Epitope Mapping Employing Antibodies Raised against Short Synthetic Peptides: A Study of the Nicotinic Acetylcholine Receptor†

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ABSTRACT: Antibodies were raised against eight synthetic peptides matching preselected portions of the amino acid sequence of nicotinic acetylcholine receptor (nAChR) from *Torpedo marmorata*. To increase the probability of obtaining antibodies specific for the exact sequence of the immunizing peptide, peptides of only five to seven amino acids in length were employed. Even under these limiting conditions some of the polyclonal rabbit immune sera showed cross-reactivity with other peptides and/or other sequence regions of the receptor. Further studies with polyclonal and monoclonal sera suggested that conformation and charge pattern rather than linear sequence are the essential determinants of antibody epitopes. Application of antibodies for topological studies therefore requires that the antibody specificity for a particular region of the antigen has been firmly established. Epitope mapping with the eight anti-peptide immune sera provides information on the accessibility to antibody of matching sequences within the receptor molecule. We find the sequence portions α81–85, α127–132, and α190–195 to be freely accessible both at membrane-bound and at purified receptor. Binding of anti-α387–392 serum does not prove accessibility of this region as the serum cross-reacts strongly with peptide fragments corresponding to the regions α165–200 and β190–200 of nAChR from *Torpedo californica*. To permit binding of anti-α137–142 immune serum, treatment of the receptor with endoglycosidase is required, showing that Asn-141 indeed is glycosylated in native nAChR. The homologous sequence of the other subunits differing only in one sequence position from α137–142 is not accessible in native nAChR to antibody, indicating clear differences in folding of the receptor polypeptides. Sequence portions α395–401 and α161–166 must first be exposed by appropriate treatment to permit binding of respective serum. These results and previous epitope mapping studies by other laboratories are discussed with respect to the limited sequence specificity of antibodies.

Our present view of the membrane topology of nicotinic acetylcholine receptors heavily depends on hydropathy plots (Kyte & Doolittle, 1982; Noda et al., 1982; Claudio et al., 1983) and epitope mapping (Lindstrom et al., 1984; Maelicke et al., 1984; Neumann et al., 1984; Ratnam & Lindstrom, 1984; Plümer et al., 1984; Young et al., 1985; Fuchs & Safran, 1986; Ratnam et al., 1986a,b). The latter is usually performed with antibodies raised against rather long synthetic peptides. We have criticized this approach (Maelicke et al., 1984; Plümer et al., 1984) as it may lead to ambiguous results: Recent structural data have established that the antigen recognition site of antibodies is quite large and that antibody–antigen interaction probably involves multipoint attachment (Amit et al., 1986). Thus, only under limiting conditions will the charge density pattern defining an antibody binding site be directly correlated to the primary structure of this particular region of the antigen.

One way of achieving such favorable conditions may be to employ synthetic peptides just long enough to evoke an immune response (Plümer et al., 1984), i.e., four to six amino acids coupled to a carrier protein. In contrast, the more a peptide exceeds this limiting size required to evoke an individual immune response, the less likely will the related antibodies recognize the *linear* sequence. As *any combination* of a few (Tzartos et al., 1981) amino acid residues of a longer peptide may form the recognition points of an epitope, the statistical probability that an anti-peptide antibody exclusively binds to sites formed by the matching sequence of the antigen is considerably lowered. The problem may be reduced by testing the sequence specificity of antibodies against shorter fragments of the original (long) peptide (Lindstrom, 1986). As the best solution, antibodies should generally be tested against a set of peptides which (i) cover overlappingly the whole primary structure of the antigen and (ii) are each sufficiently long to assume conformations expected to also exist in the intact antigen molecule.

We have tried to avoid the above discussed ambiguity in epitope specificity by raising antibodies against synthetic peptides only five to seven amino acids in length (Maelicke et al., 1984). These antibodies appeared likely to recognize at the receptor only epitopes formed by the same amino acid residues as exist in the synthetic peptide they were raised against. As we show here, even the application of "limiting-size peptides" does not fully exclude the risk of cross-reactivity with other regions of the antigen.

As an expected disadvantage, most antibodies directed against short peptides bind with rather low affinity to the corresponding sequence on the native form of antigen. This

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further limits their application in epitope mapping studies and, in particular, excludes in most cases electron microscopic studies with the colloidal gold double-labeling technique.

Our data are in general agreement with previous studies of nAChR[1] topology performed with immune sera raised against longer peptides (Ratnam et al., 1986b; Lindstrom, 1986). They suggest that the putative α-helical regions M1–M3 (Noda et al., 1982; Claudio et al., 1983) and parts of the proposed transmembrane domain M7 (Ratnam et al., 1986b) are not accessible in membrane-bound nAChR for antibody binding while parts of the proposed amphiphatic transmembrane domain M5 (Finer-Moore & Stroud, 1984; Guy, 1984) are accessible. Although in agreement with the model of Ratnam et al. (1986b), we consider these data too preliminary to justify the proposal of membrane-spanning domains in addition to those defined by hydropathy plots.

While some of the obtained anti-peptide antibodies are directed against regions of nAChR proposed to be part of the binding sites for agonists and local anesthetics [for a review, see Maelicke (1988)], none of them interfered with functional properties of nAChR.

Materials and Methods

Synthetic peptides were obtained from the following sources: PSDDV-Y, ESDRPD, and YTCCPD were from Organogen, Heidelberg, West Germany; YCEIIV was synthesized and characterized by Dr. Engelhard from our institute; FDQQNC-Y, FDWQNC-Y, KSDEES-Y, and AAEEWKY were from Biosearch, San Rafael, CA.

Live electric rays (*Torpedo marmorata*) were obtained with the help of Dr. V. P. Whittaker (Max-Planck-Institut, Göttingen, West Germany) from the Société Scientifique d'Arcachon. Balb/c mice were obtained from Zentralinstitut für Versuchstierforschung, Hannover, West Germany; New Zealand White rabbits were from Dr. Ivanovas, Gesellschaft für med. Versuchstierzuchten mbH (Kisslegg, West Germany). Histrionicotoxin was a present from Prof. Bartels-Bernal (Cali, Columbia). Peroxidase-linked anti-mouse IgG and anti-rabbit IgG were from Dakopatts GmbH (Hamburg, West Germany); the NEN-NEI 602 screening kit (New England Nuclear, Dreieichenhain, West Germany) was employed. Cell culture sera were obtained from Boehringer (Mannheim, West Germany); all radioactive materials were from Amersham-Buchler (Braunschweig, West Germany), and all other chemicals and biochemicals were from established commercial sources.

Staphylococcus aureus V8 protease and endoglycosidase H were obtained from Miles (Slough, England); trypsin and papain were from Sigma (Deisenhofen, West Germany); elastase was from Boehringer (Mannheim, West Germany, and endoglycosidase F was from NEN (Dreieichenhain, West Germany).[2]

Membrane fragments and purified nAChR from *T. marmorata* and purified and ^{3}H-labeled α-cobratoxin were obtained as described previously (Maelicke et al., 1977; Rüchel et al., 1981; Fels et al., 1982; Martin et al., 1983). Purified nAChR subunits were obtained by preparative gel electrophoresis (BRL-Gibco, Eggenstein-Leopoldshafen, West Germany) according to established procedures.

