Probing the Structure of the Nicotinic Acetylcholine Receptor with the Hydrophobic Photoreactive Probes \([^{125}\text{I}]\text{TID-BE}\) and \([^{125}\text{I}]\text{TIDPC/16}\)†

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ABSTRACT: The hydrophobic photoreactive compound 3-trifluoromethyl-3-(m-[\(^{125}\text{I}\)]iodophenyl) diazirine (\([^{125}\text{I}]\text{TID}\)) has revealed important structural information about the pore of the ion channel and lipid–protein interface of the nicotinic acetylcholine receptor (AChR). To further characterize the structure of the AChR, we have mapped the sites of photoincorporation of a benzoic acid ester analogue of TID (\([^{125}\text{I}]\text{TID-BE}\)) and a phospholipid analogue (\([^{125}\text{I}]\text{TIDPC/16}\)). For each photoreactive probe, labeled sites were identified by amino-terminal sequencing of purified tryptic fragments of individual receptor subunits. \([^{125}\text{I}]\text{TID-BE}\) reacted with \(\alpha\text{Cys-412, } \alpha\text{Met-415, and } \alpha\text{Cys-418}\) in the M4 segment of the \(\alpha\)-subunit and \(\gamma\text{Cys-451 and } \gamma\text{Ser-460}\) in \(\gamma\text{M4}\). In the M1 segment of the \(\alpha\)- and \(\beta\)-subunits, \([^{125}\text{I}]\text{TID-BE}\) labeled \(\alpha\text{Phe-227, } \alpha\text{Leu-228, and } \beta\text{Leu-234, } \beta\text{Ala-235}\), respectively. The labeling pattern in the M1 and M4 segments indicate that TID and TID-BE interact with the AChR lipid–protein interface in a similar fashion, revealing the same lipid-exposed face of each transmembrane segment. In contrast to TID, there was, however, no detectable incorporation of \([^{125}\text{I}]\text{TID-BE}\) into the channel lining \(\beta\text{M2}\) segment when the AChR was labeled in the resting state conformation. In the presence of agonist (desensitized state), \([^{125}\text{I}]\text{TID-BE}\) reacted with \(\beta\text{Leu-257, } \beta\text{Val-261, and } \beta\text{Leu-264}\) in \(\beta\text{M2}\); a labeling pattern which indicates that, in comparison to TID, the binding loci for TID-BE is located closer to the extracellular end of the channel. For \([^{125}\text{I}]\text{TIDPC/16}\), receptor labeling was insensitive to the presence of agonist and the sites of incorporation mapped to the confines of the transmembrane segments \(\alpha\text{M4, } \alpha\text{M1, and } \gamma\text{M4}\), validating previous results found with small lipophilic probes.

The nicotinic acetylcholine receptor (AChR)\(^1\) from the electric organ of Torpedo californica is the best characterized member of a superfamily of ligand-gated ion channels which includes the GABA\(_\alpha\), glycine, and 5-HT\(_3\) (serotonin) receptors (for recent reviews, see refs 1–4). The Torpedo AChR is an integral membrane glycoprotein composed of four homologous subunits in a stoichiometry of \(\alpha\beta\beta\alpha\) pseudo-symmetrically arranged about a central axis which creates the pore of the cation conducting channel. Each receptor subunit shares identical topologies (5, 1). The N-terminal half of each subunit is extracellular and contains the binding sites for agonist which are located at the interfaces of the \(\alpha\)–\(\gamma\) and \(\alpha\)–\(\delta\) subunits. The C-terminal half of each subunit consists of three hydrophobic membrane-spanning segments (M1–M3), a large cytoplasmic domain, a fourth transmembrane segment (M4), and a short C-terminal extracellular tail.

In the absence of a truly high-resolution three-dimensional structure of the AChR (6), investigators have had to rely on a host of different biochemical as well as biophysical approaches to obtain structural information about the receptor. Photoaffinity labeling studies with both AChR agonists and competitive antagonists have been instrumental in identifying amino acid residues involved in forming the agonist-binding site (reviewed in refs 7 and 8). Affinity-labeling studies with noncompetitive antagonists (NCAs) such as chlorpromazine (CPZ), triphenylmethyl-phosphonium (TPMP), and meproprafen were of central importance in determining the transmembrane \(\alpha\)-helical M2 segment of each receptor subunit forms the lining of the pore of the ion channel (9–13). One such noncompetitive antagonist of the AChR which has provided an excellent tool for identifying a number of its important structural features is the hydrophobic, photoreactive compound 3-trifluoromethyl-3-(m-[\(^{125}\text{I}\)]iodophenyl) diazirine (\([^{125}\text{I}]\text{TID}\)) (14). There are two components to the photoincorporation of TID into the AChR. First, as a potent ion channel-blocking \(\alpha\text{NCA, TID binds with micromolar affinity to both the resting and desensitized states of the receptor (15). This specific component of } [^{125}\text{I}]\text{TID incorporation is localized to the channel-lining M2 segments of each receptor subunit and is replaceable by TID itself as well as by other noncompetitive antagonists (15–17). Second, consistent with the hydro-
The phobic nature of TID, it partitions extremely effectively into the lipid bilayer and labels regions of the receptor that are exposed to lipid (18–20). While TID binds to both the resting and desensitized channel with micromolar affinity, [\textsuperscript{125}I]TID photoincorporates into the resting channel with 10-fold greater efficiency. In the resting state, [\textsuperscript{125}I]TID reacts with homologous aliphatic residues in each M2 segment which are located 9 and 13 amino acid residues C-terminal to the conserved lysine residue at the amino terminus of the M2 region (e.g., \(\beta\text{Leu}-257\) and \(\beta\text{Val}-261\)). In the presence of agonist, the pattern of [\textsuperscript{125}I]TID incorporation expands to include homologous serine residues at positions 2 and 6 (e.g., \(\beta\text{Ser}-250\) and \(\beta\text{Ser}-254\)). These results provide important information about the NCA-binding site, suggesting that the conserved leucine residues at position 9 (e.g., \(\beta\text{Leu}-257\)) may form a permeability barrier to the passage of ions in the resting channel and provide the first direct evidence of an agonist-induced rearrangement of the channel-lining M2 helices. Equally important, the identification of nonspecifically labeled residues in segments M4, M3, and M1 of each subunit define the AChR lipid–protein interface, and the periodicity of [\textsuperscript{125}I]TID-labeled residues indicate that these segments are organized as membrane-spanning \(\alpha\)-helices.

The work presented here employs two analogues of TID, a benzoic acid ester ([\textsuperscript{125}I]TID-BE), and a phospholipid ([\textsuperscript{125}I]TIDPC/16) to obtain additional structural information about the AChR and its conformational states (21). [\textsuperscript{125}I]TID-BE is the first in a potential series of structural analogues of TID which we intend to use to examine the molecular determinants of the noncompetitive antagonist binding site in the AChR channel. For the phospholipid photoactive probe ([\textsuperscript{125}I]TIDPC/16), the objectives were 2-fold: first, to further evaluate the results obtained from earlier studies identifying lipid-exposed residues in the AChR using small lipophilic probes, and second, due to the location of the photoactive group at the end of one of the acyl chains of [\textsuperscript{125}I]TIDPC/16, additional information regarding the topological disposition of labeled (lipid-exposed) residues might be obtained.

