Topological Dispositions of Lysine α380 and Lysine γ486 in the Acetylcholine Receptor from *Torpedo californica*†

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ABSTRACT: The locations have been determined, with respect to the plasma membrane, of lysine α380 and lysine γ486 in the α subunit and the γ subunit, respectively, of the nicotinic acetylcholine receptor from *Torpedo californica*. Immunoadsorbents were constructed that recognize the carboxy terminus of the peptide GVKYIAE released by proteolytic digestion from positions 378–384 in the amino acid sequence of the α subunit of the acetylcholine receptor and the carboxy terminus of the peptide KYVP released by proteolytic digestion from positions 486–489 in the amino acid sequence of the γ subunit. They were used to isolate these peptides from proteolytic digests of polypeptides from the acetylcholine receptor. Sealed vesicles containing the native acetylcholine receptor were labeled with pyridoxal phosphate and sodium [^{3}H]-borohydride. Saponin was added to a portion of the vesicles prior to labeling to render them permeable to pyridoxal phosphate. The effect of saponin on the incorporation of pyridoxamine phosphate into lysine α380 and lysine γ486 from the acetylcholine receptor in these vesicles was assessed with the immunoadsorbents. The peptides bound and released by the immunoadsorbents were positively identified and quantified by high-pressure liquid chromatography. Modification of lysine α380 in the native acetylcholine receptor in sealed vesicles increased 5-fold in the presence of saponin, while modification of lysine γ486 was unaffected by the presence of saponin. The conclusions that follow from these results are that lysine α380 is on the inside surface of a vesicle and lysine γ486 is on the outside surface. Because a majority (85%) of the total binding sites for α-bungarotoxin bind the toxin in the absence of saponin, the majority of the vesicles are right side out with the inside of the vesicle corresponding to the cytoplasmic surface and the outside of the vesicle corresponding to the extracytoplasmic, synaptic surface. Because lysine α380 and lysine γ486 lie on opposite sides of the membrane, a membrane-spanning segment must be located between the two positions occupied by these two amino acids in the common sequence of a polypeptide of the acetylcholine receptor. Within the 64 amino acids in the common amino acid sequence that separates the positions homologous to α380 and γ486 is a hydrophobic segment of sufficient length to span the membrane.

The nicotinic acetylcholine receptor is a membrane-spanning glycoprotein. Upon binding acetylcholine, it forms a cation-selective channel that connects the cytoplasm of the postsynaptic cell containing the acetylcholine receptor with the synaptic space. This channel allows Na^+ and K^+ to flow in the direction of their respective concentration gradients, and this leads to a local depolarization of the electrochemical potential across the membrane. The protein is constructed from four unique but homologous polypeptide chains (Raftery et al., 1980) designated α, β, γ, and δ in a stoichiometry of $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978), and they are arranged about a pseudo 5-fold axis of symmetry and is thought to be coincident with the channel for cations (Brisson & Unwin, 1985).

The amino acid sequences of the four polypeptides from several species have been deduced from complementary DNA. The α polypeptide from *Torpedo californica* contains 437 amino acids (Noda et al., 1982); the β polypeptide, 469 (Noda et al., 1983a); the γ polypeptide, 489 (Noda et al., 1983b; Claudio et al., 1983); and the δ polypeptide, 501 (Noda et al., 1983a). All of the polypeptides display significant similarity among their amino acid sequences, indicating that they evolved from a common ancestor (Raftery et al., 1980; Noda et al., 1983b). If so, they share a common tertiary structure. Each polypeptide contains four hydrophobic segments, numbered in order of appearance, of sufficient length, about 20 residues, to be candidates for membrane-spanning α-helices. Additionally, each contains a segment of amino acids between the third and fourth hydrophobic segments that displays the pattern predicted for an amphipathic helix. It had been suggested that each amphipathic helix from each subunit spans the membrane and that together they form the lining of the ion channel (Finer-Moore & Stroud, 1984; Guy, 1984), but it has been shown that these regions of the sequences are fully exposed to the aqueous phase (Ratnam et al., 1986b; Roth et al., 1987; Dwyer, 1988).

Central to an understanding of the way the cation channel is formed is the knowledge of the topology of the polypeptide chains as they are folded across the membrane in the native structure. By chemical modification of impermeable vesicles containing the acetylcholine receptor with the impermeant reagent pyridoxal phosphate, the locations of two lysine residues found in the hydrophilic segments flanking the fourth candidate for a membrane-spanning sequence have been determined. Antipeptide antibodies were used to construct immunoadsorbents capable of purifying peptides containing the designated lysine residues. Saponin was used to breach the sealed vesicles in order to afford labeling of the interior portions of the protein. This method was first described by Kyte et al. (1987) and subsequently used by Bayer (1990) to study the topology of the α subunit of sodium ion and potassium ion

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activated adenosinetriphosphatase [$(Na^+ + K^+)$-ATPase].[1]

Experimental Procedures

Materials. *Torpedo californica* were purchased as live specimens from Pacific Biomarine, Inglewood, CA or from Marinus, Inc., Long Beach, CA. Electric organs from freshly sacrificed specimens were immediately frozen in liquid N_2 and stored at −70 °C until use. Amino acid derivatives protected with *tert*-butoxycarbonyl (Boc) groups at their α-amino nitrogens were purchased from Bachem and Beckman; Bio-Gel A-5m, Bio-Gel A-1.5m, Aminex A-5, Affi-Gel-10, and Protein-A Affi-Gel were purchased from Bio-Rad; α-bungarotoxin, Amberlite XAD-4, sodium 3,5-(diacetamido)-2,4,6-triiodobenzoate (sodium diatrizoate), pyridoxal phosphate, saponin, and N^{α}-acetyl-L-arginine were purchased from Sigma; the endoprotease of *Staphylococcus aureus* strain V8, carboxypeptidase Y, and trypsin were purchased from Worthington; and hydrogen bromide was purchased from Aldrich. Sodium [^{3}H]borohydride ($Na[^3H]BH_4$) with a specific radioactivity of 100–500 mCi/mmol was purchased as a dry solid from Amersham. Immediately prior to use, 25 mCi of the solid material was dissolved in 0.01 M NaOH. Sodium dodecyl sulfate ($NaDodSO_4$) was recrystallized from 95% ethanol (Burgess, 1969).