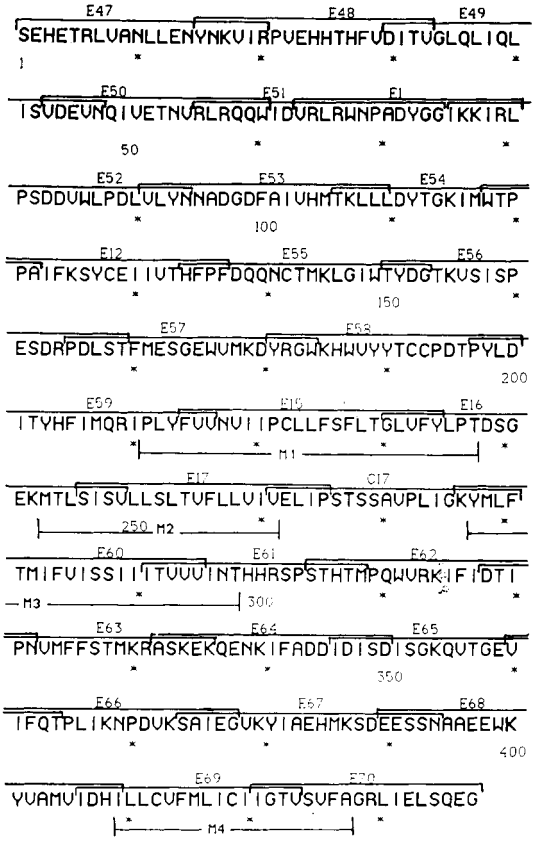

FIGURE 1: Amino acid sequence of *Torpedo* nAChR α-subunit (Noda et al., 1982, 1983). The hydrophobic segments which could form transmembrane α-helices are indicated (M1–M4). The sequences of the synthetic peptides used in the experiments aimed at verifying the sequence specificity of anti-P7 antiserum are indicated as bars above the α-subunit sequence.

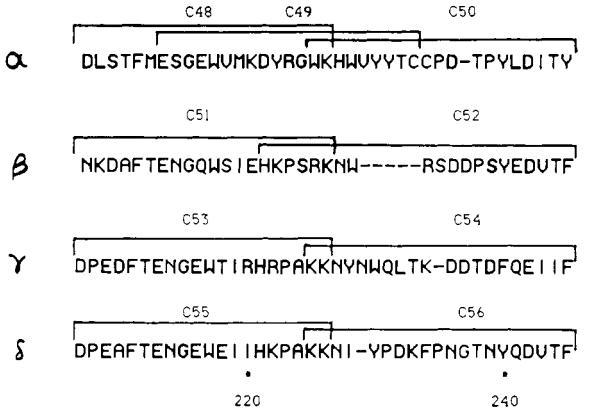

FIGURE 2: Sequence segments of *Torpedo* nAChR subunits homologous to the segment α166–203, aligned as in Noda et al. (1983). The numbers at the bottom of the sequences are as in the alignment of Noda et al. (1983). The sequences of the overlapping synthetic peptides C48–C56 corresponding to these sequences are indicated as bars.

Synthesis of Peptides Used as Immunogens. All synthetic peptides used for rabbit immunization, with the exception of hexapeptide YCEIIV, were from commercial sources. The latter was synthesized by means of a solid-phase method (Merrifield, 1963) and purified by high-pressure liquid chromatography (HPLC). Amino acid analysis and peptide sequencing were employed to further establish its purity and to check the purity of commercially obtained peptides.

Synthesis of Peptides Used for Assessment of Antisera Specificity. The specificity of the anti-peptide antisera was

[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; BSA, bovine serum albumin; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered physiological saline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

[2] Enzyme Commission numbers: *Staphylococcus aureus* V8 protease, EC 3.4.21.19; endoglycosidase H, EC 3.2.1.*x*; trypsin, EC 3.4.21.4; papain, EC 3.4.22.2; elastase, EC 3.4.21.11; endoglycosidase F, EC 3.2.1.*x*.

assessed by the use of a panel of 30 synthetic peptides, 17–21 residues long and overlapping each other by 3–6 residues, corresponding to the complete sequence of *Torpedo* nAChR α-subunit (Figure 1). Because one serum (anti-P7) showed cross-reactivity with peptides corresponding to the sequence α165–200 (see Results and Discussion), a second panel of overlapping peptides was synthesized corresponding to the region α166–203 and to the homologous segments of all the other *Torpedo* nAChR subunits (Figure 2).

Peptides were synthesized simultaneously by manual parallel synthesis (Houghten, 1985). Their purity was assayed by reverse-phase high-pressure chromatography (HPLC) using a C_{18} column (Ultrasphere ODS) and a gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. The composition of all peptides was verified by amino acid analysis, using PTH derivatives of the amino acids released by acid hydrolysis (Heinrickson & Meredith, 1981). The sequence and purity of some randomly selected peptides were further verified by gas-phase sequencing (Herrick et al., 1981).

Cross-linking of synthetic peptides with carrier proteins was achieved by the following three methods. (a) With bisdiazobenzidine (Bassiri et al., 1979): Bisdiazobenzidine in HCl was added dropwise to an ice-cooled and stirred solution of 9 μmol of peptide and 150 nmol of carrier protein in 3 mL of 0.16 M sodium borate–0.13 M NaCl, pH 9.0. The pH was kept controlled at 9 by the addition of appropriate amounts of 0.1 M NaOH. After being stirred further for 2 h, the reaction mixture was dialyzed three times against PBS. (b) With glutaraldehyde (Reichlin, 1980): A total of 0.25 mL of glutaraldehyde in PBS (0.25 v/v) was added dropwise to a stirred ice-cold solution of 2.5 μmol of peptide and 150 nmol of carrier protein in 2.0 mL of PBS. The reaction was allowed to proceed for another hour at room temperature, and the solution was dialyzed two times against PBS. (c) With carbodiimide (Tamura et al., 1983): A total of 250 nmol of carrier protein in 0.5 mL of 1 mM HCl (pH 3.0) was incubated for 15 min at 0 °C with 100 μmol of EDAC; 7 μmol of peptide dissolved in a minimal amount of 0.1 M ammonium carbonate, pH 9.0, was added, and the reaction was allowed to proceed under stirring for 3 h at room temperature. The reaction solution was then dialyzed two times against H_2O.

Cross-linking reactions were optimized by submitting aliquots of control reaction mixtures ([^{14}C]tyrosine and carrier in reaction a or labeled peptide and carrier in the other reactions) to SDS gel electrophoresis. After the gels were dried, they were exposed to a Fuji-XR film for 2 weeks. Appropriate levels of reaction were demonstrated by radioactive labeling of the carrier protein SDS band; after longer incubation times, excessive cross-linking between carrier molecules was observed (Coomassie staining showed additional SDS bands corresponding to twice or three times the apparent molecular mass of the carrier monomer).

Polyclonal Rabbit Immune Sera. Young female New Zealand White rabbits were immunized intradermally with 1–3 mg of peptide–carrier conjugate in Freund's complete adjuvant followed by booster injections (every 3 weeks) of the same amount of antigen in incomplete adjuvant. At least four booster injections were required to obtain immune sera of sufficient titer.

Monoclonal Antibodies. Young female Balb/c mice were injected intravenously with 3 mg of peptide–carrier conjugate. Three days after a booster injection, the spleens of the immunized mice were removed, and cell hybridization, cell cloning, and clone selection were performed as described previously (Watters & Maelicke, 1983).

