Identifying the cholesterol binding domain in the nicotinic acetylcholine receptor with [125I]azido-cholesterol

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Abstract

A novel photoreactive analog of cholesterol, 3α-(4-azido-3-[125I]iodosalicylic)-cholest-5-ene ([125I]azido-cholesterol), was used to label both native acetylcholine receptor (AChR)-rich membranes from *Torpedo californica* and affinity-purified *Torpedo* AChRs reconstituted into lipid vesicles. In both cases all four AChR subunits incorporated [125I]azido-cholesterol on an equal molar basis and neither the pattern nor the extent of labeling was affected by the presence of the agonist carbamylcholine. Labeled regions in each of the AChR subunits were initially mapped by *Staphylococcus aureus* V8 protease digestion to large fragments which contain the AChR transmembrane segments. Sites of [125I]azido-cholesterol incorporation were further mapped by exhaustive tryptic digestion of the V8 protease subunit fragments αV8-20 (αSer-173-Glu-338), αV8-10 (αAsn-339-Gly-439), and γV8-14 (γLeu-373-Pro-489). The digests were separated by reverse-phase high-performance liquid chromatography and labeled peptides identified by amino-terminal sequence analysis. [125I]Azido-cholesterol labeling was localized to peptides that contain almost exclusively the α-M4, α-M1 and γ-M4 membrane spanning segments. These results establish that the binding domain for cholesterol is at the lipid–protein interface of the AChR. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Nicotinic acetylcholine receptor; Photoaffinity labeling; Cholesterol

1. Introduction

The nicotinic acetylcholine receptor (AChR) isolated from the electroplacque tissue of the marine elasmobranch *Torpedo californica* is a ligand activated cation-specific ion channel. The 290 kDa membrane-imbedded pentamer consists of four unique subunits, with a stoichiometry of $\alpha_2\beta\gamma\delta$ (for recent reviews, see [1,2]). Similar hydropathy profiles and considerable sequence homology among the AChR subunits serve as the basis for a predicted folding topology consisting of four hydrophobic membrane spanning α-helical segments referred to as M1, M2, M3 and M4 [3,4]. Substantial experimental evidence

supports the pentameric subunit arrangement [5] and the M1–M4 folding of the lipid imbedded regions of AChR subunits (reviewed in [6]).

The binding of acetylcholine to sites on the extracellular domains of the AChR leads to the transient opening of a cation-selective ion channel. In the continued presence of agonist, the AChR isomerizes to a non-conducting, 'desensitized' conformation. Studies involving reconstituting purified AChRs into membranes of defined lipid composition have shown that these agonist-induced conformational transitions are highly sensitive to the presence of both cholesterol and negatively charged phospholipids [7–10]. The use of a variety of spin-labeled lipids has established that in accordance with the abundance of cholesterol and negatively charged phospholipids in native-receptor rich membranes [11,12] these lipids preferentially interact with the AChR [13–15]. The use of a brominated steroid analog in fluorescence quenching studies revealed the presence of two different lipid binding domains for the AChR [16]; annular sites (approximately 45 per AChR) which bind both sterol and phospholipids and non-annular sites (5–10 sites per AChR) which have a 20-fold higher affinity for cholesterol. Photoaffinity labeling studies with both anionic lipid [17,18] and cholesterol [19,20] analogs have further demonstrated a direct interaction of these lipids with the AChR.

The presence of cholesterol has been shown to be required for AChR function and a direct specific interaction with the receptor has been well documented. However, identification of the region(s) in the AChR primary structure which contribute to the formation of the binding site for cholesterol is an important step in obtaining a more complete understanding of the role of this lipid in regulating AChR structure and function. Here, we provide the first evidence of this type by mapping the labeling of a photoactivatable cholesterol analog, 3α-(4-azido-3-[125I]iodosalicylic)-cholest-5-ene ([125I]azido-cholesterol), to the membrane imbedded regions of the *Torpedo californica* AChR. We further provide a model of cholesterol action that involves cholesterol binding to the interfaces of the lipid-exposed transmembrane segments and by facilitating the subtle movements of these domains helping to mediate the conformational transitions of the AChR.

2. Materials and methods

2.1. Materials

3α-(4-Azido-3-[125I]-iodosalicylic)-cholest-5-ene ([125I]azido-cholesterol) was prepared by coupling 3α-aminocholest-5-ene and 4-azido salicylic acid using dicyclohexylcarbodiimide, followed by chloramine T iodination and purification by reverse-phase thin-layer chromatography (TLC) [21]. [125I]Azido-cholesterol was stored in methanol (∼10 Ci/mmol, 70 μCi/ml) at 4°C in the dark. *Staphylococcus aureus* V8 protease was obtained from ICN Biochemicals and L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin from Worthington Biochemical. Genapol C-100 (10% w/v) was purchased from Calbiochem and sodium cholate from Sigma. Asolectin (a crude soybean lipid extract) came from Avanti Polar Lipids.