Preparation of Sealed Vesicles. Thawed electroplax (400 g) was added to 500 mL of 250 mM sucrose, 1 mM ethylenediaminetetraacetate (EDTA), 0.1% β-mercaptoethanol, 30 mM sodium borate, and 30 mM sodium phosphate, pH 8.0, and homogenized in two batches in a Waring blender. Further homogenization was performed with a motor-driven Potter-Elvehjem tissue grinder with a Teflon pestle. This crude homogenate was centrifuged at 5000 rpm in a Du Pont-Sorvall GSA rotor for 10 min. The supernatant was filtered through four layers of cheesecloth and then centrifuged at 17000 rpm in a Beckman Type 21 rotor for 1 h. The membrane pellet was resuspended in 250 mM sucrose, 30 mM sodium borate, and 30 mM sodium phosphate, pH 8.0, in a volume of 36 mL. Portions (6 mL) of the membrane suspension were centrifuged over 10–22% gradients of sodium diatrizoate (24 mL/gradient). After centrifugation at 25000 rpm in a Beckman SW 27 rotor for 20 h, the floating band of vesicles was harvested and diluted with approximately five volumes of buffer containing 30 mM sodium borate, 30 mM sodium phosphate, and 250 mM sucrose, pH 8.0, and pelleted at 30000 rpm in a Beckman Ti 45 rotor for 30 min. These sealed vesicles were resuspended in a small volume of the same buffer.

Assay for Toxin Binding. The latent capacity for binding of α-bungarotoxin was measured to estimate the accessibility of binding sites in sealed vesicles in the preparation. α-Bungarotoxin was reductively methylated with $Na[^3H]BH_4$ and formaldehyde as described by Rice and Means (1971). The specific radioactivity of the [^{3}H]methyl-α-bungarotoxin ([^{3}H]-toxin) was determined to be 1.5×10^5 cpm (nmol of toxin)$^{-1}$. Triplicate samples of sealed vesicles (0.04 nmol of sites for the toxin) were incubated with an approximately 10-fold excess of [^{3}H]-toxin in buffer containing 400 mM NaCl, 1 mM EDTA, 0.1% bovine serum albumin, and 10 mM sodium phosphate, pH 7.2, (toxin-assay buffer) in a total volume of 0.2 mL at 25 °C for 1 h. Saponin was added to attain a 0.4% concentration in order to determine the total number of binding sites for the toxin. Toxin-assay buffer was added up to a volume of 1.0 mL, and the samples were spun at 50000 rpm in a Beckman TLA 100.2 rotor for 15 min. The supernatant was discarded, and the pellet was dissolved in 0.1 mL of 88% formic acid and submitted to liquid scintillation.

Labeling of Sealed Vesicles. Sealed vesicles containing the acetylcholine receptor (30 mg of protein) were divided into two pools. To one pool, saponin was added until a 0.4% solution was obtained. Pyridoxal phosphate was added to each to attain a 12 mM concentration, and the vesicles were stirred at room temperature for 15 min. Sodium [^{3}H]borohydride (25 mCi) was dissolved in 0.2 mL of 0.01 M NaOH, and half was added to each pool. The total volume of each reaction was 7.0 mL. After 30 min, the pool of vesicles labeled in the absence of saponin was reapplied to fresh gradients of sodium diatrizoate and centrifuged for 20 h at 25000 rpm in a Beckman SW 27 rotor to remove any vesicles that were broken during the labeling step. The floating band of vesicles was harvested, diluted with buffer, and then pelleted at 30000 rpm in a Beckman Ti 45 rotor for 30 min. The pool of vesicles labeled in the presence of saponin was dialyzed against 250 mM sucrose, 30 mM sodium borate, and 30 mM sodium phosphate, pH 8.0, and then pelleted at 45000 rpm in a Beckman Ti 50 rotor for 30 min.

Labeling of Membranes. Unsealed fragments of membranes enriched in the acetylcholine receptor were prepared as previously described (Dwyer, 1988) with the exception that the alkaline extraction step was omitted. A pool of membranes (50 mg of protein) was split into equal portions, saponin was added to half, and labeling was performed as just described with 12 mM pyridoxal phosphate and $Na[^3H]BH_4$. After being labeled, the membranes were pelleted.

Preparation of Labeled Proteins Free of Phospholipid. The pellets of labeled vesicles (15–25 mg of protein) were dissolved in 4 mL of a solution of $NaDodSO_4$ with at least a 5-fold excess of detergent over protein by weight in 20 mM tris(hydroxymethyl)aminomethane (Tris) sulfate, pH 8.0, and applied to a column (2.6 × 90 cm) of Bio-Gel A-1.5m equilibrated with 40 mM Tris sulfate and 0.1% $NaDodSO_4$, pH 8.0. The column was developed at a flow rate of 10 mL h^{-1}, and the fractions containing protein were pooled and lyophilized. Water was added to the lyophilizates, and they were dialyzed against 0.1 M Tris acetate, pH 8.0. Solid urea was added to attain an 8 M concentration, and the $NaDodSO_4$ was removed by adsorption and anion exchange as described by Nicholas (1984).