Internal Labeling of Monoclonal Antibodies with [^{3}H]-Leucine. Hybridoma clones were grown in minimum Eagle's medium (MEM) containing 5% dialyzed fetal calf serum (FCS). Approximately 2×10^6 cells were washed twice with leucine-free MEM containing 5% dialyzed FCS. A total of 0.4–0.5 mL of [^{3}H]leucine of high specific activity was added, and the culture was incubated overnight. The supernatant was removed and dialyzed extensively against PBS at 4 °C. The dialyzed supernatant was then employed in the binding studies described below.

Antibody Binding Assays. Enzyme-linked immunosorbent assays (ELISA) of rabbit immune sera and mouse monoclonal antibodies were performed in microtiter plates or plastic cuvette slips (Gilford/Corning, Giessen, West Germany) onto which antigen (*Torpedo* membrane vesicles, purified nAChR) was adsorbed at 4 °C overnight in 0.1 M $NaHCO_3$, pH 9 (Watters & Maelicke, 1983). To immobilize free synthetic peptides, plastic wells or cuvettes were filled with a solution of peptide in PBS (1–10 μg/mL), dried at 37 °C, and fixed with methanol (Green et al., 1982).

Excess antigen was removed by washing three times with a buffer consisting of 0.14 M NaCl, 2.5 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 0.05% Tween 20, pH 7.4 (PBS–Tween). Antisera, hybridoma supernatants, or purified antibodies were added and incubated at 37 °C for 3 h, and excess antibody was removed by three washes. The appropriate peroxidase-linked anti-IgG antibody was added and incubated at 37 °C for 1 h, excess secondary antibody was removed by three washes, and the enzyme substrate (0.2% *o*-phenylenediamine in 0.0015% H_2O_2–0.1 M sodium citrate, pH 5.0) was added. The enzymatic reaction was stopped with 0.5 M H_2SO_4 after the appropriate time, and the optical density at 490 nm was read in an EIA reader.

Competition ELISA assays (Watters & Maelicke, 1983) were performed by preincubating immobilized antigen with appropriate ligand or antibody solution. Concentration of free epitopes for the antibody under study was then determined as described above.

Radioimmune assays (RIA) were performed in flexible polyvinyl microtiter plates as follows (Watters & Maelicke, 1983). (a) With radioactive antigen: Microtiter plates were incubated consecutively (and each incubation followed by appropriate washes) with anti-mouse IgG, the monoclonal antibody under study, and ^{125}I-labeled synthetic peptide or nAChR. After appropriate time of reaction, the wells were cut out with an electrically heated wire, placed individually into counting vials, and counted in a γ-radiation counter. (b) With radioactive antibody: Flexible microtiter plates were coated with antigen and washed as described above. Various amounts of ^{3}H-labeled antibody in PBS–Tween in a total volume of 140 μL/well were incubated 3 h at 37 °C or overnight at 4 or 20 °C. The wells were washed extensively with PBS–Tween, cut out, placed in counting vials containing 10 mL of Quickszint (Zinsser, Frankfurt, West Germany) and counted in a liquid scintillation counter.

Gel Electrophoresis and Western Blots. Laemmli's (1970) gel and buffer systems were employed, and the gels were electrophoresed for 15 h at constant current of 9 mA. For Western blots, gels were equilibrated in "transfer buffer" [25 mM Tris base, 192 mM glycine, 20% (v/v) methanol], applied onto nitrocellulose paper between Scotch Brite and several layers of filter paper, and electrophoretically transferred (50–60 V, 200 mA, 3–4 h) by means of a Bio-Rad transfer apparatus (Towbin et al., 1979). The nitrocellulose paper with the transferred proteins was then incubated for 2 h at room

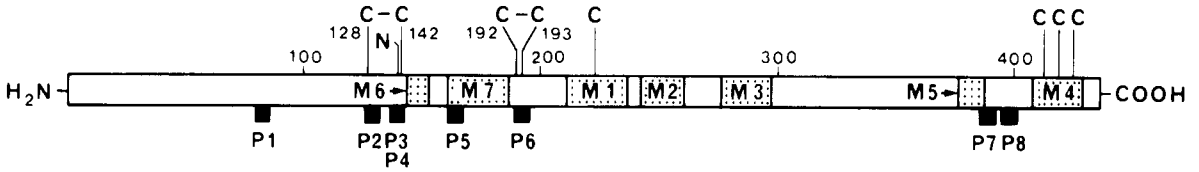

FIGURE 3: Location in the primary structure of *Torpedo* α-subunit of matching sequences of the synthetic peptides. P1–P8, matching sequence positions of synthetic peptides; M1–M7, positions of proposed transmembrane domains (Claudio et al., 1983; Noda et al., 1983; Lindstrom et al., 1984; Finer-Moore & Stroud, 1984; Guy, 1984; Ratnam et al., 1986b; Maelicke, 1988); C, cysteine residue, N, N-glycosylation site.

temperature with a solution of BSA (1%) in 150 nM NaCl–10 mM Tris-HCl, pH 7.4. After overnight incubation with antibody and extensive washing, the nitrocellulose paper was cut into slips, and these were incubated with ^{125}I-labeled secondary antibody, protein A, or α-bungarotoxin. After being washed and dried, the slips were exposed for 5–14 days to a Fuji X-ray film.

Treatment with endoglycosidases of Torpedo membrane fragments or purified nAChR was performed at 37 °C for 18–24 h. Endo H buffer: 0.2 M sodium citrate, 0.1% SDS, and 2 mM PMSF, pH 5.5; Endo F buffer: 0.1 M sodium phosphate, 50 mM EDTA, 0.05% Tween 20, 0.1% SDS, and 1% β-mercaptoethanol, pH 6.1. To check the extent of enzymatic reaction, aliquots of the reaction mixtures were submitted to Western blotting, and the apparent molecular weight of α-subunits was determined by labeling with ^{125}I-α-bungarotoxin.

Limited Proteolysis of Torpedo Membrane Fragments, Solubilized Receptor, and Purified α-Subunits. Routinely, ~0.4 mg of membrane fragments, 0.2 mg of purified receptor, or 0.1 mg of purified α-subunit was incubated at 37 °C with the following enzyms and buffers: *S. aureus* V8 protease (2–5 μg, Miles) in 1 mM EDTA, 0.1% SDS, and 125 mM Tris-HCl, pH 6.8; elastase (10 μg, porcine pancreas, Boehringer) in 0.2% SDS–PBS adjusted to pH 8.8; trypsin (0.1 μg, bovine pancreas, Sigma) in 0.1% SDS, 0.1 mM $CaCl_2$, and 100 mM NH_4HCO_3, pH 8.5. Reactions were terminated by temperature shift to 4 °C or, in the case of trypsinization, by the addition of trypsin inhibitor.

Assessment of Binding Specificity of Anti-P7 Antiserum. Because of the ambiguous results obtained when anti-P7 antiserum was used for binding to isolated nAChR subunits or to membrane-bound nAChR (see Results and Discussion), the sequence specificity of its binding was verified by the use of the panels of peptides reported in Figures 1 and 2, both by dot blot assay and by ELISA.