**EXPERIMENTAL PROCEDURES**

**Materials.** AChR-rich membranes were isolated from *T. californica* electric organ (8). Reactivials were purchased from Pierce, silica gel 60 thin-layer chromatography (TLC) plates from Merck, and [\textsuperscript{125}I]Na from American Radiolabeled Chemicals. *S. aureus* V8 protease was purchased from ICN Biochemicals and \(L\)-1-tosylamido-2-phenylethyl chloromethyl ketone treated trypsin from Worthington Biochemical Corporation. Genapol C-100 (10%) was purchased from Calbiochem. Prestained low molecular weight gel standards were purchased from Life Technologies, Inc.

[\textsuperscript{125}I]TID-BE and [\textsuperscript{125}I]TIDPC/16. [\textsuperscript{125}I]TID-BE and [\textsuperscript{125}I]TIDPC/16 were prepared by radiiododestannylation of the tin-based precursors \(4\times\text{(3-trifluoromethyl-3H-diazirin-3-yl)-2’-tributylstannylbenzyl benzoxate (TDD-BE) and 1-O-hexadecanoyl-2-O-[9\,[[(2-tributylstannyl)-4-(trifluoromethyl-3H-diazirin-3-yl)benzyl]oxy]carbonyl]mononoyl-sn-glycero-3-phosphocholine (TDDPC/16), respectively (22, 21). The tin-based precursors were a generous gift from Dr. Josef Brunner of the Swiss Federal Institute of Technology (ETH), Zurich, Switzerland. Briefly, 20 nmol of TDD-BE in ethanol/toulene (1:1) was added to a 1 mL reactivial and dried under nitrogen. The dried material was dissolved in 20 \(\mu\)L of acetic acid, and 2 mCi of [\textsuperscript{125}I]Na was then added and the iodination reaction initiated by the further addition of 5 \(\mu\)L of peracetic acid. After 10 min, the reaction was quenched by adding 3 \(\mu\)L of NaI (100 mM) and 200 \(\mu\)L of sodium bisulfite [10% (w/w)]. The radioiodated compound was extracted into 400 \(\mu\)L of ethyl acetate (upper phase), which was removed and dried under nitrogen. The dried material was dissolved in 100 \(\mu\)L of ethanol/toulene (1:1), applied to a silica gel 60 TLC plate (5 \(\times\) 20 cm), and developed with ether:hexane (1:9). The position of [\textsuperscript{125}I]TID-BE was determined by autoradiography and by comparison with the migration of TID-BE prepared with nonradioactive iodine. [\textsuperscript{125}I]TID-BE (\(R_y = 0.7\)) was scraped from the TLC plate, extracted with 1 mL of ethanol, dried under nitrogen, dissolved in 50 \(\mu\)L of ethanol, and stored at \(\leq\)20 °C. [\textsuperscript{125}I]TIDPC/16 was prepared from its tin-precursor in an identical fashion as that of [\textsuperscript{125}I]TID-BE, with the exception that following the iodination reaction [\textsuperscript{125}I]TIDPC/16 was extracted with 400 \(\mu\)L of chloroform/methanol (2:1), the TLC plate was developed with chloroform/methanol/water/acetic acid (65:25:5:5), and [\textsuperscript{125}I]TIDPC/16 was stored in ethanol/toulene (1:1) at \(\leq\)20 °C.

[\textsuperscript{125}I]TID-BE and [\textsuperscript{125}I]TIDPC/16 Photolabeling of AChR-Rich Membranes. For labeling experiments, Torpedo AChR-rich membranes [1 mg/mL in vesicle dialysis buffer (VDB), 10 mM MOPS, 100 mM NaCl, 0.1 mM EDTA, and 0.02% NaN\(_3\), pH 7.5] were incubated for 2 h at room temperature with either [\textsuperscript{125}I]TID-BE or [\textsuperscript{125}I]TIDPC/16 at a final concentration of 0.4 and 0.8 \(\mu\)M, respectively, and with or without 250 \(\mu\)M carbamylcholine chloride. For analytical-labeling experiments, approximately 1 mg of AChR membranes was used and 11–14 mg for preparative labelings. Incubations were performed in either glass test tubes or vials and under reduced lighting conditions. The samples were then irradiated with a 365 nm UV lamp (Spectroline EN-280L) for 7 min at a distance of less than 1 cm, and centrifuged at 3900g for 1 h. Pellets were solubilized in electrophoresis sample buffer and counted for 1 min using a Packard Cobra II Gamma Counter before being subjected to SDS–PAGE.

**SDS–Polyacrylamide Gel Electrophoresis.** SDS–PAGE was performed according to the method of Laemmli (23). Analytical (1.0 mm thick) and preparative (1.5 mm) separating gels were composed of 8% polyacrylamide/0.33% bisacrylamide. Following electrophoresis, gels were stained with Coomassie Blue R-250 to visualize AChR subunit bands. After soaking the gels in distilled water overnight, the \(\alpha\), \(\beta\), \(\gamma\), and \(\delta\) bands for each condition were excised and transferred to either a 1.0 or 1.5 mm (preparative) thick 15% acrylamide mapping gel (24, 19). Each gel slice was overlaid with 4 \(\mu\)g of *S. aureus* V8 protease in overlay buffer (18) or 200 \(\mu\)g of V8 protease for preparative scale mapping gels of the \(\alpha\)-subunit (\(\approx\)14 cm strip). Electrophoresis proceeded at 50 V constant voltage until the tracking dye reached the stacking/separating gel interface (\(\approx\)3 h) and then at 5 mA constant current overnight. After Coomassie Blue R-250 staining, analytical gels were dried and exposed to Kodak X-OMAT LS film with an intensifying screen at \(-80\) °C. Preparative gels were soaked in distilled water overnight and the 20 kDa (\(\alpha\)V8–20, Ser-173–Glu-338) and 10 kDa
(αV8–10, Asn-339–Gly-437) polypeptide bands were excised and, as with the β, γ, and δ subunits from the 8% gels, eluted into 15 mL of elution buffer (0.1 M NH4HCO3, 0.1% (w/v) SDS, and 1% β-mercaptoethanol, pH 7.8) for 4 days at room temperature with gentle mixing. The gel suspensions were then filtered through Whatman no. 1 paper days at room temperature with gentle mixing. The gel was subjected to a 16.5%/T6%/C Tricine SDS–PAGE gel with at least one reference lane containing prestained low molecular weight protein standards (Life Technologies, Inc). The gels were stained, destained, and dried for autoradiography. The bulk of the αV8–20 and αV8–10 tryptic digests were resolved directly by reversed-phase HPLC, while the remainder of the β, γ, and δ tryptic digests were solubilized in electrophoresis sample buffer and submitted to preparative (1.5 mm thick) scale 16.5%/T6%/C Tricine gel analysis (16, 25). Preparative Tricine gels were stained, destained, and soaked in water overnight. Using the autoradiograph from the analytical Tricine gel in conjunction with the prestained protein standards, selected bands (16, 25) were excised and eluted into 4 mL of elution buffer for 4 days at room temperature. Further separation and purification of these samples was accomplished by reversed-phase HPLC using a Brownlee Aquapore C4 column (100 × 2.1 mm) with solvent A (0.08% TFA in water) and solvent B (0.05% TFA in 60% acetonitrile/40% 2-propanol). A nonlinear elution gradient was employed (25%–100% solvent B in 80 min) and the elution of peptides was monitored by the absorbance at 210 nm. Collected fractions were counted for radioactivity and the peak protein/radioactivity-containing fractions were pooled, dried by vacuum centrifugation, and resuspended in 20 μL of 0.1 M NH4HCO3 and 0.1% (w/v) SDS, pH 7.8, for protein microsequence analysis.

