Monoclonal Antibodies against Synthetic Sequences of the Nicotinic Receptor Cross-React Fully with the Native Receptor and Reveal the Transmembrane Disposition of Their Epitopes†

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ABSTRACT: Monoclonal antibodies (mAbs) were derived from mice immunized with synthetic peptide sequence regions of the α subunit of the nicotinic acetylcholine receptor from *Torpedo* electric tissue (TAChR). Sequence-specific mAbs were obtained against the following peptides: α1–20, α291–308, α304–322, α332–350, α346–364, α360–378, α376–393, α390–409, and α420–437. The ability of mAbs to recognize native TAChR was quantitated by immunoprecipitation of TAChR solubilized in the nondenaturing detergent Triton X-100. mAbs against peptide α304–322, α332–350, and α360–378 cross-reacted with most or all Triton-solubilized TAChR molecules and, in immunoelectron microscopy experiments, bound to the cytoplasmic surface of AChR-rich postsynaptic membrane fragments. Two mAbs specific for the sequence α376–393, proposed to form an amphypathic α helix possibly involved in formation of the ion channel, recognized only ~35% of Triton-solubilized TAChR molecules and did not react with membrane-bound TAChR. All of these sequence-specific antibodies recognized SDS-denatured TAChR α subunit in Western blots. MAbs specific for the amino-terminal sequence region of the α subunit, α1–20, and for the sequences α291–308, α346–364, and α390–409 did not recognize native TAChR. A mAb directed against the carboxyl-terminal region, α420–437, recognized with low apparent titer Triton-solubilized TAChR, not membrane-bound TAChR. In conclusion, a complex membrane protein, TAChR, contains several continuous sequence segments exposed on the TAChR surface, because different mAbs raised against certain synthetic sequences recognized most or all native TAChR molecules. By analogy, it should be possible for most proteins of known sequence to raise anti-peptide antibodies fully cross-reactive with the native cognate protein.

The amino acid sequence of an increasing number of proteins is available due to the easy cloning and sequencing of their genes. Short synthetic segments of such deduced sequences are frequently used to raise antibodies (Abs)[1] to be employed in the study of the cognate proteins, whose low abundance may preclude more direct approaches, or for vaccination procedures [reviewed in Van Regenmortel (1987, 1989), Steward and Howard (1987), Allison and Gregoriadis (1990), and Bahouth et al. (1992)]. Abs against synthetic sequences of scarce membrane receptors have been used to study their cellular localization, subunit composition, transmembrane topology, and structure of their functional domains [reviewed in Bahouth et al. (1992)]. Synthetic sequences of proteins from human and cattle pathogens (viruses, bacteria) are being studied as vaccines, to induce sensitization of T cells and synthesis of Abs able to neutralize the pathogen [e.g., see Bessen and Fischetti (1990), Tanaka et al. (1991), and Wang et al. (1991)].

Due to their practical importance, it is important to properly investigate the legitimacy of these approaches, because, although they are widely used, it is still nuclear whether Abs specific for synthetic peptide sequences recognize at all the *native* cognate protein, as much occur when Abs are used for studies of the function and tridimensional structure of the cognate protein, for isolation of the native protein, or for its quantification. It has been argued that—since the epitopes for Abs against native proteins are "discontinuous", i.e., formed by residues from different, sometimes distant, regions of the antigen sequence (Barlow et al., 1986; Amit et al., 1986; Sheriff et al., 1987; Colman et al., 1987; Davis et al., 1988, 1989)—any cross-reactivity between the anti-peptide Ab and the corresponding native protein must be due to the presence of denatured molecules in preparations of "native" proteins.

In support of the above considerations, it has been demonstrated that anti-peptide Abs believed to recognize the native protein because they did so in solid-phase ELISA assays (where the protein, due to its interaction with the plastic surface, may be partially denatured) did not recognize the same native protein in solution (Spangler, 1991). That elegant study, however, investigated the properties of anti-peptide Abs against a very limited sequence region of the corresponding proteins, and it could not exclude that other synthetic sequences regions of the same proteins could be used to raise Abs fully cross-reactive with the cognate native molecule.