For the dot blot assay 1-μL aliquots of each peptide solution (60 μg/mL) were spotted onto nitrocellulose strips and allowed to dry. The strips were washed twice in 10 mM Tris–140 mM NaCl, pH 7.4, containing 0.1% Tween 20 (TBS–Tween) and incubated for 2 h on a rocking platform with the anti-peptide antiserum, diluted 1:100 or 1:250 in TBS–Tween. The strips were washed once in 10 mM Tris–500 mM NaCl containing 0.5% Tween 20 and washed twice in TBS–Tween. Horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma) diluted 1:500 in TBS–Tween was added (1 mL/strip) and incubated for 2 h on a rocking platform. The strips were washed three times with TBS–Tween and once with TBS; the substrate was added and incubated for 30 min. The strips were washed with water, dried, and scanned with a Bio-Rad scanning densitometer. All procedures were performed at room temperature.

For ELISAs, 96-well plastic plates (Dynatech) were incubated for 4 h at room temperature with 50 μL/well of 20 μg/mL multichain poly-DL-alanylpolylysine, in 0.1 M $NaHCO_3$. The plates were washed three times with 10 mM potassium phosphate buffer, pH 7. Equal volumes of peptide solutions (60 μg/mL in H_2O) and 0.25% glutaraldehyde were mixed; 50 μL was added to each well and incubated overnight at 4 °C. After three washes with potassium phosphate buffer, pH 7, the wells were blocked with 200 μL/well of 3% BSA in PBS for 3/ min at room temperature and washed twice with 0.05% Tween 20 in PBS (PBS–Tween). A total of 50 μL of antiserum, diluted 1:40 in PBS containing 0.4% BSA (PBS–BSA), was added to each well. After 2.5 h at room temperature the wells were washed three times with PBS–Tween, and 50 μL/well urease-conjugated sheep anti-rabbit IgG (Ureiase, Allelix) diluted 1:1500 in PBS–BSA was added. After 2.5 h at room temperature the wells were washed three times with PBS–Tween and three times with water, and 100 μL of urease 590 substrate (Allelix), pH 4.8, was added. After 15–60 min at room temperature the reaction was stopped by addition of 10 μL/well of 1% thymerosal (Allelix), and the plates were read with an ELISA reader at 590-nm wavelength.

Table I: Sequences and Matching Positions in nAChR Primary Structure of Synthetic Peptides[a]

peptide	sequence	matching nAChR sequence
P1	PSDDV-Y	α81–85
P2	YCEIIV	α127–132
P3	FDQQNC-Y	α137–142
P4	FDWQNC-Y	βγδ"137–142"
P5	ESDRPD	α161–166
P6	YTCCPD	α190–195
P7	KSDEES-Y	α387–392
P8	AAEEWKY	α395–401

[a] The conventional one-letter code for amino acids is used. Residues not contained in the matching receptor sequence (N-terminal tyrosines of some peptides) are separated by a hyphen from the original receptor sequence. Sequence of nAChR from *T. marmorata* is according to Devillers-Thiery et al. (1983).

Results and Discussion

Synthetic Peptides, Their Conjugates, and Production of Antibodies. Synthetic peptides were selected according to the following criteria: (i) Their sequences match regions of the primary structure of *Torpedo* nAChR predicted on the basis of hydrophilicity profiles to lie at hydrophilic surfaces (Maelicke, 1988). (ii) With the exception of peptide α81–85, the selected sequences are highly conserved between nAChRs from different species (Maelicke, 1988). (iii) All peptides carry a negative net charge. These criteria are basic requirements for sequence regions to be involved in ligand binding and in channel gating. The sequences of synthetic peptides and their positions in the primary sequence of nAChR from *T. marmorata* are shown in Table I and Figure 3.

Bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) were selected as carrier proteins as they apparently share only a few, if any, immunogenic sites. Peptides were linked via their N-terminal amino groups or via terminal tyrosine residues to the above carriers as described under Materials and Methods. Antibodies were raised by conventional immunization of rabbits or by the murine hybridoma technique. To obtain antisera of sufficient titer, at least four

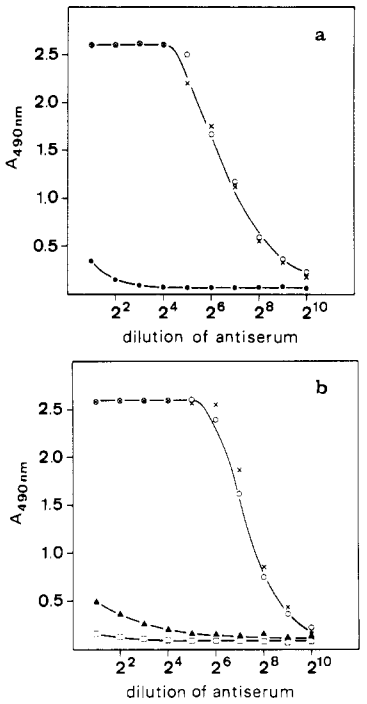

FIGURE 4: Enzyme-linked immunosorbent assays of rabbit immune sera employing peptide–carrier conjugates or nAChR as immunosorbents. (a) Cuvettes were coated with peptide P1 cross-linked with glutaraldehyde to either BSA (conjugate P1–GA–BSA) or KLH (conjugate P1–GA–KLH). Serial dilutions of rabbit anti-(P1–GA–KLH) serum, preabsorbed with KLH, with (○) P1–GA–BSA, (×) P1–GA–KLH, and (●) P6–GA–BSA as antigens. Supernatant of immune serum precipitated with KLH was employed. The data show specific reaction of anti-P1 serum with peptide P1 independent of whether BSA or KLH was employed as carrier and no cross-reactivity with peptide P6. (b) Anti-P7 serum was incubated overnight at 4 °C with or without competing antigens. 200 μL of medium was added to each cuvette of a strip of 10 coated with peptide P7–BDB–KLH conjugate. 200 μL of centrifuged incubation mixture of anti-P7 serum and competing antigen was then added to cuvette 1 and mixed, 200 μL of the content was transferred to cuvette 2, and so on (serial dilution). Further treatment was described under Materials and Methods. (○) Serial dilution in the absence of competing antigen, (▲) in the presence of excess solubilized *Torpedo* nAChR, (□) in the presence of excess P7 conjugate, and (×) in the presence of excess P1–GA–BSA conjugate.

booster injections of peptide–carrier conjugate were required. As shown in Table II, the titers of immune sera varied greatly and were orders of magnitude lower than those of anti-nAChR antisera. ELISA and RIA were employed to select antibody-producing cell clones with the desired epitope specificity and to characterize immune sera and monoclonal antibodies.

Antigen Specificity of Rabbit Anti-Peptide Immune Sera. Antigen specificity of antisera was tested as follows:

(i) All antisera studied yielded positive ELISAs when complementary conjugates (same peptide cross-linked by the same method to a different carrier protein) or the free peptide was employed as antigen (Figure 4a). Thus, the polyclonal immune sera contained antibodies exclusively directed against the *peptide* portions of the conjugates.

(ii) With the exception of immune sera raised against peptides P3 and P4 (see below), no cross-reactivity of immune sera with the other synthetic peptides was observed (Figure 4b). Thus, the majority of immune sera appeared to discriminate clearly between different peptides.