Sequence Analysis. Amino-terminal sequence analysis was performed on an Applied Biosystems model 477A protein sequencer using gas-phase cycles (in the lab of Dr. Jonathan B. Cohen, Department of Neurobiology, Harvard Medical School). Peptide aliquots (20 μL) were immobilized on chemically modified glass fiber disks (Beckman Instruments), which were used to improve the sequencing yields of hydrophobic peptides. Approximately 30% of the release PTH-amino acids were separated by an on-line model 120A PTH-amino acid analyzer, and approximately 60% was collected for determination of released 125I by γ-counting of each sample for 20 min. Initial yield (I0) and repetitive yield (R) were calculated by nonlinear least-squares regression of the observed release (M) for each cycle (n): M = I0Rn (PTH-derivatives of Ser, Thr, Cys, and His were omitted from the fit).

RESULTS

[125I]TID-BE Labeling of the AChR. Initial photolabeling experiments were designed to characterize the general pattern of [125I]TID-BE (Figure 1) photoincorporation into the AChR as well as to assess the sensitivity of the labeling to the presence of the AChR agonist carbamylcholine. AChR-rich membranes were equilibrated (2 h incubation) with [125I]TID-BE (0.4 μM) in the absence (−lane) and in the presence (+lane) of 250 μM carbamylcholine and irradiated at 365 nm (Spectrolite EN-280LT) for 7 min (at a distance of ~1 cm). Polypeptides were resolved by SDS–PAGE (1.0 mm thick, 8% polyacrylamide gel), visualized by Coo massie Blue R-250 staining and subjected to autoradiography (2–3 day exposure with intensifying screen). Labeled lipid and free photolysis products were electrophoresed from the gel with the tracking dye. The migration of individual AChR subunits is indicated on the left.

FIGURE 1: Chemical structure of [125I]TID-BE.
Inspection of the autoradiograph of the V8 protease digests overlapping fragments for each receptor subunit (20).

Limited V8 digestion reproducibly generates a set of non-

repetitive increase (30%, n = 6) in the extent of incorporation into the β-subunit.

[125I]TID-BE incorporation within each of the AChR subunits was examined by limited digestion with S. aureus V8 protease in a 15% acrylamide mapping gel (24). Limited V8 digestion reproducibly generates a set of non-overlapping fragments for each receptor subunit (20).

Inspection of the autoradiograph of the V8 protease digests (Figure 3) reveals significant [125I]TID-BE incorporation into the following fragments, using the nomenclature of Blanton and Cohen (20): αV8–20 (Ser-173–Glu-338); αV8–10 (Asn-339–Gly-437); βV8–22 (Ile-173/Asn-183–Glu-383); γV8–24 (Ala-167/Trp-170–Glu-372); γV8–14 (Leu-373/Ile-413–Pro-489); γV8–12 (Ala-1 of the β-subunit of the Torpedo Na/K ATPase); ÏV8–12 (Ile-192–Glu-280); ÏV8–11 (Lys-436–Ala-501).

FIGURE 3: Proteolytic mapping of the sites of [125I]TID-BE incorporation into AChR subunits using S. aureus V8 protease. AChR-rich membranes were labeled with [125I]TID-BE in the absence (+lanes) or presence (−lanes) of 250 μM carbamylcholine and subjected to SDS–PAGE on a 1.0 mm thick 8% slab gel. AChR subunit bands were excised following identification by staining, transferred to the wells of a 15% mapping gel and overloaded with 6 μg of S. aureus V8 protease (see Experimental Procedures). AChR subunits were proteolytically digested as they migrated through the 4.5% acrylamide stacking gel (50 V constant voltage, transit time ~3 h) and the resulting fragments were then resolved on the lower 15% acrylamide separating gel. Following electrophoresis, the mapping gel was stained with Coomassie Blue R-250 and subjected to autoradiography (2 week exposure with intensifying screen). The mapping gel was stained with Coomassie Blue R-250 and subjected to autoradiography (2 week exposure with intensifying screen). The following fragments, using the nomenclature of Blanton and Cohen (20), are αV8–20 (Ser-173–Glu-338); αV8–10 (Asn-339–Gly-437); βV8–22 (Ile-173/Asn-183–Glu-383); γV8–24 (Ala-167/Trp-170–Glu-372); γV8–14 (Leu-373/Ile-413–Pro-489); γV8–12 (Ala-1 of the β-subunit of the Torpedo Na/K ATPase); ÏV8–12 (Ile-192–Glu-280); ÏV8–11 (Lys-436–Ala-501).

FIGURE 4: Reversed-phase HPLC purification and sequential Edman degradation of a [125I]TID-BE labeled fragment containing αM4. The [125I]TID-BE-labeled 10 kDa V8 protease fragment of the AChR α-subunit (αV8–10, isolated from AChR labeled in the presence of carbamylcholine) was further digested in solution with trypsin and the labeled material purified by reversed-phase HPLC (panel A) on a Brownlee Aquapor C4 column (100 × 2.1 mm) as described in the Experimental Procedures. The elution of peptides was monitored by absorbance at 210 nm (solid line) and elution of [125I] by γ-counting of each 500 μL fraction (○). HPLC fractions 36–39 were pooled and subjected to automated sequential Edman degradation (panel B). Sixty percent of each cycle of Edman degradation was analyzed for released [125I] (●) and 30% for released PTH-amino acids (△) with the dashed line corresponding to the exponential decay fit of the amount of detected PTH-amino acids. A primary peptide was detected beginning at Tyr-401 of the α-subunit (initial yield, 78 pmol; repetitive yield, 89.5%; 14 200 cpm loaded on sequencing filter; 6150 cpm remaining after 33 cycles). The amino acid sequence of the peptide is shown above panel B with the solid line indicating the limits of the M4 region.

significant difference (>10%) was detected in the extent of incorporation into any of the other V8 fragments for either the β-subunit or α–α or δ-subunits. The relative incorporation of [125I]TID-BE into αV8–20/αV8–10 and γV8–24/γV8–14 was very similar for each labeling condition, 46%/54% and 49%/51%, respectively.