In the present study we used the well-characterized nicotinic acetylcholine receptor from *Torpedo* electric tissue (TAChR) to quantify the ability of monoclonal Abs (mAbs) raised

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[1] Abbreviations: Ab, antibody; mAb, monoclonal antibody; TAChR, nicotinic acetylcholine receptor from *Torpedo* electric tissue; α-BGT, α-bungarotoxin; [^{125}I]-α-BGT, radiolabeled α-bungarotoxin; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; HPLC, high-pressure liquid chromatography; PBS, 10 mM sodium phosphate buffer (pH 7.4) containing 140 mM NaCl; PBS-Tween, PBS containing 0.01% Tween; ECDI, 3-ethyl-(3-ethylenediamine)carbodiimide; FA, Freund's adjuvant; BSA, bovine serum albumine; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; TBS-T, 10 mM Tris, 140 mM NaCl, pH 7.4, containing 0.1% Tween 20; IVH, influenza virus hemagglutinin; M1, M2, M3, M4, sequence regions of the TAChR subunits proposed to form transmembrane α helices; MA, sequence region of the TAChR subunits proposed to be involved in ion channel formation because of its characteristic of amphipatic α-helix periodicity.

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against different 20-residue synthetic TAChR sequences to recognize native TAChR. The synthetic peptides used corresponded to a large part of the sequence of the TAChR α subunit, including the amino- and carboxyl-terminal segments, and the sequence region (residues α214–427) comprising four putative transmembrane regions (M1–M4) and a putative cytoplasmic region (residues α397–408), for which either cytoplasmic, extracellular, or transmembrane dispositions have been proposed (Maelicke, 1988; Claudio, 1989; Stroud et al., 1990; Moore et al., 1991). The ability of mAbs to recognize all or part of the native TAChR molecules was determined by quantitative immunoprecipitation of TAChR complexes with radiolabeled α-bungarotoxin (α-BGT), solubilized in the nondenaturing detergent Triton X-100. The ability of mAbs to recognize native TAChR in the postsynaptic membrane was determined by immunoelectron microscopy, which also indicated the transmembrane topology of the corresponding sequence regions.

MATERIALS AND METHODS

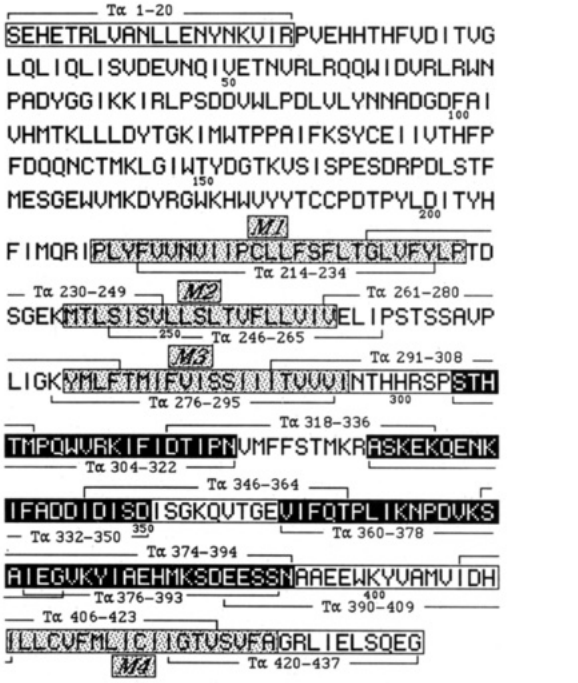
Rationale. The goals of the present paper were (i) to determine, using a well-characterized protein, TAChR, whether Abs against synthetic sequence regions can recognize native forms of the cognate protein and (ii) to gain information about the transmembrane disposition of the sequence region of the TAChR α subunit comprised between the two putative transmembrane segments M3 and M4, since conflicting conclusions about its transmembrane topology have been reported.

Toward these goals, we synthesized overlapping peptides corresponding to the complete sequence of *Torpedo* AChR α subunit. MAbs were raised against peptides corresponding to the amino-terminal and carboxyl-terminal sequences and to the regions joining the different putative transmembrane segments, M1–M4 (Figure 1).