2.2. AChR-rich membranes

AChR-rich membranes were isolated from the electric organ of *Torpedo californica* (Aquatic Research Consultants, San Pedro, CA) according to the procedure of Sobel et al. [22], with the modifications described previously [23]. The final membrane suspensions in 36% sucrose/0.02% NaN₃ were stored at −80°C.

2.3. Affinity column purification and reconstitution of AChR membranes

The *Torpedo* AChR was isolated from detergent (cholate) extracts of crude AChR membranes by affinity chromatography in the presence of asolectin lipids. Affinity column purification was performed using an acetylcholine affinity matrix according to the procedure of Ellena et al. [14] with several modifications according to the methods outlined in Blanton and Wang [17]. Briefly, the affinity column matrix was prepared by coupling cystamine to Affi-gel 10 (Bio-Rad), reduction with dithiothreitol and final modification with bromoacetylcholine bromide. Affinity-purified AChRs were reconstituted with asolectin at a lipid protein ratio of 800:1 on a mole per mole basis and stored at −80°C.

2.4. [^{125}I]Azido-cholesterol labeling of AChR membranes

To introduce the photoaffinity probe into AChR-rich membranes, typically 20 μCi (~1.25 nmol) of [^{125}I]azido-cholesterol (methanolic stock solution) was dried on the inside of a 10×75 mm glass test tube with a stream of nitrogen. AChR-rich membranes (4 mg/ml) in vesicle dialysis buffer (VDB, 100 mM NaCl, 0.1 mM EDTA, 0.02% NaN$_3$, 10 mM MOPS pH 7.5) were solubilized with sodium cholate at a final concentration of 1% (2 mg/ml protein) at 4°C for 1 h (constant stirring). Insoluble material was removed by centrifugation at $39000 \times g$ for 1 h at 4°C. The solubilized material (supernatant) was added to the test tube containing a dried layer of [^{125}I]azido-cholesterol (final concentration ~2.5 μM) and mixed thoroughly. Following a 30 min incubation, the solution was divided into two aliquots and carbamylcholine (250 μM) added to one sample. Following a 1 h incubation the membrane suspensions were irradiated at 365 nm (Spectroline EN-280L) for 7 min at a distance of < 1 cm. All operations, unless specified otherwise, were performed under reduced lighting conditions and at room temperature. Photolyzed material was then solubilized in electrophoresis sample buffer and subjected to SDS-PAGE.

An alternate method was used to incorporate [^{125}I]azido-cholesterol into affinity-purified AChRs that have reconstituted into lipid (asolectin) vesicles. Asolectin (1 mg in dichloromethane) was dried onto the surface of a glass test tube with a stream of nitrogen. To this was added either 20 μCi (analytical labeling) or 100 μCi (preparative labeling) of [^{125}I]azido-cholesterol and the organic solvent removed under a stream of nitrogen. VDB buffer was then added, the solution layered with nitrogen, and the tube sealed. The solution was vortexed for 15 min followed by 60 min of gentle sonication. Affinity purified reconstituted AChRs (0.2 mg for analytical and 1.6 mg for preparative labelings) were added to the tube containing [^{125}I]azido-cholesterol. The test tube was then sealed in the presence of nitrogen and the membrane suspension incubated overnight at 5°C. Following overnight incubation, carbamylcholine

(250 μM) was added to appropriate samples and allowed to equilibrate for 1 h. The samples were irradiated as described above, the membranes pelleted ($39000 \times g$ for 1 h), solubilized in electrophoresis sample buffer and subjected to SDS-PAGE.