Identification of Sites of [125I]TID-BE Incorporation in the M4 Segments of the α- and γ-Subunits. The M4 segment of the α-subunit was isolated from a tryptic digest of the 10 kDa V8 protease fragment of the AChR α-subunit (αV8–10, Asn-339–Gly-437). The tryptic digest of αV8–10 was separated by reversed-phase HPLC (Figure 4A). In the HPLC chromatogram, there is a single peak of [125I] counts per minute (fraction 38) and a corresponding peak in absorbance (93 min). When HPLC fractions 36–39 were pooled and subjected to N-terminal amino acid sequence analysis, the major sequence detected began at αTyr-401 (initial yield, 78 pmol; repetitive yield, 89.5%). In addition to the αTyr-401 sequence, an overlapping fragment beginning at αSer-388 was also detected (initial yield, 54 pmol; repetitive yield, 87.5%). In the 125I release profile shown in Figure 4B, the
largest release occurs in cycle 12, with additional release in cycles 18, 25, and perhaps cycle 15. Comparison of the pattern of release with the corresponding identified amino acids indicates that the labeled residues include αCys-412 (12.2 cpm/pmol), αMet-415, αCys-418, and αVal-425. However, some or all of the release in cycle 25 (αVal-425) may result from release from αCys-412 (11.26 cpm/pmol) in the αSer-388 peptide.

The M4 segment of the γ-subunit was isolated from a tryptic digest of the intact subunit. When the tryptic digest was resolved on a 1.5 mm thick 16.5%T/6%C Tricine SDS–PAGE gel, Coomassie Blue R-250 stained bands migrating with apparent molecular masses of 7 kDa (γT-7K) and 5 kDa (γT-5K), which have previously been shown to contain γM4 (20), were excised. These two fragments are also the most prominently labeled bands present in the autoradiograph of analytical (1.0 mm thick) Tricine SDS–PAGE gel of the [125I]TID-BE labeled γ-subunit tryptic digest. Material eluted from the γT-7K and γT-5K bands were further purified by reversed-phase HPLC. For γT-7K and γT-5K there were peaks of 125I counts per minute (fractions 37 and 35) and absorbance (90 and 87.5 min), respectively. Fractions 36–38 were pooled for γT-7K and subjected to N-terminal sequence analysis. A single peptide was detected beginning at γGlu-429 and extending through γM4 (initial yield, 53.4 pmol; repetitive yield, 88.2%). Release of 125I in cycle 23 indicated that [125I]TID-BE was incorporated into γCys-451 (10.4 cpm/pmol) within γM4 (7709 cpm loaded; 6509 cpm remaining after 32 cycles). Sequence analysis of the pool of HPLC fractions (fractions 35–38) for γT-5K revealed the presence of a primary sequence beginning at γVal-446, 17 residues C-terminal to the start of γT-7K, and extending through γM4 (initial yield, 26 pmol; repetitive yield, 73%). Release of 125I in cycles 6 and 15 confirmed incorporation of [125I]TID-BE into γCys-451 (10 cpm/pmol) and indicated that there was also incorporation into γSer-460.

**Identification of Sites of [125I]TID-BE Incorporation in the M1 Segment of the α- and β-Subunit.** The M1 segment of the α-subunit was isolated from a tryptic digest of the 20 kDa V8 protease subunit fragment (αV8–20, Ser-173-Glu-338). The tryptic digest was separated by reversed-phase HPLC (Figure 5A). HPLC fractions 35–37, which contain both a peak of 125I counts per minute (fraction 36) and absorbance (89 min), were pooled based on previous work establishing the point of elution of an ~3.4 kDa tryptic fragment containing αM1 (27). Sequence analysis of the pooled fractions revealed the presence of a single peptide beginning at αIle-210 (Figure 5B), extending through the transmembrane segment M1 and, in all likelihood, terminating at αLys-242, which is at the amino-terminal end of the M2 segment (initial yield, 47 pmol; repetitive yield, 92.9%). The [125I]TID-BE released in cycles 18 and 19 is consistent with [125I]TID-BE incorporation into αPhe-227 and αLeu-228 within the M1 segment of the α-subunit (1400 cpm loaded onto filter; 689 cpm remaining after 30 cycles).

The M1 segment of the β-subunit was isolated from a tryptic digest of the intact subunit. When the tryptic digest was resolved on a 1.5 mm thick 16.5%T/6%C Tricine SDS–PAGE gel, Coomassie Blue R-250 stained band migrating with an apparent molecular weight of 10 kDa (βT–10K), and which was previously shown to begin at βLys-216 and contain the M1-M2-M3 segments of the β-subunit (16, 27), was excised. Material eluted from the βT–10K band was further purified by reversed-phase HPLC and the pooled fractions (38–40) were sequenced. Sequence analysis revealed the presence of a single peptide beginning at βLys-216 (initial yield, 20 pmol; repetitive yield, 92%) with 125I release in cycles 19 and 20 (2200 cpm loaded, 1350 cpm remaining after 26 cycles). Comparison of the pattern of 125I release with the corresponding identified amino acids indicate that βLeu-234 and βAla-235 are the primary sites of [125I]TID-BE labeling in βM1.

**Identification of the Sites of [125I]TID-BE Incorporation in βM2.** The β-subunits isolated from AChRs labeled with [125I]TID-BE in the absence and in the presence of 250 μM carbamylcholine were digested with 20% (w/w) trypsin for 4 days. An aliquot of each digest was resolved on a 1.0 mm thick 16.5%T/6%C Tricine SDS–PAGE gel. The corresponding autoradiograph revealed the presence of two bands migrating with apparent molecular weights of 10 kDa (βT–10K) and 7 kDa (βT–7K) in which there was enhanced labeling in the presence of carbamylcholine. These fragments have been identified in previous studies (16, 25).
The amino-terminus of βT–10K is β-Lys-216, with the peptide extending through transmembrane segments M1, M2 and M3, and terminating at or near β-Arg-307 (M1–M2–M3). The C-terminus of βT–7.7K is also likely to be at or near β-Arg-307, while the amino-terminus, β-Met-249, is at the N-terminal end of the M2 segment (M2–M3). In the autoradiograph of the β-subunit digest, the lack of carbamylcholine-enhanced labeling of an approximately 5 kDa fragment (βT–5K), known to contain only the transmembrane segment M1 (unpublished results), indicates that the agonist-sensitive [125I]TID-BE labeling is contained within either the M2 or M3 transmembrane segment.