To sensitize B cells with the broadest spectrum of possible peptide conformations, and to increase the chance of obtaining anti-peptide mAbs cross-reactive with native TAChR, we immunized mice with peptides coupled to a carrier [keyhole limpet hemocyanin (KLH)] or with uncoupled peptides. We also used synthetic peptides containing both a T epitope for the mouse strain used and the TAChR sequence region toward which we sought to obtain mAbs.

Two mice strains or different major histocompatibility complex (MHC) haplotype were used, because they are expected to recognize different B epitopes on the same protein antigen.

Peptide Synthesis and Characterization. Overlapping peptides 18–21 residues long, corresponding to the complete *Torpedo* α-subunit sequence [as reported by Noda et al. (1983); see Figure 1], were synthesized (Houghten, 1985). They are indicated with codes that include Tα for *Torpedo* AChR α subunit and two numbers which indicate the position on the α-subunit sequence of the first and last residue of the peptide. They corresponded to the following sequence segments: Tα1–20, Tα15–33, Tα30–47, Tα43–60, Tα55–74, Tα63–80, Tα75–94, Tα91–110, Tα106–122, Tα118–137, Tα126–145, Tα134–153, Tα150–169, Tα165–184, Tα181–200, Tα197–216, Tα214–234, Tα230–249, Tα246–265, Tα261–280, Tα276–295, Tα291–308, Tα304–322, Tα318–336, Tα332–350, Tα346–364, Tα360–378, Tα374–394, Tα390–409, Tα406–423, Tα420–437. We also synthesized three peptides which included the sequence Tα304–322, which forms an immunodominant T helper epitope in Balb/C mice (Bellone et al., 1991), followed by one of the following three sequence segments of the *Torpedo* AChR α subunit: Tα234–244, Tα266–276, and Tα349–359. The sequences of the last three peptides are given in Figure 1.



Tα304-322/234-244	STHTMPQWVRKIFIDTIPNYLPTDSGEKMT
Tα304-322/266-276	STHTMPQWVRKIFIDTIPNSTSSAVPLLGK
Tα304-322/349-359	STHTMPQWVRKIFIDTIPNSDISGKQVTGE

FIGURE 1: Synthetic peptides used to immunize mice for mAb production, aligned on the sequence of TAChR α subunit [as reported by Noda et al. (1982)]. The peptides are indicated by their code and by a line above or below the α-subunit sequence, which includes the α-subunit residues comprised by each peptide. The peptide code includes Tα, for *Torpedo* α subunit, and two numbers, which indicate the position on the α-subunit sequence of the first and last residues of the peptide. The sequence regions enclosed in dotted boxes are the putative transmembrane segments M1–M4. Sequence regions enclosed in white boxes correspond to peptides for which sequence-specific mAbs unable to cross-react with native TAChR were obtained; residues in black boxes correspond to peptides which induced synthesis of sequence-specific mAbs cross-reactive with native TAChR. The box at the bottom of the figure indicates the sequence of three peptides which included an α-subunit region forming an immunodominant T helper epitope (sequence α304–322), plus an additional α-subunit sequence segment, as indicated by their codes.

Peptide purity was assessed by reversed-phase high-pressure liquid chromatography (HPLC) and, for a few randomly selected peptides, by amino-terminal gas-phase sequencing (Applied Biosystems, Foster City, CA). One main peak and the expected sequence were consistently found. Amino acid composition (Heinrickson and Meredith, 1983) of the synthetic peptides yielded excellent correspondence between expected and experimental values for all peptides.

Coupling of Peptides to KLH. Some peptides (see below) were coupled to the protein carrier KLH, to increase their antigenicity. Two milligrams of peptide in 500 μL of 50% dioxane–water was mixed with 3 mg of KLH in 150 μL of H_2O and 0.2 mg of 3-ethyl-(3-ethylenediamine)carbodiimide (ECDI) and incubated at room temperature for 12 h with stirring. Another 6.2 mg of ECDI was added and the incubation continued for 12 h. The peptide/KLH conjugates were dialyzed against 10 mM sodium phosphate buffer (pH 7.4) containing 140 mM NaCl (PBS) and stored frozen.

