion by carboxypeptidase Y as described under Experimental Procedures. Time aliquots representing 120 pmol of peptide were removed from a stock digestion mixture and treated with diisopropyl phosphofluoridate (1 mM final concentration) to terminate digestion. Free amino acids in the aliquots were identified by derivatization with phenyl isothiocyanate (see Experimental Procedures). The net number of picomoles of each residue released represents the number of picomoles detected at the times indicated minus the picomoles present in a 0-h digest control. Four residues were released at elevated levels: serine (●), glutamate (▲), isoleucine (▼), and proline (◆). All other residues showed release of less than 9 pmol.

at residue Lys-336. V8-4, with its major sequence beginning at Ser-1, clearly contained the amino terminus of the α-subunit. A minor contaminating sequence (present at roughly $^1/_{10}$ the level of the major sequence) began at Asn-339 and presumably resulted from further degradation of V8-10. The results for V8-20 and V8-18 conform reasonably well with those reported by Pedersen et al. (1986). The major sequence of V8-20 began at Ser-173, and minor sequences began at Ser-162 and Thr-52. The major sequence of V8-18 began at Thr-52, a result differing slightly from that previously reported (Val-46). The sequence beginning at Val-46 was present in V8-18 as a minor component, as was the major sequence of V8-20.

Characterization of the Carboxyl Termini of V8-18 and V8-10. The isolated fragments of the α-subunit generated by digestion with V8 protease were subjected to carboxyl-terminal digestion with carboxypeptidase Y. V8-4 and V8-20 proved resistant to digestion with this enzyme under a variety of conditions (data not shown), but both V8-18 and V8-10 showed significant release of amino acids. The reactions were carried out as described under Experimental Procedures.

The time course of release of amino acids for V8-18 is shown in Figure 9. Serine, glutamate, isoleucine, and proline were the only residues released in significant amounts, with about twice as much Ser released at each time point as the other three residues. Examination of the potential V8 cleavage sites on the α-subunit revealed that the only sequence compatible with this profile of amino acid release extends from Ser-157 to Glu-161 (-Ser-Ile-Ser-Pro-Glu). Further cleavage of the peptide by carboxypeptidase Y was evidently inhibited by the appearance of lysine (Lys-155) in the penultimate position (Nelson-Jones, 1986). The assignment of the carboxyl terminus of V8-18 to Glu-161 is consistent with (a) the presence of the glycosylated residue Asn-141 within this fragment, (b) the location of the two amino termini of V8-20 (Ser-162 and Ser-173), and (c) the amino acid composition of V8-18 (Table IV).

Table IV: Amino Acid Composition of the AChR α-Subunit Fragments V8-18 and V8-10 and Digestion of V8-10 with Carboxypeptidase Y

	amino acid composition[a]		carboxypeptidase Y digest of V8-10[b] (pmol released)
	V8-18 (mol %)	V8-10 (mol %)	
Asp	12.1 (14.6)	10.9 (10.4)	6
Glu	7.1 (5.8)	12.5 (12.5)	48
Asn			7
Ser	5.9 (3.9)	6.6 (8.3)	29
Gln			16
Gly	8.2 (5.8)	8.1 (6.2)	47
His	2.1 (1.9)	1.9 (2.1)	1
Arg	4.7 (4.8)	3.3 (1.0)	14
Thr	6.8 (7.8)	3.9 (3.1)	14
Ala	3.8 (3.9)	9.1 (7.3)	26
Pro	6.9 (6.8)	3.8 (2.1)	8
Tyr	4.1 (4.8)	2.4 (2.1)	10
Val	6.2 (6.8)	8.3 (9.4)	29
Met	2.7 (2.9)	2.0 (3.1)	8
Ile	8.3 (9.7)	9.4 (14.6)	39
Leu	10.3 (9.7)	7.1 (6.2)	45
Phe	4.4 (3.9)	3.7 (4.2)	19
Lys	6.3 (6.8)	7.0 (7.3)	3