When the bulk of the tryptic digests of the β-subunit was resolved on 1.5 mm thick Tricine SDS–PAGE gels, the βT–7.7K fragment from each labeling condition were isolated and the eluted material further purified by reversed-phase HPLC. For each labeling condition, the majority of [125I]counts eluted in a peak contained in fraction 37 with a corresponding peak of absorbance eluting at 93 min. HPLC fractions 36–39 were pooled and subjected to N-terminal sequence analysis (Figure 6). In each labeling condition, sequence analysis revealed the presence of a major sequence beginning at β-Met-249 of the β-subunit, present at nearly identical mass levels and that sequenced with similar efficiency (initial yields of 21, 22 pmol; repetitive yields of 93.3 and 93.6%). In each condition, a minor sequence beginning at β-Ser-199 was also present at about one-quarter the amount of the β-Met-249 peptide. For the sample labeled in the absence of agonist (Figure 6A), there was no significant release of [125I]counts per minute (above background) in any of the 20 sequencing cycles (2420 cpm loaded; 2150 cpm remaining after 20 cycles). While there were no apparent sites of [125I]TID-BE incorporation into βM2 (labeled in the absence of carbamylcholine), the [125I]counts per minute associated with the βT–TID-BE labeling suggests that there are sites of labeling further downstream (C-terminal) of the M2 segment, in all likelihood within the M3 segment. For the βT–7.7K sample labeled in the presence of agonist (Figure 6B), there are sites of [125I]release in cycles 9, 13, and 16 (5519 cpm loaded on sequencing filter; 3409 cpm remaining after 34 cycles). The pattern of [125I]release corresponds to [125I]TID-BE incorporation into β-Leu-257 (0.66 cpm/pmol), β-Leu-264 (0.6 cpm/pmol), and the primary site of labeling β-Val-261 (1.52 cpm/pmol). In addition, while the levels of [125I]counts per minute in cycles 10–12 are elevated above that of the background observed in cycles 1–8, it is unclear whether this is the result of increased wash-off of the labeled peptide, preview of the release in cycle 13, or incorporation into one or more of the corresponding β-M2 residues (β-Ala-258, β-Val-259, β-Thr-260).

[125I]TIDPC/16 Labeling of the AChR. Initial photolabeling experiments were designed to assess the ability to introduce the phospholipid probe (ethanolic stock solution) by simple incubation with AChR-rich membranes as well as to characterize the general pattern and agonist-sensitivity of [125I]TIDPC/16 (Figure 7) photoincorporation into the AChR. AChR-rich membranes were equilibrated for increasing periods of time with [125I]TIDPC/16 (0.8 μM) in the absence and in the presence of 250 μM carbamylcholine, exposed to UV light (365 nm) and the membrane suspensions pelleted. With a 2 h incubation, greater than 86% of the 

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**Figure 6:** Radioactivity and mass release upon amino-terminal sequence analysis of a [125I]TID-BE labeled fragment containing βM2. The AChR β-subunit isolated from receptors labeled in the presence and absence of carbamylcholine was digested in solution with trypsin, the digests separated on 1.5 mm thick 16.5%T/6%C Tricine SDS–PAGE gels and an approximately 7.7 kDa fragment (βT–7.7K) isolated (see Experimental Procedures). The βT–TID-BE fragment was further purified by reversed-phase HPLC with fractions 36–39 pooled and subjected to automated Edman degradation. Sixty percent of each cycle of Edman degradation was analyzed for released [125I] (•) and 30% for released PTH-amino acids (□) with the dashed line corresponding to the exponential decay fit of the amount of detected PTH-amino acids. The amino acid sequence of the sequenced peptide containing the M2 region is shown above each panel. (Panel A) [125I] release from β-TID-BE labeled in the absence of carbamylcholine (initial yield, 21 pmol; repetitive yield, 93.6%; 2420 cpm loaded on sequencing filter; 2150 cpm remaining after 20 cycles). (Panel B) [125I] release from β–TID-BE labeled in the presence of carbamylcholine (initial yield, 21 pmol; repetitive yield, 93.3%; 5519 cpm loaded on sequencing filter; 3409 cpm remaining after 20 cycles).

**Figure 7:** Chemical structure of [125I]TIDPC/16.

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[125I]counts per minute remained with AChR-rich membranes and longer incubation times of up to 20 h resulted in only a minor increase in the extent of [125I]TIDPC/16 membrane partitioning. Following centrifugation and γ-counting.

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2 The pattern of [125I]TIDBC/16, [125I]TID, and [125I]TID-BE incorporation into AChR subunits and subunit proteolytic fragments was also determined under conditions in which the photoreactive probe was incubated with detergent (cholate) solubilized AChRs and with or without carbamylcholine. The pattern and extent of labeling for each photoreactive compound was found to be identical to that observed when the probe was added to AChR-rich membranes. One implication of these results is that it indicates that cholate solubilized AChRs are still capable of undergoing agonist-induced conformational transitions and that in the absence of agonist the receptor remains in the resting state.
Figure 8: Photoincorporation of [125I]TIDPC into AChR-rich membranes in the absence and presence of carbamylcholine. AChR-rich membranes were equilibrated (2 h incubation) with [125I]TIDPC/16 (0.8 μM) in the absence (−lane) and in the presence (+lane) of 250 μM carbamylcholine and irradiated at 365 nm for 7 min. Polypeptides were resolved by SDS-PAGE (1.0 mm thick, 8% polyacrylamide gel), visualized by Coomassie Blue R-250 staining and subjected to autoradiography (24 h exposure with intensifying screen). Labeled lipid and free photolysis products were electrophoresed from the gel with the tracking dye. The migration of individual AChR subunits is indicated on the left. Significant [125I]TIDPC/16 incorporation is also evident in the α-subunit of the Na/K ATPase (≈95 kDa) and in the CLC-0 chloride channel (≈90 kDa).

Membrane pellets were solubilized in electrophoresis sample buffer and the pattern of incorporation into AChR subunits monitored by SDS-PAGE followed by autoradiography. The autoradiograph of an 8% polyacrylamide slab gel (Figure 8) reveals that there is substantial incorporation of [125I]TIDPC/16 into each of the AChR subunits. In addition to the AChR subunits, there is also labeling of the receptor-associated peripheral protein, Rapsyn, of M, 43000 (28) which migrates with slightly slower electrophoretic mobility than the α-subunit. There is also substantial [125I]TIDPC/16 incorporation into the α-subunit of the Na/K ATPase (≈95 kDa) and to a lesser extent into the CLC-0 chloride channel protein (≈89 kDa). Neither the pattern of incorporation into individual AChR subunits nor the overall labeling pattern was effected by inclusion of 250 μM carbamylcholine (Figure 8, +lane). Upon the basis of γ-counting of excised gel bands, the relative incorporation of [125I]TIDPC/16 into the AChR subunits is 0.57:1.05:1:1.07 in the absence of agonist and 0.58:1.05:1:0.92 in the presence of 250 μM carbamylcholine (α, β, γ, δ, n = 4).