2.5. SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli [24] with analytical (1.0 mm thick) and preparative (1.5 mm) separating gels comprised of 8% polyacrylamide/0.33% bis-acrylamide. Following electrophoresis, AChR subunits were visualized by staining with Coomassie blue R-250 (0.25% (w/v) in 45% methanol, 10% acetic acid, 45% dH$_2$O) and destaining (25% methanol, 10% acetic acid, 65% dH$_2$O). Stained gels were soaked in distilled water overnight and the AChR α, β, γ, and δ bands for each labeling condition (−/+ carbamylcholine) were excised and transferred to either a 1.0 or 1.5 mm (preparative) thick 15% acrylamide mapping gel [25,23]. For analytical labelings, each gel slice was overlaid with a solution containing *S. aureus* V8 protease (6 μg) and electrophoresed at 50 V constant voltage for ~3 h and then at 5 mA constant current overnight. After Coomassie blue R-250 staining (2 h) and destaining (3–4 h), analytical gels were dried and exposed to Kodak X-OMAT film with an intensifying screen at −80°C. Preparative 8% acrylamide gels (1.5 mm thick) were soaked in distilled water overnight and the α- and γ-subunits excised as an approximately ~14 cm strip. The strips were transferred to individual 15% acrylamide mapping gels and overlaid with 200 μg V8 protease. Following electrophoresis and staining (same as analytical gel conditions), the subunit fragment bands αV8-20 (αSer-173-Glu-338), αV8-10 (αAsn-339-Gly-437), and γV8-14 (γLeu-373-Pro-489) were excised and the polypeptides eluted into 15 ml of Elution buffer (0.1 M NH$_4$HCO$_3$, 0.1% (w/v) SDS, 1% β-mercaptoethanol, pH 7.8) for 4 days at room temperature with gentle mixing [26,27]. The gel suspensions were then filtered through Whatman no. 1 paper and concentrated using a Centriprep-10 (Amicon). Excess SDS was removed by acetone precipitation (85% acetone at −20°C for ~18 h).

2.6. Isolation of [¹²⁵I]azido-cholesterol labeled fragments

For digestion with trypsin, acetone precipitated V8 protease subunit fragments, αV8-20, αV8-10, and γV8-14 were resuspended in approximately 300 μl of 0.1 M NH_4HCO_3, 0.02% (w/v) SDS, 0.5% Genapol C-100, pH 7.8 (1–2 mg/ml protein). Trypsin was added at a 100% (w/w) enzyme to substrate ratio and the digestion allowed to proceed 4 days at room temperature. The digests were then separated by reverse-phase HPLC using a Brownlee Aquapore C_4 column (100×2.1 mm) with solvent A (0.08% TFA in water), solvent B (0.05% TFA in 60% acetonitrile/40% 2-propanol). A non-linear 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 in a Packard Cobra II Gamma Counter and the peak protein/radioactivity-containing fractions were pooled, dried by vacuum centrifugation, and resuspended in 20 μl of 0.1 M NH_4HCO_3, 0.1% (w/v) SDS, pH 7.8 for protein sequence analysis.

2.7. Sequence analysis

Amino terminal sequence analysis was performed on a Beckman Instruments (Porton) 20/20 automated protein sequencer using gas phase cycles (Texas Tech Biotechnology Core Facility). 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, and subjected to 10 sequencing cycles. The α-M4 peptide, from HPLC purified tryptic digest of αV8-10, was radiosequenced on an Applied Biosystems model 477A protein sequencer using gas phase cycles (in the laboratory of Dr. Jonathan B. Cohen, Department of Neurobiology, Harvard Medical School). 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 45 min. Initial yield (I_o) and repetitive yield (R) were calculated by non-linear least-squares regression of the observed release (M) for each cycle (n): $M = I_o R^n$ (PTH-derivatives of Ser, Thr, Cys, and His were omitted from the fit).

3. Results

3.1. Characterization of [¹²⁵I]azido-cholesterol labeling of AChR subunits

Initial attempts to introduce the cholesterol photo-affinity analog [¹²⁵I]azido-cholesterol (Fig. 1) into AChR-rich membranes by addition of an aliquot of a methanolic stock solution proved unsatisfactory. With a 2 h incubation less than 25% of the [¹²⁵I]azido-cholesterol probe remained associated with the AChR-rich membranes following irradiation and centrifugation. Subsequent experiments revealed that the labeling efficiency was greatly improved by incubating probe that has been dried against the walls of a glass test tube with detergent (cholate)-solubilized AChR-rich membranes. Previous studies have shown that cholate solubilized AChRs retain their ability to undergo agonist-induced conformational transitions and in the absence of agonist the receptor remains in the resting state ([28]; M. Blanton, unpublished data). Following irradiation, cholate-solubilized AChR-rich membrane polypeptides were resolved by SDS-PAGE. The autoradiograph of an 8% polyacrylamide slab gel (Fig. 2) shows there is significant incorporation of [¹²⁵I]azido-cholesterol into each of the AChR subunits. In addition, there is substantial labeling of the α-subunit of the Na/K-ATPase (α_{NK}, ~95 kDa) and the CLC-0 chloride channel (~89 kDa). Addition of the agonist carbamylcholine (Fig. 2, + lane) had no effect on either the pattern of incorporation into individual AChR subunits or the overall labeling pattern of AChR-rich membrane polypeptides. Based upon γ-counting of excised gel bands, [¹²⁵I]azido-cholesterol incorporates into each of the AChR subunits on an equal molar basis. In the absence of agonist the labeling stoichiometry is: (1.03):(0.95):(1):(0.85);
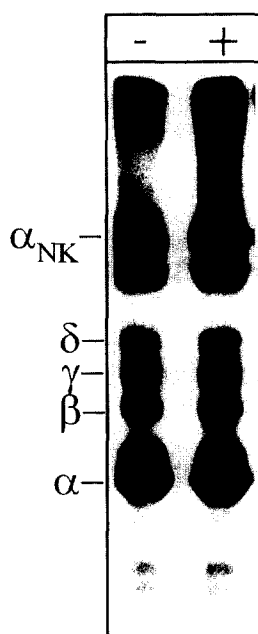