(iii) All anti-peptide sera recognized *Torpedo* nAChR as antigen. This was not the result of cross-reactivities between carrier protein and nAChR, as antisera raised against BSA or KLH or against "nonsense" conjugates did not bind to

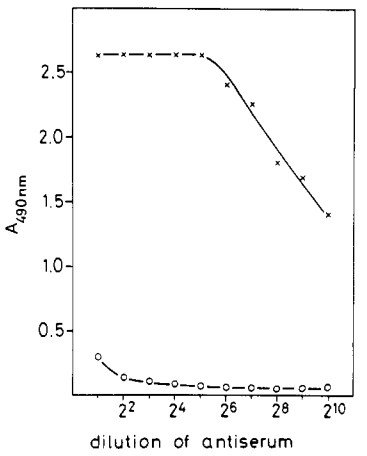

FIGURE 5: Enzyme-linked immunosorbent assay of rabbit anti-P2 immune serum with purified nAChR subunits as immunosorbents. Plastic cuvettes were coated with purified nAChR subunits and incubated with immune serum as described under Materials and Methods. (×) Purified α-subunit; (○) purified δ-subunit. Binding to δ- (shown) and β- and γ- (not shown) subunits was within background levels.

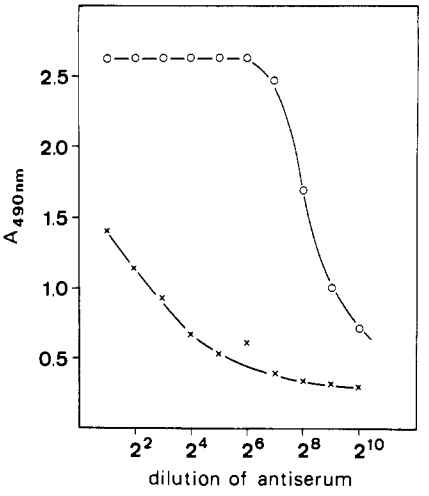

FIGURE 6: ELISA of rabbit anti-P3 immune serum with endoglycosidase F treated nAChR. Purified solubilized *Torpedo* nAChR before and after treatment with endoglycosidase F was extensively dialyzed against 0.1 M $NaHCO_3$ prior to being applied to ELISA cuvettes. Serial dilutions with (×) untreated and (○) endo F treated nAChR. The observed rather high background readings (×) are due to receptor denaturation at the buffer conditions of enzyme treatment (see Materials and Methods).

receptor preparations. In some cases (see below), however, antibody binding required pretreatment of nAChR by enzymes (endoglycosidases, proteases) and/or denaturation. nAChR and free peptides competed for antibody binding supporting the notion that identical epitopes existed in the synthetic peptides and the macromolecule.

(iv) nAChR subunit specificity of the anti-peptide sera was determined by means of Western blots of *Torpedo* membrane fragments and ELISAs with purified nAChR subunits as antigen (Figure 5). Because most anti-peptide antisera exhibited relatively low affinity for their antigens (see below), solid-phase binding tests (ELISA) yielded more reliable results. Antisera raised against peptides P2, P6, and P8 bound exclusively to α-subunits; P1 bound to α- and, more weakly, to β-subunits; P7, almost equally well to α- and β-subunits. Anti-P3 serum recognized α-subunits of endoglycosidase-treated nAChR (Figure 6). Anti-P4 serum was specific for the heavier subunits (δ-subunit) of the receptor. Proteolytic treatment of solubilized, denatured nAChR (see Materials and

Table II: Composition of Peptide–Carrier Conjugates and Relative Titer of Anti-Peptide Immune Sera for Different Antigen Preparations[a]

			relative titers				
peptide	carrier protein	coupling reagent	complementary conjugate	free peptide	membrane fragments	solub. AChR	α-subunit
P1	BSA	BDB	nd	0.1	+	+	0.005
P1	BSA	GA	0.2	0.06	+	+	0.02
P1	KLH	GA	0.2	0.06	+	+	0.005
P2	BSA	GA	0.06	0.13	0.16	0.16	0.04
P3	KLH	EDAC	0.13	0.13	–	(+)	–
P3	KLH	BDB	0.13	0.25	–	(+)	–
P3	BSA	BDB	0.06	0.25	–	(+)	–
P4	KLH	BDB	0.13	0.2	–	–	
P5	KLH	EDAC	0.006	0.01	–	–	–
P6	BSA	GA	0.2	0.06	+	+	0.005
P6	BSA	BDB	0.5	0.25	+	–	0.003
P7	KLH	BDB	1	0.2	0.02	0.32	0.08
P8	KLH	BDB	0.1	0.02	–	0.01	0.01
nAChR			–	–	>32	>32	>32

[a] The following additional abbreviations are used: BDB, bisdiazobenzidine; GA, glutaraldehyde; nd, not determined. + indicates weak positive ELISA, (+) indicates positive ELISA after treatment with endoglycosidase F, and – indicates ELISA within background levels. Immobilized antigens: free peptide, peptide–carrier conjugate, *Torpedo* membrane fragments, solubilized and purified *Torpedo* AChR, and purified nAChR subunits as described under Materials and Methods. Titers are expressed in comparison to ELISA reading of rabbit anti-P7 serum with P7–BDB–KLH conjugate as immunosorbent, as serum concentrations required for half-maximal ELISA readings (1.3 A_{490} units). Standard reading was obtained with a 1:8000 dilution of anti-P7 serum.

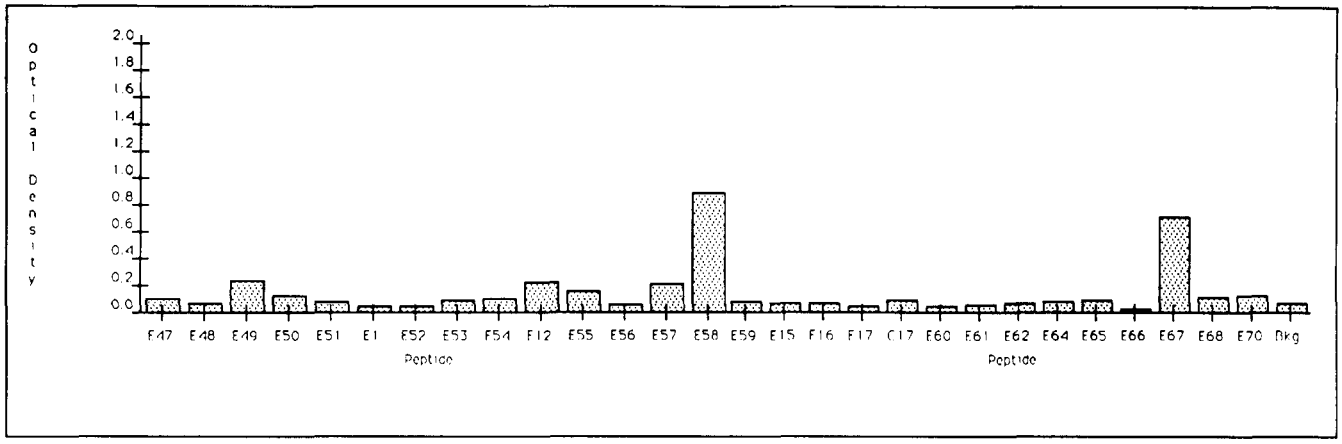

FIGURE 7: ELISA of anti-P7 antiserum, tested with synthetic peptides corresponding to the complete α-subunit sequence. The sequence of the peptides is reported in Figure 1. After 15-min incubation, peptide E67, which contains the immunizing sequence segment KSDEES, and peptide E58, which corresponds to the apparently unrelated sequence α181–200, are recognized to a similar extent. Upon more prolonged incubations (up to 60 min) also the recognition of peptide E57, which corresponds to the sequence α165–184, became evident (not reported in this figure).