[125I]TIDPC/16 incorporation within each of the AChR subunits was next mapped by limited enzymatic digestion with S. aureus V8 protease (24, 26). As described previously, limited V8 digestion reproducibly generates a set of nonoverlapping fragments for each receptor subunit (20). Inspection of the autoradiograph of the 15% acrylamide mapping gel containing the V8 protease digests of each receptor subunit (Figure 9) reveals significant [125I]TIDPC/16 incorporation into the following fragments: αV8=20; βV8=10; γV8=22; δV8=12; V8=14; αV8=10; βV8=22; γV8=14; γV8=12; δV8=12; and δV8=11 (see legend to Figure 3).

Figure 9: Proteolytic mapping of the sites of [125I]TIDPC/16 incorporation into AChR subunits using S. aureus V8 protease. AChR-rich membranes were labeled with [125I]TIDPC/16 in the absence (−lanes) or presence (+lanes) of 250 μM carbamylcholine and subjected to SDS-PAGE on a 1.0 mm thick 8% polyacrylamide slab gel. [125I]TIDPC/16-labeled AChR subunit bands were excised for digestion with 6 μg of S. aureus V8 protease in a proteolytic mapping gel as described in the Experimental Procedures and in the legend to Figure 3. The mapping gel was stained with Coomassie Blue R-250 and subjected to autoradiography (1 week exposure with intensifying screen). The principal [125I]TIDPC/16-labeled fragments are αV8=20; αV8=10; βV8=22; γV8=24; γV8=14; γV8=12; δV8=12; and δV8=11 (see legend to Figure 3). Direct counting of the excised gel bands did not reveal any significant difference (>5%) in the extent of [125I]TIDPC/16 incorporation into any of the AChR subunit V8 protease fragments. Of the four fragments generated by V8 digestion of the AChR α-subunit (αV8=20, Ser-173–Glu-338; αV8=18, Thr-52–Glu-172; αV8=10, Asn-339–Gly-437; and αV8=4, Ser-1–Glu-51) greater than 92% of the [125I] counts per minute was contained within αV8=20 and αV8=10, the relative incorporation being 42 and 58%, respectively. The homologous set of fragments generated by V8 protease digestion of the γ-subunit, γV8=24/γV8=14, incorporated [125I]TIDPC/16 in relative amounts that were nearly identical (41 and 59%, respectively) to that of αV8=20 and αV8=10.

Mapping the Sites of [125I]TIDPC/16 Incorporation to the M4 Segments of the α- and γ-Subunits. The M4 segment of the α- and γ-subunits was isolated from tryptic digests of αV8=10 (Asn-339–Gly-437) and γV8=14 (Leu-373–Pro-489). The tryptic digest of αV8=10 was separated by reversed-phase HPLC and the M4 peptide eluted as a single peak of [125I] counts per minute (fraction 38) and a corresponding peak in absorbance (92.5 min). In the HPLC chromatogram of the tryptic digest of γV8=14, the γM4 peptide eluted in a peak of [125I] counts per minute (fraction 36/37) and absorbance (90 min). When HPLC fractions 36–39 were pooled for the tryptic digest of αV8=10 and subjected to N-terminal amino acid sequence analysis, the major sequence detected began at αTyr-401 (initial yield, 89 pmol; repetitive yield, 93%). In the [125I] release profile, there was sporadic release of counts throughout the 30 sequencing cycles. While the primary site of release was in cycle 12 (0.32 cpm/pmol) corresponding to [125I]TIDPC/16 incorporation into αCys-412 in αM4, the complex labeling pattern did not allow for a definitive identification of any.
one site of labeling. This result was not entirely unexpected; there was considerable concern that because of the zwitterionic nature of \([^{125}\text{I}]\text{TIDPC/16}\), labeled amino acid residues might not be efficiently extracted by the solvents used in automated Edman degradation (Dr. J. Brunner, personal communication). Sequence analysis of the pool of HPLC fractions (35–39) for the tryptic digest of \(\gamma\text{V8–14}\) resulted in a similar \([^{125}\text{I}]\)release profile. Despite the presence of a fairly abundant peptide beginning at \(\gamma\text{Val-446}\) and which sequenced with high efficiency (initial yield, 45 pmol; repetitive yield, 92%), there was sporadic release of \([^{125}\text{I}]\) counts per minute with no clear primary site of incorporation.

The M1 segment of the \(\alpha\)-subunit was isolated from a tryptic digest of \(\alpha\text{V8–20}\) (Ser-173–Glu-338). The digest was resolved by reversed-phase HPLC with a peak of \([^{125}\text{I}]\) counts per minute in fraction 37 and a corresponding peak in absorbance eluting at 91 min. HPLC fractions 36–38 were pooled and sequenced for five cycles in order to confirm the identity of the labeled peptide (\(\alpha\text{M1, Ile-210-Lys-242}\)).

**DISCUSSION**

The purpose of the work presented here was to probe the structure of both the AChR ion channel as well as lipid–protein interface with the hydrophobic photoreactive compounds \([^{125}\text{I}]\text{TID-BE}\) and \([^{125}\text{I}]\text{TIDPC/16}\). For both probes, the majority of photoincorporation into the AChR was insensitive to the presence of agonist, hence, the labeling was the same in either the resting or the desensitized conformational state of the receptor. The agonist-insensitive labeling for both \([^{125}\text{I}]\text{TID-BE}\) and \([^{125}\text{I}]\text{TIDPC/16}\) mapped to regions and individual amino acid residues which previous studies have shown are exposed to the lipid bilayer (reviewed in ref 25). In addition, for \([^{125}\text{I}]\text{TID-BE}\), there was enhanced labeling (~30%) of the \(\beta\)-subunit when the AChR was labeled in the presence of agonist and this agonist-induced labeling was restricted to residues in the channel lining \(\beta\text{M2}\) segment. Thus, there exist two components to the labeling of the AChR by \([^{125}\text{I}]\text{TID-BE}\): an agonist-insensitive component consistent with incorporation into residues situated at the receptor lipid–protein interface and a specific, agonist-induced component reflecting labeling of the desensitized conformation of the AChR ion channel.