Fig. 1. Chemical structure of [¹²⁵I]azido-cholesterol.

Fig. 2. Photoincorporation of [^{125}I]azido-cholesterol into detergent (sodium cholate) solubilized AChRs in the absence and presence of carbamylcholine. Detergent (sodium cholate)-solubilized AChRs were equilibrated (1 h incubation) with [^{125}I]azido-cholesterol in the absence (− lane) and in the presence (+ lane) of 250 µM carbamylcholine and irradiated at 365 nm (Spectroline EN-280L) for 7 min (at a distance of < 1 cm). Polypeptides were resolved by SDS-PAGE (1.0 mm thick, 8% polyacrylamide gel), visualized by Coomassie blue R-250 staining and subjected to autoradiography (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. Significant [^{125}I]azido-cholesterol incorporation is also evident in the α-subunit of the Na/K-ATPase (α$_{NK}$, ∼95 kDa) and in the CLC-0 chloride channel (∼90 kDa).

and (0.95):(0.90):(1):(0.89) in the presence of agonist (α:β:γ:δ, n = 3).

A second method was used to introduce [^{125}I]azido-cholesterol into affinity-purified AChR lipid reconstituted vesicles. [^{125}I]azido-cholesterol containing liposomes were formed by drying asolectin lipids on the surface of a thick walled glass tube followed by a dried layer of [^{125}I]azido-cholesterol. Aqueous buffer was then added and the solution vortexed and sonicated. Greater than 98% of [^{125}I]azido-cholesterol was transferred to lipid/probe vesicles by this process. The [^{125}I]azido-cholesterol laden liposomes were combined with affinity purified reconstituted receptors and stirred over night at 5°C.

Following incubation with cholinergic ligands, irradiation and centrifugation, greater than 80% of the [^{125}I]azido-cholesterol probe remained associated with the membrane pellet. The labeling of AChR subunits with [^{125}I]azido-cholesterol was confirmed by autoradiographic analysis of dried 8% acrylamide SDS-PAGE gels. As with the labeling of cholate-solubilized AChR-rich membranes, each of the AChR subunits incorporated [^{125}I]azido-cholesterol on a equal molar basis and neither the pattern nor the extent of subunit labeling was affected by the presence of carbamylcholine.
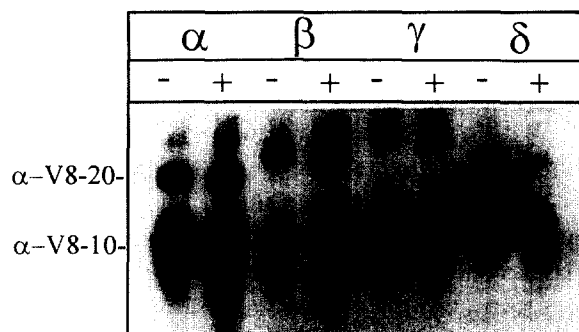

Fig. 3. Proteolytic mapping of the sites of [^{125}I]azido-cholesterol incorporation into AChR subunits using S. aureus V8 protease. Detergent (sodium cholate) solubilized AChRs were labeled with [^{125}I]azido-cholesterol in the absence (− lanes) or presence (+ lanes) of 250 µM carbamylcholine and subjected 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 overlaid with 6 µg of S. aureus V8 protease (see Section 2. 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 principal [^{125}I]azido-cholesterol-labeled proteolytic fragments, following the nomenclature of Blanton and Cohen [26], are: αV8-20 (Ser-173-Glu-338); αV8-10 (Asn-339-Gly-437); βV8-22 (Ile-173/Asn-183-Glu-383); βV8-12 (Met-384-Ala-469); (faint band) 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); (faint band) δV8-20 (Ile-192-Glu-345); δV8-12 (Ile-192-Glu-280); δV8-11 (Lys-436-Ala-501).