Enzymatic Digestions. Labeled protein, stripped of phospholipid and $NaDodSO_4$, was dialyzed against 0.1 M sodium phosphate, pH 7.8. Trypsin or the endoprotease from *S. aureus* strain V8 (Drapeau, 1977) was added at a concentration of 5% relative to the weight of the substrate. Digestions proceeded overnight at 37 °C. The synthetic peptide covalently attached to Affi-Gel-10 was suspended in 10 mL of 0.1 M pyridinium acetate, pH 5.5, and 0.1 mL was taken for analysis by digestion with carboxypeptidase Y (final concentration of 150 μg mL^{-1}). The amount and identity of each amino acid cleaved from the peptide resin were determined by amino acid analysis.

[1] Abbreviations: EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; Boc, *tert*-butoxycarbonyl; $Na[^3H]BH_4$, sodium [^{3}H]borohydride; HPLC, high-pressure liquid chromatography; sodium diatrizoate, sodium 3,5-(diacetamido)-2,4,6-triiodobenzoate; PBS, phosphate-buffered saline, which contains 150 mM NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4; Na HEPES, sodium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; [^{3}H]-toxin, [^{3}H]-methyl-α-bungarotoxin; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; C_{18}, octadecylsilane; PAP, pyridoxamine phosphate; toxin-assay buffer, consisting of 400 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 0.1% bovine serum albumin, pH 7.2; $(Na^+ + K^+)$-ATPase, sodium ion and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3).

Preparation of an Immunoadsorbent Specific for -YVP. The peptide KYVP was prepared by solid-phase peptide synthesis (Stewart & Young, 1969). Chloromethylstyrene beads (3 g) were swollen in 30 mL of dimethylformamide, and 0.73 g of *N*-Boc-L-proline and 0.77 g of potassium fluoride were added to the slurry of beads (Horiki et al., 1978). The mixture was heated at 80 °C for 24 h, and the beads were washed. The amount of *N*-Boc-L-proline attached was determined by amino acid analysis to be 1.4 mmol (g of beads)$^{-1}$. Successive extensions of the peptide chain were accomplished by using the *N*-Boc-amino acids (8 mmol), diisopropylcarbodiimide (8 mmol), and hydroxybenzotriazole (8 mmol) with *N*-methylpyrrolidinone (20 mL) as the solvent. Couplings proceeded for 2 h at room temperature. A portion (1 g) of the uncleaved undeprotected resin, after the coupling of N^{α}-Boc-N^{ϵ}-(benzoxycarbonyl)-L-lysine and deblocking, was modified at the free amino terminus of the attached protected peptide with N^{α}-acetyl-L-arginine. The coupling was performed with N^{α}-acetyl-L-arginine (3 mmol), hydroxybenzotriazole (3 mmol), and diisopropylcarbodiimide (3 mmol) in 15 mL of dimethyl sulfoxide. Another portion (0.1 g) of the same resin was acetylated at the amino terminus of the attached peptide with acetic anhydride. Anhydrous hydrogen bromide was passed through slurries of the different resins in trifluoroacetic acid (TFA) in order to remove and deprotect the finished peptides. Crude peptides were purified by preparative high-pressure liquid chromatography (HPLC) on a Vydac octadecylsilane (C_{18}) column (2.2 × 25 cm). Purified peptides were characterized by amino acid analysis of an acid hydrolyzate as well as exopeptidase digests generated with carboxypeptidase Y [0.4 μg (μg of peptide)$^{-1}$] and leucine aminopeptidase [0.08 μg (μg of peptide)$^{-1}$].

A conjugate of KYVP and bovine serum albumin was prepared by the method of Kagen and Glick (1979). Glutaraldehyde was added to attain a final concentration of 6.7 mM in a solution of bovine serum albumin (6.7 mg mL^{-1}) and the synthetic peptide (5 mM) in 67 mM sodium phosphate, pH 7.5. After 30 min at room temperature, glycine was added, at a final concentration of 77 mM, to block unreacted glutaraldehyde.

Antibodies were produced in white New Zealand rabbits. A 1:1 suspension of the conjugate (5 mg mL^{-1}) and Freund's incomplete adjuvant was injected into the lymph nodes of the hind legs initially, and a subsequent boosting dose was given subcutaneously after four weeks.

An affinity column was prepared with KYVP and succinylated agarose activated with *N*-hydroxysuccinimide (Affi-Gel-10). Crude KYVP (20 mg) was dissolved in 0.1 M sodium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethane sulfonate (Na HEPES), pH 7.5, and 2 mL of washed beads were added. The slurry was shaken overnight at 4 °C. The beads were washed and suspended in 0.1 M pyridinium acetate, pH 5.5. A portion (0.2 mL) of the slurry was removed and mixed with carboxypeptidase Y (10 units), and the enzymatic digestion proceeded for 5 h at 37 °C. The digest was submitted to amino acid analysis, from which the capacity of the peptide affinity resin was determined to be 3.7 μmol of peptide (mL of resin)$^{-1}$.

Fresh antiserum was applied to the peptide affinity column in order to purify anti-YVP antibodies. Nonspecifically bound proteins were washed from the column with PBS, which contains 150 mM NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4, and the specifically bound antibodies were eluted with 0.1 M sodium phosphate, pH 2.5. The purified antibodies were dialyzed against PBS, precipitated with 50% ammonium sulfate, and stored at 4 °C until use. Precipitated antibodies were dissolved in PBS and dialyzed against PBS to remove ammonium sulfate. The reconstituted antibodies (5 mg in 2.5 mL) were attached to 2 mL (settled volume) of Sepharose beads activated with CNBr (March et al., 1974). The capacity of the immunoadsorbent (3 nmol) was determined by binding an excess of the synthetic peptide, rinsing the column, eluting the bound peptide with 0.1 M sodium phosphate, pH 2.5, and determining the yield by amino acid analysis.