**Agonist-Induced \([^{125}\text{I}]\text{TID-BE}\) Photoincorporation into \(\beta\text{M2}\).** In the absence of agonist, 3-trifluoromethyl-3-(m-\([^{125}\text{I}]\)iodophenyl)diazirine (\([^{125}\text{I}]\text{TID}\), the parent compound for \([^{125}\text{I}]\text{TID-BE}\), photoincorporates extremely efficiently into homologous aliphatic residues (e.g., \(\beta\text{Leu-257, }\beta\text{Val-261}\)) in the channel-lining M2 segments of each AChR \(\beta\)-subunit (16). At the subunit level, greater than 70% of the total \([^{125}\text{I}]\)TID labeling reflects incorporation into the M2 subunit. In contrast, there was no detectable \([^{125}\text{I}]\text{TID-BE}\) photoincorporation into any residue within the M2 segment of the \(\beta\)-subunit. Furthermore, addition of nonradioactive TID-BE had very little effect on the extent of photoincorporation of \([^{125}\text{I}]\text{TID}\) into the resting AChR (IC\(_{50}\) > 120 \(\mu\text{M}\); data not shown). While TID binds to the AChR channel with micromolar affinity (15), these results indicate that TID-BE binds to the channel very weakly if at all. The inability of TID-BE to interact with the resting channel is not apparently a simple reflection of a difference in the size of the two compounds. The hydrophobic photoreactive compound, \([^{3}\text{H}]\) diazofluorene (DAF), is similar in size to \([^{125}\text{I}]\text{TID-BE}\), yet binds with micromolar affinity to the resting AChR channel, photoincorporating into \(\beta\text{Val-261}\) and \(\delta\text{Val-269}\) within the M2 segment of the \(\beta\)- and \(\delta\)-subunits (25). Clearly, the physiochemical differences between TID and TID-BE are critically important for binding to the pore of the resting ion channel. These differences provide important clues about the molecular environment of the ligand-binding site and insight regarding the binding site determinants. Among the possible explanations for the differences in binding affinity is that either the polarity of the ester linkage and/or the increased torsional flexibility of the molecule introduced by the ester linkage adversely affects the interaction of TID-BE with the resting channel pore. Along these lines, we are currently examining the pattern of photoincorporation into the AChR of both an alcohol (\([^{125}\text{I}]\text{TIDBA}\) and an acetate (\([^{125}\text{I}]\text{TIDBAC}\)) derivative of \([^{125}\text{I}]\text{TID}\) (21). Preliminary studies indicate that \([^{125}\text{I}]\text{TID}\) and its alcohol analogue (\([^{125}\text{I}]\text{TIDBA}\)) interact with the resting AChR channel in a similar fashion: (1) the pattern and extent of incorporation into AChR subunits is nearly identical for the two probes as is the sensitivity of the labeling to the addition of the agonist carbamylcholine; (2) nonradioactive TIDBA inhibits \([^{125}\text{I}]\text{TID}\) photoincorporation into the resting AChR in a dose-dependent fashion (IC\(_{50}\) = 4.8 \(\mu\text{M}\)) with a reported \(K_i\) value for nonradioactive TID inhibition of 4 \(\mu\text{M}\) (15); (3) Finally, the AChR NCA 3,4,5-trimethoxybenzoic acid \(8\)-(diethylamino) octyl ester (TMB-8) inhibits photoincorporation of both \([^{125}\text{I}]\text{TID}\) and \([^{125}\text{I}]\text{TID}\) into the AChR \(\gamma\)-subunit by greater than 85% and with nearly identical IC\(_{50}\) values, 2.75 and 3.1 \(\mu\text{M}\), respectively. Examination of the interaction of the acetate analogue of TID (\([^{125}\text{I}]\text{TIDBAC}\)) with the AChR will likely provide critical information regarding the structure–activity relationship for TID binding to the resting channel.

In the presence of agonist, \([^{125}\text{I}]\text{TID-BE}\) photoincorporates into the aliphatic residues \(\beta\text{Leu-257, }\beta\text{Val-261, and }\beta\text{Leu-264}\) in the M2 region of the AChR \(\beta\)-subunit. Under nearly identical labeling conditions, \([^{125}\text{I}]\text{TID}\) incorporates into \(\beta\text{Ser-250, }\beta\text{Ser-254, }\beta\text{Leu-257, and }\beta\text{Val-261}\) (16). As illustrated in Figure 10, for each photoreactive probe, labeled residues are located over a fairly broad expanse of the M2 segment, between three and four consecutive \(\alpha\)-helical turns. It is also evident, that while the binding sites for TID and TID-BE overlap, the binding loci for TID-BE is located closer to the extracellular end of the M2 segment (i.e., channel pore). These results reinforce earlier conclusions that different NCAs bind to unique regions of the channel pore even when the AChR is in the same conformational state (reviewed in ref 7). For example, in the desensitized channel, NCAs such as TID (16) and chlorpromazine (9–11) bind close to the intracellular end of the channel (i.e., near \(\beta\text{Ser-250}\) and \(\beta\text{Ser-254}\)), TID-BE and DAF (25) bind closer to the middle or upper half of the channel (i.e., near \(\beta\text{Leu-257, }\beta\text{Val-261, and }\beta\text{Leu-264}\)), and finally, the NCA mepradifen (13) binds.

\(^3\) An alternative explanation for \([^{125}\text{I}]\text{TID}\) labeling of \(\beta\text{Leu-257 and }\beta\text{Val-261}\) in the presence of agonist (50 \(\mu\text{M}\) carbamylcholine) is the presence of a small fraction (<10%) of AChRs remaining in the resting state. The observed \([^{125}\text{I}]\text{TID}\) labeling pattern would then reflect a mixture of patterns resulting from AChRs in both the resting and desensitized state. With labeling of \(\beta\text{Ser-250 and }\beta\text{Ser-254 in }\beta\text{M2}\) resulting from \([^{125}\text{I}]\text{TID}\) binding to the desensitized channel (16).
Photoreactive Probes of AChR Structure

**Figure 10:** Helical representation of the \( \beta-M2 \) segment with the binding loci of TID and TID-BE indicated. A helical representation of the M2 segment of the AChR \( \beta \)-subunit is shown. Amino acids which incorporate \([^{125}I]TID-BE \) when the AChR is labeled in the presence of agonist, i.e., the desensitized state, include \( \beta \)-Leu-257, \( \beta \)-Val-261, and \( \beta \)-Leu-264; those labeled by \([^{125}I]TID \) in the presence of carbachol include \( \beta \)-Ser-250, \( \beta \)-Ser-254, \( \beta \)-Leu-257, and \( \beta \)-Val-261 (16). The \( \beta \)M2 helix is oriented with the N-terminus at the bottom reflecting the extracellular location of the carboxy-terminal. The solid and dotted boxes to the right of the M2-helix are meant to illustrate that TID-BE and TID, respectively, have unique though overlapping binding site loci.

near the extracellular end of the channel (i.e., near \( \beta \)-Asp-268). It is also noteworthy that the enhanced labeling of the AChR observed in the presence of agonist is restricted to the M2 segment of the \( \beta \)-subunit. While it is possible that the M2 segments of the \( \alpha \)-, \( \gamma \)-, and \( \delta \)-subunits also incorporate \([^{125}I]TID-BE \) at some low level, residues within \( \beta \)M2 are nonetheless preferentially labeled. This result is surprising given that, for the residues labeled by \([^{125}I]TID-BE \) in \( \beta \)M2 (\( \beta \)-Leu-257, \( \beta \)-Val-261, and \( \beta \)-Leu-264), identical residues are present at the homologous positions in the M2 segment of the \( \alpha \)- and \( \delta \)-subunits. The preferential labeling of \( \beta \)M2 residues is then not likely the result of differences in the reactivity of these residues in the other M2 segments, rather it indicates that within the pore of the ion channel the photoreactive diazirine moiety of \([^{125}I]TID-BE \) is preferentially oriented toward \( \beta \)M2. This result suggests the presence of a certain degree of structural asymmetry within the pore of the desensitized channel. A similar conclusion was reached about the structure of the resting ion channel based on the pattern of M2 labeling by the uncharged photoreactive NCA, \( [^{3}H]DAF \) (25).