3.2. Mapping the sites of [^{125}I]azido-cholesterol labeling in each AChR subunit

Limited digestion of each AChR subunit using *S. aureas* V8 protease reproducibly generates a set of non-overlapping fragments [23,26]. For the AChR α-subunit four principal fragments are generated with apparent molecular weights of 20 kDa (αV8-20, Ser-173-Glu-338), 18 kDa (αV8-18, Thr-52-Glu-172), 10 kDa (αV8-10, Asn-339-Gly-437), and 4 kDa (αV8-4, Ser-1-Glu-51) [23,29]. Inspection of the autoradiograph of a 15% acrylamide mapping gel containing the V8 protease digests of each receptor subunit (Fig. 3) reveals that [^{125}I]azido-cholesterol incorporation within the α-subunit is restricted to the fragments αV8-20 and αV8-10. The αV8-20 (Ser-173-Glu-338) fragment contains the hydrophobic membrane spanning segments M1, M2 and M3 while αV8-10 (Asn-339-Gly-437) contains a large portion of the subunits' cytoplasmic loop as well as the transmembrane segment M4. Inclusion of the agonist carbamylcholine does not alter the pattern of [^{125}I]azido-cholesterol labeling of the α-subunit which remains restricted to αV8-20 and αV8-10 (Fig. 3, + lane). Direct counting of excised gel bands shows that in the absence of agonist 26% of [^{125}I]azido-cholesterol incorporation into the α-subunit is restricted to αV8-20 and 74% to αV8-10. In the presence of agonist, the relative incorporation is very similiar, 20 and 80% respectively. The similarity in the distribution of [^{125}I]azido-cholesterol labeling between the αV8-10 and αV8-20 fragments of the carbamylcholine treated and untreated samples indicates that [^{125}I]azido-cholesterol labeling is not sensitive to the conformational state of the receptor.

Inspection of the autoradiograph of the V8 protease digests of the β-, γ-, and δ-subunits (Fig. 3) reveals significant [^{125}I]azido-cholesterol incorporation into the following fragments, using the nomenclature of Blanton and Cohen [26]: βV8-22 (Ile-173/Asn-183-Glu-383); βV8-12 (Met-384-Ala-469); (faint band) 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); δV8-12 (Ile-192-Glu-280); δV8-11 (Lys-436-Ala-501). Fragments βV8-22 and γV8-24 are homologous to αV8-20 in that they contain the same stretch of amino acid residues in the aligned sequences of each subunit,
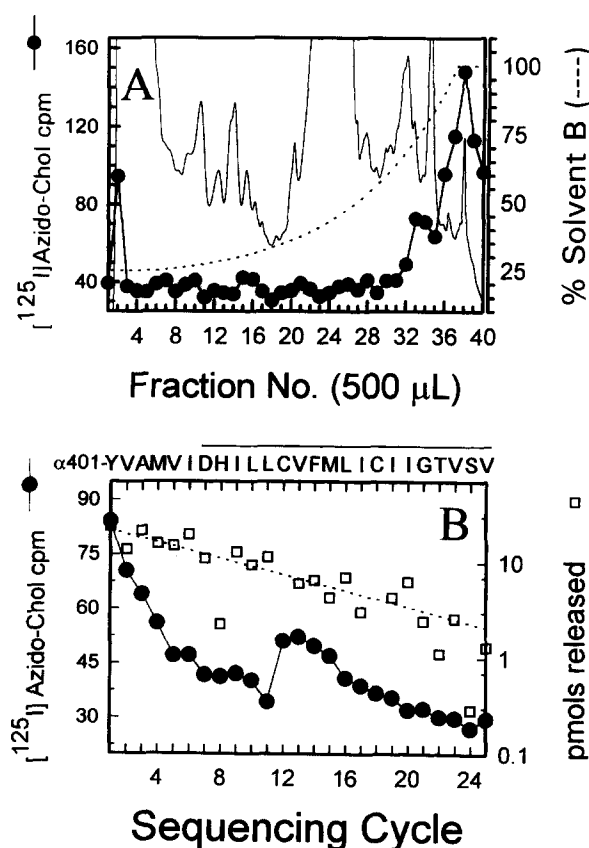