[a] Proteolytic fragments of the AChR α-subunit were generated by digestion with *S. aureus* V8 protease and isolated as described under Experimental Procedures. One- and two-microgram samples of each fragment were acid hydrolyzed at 108 °C for 24 h, and free amino acids were derivatized with phenyl isothiocyanate for quantitation (see Experimental Procedures). The average mole percents for each residue (designated according to the conventional three-letter code) are given. In parentheses are the theoretical mole percents of peptides spanning α-subunit residues 52–161 (V8-18) and 339–437 (V8-10). Calculation of the latter values neglected tryptophan and cysteine residues (which are destroyed under the conditions used in these experiments). Asparagine and glutamine residues were treated as aspartate and glutamate residues, respectively. [b] Approximately 200 pmol of fragment V8-10 were digested for 4 h with carboxypeptidase Y as described under Experimental Procedures. The number of picomoles released represents the difference between the number of picomoles detected after 4 h of digestion and the number of picomoles present in a 0-h digest control.

Digestion of V8-10 with carboxypeptidase Y resulted in a more complicated pattern of released amino acids. The net number of picomoles of each amino acid released after 4 h of digestion is displayed in Table IV. The predominant release

of glycine and glutamate is consistent with extension of V8-10 to the end of the α-subunit, which terminates at Gly-437 with the sequence Gly-Arg-Leu-Ile-Glu-Leu-Ser-Gln-Glu-Gly. The high release of both Leu and Ile suggests that a peptide terminating at Glu-432 is also present. A primary interest was to determine whether the putative transmembrane region M4 was contained in V8-10. Presence of this region, which extends from Ile-409 to Ala-427, might account for the carbamylcholine-insensitive incorporation of [^{125}I]TID into V8-10. The abundance of leucine among the released amino acids (Table IV) strongly suggests that V8-10 does contain M4. Of the six leucine residues lying between the amino terminus of V8-10 (Asn-339) and the carboxyl terminus of the α-subunit, five are located in or after M4 (Leu-410, -411, -416, -430, and -433). Similarly, the release of arginine in the carboxypeptidase digestion indicates that V8-10 contains M4 since the only possible source of arginine between Asn-339 and Gly-437 is Arg-429 on the carboxyl side of M4. Acid hydrolysis of V8-10 (Table IV) confirmed the presence of both leucine and arginine, and the composition (Table IV) agreed reasonably well with the calculated composition of the sequence from residue 339 to 437 (Table IV). The estimate for arginine was somewhat high, but the generally close agreement for leucine and most other residues supports the conclusion that V8-10 extends through M4. The unexpectedly low levels of isoleucine in the compositional analysis are probably due in part to deficient cleavage of bonds between isoleucine and other long- or branched-chain aliphatic residues (Glazer et al., 1975).

Discussion

The studies presented here provide an initial characterization of AChR labeling by the small, lipophilic photoreagent [^{125}I]TID. This probe partitions efficiently into *Torpedo* AChR-rich membranes ($P_L = 1.3 \times 10^5$ μL/mg), with 99% associated with membrane at the concentration used for labeling (1 mg of protein/mL). Upon photolysis it reacts with both lipid and the principal transmembrane polypeptides of the *Torpedo* membranes: the four AChR subunits and the α-subunit of the Na^+/K^+-ATPase. Peripheral membrane proteins are labeled to a much smaller extent, if at all, and the pattern of protein labeling is unaltered by 50 mM glutathione, an aqueous scavenger.

Surprisingly, the degree of incorporation into the AChR subunits is profoundly sensitive to certain cholinergic ligands. The agonist carbamylcholine and the noncompetitive antagonist H_{10}-HTX both specifically inhibit [^{125}I]TID labeling of the AChR by roughly 75%. The concentrations of these ligands that produce half-maximal inhibition correlate well with their known equilibrium binding constants, and the inhibitory effects of each are antagonized by competing ligands: α-BgTx, a competitive antagonist, and phencyclidine, a noncompetitive antagonist, block inhibition by carbamylcholine and H_{10}-HTX, respectively. Neither α-BgTx nor phencyclidine has marked inhibitory effects on its own.

It is unlikely that the ligand-sensitive component of AChR labeling results from [^{125}I]TID in the aqueous phase. The probe's high partition coefficient, the insensitivity of AChR labeling to glutathione, and the apparent balance between AChR and lipid labeling (such that a reduction in one is accompanied by an enhancement of the other) speak against this possibility. This component of labeling must thus result either from (1) free [^{125}I]TID in the lipid phase or (2) [^{125}I]-TID bound to the AChR itself (perhaps at a lipid-accessible site). It would arise in the first case from ligand-dependent changes in the number or reactivity of AChR regions exposed to lipid and in the second from ligand-dependent changes in the structure (and presumably affinity) of the [^{125}I]TID binding site.