**Figure 11:** CPK model of the \( \alpha \)-M4 and \( \alpha \)-M1 helices with \([^{125}I]TID \)-labeled amino acid residues. CPK helical representations of the \( \alpha \)-M4 and \( \alpha \)-M1 regions indicating the residues previously shown to be labeled by \([^{125}I]TID \) [shaded (20)]. The model illustrates that the residues labeled by \([^{125}I]TID-BE \) in \( \alpha \)M4 (Cys-412, Met-415, and Cys-418) and in \( \alpha \)-M1 (Phe-227 and Leu-228) represent a subset of the \([^{125}I]TID \)-labeled, lipid-exposed residues in each transmembrane helix. Note: \( \alpha \)-His-408 is not labeled by \([^{125}I]TID \) but has been identified as a lipid-exposed residue by reaction with other lipophilic probes (25, 29).

In Figure 11, the residues labeled by \([^{125}I]TID-BE \) in \( \alpha \)-M4 (and in \( \gamma \)-M4) represent a subset of the residues previously identified for \([^{125}I]TID \) incorporation into M4 segments (20). For both \( \alpha \)-M4 and \( \gamma \)-M4, \([^{125}I]TID-BE \)-labeled residues are situated within the same lipid-exposed face of each \( \alpha \)-helix (Figure 11) identified by the lipophilic probes \([^{125}I]TID \) (20), \( [^{3}H]diazofluorene \) (25), and \( [^{3}H]promegestone \) (29). As with each of these other probes, positional effects rather than the intrinsic reactivity of the side chains of individual amino acid residues have a dominant effect on the pattern of \([^{125}I]TID-BE \) incorporation. For example, in \( \alpha \)-M4 \([^{125}I]TID-BE \) reacts with Cys-412 (12.2 cpn/pmol) with 5-fold greater efficiency than with Cys-418 (2.47 cpn/pmol). One interpretation of these results is that TID-BE as well as other lipophilic probes tend to orient in the bilayer and/or preferentially interact with this region in each of the M4 segments.

It was hoped that the phospholipid analogue of TID (\([^{125}I]TIDPC/16 \) might provide additional topological information regarding the disposition of some of these labeled (lipid-exposed) residues relative to that of the lipid bilayer. Within \([^{125}I]TIDPC/16 \), the photoreactive group is located at the end of one of the fatty acyl chains and in the lipid bilayer would be situated near the center of the bilayer. While it is likely that the fatty acyl-attached photoreactive TID group would have some transverse mobility in the lipid bilayer, the expectation was that the pattern of \([^{125}I]TIDPC/16 \) incorporation into residues in the M4 segment would at least partially reflect the transbilayer disposition of the labeled residues. Unfortunately, while \([^{125}I]TIDPC/16 \) incorporation into the AChR was mapped to the confines of at least the M4 segment of the \( \alpha \)- and \( \gamma \)-subunit (as well as \( \alpha \)-M1), due to technical reasons it was not possible to obtain information about incorporation into individual residues using automated N-terminal sequence analysis. We are currently exploring alternative strategies for determining which residues within these transmembrane segments are labeled by \([^{125}I]TIDPC/
16. Nonetheless, that the sites of $^{[125]}$I-TIDPC/16 incorporation map to the same transmembrane segments previously identified using small lipophilic probes such as $^{[125]}$I-TID and $[^3]$H-DAF provides further validation of the results obtained with these probes, and, diminishes the possibility that these small lipophilic probes are identifying hydrophobic pockets as opposed to regions which are directly in contact with lipid.

In the M1 segments of the α- and β-subunits, it was determined that $^{[125]}$I-TID-BE reacted with α-Phe-227, α-Leu-228 (Figure 5B, αM1) and β-Leu-234, β-Ala-235 (βM1). As is illustrated in Figure 11, the residues which incorporate $^{[125]}$I-TID-BE in αM1 represent a subset of the residues previously shown to be labeled by $^{[125]}$I-TID (20). As with TID (αM1), the $^{[125]}$I-TID-BE labeled residues are located in the C-terminal portion of the αM1 and βM1 segments (after αPro-221), suggesting that only this part of M1 is exposed to lipid. While it does not provide direct support, this conclusion is also consistent with the results of Akbas and Karlin (30) which indicate that select residues in the amino-terminal third of αM1 and βM1 (31) are exposed to the aqueous lumen of the ion channel pore. It is particularly striking there is no detectable $^{[125]}$I-TID-BE incorporation into αCys-222, as this residue was labeled by $^{[125]}$I-TID and cysteine residues are extremely reactive to diazirines (32). The implication of this result is that the larger TID-BE molecule is restricted in how it can interact with the lipid-exposed portion of the M1 segment. $^{[125]}$I-TID-BE can only associate with the M1 segment with the photoreactive diazirine group oriented away from (αCys-222 and α-Leu-223) and toward (α-Phe-227 and α-Leu-228). Given the limited lipid exposure of the M1 segment and that the extracellular portion of M1 is exposed to the channel lumen as well as presumably making protein:protein contacts, it is tempting to speculate that the labeled portion of the M1 segment may represent a lipid-exposed pocket.

Finally, from the limited number of labeled residues in either αM1 or βM1, no definitive conclusion can be reached regarding the secondary structure of the M1 segment, which may represent either a distorted α-helix (27) or β-sheet (6) and contain stretches of unordered secondary structure (30, 33, 34).

CONCLUSIONS

Our results demonstrate that there are two components to the labeling of the AChR for both TID and TID-BE: a specific agonist-sensitive component reflecting labeling of the channel-lining M2 segments and a nonspecific agonist-insensitive component consistent with incorporation into residues situated at the lipid–protein interface. Only the latter component is present for the phospholipid analogue of TID (TIDPC/16). While TID, TID-BE, and TIDPC/16 interact in a similar fashion with the transmembrane segments of the AChR which are in contact with lipid, TID and TID-BE interact very differently with the AChR ion channel. While TID binds with micromolar affinity to the resting channel, photoincorporating with high efficiency into M2 residues, TID-BE does not appear to bind to the resting channel. Both TID and TID-BE bind to the channel in the desensitized state, however, the two compounds have distinct binding loci with TID-BE binding closer to the extracellular end of the channel. These differences provide important clues about the structure and environment of the binding site(s) for noncompetitive antagonists in both the resting and desensitized channel and provide the foundation for future studies aimed at better understanding the molecular determinants of the NCA-binding site.

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