Immunoadsorption. Digests containing between 0.5 and 2 nmol of the indigenous peptide in 2 mL of 0.1 M sodium phosphate, pH 7.8, were applied to an immunoadsorbent. The nonspecifically bound peptides were rinsed from the immunoadsorbent with 10 mL of PBS. Specifically bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5, and the first 2 mL of acidic eluate were submitted directly to HPLC. The Vydac C_{18} column (0.46 × 25 cm), equilibrated in 0.05% aqueous TFA, was developed with a linear gradient of 0.02% TFA in acetonitrile at a rate of increase of 1% min^{-1}. Fractions containing the purified peptides were counted by liquid scintillation.

Analytical Methods. Amino acid analysis was performed on a Beckman 118C amino acid analyzer. Samples were hydrolyzed in 6 M HCl under vacuum for 24 h at 110 °C or for 40 min at 155 °C. Radioactivity was measured with a Beckman LS-250 or a Beckman LS-1701 liquid scintillation counter. High-pressure liquid chromatography was performed on a system comprising two Waters M6000 pumps, a Waters 680 gradient controller, a Waters 440 detector fitted with an external wavelength module that operates at 229 nm, and a Waters U6K injector. Vydac C_{18} columns (0.46 × 25 cm and 2.2 × 25 cm) were used for HPLC. Amino acid sequences were determined with an Applied Biosystems gas-phase sequenator.

Results

Sealed Vesicles. The determination of the topology of a membrane-spanning protein by vectorial chemical modification requires a preparation of vesicles predominantly right side out or inside out and impermeable to the reagent used for chemical modification. Kyte et al. (1987) used a method initially described by Forbush (1982) to prepare sealed right-side-out vesicles from microsomes derived from the canine renal medulla in order to demonstrate the cytoplasmic location of lysine 501 from the α subunit of (Na^+ + K^+)-ATPase. In this procedure, sealed vesicles float during centrifugation at 25 000 rpm in a Beckman SW 27 rotor on a gradient of the radioopaque salt sodium diatrizoate, while unsealed vesicles sink. This method can also be used to prepare sealed vesicles containing the acetylcholine receptor from *T. californica* electroplax. Radioactive α-bungarotoxin was used to determine the relative amounts of vesicles containing the acetylcholine receptor that float on or sink through gradients of sodium diatrizoate.[2] Only a fraction (20%) of the binding sites for the toxin present in the microsomes derived from the electroplax were in vesicles that floated on the gradients of sodium diatrizoate. When these vesicles that had floated were submitted to a second centrifugation on a gradient of sodium diatrizoate, after treatment with 0.4% saponin, almost none (1%) of the binding sites for the toxin were still in vesicles that floated on the gradient. In a control sample that was not exposed to saponin, almost all (90%) of the binding sites for toxin were in vesicles that still floated during the second

[2] K. Ewalt, personal communication.

centrifugation. These results are consistent with the conclusions that vesicles containing the acetylcholine receptor float on top of the gradients of sodium diatrizoate because they are sealed and that the addition of saponin, which renders the vesicles permeable, causes the previously sealed vesicles to sink.

Saponin is a heterogeneous preparation of botanical glycosides that creates holes (Bangham & Horne, 1962) in membranes that are large enough to allow ferritin to pass through while having no effect on the ability of the acetylcholine receptor to bind α-bungarotoxin (St. John et al., 1982). The enzymatic activity of $(Na^+ + K^+)$-ATPase, which is inactivated by deoxycholate at concentrations greater than 2 mg mL^{-1}, is insensitive to saponin at concentrations of 10 mg mL^{-1} or less (Kyte et al., 1987), supporting the claim that saponin does not alter the tertiary structure of a membrane protein.

α-Bungarotoxin binds to the extracytoplasmic surface of the acetylcholine receptor and can be used to measure the accessibility of this surface of the protein in the sealed vesicles. In the absence of saponin, the number of binding sites for the toxin on the outside of the sealed vesicles can be measured; and in the presence of saponin the total number of binding sites for the toxin can be measured. When sealed vesicles were assayed with [^{3}H]methyl-α-bungarotoxin, a majority of the binding sites for the toxin were found to be accessible from the outside. This assay was repeated for several preparations of sealed vesicles, and the range of the percentage of accessible binding sites was 70–85%. α-Bungarotoxin can also be used to determine the amount of acetylcholine receptor present in the membrane preparation relative to other proteins. In these vesicle preparations, the concentration of binding sites for the toxin was determined to be 1 nmol $(2.5 \times 10^6$ ng protein$)^{-1}$.

Preparation of the Immunoadsorbents. The immunoadsorbent that binds peptides containing the carboxy-terminal sequence -YIAE and that was used to isolate the peptide α(378–384),[3] GVKYIAE, from digests of the acetylcholine receptor performed with the endoprotease from *S. aureus* strain V8 has been described previously (Dwyer, 1988). Briefly, affinity-purified polyclonal antipeptide antibodies, obtained from rabbits that had been immunized with a conjugate of bovine serum albumin and the synthetic peptide KYIAE, were covalently attached to Sepharose beads (March, 1974). The capacity of this immunoadsorbent is 14 nmol (mL of packed agarose)$^{-1}$, and the same immunoadsorbent has been used repeatedly over four years.