Fig. 4. Reverse-phase HPLC purification and sequential Edman degradation of a [^{125}I]azido-cholesterol labeled fragment containing αM4. The [^{125}I]azido-cholesterol-labeled 10 kDa V8 protease fragment of the AChR α-subunit (αV8-10, isolated from AChR labeled in the absence of carbamylcholine) was further digested in solution with trypsin and the labeled material purified by reverse-phase HPLC (A) on a Brownlee Aquapore C4 column (100×2.1 mm) as described in Section 2. The elution of peptides was monitored by absorbance at 210 nm (solid line) and elution of ^{125}I by γ-counting of each 500 μl fraction (●). HPLC fractions 36–39 were pooled and subjected to automated sequential Edman degradation (B). Sixty percent of each cycle of Edman degradation was analyzed for released ^{125}I (●) 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, 22 pmol; repetitive yield, 90.6%; 6430 cpm loaded on sequencing filter; 1410 cpm remaining after 25 cycles). The amino acid sequence of the peptide is shown above B with the solid line indicating the limits of the M4 region.

which includes the transmembrane segments M1, M2, and M3. Similarly, βV8-12, γV8-14, and δV8-11 are homologous to αV8-10 and contain the transmembrane segment M4.

3.3. Mapping the sites of [^{125}I]azido-cholesterol incorporation to the M4 segments of the α- and γ-subunits and to the M1 segment of the α-subunit

The sites of [^{125}I]azido-cholesterol labeling within the V8 protease subunit fragments αV8-20, αV8-10, and γV8-14 were further defined by exhaustive proteolytic digestion with trypsin and separation of the digests by reverse-phase HPLC. In the HPLC chromatograph of the tryptic digest of αV8-10 (Fig. 4A) the vast majority of ^{125}I cpm elutes in a single peak centered in fraction 38 with a corresponding peak in absorbance eluting at ~93 minutes. 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, 22 pmol; repetitive yield, 90.6%). A secondary sequence was also present beginning at αSer-388, 13 residues upstream (N-terminal) of αTyr-401 (initial yield, 15 pmol; repetitive yield, 80%). In the ^{125}I-release profile (Fig. 4B) 'peptide washoff', that is removal of the [^{125}I]azido-cholesterol labeled peptide from the glass fiber disk (see Section 2, can be seen as a cycle to cycle decline in the level of detected ^{125}I cpm (6430 cpm loaded onto sequencing filter; 1410 cpm remaining after 25 cycles). In cycle 12, there is a small, but significant, increase in the amount of detected ^{125}I cpm which corresponds to [^{125}I]azido-cholesterol incorporation into αCys-412 (2.3 cpm/pmol) within the membrane-spanning segment αM4. Following cycle 12, the level of ^{125}I cpm remains elevated in cycle 13 and this is followed by a rather a slow decline in cpm in cycles 14 and 15. The ^{125}I release in cycles 13–15 may represent [^{125}I]azido-cholesterol incorporation into one or more of the residues within αM4: αVal-413, αPhe-414, and αMet-415. Alternatively, the ^{125}I release may instead reflect the inability of the solvents used in automated sequencing to efficiently extract the [^{125}I]azido-cholesterol labeled PTH-cysteine residue released from the peptide in cycle 12. No other sites of labeling are apparent within the αM4 peptide.

When the tryptic digest of αV8-20 was separated by reverse-phase HPLC (Fig. 5A) the majority of ^{125}I cpm elutes in a peak centered in fraction 38 with a corresponding peak in absorbance eluting at ~92 minutes. HPLC fractions 36–39 were pooled and
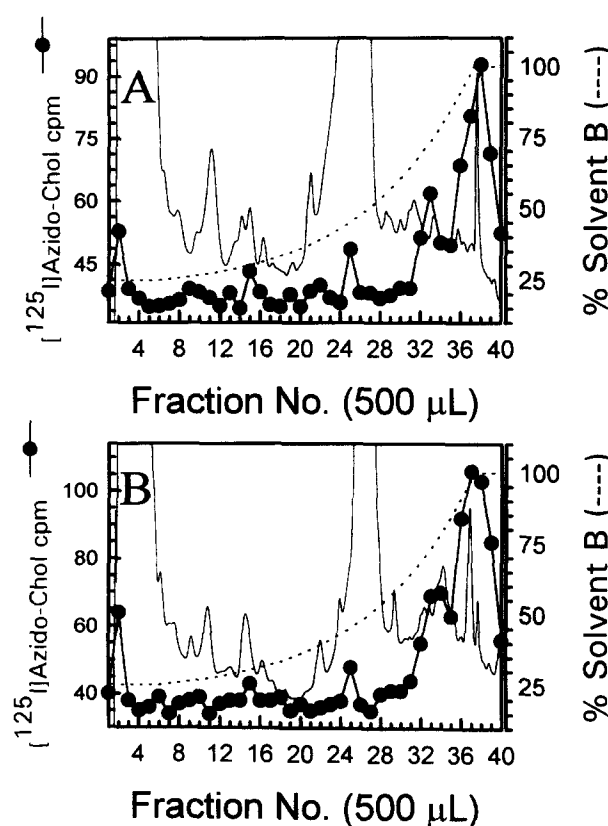