The first possibility is rendered unlikely by the apparent failure of hydrophobic probes other than [^{125}I]TID to label the AChR in an agonist-dependent fashion. 5-[^{125}I]Iodonaphthyl 1-azide (Tarrab-Hazdai et al., 1980), [^{3}H]-adamantanediazirine (Middlemas & Raftery, 1983), two photoreactive arylazido phospholipids (Giraudat et al., 1985), and a cholesterol photoaffinity probe (Middlemas & Raftery, 1987) have all been reported to label the AChR in a carbamylcholine-insensitive manner. The sensitivity of these probes to highly reactive regions of protein should exceed that of [^{125}I]TID, which exhibits only slight selectivity toward different types of amino acid residue (Hoppe et al., 1984).

It thus seems likely that the ligand-sensitive component of labeling conforms to the second possibility mentioned above and represents labeling of an [^{125}I]TID binding site (or sites) on the AChR whose affinity for [^{125}I]TID is modulated by the binding of other ligands. Additional studies requiring nonradioactive TID will be necessary to demonstrate the presence of such a site. [^{125}I]TID has been reported to affinity label hydrophobic pockets of several calcium-binding proteins (Buerkler & Krebs, 1985; Krieg et al., 1987; Van Ceunebroeck et al., 1986) and the substrate-binding site of rat hepatic cytochrome P-450 form PB-4 (Frey et al., 1986). If [^{125}I]TID binds specifically to the AChR, its binding site(s) must be distinct from either of the previously characterized binding sites, since ligands exist for both sites (α-BgTx and *d*-tubocurarine for the agonist site and phencyclidine for the noncompetitive antagonist site) that only modestly inhibit labeling. Any [^{125}I]TID binding site must, however, be coupled to both of these other sites.

While neither α-BgTx nor *d*-tubocurarine significantly inhibited the photoincorporation of [^{125}I]TID into AChR, agonists were uniformly found to do so. Acetylcholine, suberyldicholine, and phenyltrimethylammonium each inhibited [^{125}I]TID incorporation to the same extent as carbamylcholine (data not shown). The lack of inhibition by competitive antagonists in contrast to agonists might reflect the fact that agonists but not antagonists bind with high selectivity to the desensitized state of the AChR so that receptors occupied by agonists are fully desensitized [reviewed in Taylor (1983)]. However, diminished incorporation of [^{125}I]TID into desensitized AChRs cannot account for the inhibition produced by HTX, a noncompetitive antagonist that produces less receptor desensitization than phencyclidine (Heidmann et al., 1983) or *d*-tubocurarine (Boyd & Cohen, 1984; Cohen & Strnad, 1987).

One of our principal interests in labeling the AChR with [^{125}I]TID was to localize regions of the AChR subunits exposed to the lipid bilayer. As an initial approach we mapped [^{125}I]TID incorporation into the AChR α-subunit by digestion with *S. aureus* V8 protease. Two of the four fragments generated by this procedure were found to contain sites of [^{125}I]TID labeling: V8-20, which begins at Ser-173, and V8-10, which extends from Asn-339 through the hydrophobic region M4. Labeling of V8-20 is strongly inhibited (~85%) by 100 μM carbamylcholine, but not completely abolished. Fragments V8-18, which spans residues α52–161, and V8-4, which begins at Ser-1, fail to incorporate a detectable amount of label. This pattern of incorporation is consistent with the distribution of the hydrophobic regions proposed to span the membrane (Claudio et al., 1983; Devillers-Thiery et al., 1983; Noda et al., 1983b): V8-10 includes the hydrophobic region M4, and V8-20 is sufficiently long to contain the regions M1–3

that span the sequence α210–298.