Another immunoadsorbent was constructed in order to isolate the tryptic peptide K(X)YVP, where X indicates that the lysine is modified. This peptide, γ(486–489), comprises the carboxy terminus of the γ polypeptide of the acetylcholine receptor. The peptide KYVP was synthesized by solid-phase methods and purified by preparative HPLC. The amino acid composition of the purified peptide following hydrolysis with acid was $K_{0.89}$ $Y_{0.94}$ $V_{1.06}$ $P_{1.08}$, following digestion with carboxypeptidase Y was $K_{0.02}$ $Y_{0.08}$ $V_{0.85}$ $P_{1.00}$, and following digestion with leucine aminopeptidase was $K_{1.00}$ $Y_{1.26}$ $V_{0.09}$ $P_{0.09}$. A conjugate of bovine serum albumin and the synthetic peptide was constructed by using glutaraldehyde (Kagen & Glick, 1979). A total of 8 nmol of peptide was covalently attached to every nanomole of bovine serum albumin, as determined from total amino acid analysis. This conjugate was used to immunize rabbits for the production of polyclonal antibodies. The anti-KYVP antibodies were isolated from freshly collected serum by affinity chromatography using agarose beads to which the synthetic peptide had been covalently attached. Attachment of the purified polyclonal antibodies to agarose (March et al., 1974) yielded an immunoadsorbent with a capacity of 3 nmol (1.5 mL of packed beads)$^{-1}$.

[3] Peptides digested from intact polypeptides of the acetylcholine receptor are identified by their sequence positions in the subunit from which they were derived.

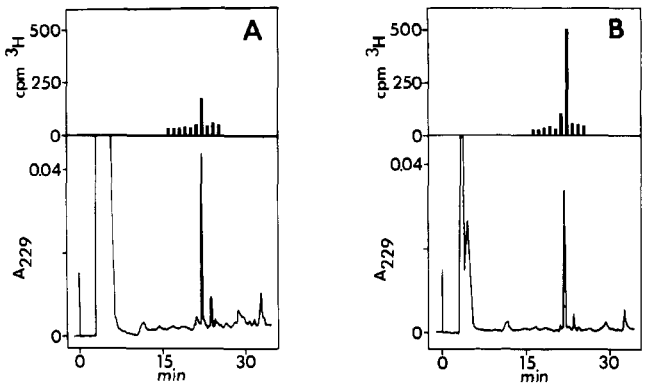

FIGURE 1: Labeling of lysine α380 in the native acetylcholine receptor. Portions of phospholipid-free membrane proteins obtained from sealed right-side-out vesicles labeled with pyridoxal phosphate and Na[^{3}H]BH_4 in the absence (A) or presence (B) of saponin were digested with the endoprotease from *S. aureus* strain V8, and a portion of the digests was submitted to immunoadsorption on the immunoadsorbent that recognizes the carboxy-terminal sequence -YIAE. The unbound peptides were rinsed away with PBS, and the bound peptides were eluted from the immunoadsorbent with 0.1 M sodium phosphate, pH 2.5. The eluates were submitted to HPLC on a Vydac C_{18} column (0.46 × 25 cm) equilibrated in 0.05% TFA in water. The column was eluted with a linear gradient increasing at 1% acetonitrile min^{-1} between solvent A (0.05% TFA in water) and solvent B (0.02% TFA in acetonitrile) in 40 min at a flow rate of 1 mL min^{-1}. The absorbance as a function of time is presented in the lower panels. The effluent from the flow cell of the spectrophotometer was collected in 1-mL fractions that were then submitted to liquid scintillation (top panels). The top and bottom panels in each graph are from the same chromatographic run.

Specific Radioactivity of the Peptide α(378–384) Isolated from Vesicles Labeled with Pyridoxal Phosphate. The amino acid sequence surrounding lysine α380 in the α polypeptides of the acetylcholine receptor from *T. californica* is -AIEGVKYIAEHM-. Digestion of the α polypeptide with the endonuclease from *S. aureus* strain V8 produces the peptide GVKYIAE that can be isolated by immunoadsorption (Dwyer, 1988). Sealed and predominantly right-side-out vesicles were labeled with 12 mM pyridoxal phosphate and Na[^{3}H]BH_4 in the presence and absence of 0.4% saponin. The labeled protein was separated from the phospholipid by molecular-exclusion chromatography in 0.1% $NaDodSO_4$ and 40 mM Tris sulfate, pH 8.0, and subsequently stripped of $NaDodSO_4$. Pools of the protein were then digested with the endoprotease from *S. aureus* strain V8. Samples of each pool were applied to the immunoadsorbent that recognized the carboxy-terminal sequence, -YIAE, and the specifically bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5. The acidic eluates were submitted to HPLC on a Vydac C_{18} column developed with a linear gradient between 0.05% TFA in water and 0.02% TFA in acetonitrile. The first chromatogram (Figure 1A) is that of the peptides obtained from vesicles labeled in the absence of saponin. The major peak of absorbance, which eluted at 21% acetonitrile, has been identified as GVKYIAE (Dwyer, 1988) by submitting another sample of this peptide, obtained by immunoadsorption from a digest of the acetylcholine receptor isolated by the procedure of Elliot et al. (1980), to automated Edman degradation [(cycle number) identity, and picomoles of all phenylthiohydantoin (PTH) derivatives observed were (1) G, 217; P, 4.4; (2) V, 256; G, 19; (3) K, 275, V, 22; (4) Y, 286; K, 23; (5) I, 219; Y, 20; (6) A, 219; I, 19; and (7) E, 72; A, 25]. The specific radioactivity of this major

Table I: Specific Radioactivities of [^{3}H]Pyridoxamine Phosphate Containing Target Peptides[a]

	GVKYIAE			KYVP			
expt	−saponin	+saponin	+saponin / −saponin	−saponin	+saponin	+saponin / −saponin	% of outside-accessible toxin-binding sites[b]
1[c]	1250	6900	5.5	19000	21000	1.1	ND[e]
2[c]	3400	17000	5.0	10900	11600	1.1	87
3[c]	3050	11400	3.7	ND	ND	ND	84
4[c]	3700	19100	5.2	ND	ND	ND	84
5[d]	24700	27900	1.1	ND	ND	ND	ND