Fig. 5. Reverse-phase HPLC purification of [^{125}I]azido-cholesterol labeled fragments containing αM1 and γM4. The [^{125}I]azido-cholesterol-labeled 20 kDa V8 protease fragment of the AChR α-subunit (αV8-20) and 14 kDa V8 fragment of the γ-subunit (γV8-14) were further digested in solution with trypsin and the labeled material purified by reverse-phase HPLC as described in Section 2 and in the legend to Fig. 4. Elution of ^{125}I was determined by γ-counting of each 500 μl fraction (●) and the elution of peptides by absorbance at 210 nm (solid line). (A) HPLC chromatograph of αV8-20 tryptic digest; (B) HPLC chromatograph of γV8-14 tryptic digest.

subjected to 10 cycles of automated Edman degradation. N-Terminal sequencing identified a single sequence beginning at αIle-210 and extending into the membrane-spanning segment αM1. Based on previous studies establishing the point of elution of an ~3.4 kDa tryptic fragment of αV8-20 containing αM1 [26,27], the αIle-210 peptide extends through αM1 and terminates at αLys-242, which is at the amino-terminal end of the M2 segment. From the HPLC chromatograph of the tryptic digest of γV8-14 (Fig. 5B) HPLC fractions 35–39 were pooled (^{125}I cpm peak in fractions 37/38) and sequenced for 10 cycles. Sequence analysis revealed the presence

of a primary sequence beginning at γVal-446. The γVal-446 peptide extends through the transmembrane segment M4, terminating at either γArg-485 the next available tryptic cleavage site or possibly at the subunit C-terminus (Pro-489). Sequence analysis also revealed the presence of a minor secondary sequence beginning at γGlu-429, 17 residues upstream (N-terminal) to the start of the γVal-446 peptide and also presumably extending through γM4.

4. Discussion

Cholesterol, an essential constituent of most eukaryotic cells, is also necessary for the functional activity of many membrane proteins, including the nicotinic acetylcholine receptor [7,9,30]. While the requirement for cholesterol or more generally sterol [31] for AChR function is well established, the mechanism by which cholesterol modulates the structure/function of the receptor is by no means well understood. Theories of regulation based on membrane fluidity or bilayer structure have for the most part been discounted [32–34]. Cholesterol has also been proposed to stabilize transmembrane segment secondary structure by packing of the rigid sterol ring structure into grooves of transmembrane helices [35,36]. However in more recent structural studies of the AChR no significant differences in receptor secondary structure have been detected in the absence of cholesterol [37]. Obtaining a better understanding of the role of cholesterol in AChR structure/function is further complicated by the lack of a defined binding domain for sterol. It has been proposed that there are non-annular or interstitial binding sites for cholesterol which are distinct from the lipid–protein interface [16] and even extramembrane binding sites [15].

In the present study, we have employed a photoreactive analog of cholesterol [3α-(4-azido-3-[^{125}I]iodosalicylic)-cholest-5-ene ([^{125}I]azido-cholesterol)], to identify the binding domain for cholesterol in the AChR. Based upon the extremely lax structure–activity requirements for cholesterol [31,33,34], [^{125}I]azido-cholesterol serves not only as a probe on the cholesterol binding domain, but is probably also a functional substitute for cholesterol. For example, in reconstitution studies, cholesterol analogs derivat-

ized at the 3′ position with substituents as small as sulfate and as large as phosphatidylcholine, were shown to support AChR activity [34]. Sunshine and McNamee [31] reported that neutral lipids as structurally dissimiliar to cholesterol as vitamin D were also found to support AChR function. In addition, supportive evidence that azido-cholesterol is a functional substitute for cholesterol was obtained by examining the ability of the AChR to undergo conformational transitions in the presence of the cholesterol probe. The pattern and extent of photoincorporation of 3-trifluoromethyl-3-(m[^{125}I]iodophenyl) diazirine ([^{125}I]TID) into the AChR can be used as a diagnostic indicator of the conformational state of the AChR [28,29]. In the presence of 100 μM azido-cholesterol and in the absence of agonist, the pattern and extent of [^{125}I]TID incorporation into receptor subunits was consistent with the AChR being in the resting state conformation (data not shown). The implication of this result is that azido-cholesterol does not shift the conformational equilibrium of the AChR. In addition, as judged by the pattern of [^{125}I]TID incorporation in the presence of both agonist and azido-cholesterol, the cholesterol probe did not inhibit the ability of the receptor to undergo conformational transitions. Together, these results provide indirect evidence that azido-cholesterol supports AChR activation.