Methods) was required to expose the epitope for anti-P5 serum.

The above data confirmed that the anti-peptide immune sera contained antibodies specifically directed against the synthetic peptides employed as conjugates in rabbit immunization and that they also recognized nAChR as antigen. As shown below, however, these criteria do not suffice to ensure that anti-peptide antibodies *exclusively* bind to the respective sequence portions at the receptor.

Sequence Specificity of Immune Sera. A first indication of anti-peptide immune sera having only limited sequence specificity was obtained from cross-reactivity studies with peptides P3 and P4 which differ in a single sequence position (Trp rather than Gln in P4). In ELISAs with the free peptides, ~50-fold higher concentrations of anti-P3 serum (100-fold higher concentration of anti-P4 serum) were required to obtain the same reading with peptide P3 (P4) as with the immunizing peptide. Thus, either there existed in the two sera antibodies which do not use the exchanged sequence position as epitope determinant, or antigen–antibody interaction is by multipoint attachment with a missing attachment point reducing affinity but not completely eliminating binding. In view of the available structural data on antigen–antibody complexes (Amit et al., 1986), the latter explanation appears more likely. It agrees with the notion that antibodies in general recognize three-dimensional rather than linear epitopes.

Further evidence of cross-reactivity of anti-peptide antisera with related and even unrelated sequences was provided by dot blot and ELISA studies employing as antigens a library of peptides covering the whole primary structures of nAChR α-subunit and selected segments of all nAChR subunits from *Torpedo californica* (Figures 1 and 2). In dot blots of 100- or 250-fold dilutions, anti-P7 antiserum recognized peptides E57 and E58 of *Torpedo* α-subunit but not E67, which contains the immunizing sequence. By use of ELISA and a 40-fold dilution of the serum, peptides E58 and E67 were recognized (Figure 7). E57 and E58 correspond to the sequence α165–200 (Noda et al., 1982, 1983) which does not have any obvious homology with the immunizing peptide. When anti-P7 was tested with the panel of peptides corresponding to the sequence α166–203 and the homologous segments of the other *Torpedo* nAChR subunits, peptides C50 and C52 were strongly recognized (Figure 8). C50 corresponds to the sequence α184–203 which has been proposed to contain elements of the transmitter binding site. Again, this region does not exhibit significant sequence homology to the immunizing peptide. In contrast, peptide C52 corresponds

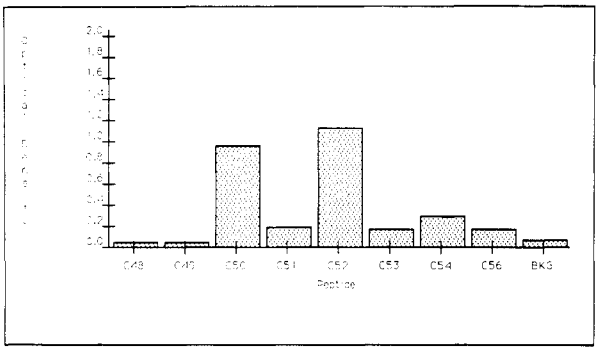

FIGURE 8: ELISA of anti-P7 antiserum using peptides C48–C56 of the second panel of peptides. For experimental details, see Materials and Methods. Peptides C50 and C52 are recognized.

to the sequence β190–209 (Noda et al., 1983) which contains a sequence quite similar to that of peptide P7, i.e., RSDDPSY instead of KSDEESY. Controls with preimmune serum from the same rabbits indicate that the observed recognition of receptor peptides other than E67 indeed is due to cross-reactivity of anti-P7 antibodies. In conclusion, anti-peptide antibodies do not necessarily possess the sequence specificity often assumed in epitope mapping studies.

Properties of Monoclonal Antibodies. The murine hybridoma technique was employed to produce monoclonal antibodies directed against synthetic peptides. As published in full for monoclonal anti-P2 antibodies (Plümer et al., 1984), they all recognized as antigens their specific peptide–carrier conjugate but not conjugates of other peptides (nonsense peptides) linked by the same cross-linker to the same carrier. Hybridoma clones studied in further detail produced antibodies exhibiting an antigen specificity comparable to those of the respective polyclonal rabbit immune sera. Several monoclonals obtained from immunizations with either peptide P3 or P4 were tested as described before for their ability to cross-react with other peptides. ELISA readings above background were obtained only with P4 and P3, respectively, but not with the other synthetic peptides available. Again, the monoclonal antibodies also recognized the homologous peptide, but recognition was generally weaker than for the immunizing one. This strongly supports the notion that exchange of just one out of six amino acids sufficiently changes the charge density pattern to result in weaker interaction (loss of attachment points) with the noncorresponding monoclonal.

Monoclonal anti-peptide antibodies provide a good means to determine their binding affinity for immobilized antigen (Watters & Maelicke, 1983). By employing this procedure (see Materials and Methods), the apparent equilibrium dissociation constant of monoclonal anti-P2 antibody produced by hybridoma clone C2-NI-C2 for the (immobilized) P2–BSA conjugate it was raised against was (2–6) $\times 10^{-8}$ M, i.e, $\sim$1 order of magnitude lower than the affinity of anti-nAChR mAbs for their antigen (Watters & Maelicke, 1983). Binding affinity of anti-P2 serum for *Torpedo* membrane fragments or purified receptor was even lower.

Accessibility of Peptide Epitopes in Native nAChR. Although we show here that polyclonal anti-peptide antibodies often possess only limited sequence specificity, some of the immune sera provide further evidence as to the accessibility of matching sequence portions in nAChR structure. This applies in particular to sera raised against peptides P3, P4, P5, and P8 as their binding requires selective treatment of nAChR.

As summarized in Table II, the immune sera raised against peptides P2 and P7 exhibited high titers for nAChR in membrane-bound and purified forms. Consequently, their epitopes lie at a hydrophilic surface of nAChR. Assuming that the matching sequence indeed provides a binding site for the corresponding antibody, the results for P2 (α127–132) agree with previous suggestions (Criado et al., 1986; Barkas et al., 1987). In the case of P7 (α387–392), the data could superficially be interpreted as arguing against the existence in this region of membrane-spanning amphipathic helix M5 (Lindstrom et al., 1984; Lindstrom, 1986). Due to strong cross-reactivity of this serum with other regions of nAChR (see above), however, this conclusion cannot be drawn.

Anti-P1 and anti-P4 immune sera exhibited considerably lower titers (lower affinity) for native nAChR. As both sera bound stronger to purified α-subunit, the related sequence portions may exist in different conformations in native nAChR as compared to those in the free peptides. This seems quite likely as both peptides contain proline residues, and P6 in addition contains two cysteines suggested to form a disulfide bridge in native nAChR (Kao & Karlin, 1986; Neumann et al., 1986). In summary, the regions α81–85 and α190–195 probably are at a hydrophilic surface of nAChR.

Anti-P8 antibodies did not recognize native *Torpedo* membranes even if these were permeabilized (Young et al., 1985) or the incubation mixture was sonicated. In contrast, they bound quite well to detergent-solubilized nAChR and to purified α-subunits (Table II). Thus, accessibility of the hydrophilic and charged region α395–401 apparently depends sensitively on nAChR conformation, i.e., whether the receptor is in membrane-bound or solubilized form.