[a] The cpm of tritium associated with the appropriate peaks in chromatograms analogous to those displayed in Figure 1 and Figure 2 (which themselves comprise experiment 2) were divided by the height of the peak of absorbance associated with the corresponding unlabeled peptides. [b] The percent of outside-accessible toxin-binding sites is the ratio of the number of toxin-binding sites measured in the absence of saponin divided by the number of toxin-binding sites measured in the presence of saponin times 100. [c] These experiments were performed with sealed, right-side-out vesicles. [d] This experiment was performed with microsomes derived from *T. californica* electroplax that are mostly unsealed as judged by their inability to float on a solution of sodium diatrizoate. [e] Not determined.

peptide, bound and released by the immunoadsorbent and eluting at 21% acetonitrile (21 min) during HPLC, was determined to be 3400 cpm A_{229}^{-1}, where A_{229} is the maximum absorbance of the peak on the HPLC profile. Treatment of the sealed vesicles with saponin prior to labeling resulted in an increase in the specific radioactivity of the purified peptide (Figure 1B) of 5-fold to 17000 cpm A_{229}^{-1}. Both the radioactivity and the absorbance at 229 nm that cochromatographed with it were shown to be specifically bound by the immunoadsorbent since addition of a large excess (500 nmol) of the synthetic peptide KYIAE to a portion of the digest caused both to disappear (<7% remained). The results from several experiments are summarized in Table I.

Specific Radioactivity of the Peptide γ(486–489) Isolated from Vesicles Labeled with Pyridoxal Phosphate. The amino acid sequence at the carboxy terminus of the γ polypeptide of the acetylcholine receptor from *T. californica* is -GDPRKYVP, in which the lysine is lysine γ486. Digestion with trypsin of the γ polypeptide modified by a reagent X at lysine γ486 should produce the peptide K(X)YVP, and digestion with trypsin of the unmodified γ polypeptide should produce the peptide YVP. In order to determine the retention times of the products expected from immunoadsorption of a tryptic digest of the modified and unmodified γ polypeptide, the peptides N^{α}-acetyl RKYVP and N^{α}-acetyl KYVP were synthesized. Cleavage of N^{α}-acetyl KYVP with trypsin yielded one product, identified by amino acid analysis as YVP. Even though it could be resolved, this tripeptide migrated on HPLC less than 0.5 min faster than the peptide KYVP. Cleavage of N^{α}-RKYVP with trypsin yielded two products, the major one (75%) being YVP and the minor one (25%) being KYVP. The synthetic peptide N^{α}-acetyl RKYVP was modified with pyridoxal phosphate (1.6 M, pH 8.0, 15 min) and sodium cyanoborohydride (3.2 M, 30 min) and subsequently digested with trypsin. The product of the digestion, presumably K-(PAP)YVP, where PAP indicates that pyridoxamine phosphate is covalently attached to the lysine, was bound and released by the immunoadsorbent that recognizes the carboxy terminus -YVP[2] and migrated in the same position of the chromatogram as KYVP. It displayed an absorbance at 313 nm as expected of a pyridoxylated peptide. Therefore, the addition of pyridoxamine phosphate to the peptide KYVP does not alter its retention time on HPLC. The results of these experiments indicate that KYVP and K(PAP)YVP migrate in the same place on HPLC and that they migrate within 0.5 min of YVP.

The fact that addition of pyridoxamine phosphate to the peptide KYVP has no effect on its mobility on HPLC provides an explanation for the coincidence of the peak of cpm, from the peptide GVK([^{3}H]PAP)YIAE, and the peak of A_{229}, from

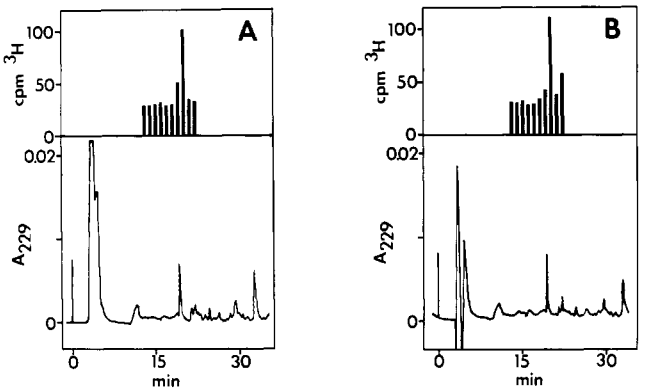

FIGURE 2: Labeling of lysine γ486 in the acetylcholine receptor. Portions of phospholipid-free membrane proteins obtained from sealed right-side-out vesicles labeled with pyridoxal phosphate and Na[^{3}H]BH_4 in the absence (A) or presence (B) of saponin used in the experiment described in Figure 1 were treated with trypsin and submitted to immunoadsorption on the immunoadsorbent that recognizes the carboxy-terminal sequence -YVP. The specifically bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5. The eluates were submitted to HPLC on a Vydac C_{18} column (0.46 × 25 cm) equilibrated in 0.05% TFA in water. The column was eluted with a linear gradient increasing at 1% acetonitrile min^{-1} between solvent A (0.05% TFA in water) and solvent B (0.02% TFA in acetonitrile), in 40 min at a flow rate of 1 mL min^{-1}. The absorbance as a function of time is presented in the lower panels. The effluent from the flow cell of the spectrophotometer was collected in 1-mL fractions that were then submitted to liquid scintillation (top panels). The top and bottom panels in each graph are from the same chromatographic run.

the peptide GVKYIAE, on the chromatograms in Figure 1. Evidently, the mobility on the reversed-phase chromatographic medium is more strongly influenced by the hydrophobic amino acids in the peptides than by the hydrophilic pyridoxamine phosphate.