In the AChR α-subunit, sites of [^{125}I]azido-cholesterol incorporation are restricted to the membrane-spanning segments αM1 and αM4. Previous studies have established that the M1, M3, and M4 segments of each receptor subunit define the lipid–protein interface of the AChR [26,38,39]. The observation that [^{125}I]azido-cholesterol incorporates into the AChR subunits equally on a molar basis for both native AChR membranes and affinity purified reconstituted receptors indicate that the labeling is proportional to mass and, by implication, to the surface area exposed to lipid. Incorporation of [^{125}I]azido-cholesterol into the α-subunit of the Na/K-ATPase and the CLC-0 chloride channel (Fig. 2), both multi-membrane spanning polypeptides, provides additional support that labeling is proportional to the surface area exposed to lipid. While these results support the existence of a cholesterol binding domain at the AChR lipid–protein interface, they do not support the existence of non-annular binding sites for cholesterol

that are distinct from the lipid–protein interface nor are they consistent with the presence of extramembrane sterol binding sites.

For AChRs labeled with [^{125}I]azido-cholesterol in the presence and in the absence of the agonist carbamylcholine, no significant differences in either the pattern or extent of incorporation into AChR subunits or subunit proteolytic fragments were detected. Similar results were observed with the cholesterol photoaffinity probe [^{3}H]cholesteryl diazoacetate ([19]; but see Fernandez et al. [20]). These data suggest that structural transitions in the receptor upon addition of agonist do not significantly change the surface area of the receptor exposed to the steroid. In addition, cholesterol does not appear to induce any significant secondary structural changes to the AChR transmembrane segments [37]. Therefore, while the presence of cholesterol (sterol) is necessary for the receptor to undergo conformational transitions (reviewed in [30]), these results suggest a mechanism of cholesterol modulation that involves subtle changes in the structure and/or orientation of the lipid-exposed segments. One possibility is that cholesterol may affect the interaction/packing of transmembrane segments [35] and these interactions are in all likelihood critical in facilitating the conformational transitions of the AChR. Localized interactions between the lipid-exposed segments M4, M3, and M1 and the channel-lining M2 segment are clearly important in mediating the agonist-induced transitions necessary for channel opening [40,41]. Substitution of lipid-exposed residues [42] as well as the interaction of agents with the lipid–protein interface [43,44] also appear to modulate these interactions as judged by effects on AChR channel properties. Cholesterol does not appear to act by altering the secondary structure of the membrane-spanning segments in any significant fashion [37], but may instead act as a sort of 'molecular grease' in facilitating subtle structural movements of the transmembrane segments that occur during conformational transitions. A sterol binding site at the interfaces of membrane-spanning segments [45] is consistent with the results of Dreger et al. [15]. In this study, it was found that proteolytic removal of the extramembrane portions of the AChR resulted in a loss of receptor-specific binding sites for the spin-labeled sterol adrostane. The authors interpreted these re-

sults as suggesting the existence of extramembrane sterol binding sites. An alternative explanation is that extramembrane regions and in particular the loops connecting the transmembrane segments are critically important in stabilizing the interaction/packing of membrane-spanning segments. Removal of the loops connecting the transmembrane segments would serve to destabilize their packing and result in a disruption of cholesterol binding sites. Along these lines, future cross-linking studies with analogs of cholesterol may be useful in further defining the sterol binding domain as well as the structure of the AChR transmembrane domain.

In summary, this study represents an important step in elucidating the molecular details of the cholesterol–AChR interaction. Sites of incorporation of the photoactivatable cholesterol analog [^{125}I]azido-cholesterol were localized to transmembrane segments which form the lipid–protein interface of the AChR. Additional work will be necessary to identify specific amino acid residues in each lipid-exposed transmembrane segment which interact with cholesterol. Nonetheless, these results support a possible mechanism for the requirement of cholesterol for AChR function that involves sterol facilitating the packing/interaction of membrane-spanning α-helical segments.

Acknowledgements

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