As Asn-141 is generally assumed to be glycosylated in native nAChR (Maelicke, 1988), it is not surprising that antibodies raised against the (nonglycosylated) peptides P3 and P4 did not recognize native nAChR as antigen. After treatment with endoglycosidase F, anti-P3 serum bound to nAChR and to purified α-subunit (Figure 6). The same enzyme treatment did not suffice to render nAChR an antigen for anti-P4 serum. Consequently, the sequence α137–142 probably is at a hydrophilic surface of nAChR while the homologous sequence of the other three subunits is situated closer to the hydrophobic core of the receptor. This agrees with model considerations concerning the exchange of Trp for Gln in position 139 of this region (Smart et al., 1984).

nAChR must be denatured and partially digested (see Materials and Methods) to expose epitope(s) for anti-P5 serum, suggesting that the region α161–166 is situated within the hydrophobic core of native nAChR. This agrees with previous findings by Ratnam et al. (1986a).

All anti-peptide antisera were tested as to their ability to compete in antigen binding with antibodies raised against the whole receptor protein. None of the monoclonal antibodies of our library (Watters & Maelicke, 1983) competed with the anti-peptide antibodies for binding to native nAChR. Furthermore, none of the monoclonal antibodies recognized any of the synthetic peptides or peptide conjugates as antigen. In contrast, a rabbit anti-nAChR immune serum showed limited competition with anti-P2 mAbs (Figure 9) and with immune sera raised against peptides P1, P2, and P7 for binding to their antigen.

Anti-Peptide Antibodies in Functional Studies of nAChR. Regions α137–142 and α190–195 have been proposed to be part of the transmitter binding sites at the receptor (Noda et al., 1982; Smart et al., 1984; Kao et al., 1984; Mishina et al., 1986). The latter suggestion is supported by results of affinity labeling experiments (Kao et al., 1984). Region $\beta\gamma\delta$"137–142" has been proposed to be part of local anesthetics and/or low-

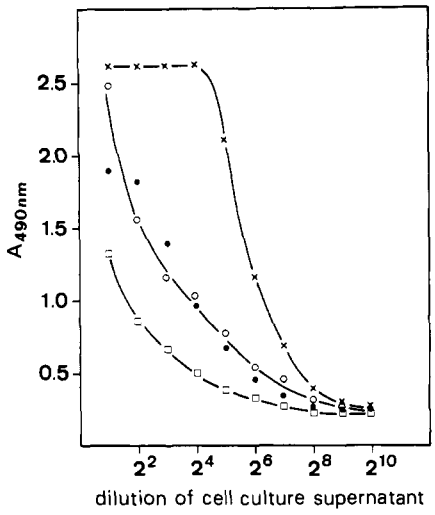

FIGURE 9: ELISA of monoclonal antibody C2–NI–C2 in the presence and absence of rabbit immune sera. ELISA cuvettes were coated with free peptide P2 as described under Materials and Methods. After incubation for 1 h at room temperature with buffer (×), rabbit anti-(P2–GA–KLH) immune serum (●), rabbit anti-(P2–GA–BSA) immune serum, (□) or rabbit anti-AChR immune serum (○) and three extensive washes, binding of culture supernatant of mouse hybridoma clone C2–NI–C2 was tested as described under Materials and Methods.

affinity transmitter binding sites (Noda et al., 1982) and region α161–166 to be part of the main immunogenic region (MIR) of nAChR (Noda et al., 1982). Recent antibody binding data established, however, that the MIR is within a segment of 80 residues at the amino-terminal part of the α-subunit (Barkas et al., 1987), with a main constituent between residues 67 and 76 in the case of human muscle nAChR (Tzartos et al., 1988a,b).

To probe these suggestions, anti-peptide antibody binding to peptide–carrier conjugates and native nAChR was studied in the presence and absence of representative cholinergic ligands including local anesthetics and the channel toxin histrionicotoxin (Figure 10). None of these, even if applied in large excess over antibody, was capable of affecting antibody binding to either the immunizing peptides, their conjugates, or nAChR. In addition, monoclonal antibodies previously shown to compete with cholinergic ligands for receptor binding (Watters & Maelicke, 1983; Fels et al., 1986) did not affect anti-peptide antibody binding to their conjugates or nAChR. Furthermore, anti-peptide antibodies had no effect on agonist-induced influx of Tl^+ into *Torpedo* membrane vesicles (Fels et al., 1986). As a general observation, none of the rabbits immunized with peptide conjugates showed any myasthenic symptoms. Rather than excluding the regions defined by the sequences of synthetic peptides as parts of functional sites of nAChR, these studies suggest that functional sites have more complicated structural features than can be mimicked by (short) synthetic peptides. Alternatively in some studies (Tl^+ influx, myasthenic symptoms), low antiserum titer may have excluded detection of functional sites.

General Discussion

Technical Comments. As a major conclusion of this study, antibodies raised against synthetic peptides are not necessarily limited to recognizing only matching sequences in a larger polypeptide. By employing *very short* immunizing peptides, the narrow limits of sequence specificity of anti-peptide antibodies indeed are clearly exposed: Homologous peptides (see data on the pairs FDQQNCY, FDWQNCY and RSDDPSY, KSDEESY) may be recognized better or worse than the immunizing peptide. Cross-reactivity may also occur with peptides without any significant sequence homology (see Figures 7 and 8).

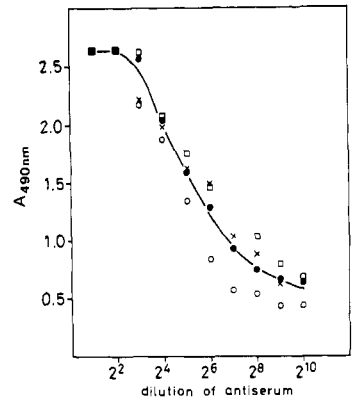

FIGURE 10: ELISA of anti-P6 immune serum in the presence and absence of nAChR ligands. Plastic cuvettes were coated with P6 conjugate as described under Materials and Methods. Serum was incubated with buffer (○) or ligands for 1 h at room temperature, and serial dilutions were performed. (●) 10^{-6} M α-cobratoxin; (□) 10^{-3} M carbamoylcholine; (×) 10^{-3} M tubocurarine. Similarly (not shown), decamethonium, acetylcholine, hexamethonium, procaine, quinacrine, chloropromazine (each 10^{-3} M), or histrionicotoxin (10^{-5} M) did not affect the ELISA for anti-P6 serum.

The longer an immunizing peptide, the less it appears likely that epitope specificity *linearily* correlates with the primary structure of the antigen. Further evidence against this assumption is provided by the demonstration of "discontinuous" epitopes (Amid et al., 1986) and the general observation that antibody affinity often increases with increasing size of the immunizing peptide. Another limitation of antibodies raised against long peptides comes from studies with anti-nAChR monoclonal antibodies (Fels et al., 1986), some of which have been shown to be capable of allosteric action; i.e., they induce conformational changes of their antigens. Antibodies raised against peptides may therefore induce or favor antigen conformations that do not significantly contribute to the equilibrium state of conformations of the antigen under study [for further examples, see Getzoff et al. (1987)]. Taken together, these are serious reservations agains all epitope mapping data (and predictions of membrane topology based on them) obtained with antibodies suggested, but not proven by appropriate methods, to have specificity for matching sequences (Young et al., 1985; Ratnam et al., 1986a,b; Lindstrom, 1986).