Portions of the phospholipid-free proteins obtained from sealed vesicles labeled with pyridoxal phosphate and Na[^{3}H]BH_4 in the presence and absence of saponin, which were used in the experiment described above, were digested with trypsin. The digests were applied to the immunoadsorbent that recognizes the carboxy-terminal sequence -YVP, eluted with 0.1 M sodium phosphate, pH 2.5, and submitted (Figure 2) to HPLC on a Vydac C_{18} column developed with a linear gradient between 0.05% TFA in water and 0.02% TFA in acetonitrile. Both the major bound and eluted peptide and the major bound and eluted peak of counts of tritium eluted within 1 min of each other and in the same place (19 min) on the HPLC profile as the synthetic peptide KYVP. Addition of the synthetic peptide KYVP (500 nmol) to a sample of a digest prior to immunoadsorption eliminated all of the bound radioactivity (<5% remained). This result offers evidence that

the bound tritium was attached to a peptide that had the carboxy-terminal sequence -YVP. The specific radioactivities calculated for the labeled peptide γ(486–489) isolated from vesicles labeled in the absence (Figure 2A) or presence (Figure 2B) of saponin were 10900 cpm A_{229}^{-1} and 11600 cpm A_{229}^{-1}, respectively. Table I presents the results of two independent experiments.

Specific Radioactivity of the Peptide α(378–384) Isolated from Membranes Labeled with Pyridoxal Phosphate. Since labeling of lysine 380 of the α subunit in sealed vesicles increases upon the addition of saponin, the possibility that saponin caused an increase in the reactivity of lysine 380 by partially denaturing the native protein had to be considered. Microsomes containing the acetylcholine receptor that were predominately unsealed were labeled. The conclusion that the majority of membranes containing the acetylcholine receptor (80%) in the microsomal pool were unsealed comes from the fact that the majority of the binding sites for α-bungarotoxin in microsomes from *T. californica* electroplax do not float when submitted to centrifugation on gradient of sodium diatrizoate. Virtually no difference in specific radioactivity (Table I, experiment 5) were measured for the peptide α(378–384) labeled in the native protein in these unsealed membranes in the presence or absence of saponin.

Discussion

It is possible to determine whether or not a segment of a protein spans the membrane by chemical modification of sealed, oriented vesicles containing the membrane-bound protein in its native state. Impermeant electrophiles, bearing either a chromophore or radioactivity that permits easy detection, may be used to modify amino acids found in the hydrophilic regions that flank the membrane-spanning segments. Lysine is the most obvious nucleophile to act as a target since it has been shown to be located almost exclusively in regions of proteins exposed to water (Chothia, 1976). Should a pair of lysines be shown to exist on opposite sides of the lipid bilayer, then it must be the case that a membrane-spanning segment separates the pair.

Lysine α380 is found in the long hydrophilic region that separates the third and fourth candidates for membrane-spanning segments in the α subunit of the acetylcholine receptor. Addition of 0.4% saponin to sealed vesicles results in a 5-fold increase in incorporation of [^{3}H]pyridoxamine phosphate into lysine α380 (Table I). Conversely, when predominantly open membranes were labeled in the presence and absence of saponin, no difference in incorporation was seen (Table I, experiment 5). The results of this second experiment, in addition to that fact that 0.4% saponin does not decrease the maximal activity of the membrane-spanning enzyme, (Na^+ + K^+)-ATPase (Kyte et al., 1987), lead to the conclusion that saponin simply makes holes in the membrane and the only effect of these holes is to provide the label with equal access to the two sides of the membrane. Since a majority of the toxin-binding sites, which are markers for the synaptic side of the acetylcholine receptor, are accessible to [^{3}H]-toxin in the absence of saponin, the majority of the sealed vesicles used in these experiments were right side out. Because saponin significantly increases the incorporation of [^{3}H]pyridoxamine phosphate into lysine α380, this amino acid residue must be located on the cytoplasmic surface of the acetylcholine receptor.

Lysine γ486 is contained within the last five amino acids, -RKYVP, of the γ subunit of the acetylcholine receptor. Two potential cleavage sites for trypsin are contained within this sequence: arginine γ485 and lysine γ486. Proteolytic digestion of the γ polypeptide with trypsin could occur at either of these two sites when lysine γ486 is unmodified, but when lysine γ486 is modified with pyridoxamine phosphate, only cleavage at arginine γ485 can occur. Consequently, digestion of a mixture of γ polypeptides, some labeled and some unlabeled at lysine γ486, would be expected to yield the following peptides that contain the carboxy-terminal sequence YVP: YVP, KYVP, and K(PAP)YVP. All of these peptides, however, have mobilities within 0.5 min of each other on HPLC. The counts of radioactivity that migrate with the peak of absorbance (Figure 2) appear in the same position of the chromatogram as does the synthetic peptide KYVP. Furthermore, addition of a large excess of KYVP to the digest prior to immunoadsorption eliminates these counts of radioactivity from the chromatogram. Modification of KYVP by pyridoxamine phosphate does not alter its retention time in reversed-phase HPLC. There is little doubt that the counts of tritium and the peaks of absorbance in Figure 2 represent peptides containing the carboxy-terminal -YVP and lysine γ486. Since no significant increase in incorporation of [^{3}H]pyridoxamine phosphate into lysine γ486 was observed when saponin was present during labeling (Figure 2, Table I) in the same vesicles in which the labeling of lysine α380 increased 5-fold, lysine γ486 must be located on the outside surface of the right-side-out vesicles.