As suggested earlier, the ligand-dependent incorporation of [^{125}I]TID into V8-20 is likely to arise because regions of this fragment contribute to an [^{125}I]TID binding site whose affinity for (and hence labeling by) [^{125}I]TID is reduced by the binding of agonists and some noncompetitive antagonists. Coupling of such an [^{125}I]TID binding site to both the agonist and noncompetitive antagonist sites would be consistent with the localization of all three sites to the V8-20 fragment. It also seems likely, however, that at least some of the V8-20 labeling—in particular, the carbamylcholine-insensitive component—represents [^{125}I]TID incorporation into lipid-exposed regions. Two observations support the hypothesis that [^{125}I]TID labeling of AChR subunits in the presence of carbamylcholine represents labeling of this sort: (1) the relative incorporation of label corresponds roughly to the subunit stoichiometry of 2:1:1:1 (α:β:γ:δ) and does not exhibit the preferential labeling of the γ-subunit seen in the absence of agonist, and (2) in the presence of carbamylcholine the labeling of each AChR subunit is similar to that of the Na^+/K^+-ATPase α-subunit which appears (by Coomassie Blue staining) to be present in roughly equal amounts. This latter protein is thought to have at least six transmembrane regions (Kawakami et al., 1985). Both observations indicate that AChR labeling in the presence of carbamylcholine is proportional to mass and, by implication, to the surface area exposed to lipid.

The labeling of V8-10, which is unchanged by 100 μM carbamylcholine and accounts for nearly three-fifths of the total label incorporated into the α-subunit in the presence of agonist, is thus likely to represent labeling of regions exposed to lipid. The distribution of label within V8-10 has not yet been determined, but incorporation into the hydrophobic region M4 seems probable. Evidence from labeling with photoreactive phospholipids (Giraudat et al., 1985) and substitution of alternative hydrophobic domains (Tobimatsu et al., 1987) also supports the conclusion that M4 is in contact with lipid. It is significant that in the presence of carbamylcholine V8-10 incorporates more [^{125}I]TID than V8-20, which contains the hydrophobic sequences M1–M3. If labeling of V8-10 is restricted to M4, our results indicate that this region of the α-subunit is in greatest contact with lipid. This conclusion is consistent with the observation that the amino acid sequence of M4 is less conserved across *Torpedo* AChR subunits than those of the other hydrophobic regions M1–M3 [see Popot and Changeux (1984)]. The lack of conservation suggests that each M4 segment is more likely to interact with solvent (i.e., lipid) than with other regions of protein. Further experiments are necessary to determine the distribution of [^{125}I]TID within M1–M3 to identify which of these regions may also contact lipid.

Immunological studies by Ratnam et al. (1986) indicate that epitopes on both sides of M4, including most of the proposed amphipathic transmembrane helix, M5 (α364-399; Finer-Moore & Stroud, 1984; Guy, 1984) are cytoplasmically exposed. The authors infer a cytoplasmic location of M4 and propose a topological model that places the entire carboxyl-terminal third of the α-subunit on the cytoplasmic side of the membrane. The incorporation of [^{125}I]TID into V8-10 fails to support this model.

The failure of fragments V8-18 and V8-4 to incorporate [^{125}I]TID places further constraints on topological models of the AChR subunits. Together these fragments represent roughly the first third of the α-subunit, and their failure to incorporate label makes it extremely unlikely that this region is exposed to the lipid bilayer. While most proposed models place the amino terminus of the α-subunit (through α210) on the extracellular side of the membrane [for review see Guy and Hucho (1987)], Criado et al. (1985) have suggested the existence of membrane-spanning regions between residues α141–152 and α159–192 on the basis of epitope mapping with monoclonal antibodies directed against a synthetic peptide corresponding to the sequence α152–167. The first of these proposed transmembrane regions lies within V8-18, which terminates at Glu-161, and the failure of V8-18 to incorporate [^{125}I]TID suggests that if residues α141–152 do cross the membrane, they are not in direct contact with lipid.

In conclusion, our results demonstrate that [^{125}I]TID can be used to identify regions of the AChR in contact with lipid. More detailed mapping of the regions incorporating label should yield important structural information about the transmembrane topology of the AChR subunits and their degree of exposure to the lipid bilayer. In addition, [^{125}I]TID should allow identification of a novel conformationally sensitive domain shared by, or common to, all AChR subunits. This domain appears to represent a specific binding site for [^{125}I]-TID that is allosterically linked to both the agonist and noncompetitive antagonist binding sites.

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Registry No. α-BgTx, 11032-79-4; H_{10}-HTX, 81521-85-9; 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine, 81340-56-9; carbamylcholine, 462-58-8; phencyclidine, 77-10-1; *d*-tubocurarine, 57-95-4.

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