With their epitope specificity established, however, antibodies raised against long peptides certainly are advantageous. They are likely to recognize with high affinity a single stable conformation of their antigen, thus providing high epitope specificity together with the requirements for application of the electron microscopy immuno gold labeling technique.

Transmembrane Topology of Torpedo α-Polypeptide. We are aware of the fact that also antibodies raised against *short* immunizing peptides show only limited sequence specificity. The conclusions drawn below appear justified, however, because (i) they were obtained with antibodies of at least partially defined epitopes specificty and (ii) they address rather limited aspects of topology. Thus, our conclusions are based on the assumption that the respective matching sequences represent at least a significant part of available epitopes for the employed antibodies.

The sequence regions α81–85 (P1), α127–132 (P2), and α190–195 (P6) of native nAChR all appear more or less freely accessible for antibody binding. Recognition of nAChR by anti-P7 serum does not permit the same conclusion as this serum also binds to other regions than that defined by the

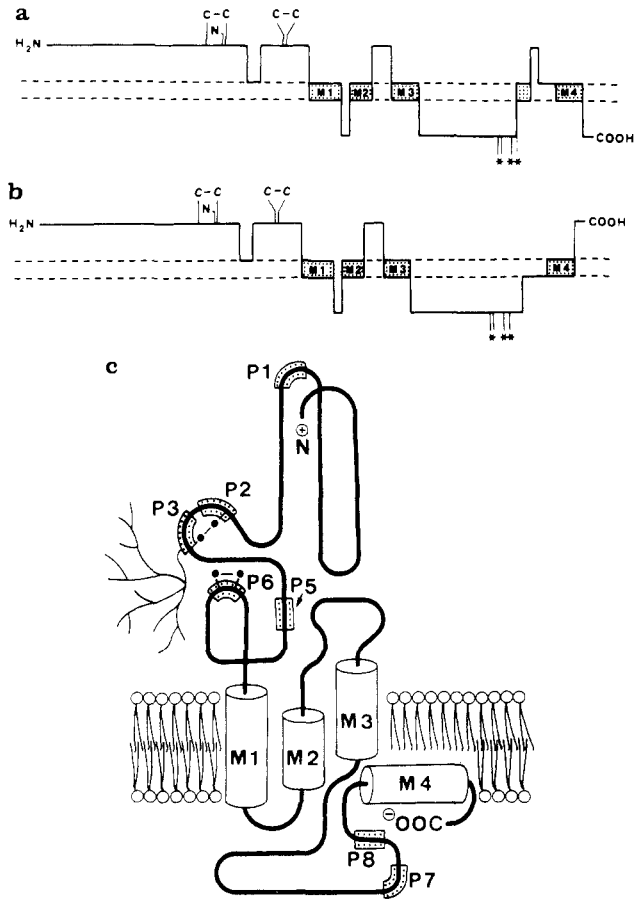

FIGURE 11: Models of the transmembrane topology of α-subunit for *Torpedo* nAChR with three, four, or five transmembrane domains. In schemes a and b, M1–M4 are putative α-helical domains predicted by hydrophobicity plots of α-subunit amino acid sequence. Asterisks indicate the (cytoplasmic) phosphorylation sites. Scheme a considers the C-terminus to be cytoplasmic (Young et al., 1985; Ratnam et al., 1986a). This would require one to assume one or more additional membrane-spanning domains (Finer-Moore & Stroud, 1984; Guy, 1984; Ratnam et al., 1986b). Scheme b alternatively considers an extracellular (possibly membrane associated) C-terminus which would also agree with the epitope mapping data of Young et al. (1985) and Ratnam et al. (1986a). Scheme c is another alternative.

matching sequence α387–392 (Figures 7 and 8). As endoglycosidase-treated but not untreated nAChR is recognized by anti-P3 serum, this serum is likely to be directed against α137–142 with this region being exposed. Thus, the sequence portions matching the peptides P1, P2, P3, and P6 probably all lie at hydrophilic surfaces of the receptor.

The sequence regions α161–166 (P5) and α395–401 (P8) are not accessible for antibody binding in native nAChR. Thus, they may be part of membrane-spanning domains as was suggested for parts of α161–166 (Ratnam et al., 1986a), or they may exist in native nAChR in a conformation not matched by the synthetic peptide. As anti-P8 serum bound to solubilized nAChR and to purified α-subunits, the region α395–401 appears to be in a detergent-sensitive environment, i.e., associated to hydrophobic domain M4 or the plasma membrane. This agrees with previous findings (Lindstrom et al., 1984; Young et al., 1985; Ratnam et al., 1986b) showing that the C-terminus of *Torpedo* α-polypeptide is not freely accessible to antibody binding in membrane-bound receptor.

Detergent treatment of native nAChR did not suffice to permit binding of anti-P5 serum. Similarly, treatment with urea, guanidinium, or dithiothreitol was insufficient to expose this epitope. The sequence α161–166 therefore appears to be located in the core of the nAChR structure. It may be part of a membrane-spanning domain (Ratnam et al., 1986b). Alternatively, it may be involved in interloop interactions.

In view of the limited sequence specificity of anti-peptide antibodies, we do not consider any of the models of nAChR topology based on these (Young et al., 1985; Ratnam et al., 1986a,b; Lindstrom, 1986) as sufficiently founded. This applies to location (extracellular or cytoplasmic side) of the C-terminal region and the evidence for or against transmembrane domains in addition to those suggested by hydropathy profiles. Thus, the available antibody binding data so far do not suffice to establish or reject any one of the alternative schemes of transmembrane topology depicted in Figure 11. With libraries of high-affinity antibodies for native nAChR available, however, it may only require an appropriate analysis of their epitope specificity (see Figures 7 and 8) before more reliable data on receptor topology will emerge.

ACKNOWLEDGMENTS

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Registry No. C17, 117861-13-9; C48, 117861-23-1; C49, 117861-24-2; C50, 117861-25-3; C51, 117861-26-4; C52, 117861-27-5; C53, 117861-28-6; C54, 117861-29-7; C55, 117861-30-0; C56, 117861-31-1; E1, 117861-01-5; E12, 115521-13-6; E15, 117861-10-6; E16, 117861-11-7; E17, 117861-12-8; E47, 91400-94-1; E48, 117860-97-6; E49, 117860-98-7; E50, 117860-99-8; E51, 117861-00-4; E52, 117861-02-6; E53, 117861-03-7; E54, 117861-04-8; E55, 117861-05-9; E56, 117861-06-0; E57, 117861-07-1; E58, 117861-08-2; E59, 117861-09-3; E60, 117872-87-4; E61, 117861-14-0; E62, 117861-15-1; E63, 117861-16-2; E64, 117872-88-5; E65, 117861-17-3; E66, 117861-18-4; E67, 117861-19-5; E68, 117861-20-8; E69, 117861-21-9; E70, 117861-22-0; P1, 117860-91-0; P2, 117860-92-1; P3, 117860-93-2; P4, 117860-94-3; P5, 95758-98-8; P6, 117860-95-4; P7, 117872-86-3; P8, 117860-96-5.

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