Proteins homologous in sequence always share structural homology because homology in sequence disappears during evolution before structural homology. This fact is illustrated by a comparison of crystallographic molecular models and the amino acid sequences of dihydrofolate reductases from *Escherichia coli* and *Lactobacillus casei* (Bolin et al., 1982). Although these two proteins share only 27% identical residues, their crystallographic molecular models are virtually indistinguishable. The topological results reported in the present study are for different subunits of the acetylcholine receptor. Because of the homology in sequence (an average of 40% identity in pairwise comparisons) displayed among the four polypeptides (Noda et al., 1983b), the topological assignment made for a particular amino acid in one subunit may be extended to the homologous regions of the others. Asparagine γ421 is located in the amino acid sequence of the γ polypeptide at the position homologous to that occupied by lysine α380 in the α polypeptide. Between asparagine γ421 and lysine γ486 are 64 amino acids. Within this segment of the amino acid sequence in each of the polypeptides is a conserved hydrophobic segment of sufficient length to span the lipid bilayer as an α-helix. This is the segment referred to as M4 by Noda et al. (1983b). Because lysine α380 is on the cytoplasmic surface and lysine γ486 is on the extracytoplasmic surface and the same must be true for the homologous positions in all of the subunits, this candidate for a membrane-spanning segment must span the membrane in every subunit in the native protein.

It has been reported that antipeptide antisera have, on occasion, displayed affinity for large polypeptides dissimilar in sequence to the small peptide used as the immunogen (Maelicke et al., 1989). In order to minimize this possibility, the antipeptide immunoglobulins that were used to construct the immunoadsorbents used here were purified by affinity chromatography on synthetic peptides immobilized on beaded agarose and the modified products were identified chromatographically following the immunoadsorption. Furthermore, both the antigens and the immobilized peptides used to purify the immunoglobulins were identical in structure to the portion of the indigenous peptide that was recognized by the final immunoadsorbent.

Several experimental approaches have been used to identify the segments of the amino acid sequences of the subunits of the acetylcholine receptor that span the membrane. These include chemical modification, identification of sites of posttranslational modification, and the use of antipeptide antibodies as probes for determining the relative positions of the hydrophilic regions that flank the membrane-spanning segments. The locations of some of the regions of the subunits can be assigned on the basis of the results of protein chemical studies. Using the affinity label [4-(*N*-maleimido)benzyl]-tri[^{3}H]methylammonium iodide, Kao et al. (1984) demonstrated that cysteine α192 or cysteine α193 or both were modified after reduction and are within the binding site for agonists. All of the subunits of the acetylcholine receptor have been shown to contain a conserved cystine immediately adjacent to a conserved and glycosylated asparagine in locations homologous to cysteine α128, asparagine α141, and cysteine α142 (Kao & Karlin, 1986; Kellaris & Ware, 1989). The immunogenic region responsible for the autoimmune disorder myasthenia gravis has been mapped to amino acid residues 67–76 of the α subunit (Tzartos et al., 1988). The conclusion that follows from these results is that the region amino terminal to the first candidate for a membrane-spanning sequence is in the extracytoplasmic portion of the protein. This conclusion, however, has been challenged by some immunochemical evidence suggesting that a region between isoleucine α148 and cysteine α192 is on the cytoplasmic surface of the protein (Criado et al., 1985; Pederson et al., 1990).

Lipophilic precursors of nitrenes and carbenes have been used in an attempt to modify the membrane-spanning domains of the acetylcholine receptor. Synthetic phospholipids containing arylazide functional groups were used to modify the membrane-bound acetylcholine receptor from *Torpedo marmorata* and *T. californica* (Giraudet et al., 1985). While all of the polypeptides from both species become modified, the α polypeptide of *T. marmorata* was labeled more than the other three. The location of the label was restricted to a peptide 70 amino acids in length believed to contain the carboxy terminus, but no unequivocal identification of the modified residues was made. White and Cohen (1988) used 3-trifluoro-3-(*m*-[^{125}I]iodophenyl)diazirine to label membranes rich in the acetylcholine receptor and isolated two labeled peptides that included the second, third, and fourth candidates for membrane-spanning segments, but none of the labeled residues was identified.

The location of the carboxy termini of the subunits is a matter of uncertainty. Immunological evidence places the carboxy termini on the cytoplasmic surface of the protein (Lindstrom et al., 1984; Young et al., 1985; Ratnam et al., 1986a,b). The disulfide bond that exists between pairs of δ subunits has been shown to be on the extracytoplasmic surface (McCrea et al., 1987; DiPaola et al., 1989), and this disulfide has been shown to be formed from two penultimate cysteines of the two linked δ polypeptides (DiPaola et al., 1989). The region between the third and fourth candidates for membrane-spanning sequences is generally believed to be on the cytoplasmic surface (LaRochelle et al., 1985; Ratnam et al., 1986b), but an extracytoplasmic assignment for this region has been reported (Moore et al., 1989).

Several topological models have been proposed for the nicotinic acetylcholine receptor, and most include the fourth hydrophobic segment as spanning the membrane (Devillers-Thiery et al., 1983; Noda et al., 1983b; Guy, 1984; Finer-Moore & Stroud, 1984; Criado et al., 1985), though it has been placed elsewhere by some (Ratnam et al., 1986b; Maelicke et al., 1988). Given that both the amino terminus and the carboxy terminus of each subunit lie on the extracytoplasmic side of the membrane, there must be an even number of membrane-spanning segments. This would seem to exclude those models that contain an odd number of membrane-spanning segments.

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CORRECTION

Oligomeric Protein Associations: Transition from Stochastic to Deterministic Equilibrium, by Leonardo Erijman and Gregorio Weber*, Volume 30, Number 6, February 12, 1991, pages 1595–1599.

Page 1597. Equations 14a and 14b should be replaced by

$$d\alpha = [1 - \alpha - 4\alpha^2(C/K_0)\exp(p_{1/2}\Delta V/RT)] \times [k_- \exp(fp_{1/2}\Delta V/RT)C]\, dt \quad (14)$$

Further reference in the text to eq 14b should be to eq 14.