We also used a peptide/peptide conjugate of the synthetic sequence Tα360–378, which forms an immunodominant T

Table I: Sequence-Specific Anti-Peptide MABs Used in This Study

mABs(s)	immunogen[a]	strain	reactivity with solubilized AChR	reactivity with membrane-bound AChR[b]
Vα1–20	Tα1–20	Balb/C	–	NT[c]
Vα291–308/1/2/3	pool 2[d]	C57BL/6	–	NT[c]
Vα304–322/1/2	Tα304–322	Balb/C	+	+
Vα304–322/3	Tα304–322/349–359[e]	Balb/C	+	+
Vα332–350/1/2	Tα332–350	Balb/C	+	+
Vα332–350/3	Tα332–350	Balb/C	–	NT
Vα332–350/4	Tα332–350	Balb/C	–[f]	NT
Vα346–364/1	Tα346–364	Balb/C	–	NT
Vα346–364/2	Tα346–364–KLH	Balb/C	–	NT
Vα360–378/1	Tα360–378	C57BL/6	+	+
Vα360–378/2	Tα360–378/261–280[g]	C57BL/6	–	NT
Vα376–393/1/2	pool 1[h]	Balb/C	+	–
Vα390–409/1	Tα390–409	Balb/C	–	NT
Vα390–409/2/3/4	pool 3[i]	Balb/C	–	NT
Vα420–437/1/2	Tα420–437	Balb/C	–	NT
Vα420–437/3	Tα420–437	Balb/C	±[j]	–

[a] Unless specified, the peptides were used for immunization without coupling to KLH. [b] Tested by immunoelectron microscopy. See text for experimental details. [c] NT, not tested. [d] Pool 2 was an equimolar mixture of three peptides: Tα291–308, Tα304–322, Tα318–336, not coupled to KLH. [e] The peptide had a sequence corresponding to the two sequence regions of *Torpedo* AChR α subunit, as indicated (see also Figure 1). [f] This mAb cross-reacted, albeit to a lesser extent, with peptide Tα304–322, which contains the sequence KIFID, very similar to the sequence KIFAD, contained within the immunizing peptide Tα332–350. Therefore, this mAb seems to recognize a linear epitope which includes this sequence region, of which three residues—K, I/A, and F—are hydrophobic and presumably buried in the core of the AChR molecule, hence its lack of reactivity with nondenatured AChR. [g] Obtained by coupling peptides Tα360–378 and Tα261–280. See text for experimental details. [h] Pool 1 was an equimolar mixture of 10 peptides: Tα230–249, Tα261–280, Tα291–308, Tα304–322, Tα318–336, Tα346–364, Tα360–378, Tα374–394, Tα376–393, Tα390–409, not coupled to KLH. [i] Pool 3 was an equimolar mixture of three peptides: Tα346–364, Tα360–378, Tα390–409, not coupled to KLH. [j] This mAb cross-reacted with most or all of the synthetic peptides with which it was tested in SPRIA experiments (32 peptides, corresponding to the complete α-subunit sequence) when tested at low ionic strength (PBS). When tested in the presence of 0.5 M NaCl, it only recognized peptide Tα406–423, which overlaps the immunizing peptide Tα420–437 by five residues, IIGTV, and—to a low degree—peptide Tα118–137, which contains the sequence IIVT. Further, this mAb recognized nondenatured, Triton X-100 solubilized AChR inefficiently, while it specifically recognized the denatured α subunits in Western blots (data not shown). All of this suggests that it recognizes a linear, largely hydrophobic epitope which includes residues IIVT. See text for further details.

Table II: Anti-Peptide MAbs Cross-Reacting with a Number of Unrelated Synthetic Sequences[a]

mAb(s)	immunogen[b]	strain	reactivity with solubilized AChR
PVα230–249/1/2/3/4[c]	Tα230–249	Balb/C	no
PVα230–249/5/6/7/8/9	Tα230–249–KLH	Balb/C	no
PVα261–280/1/2/3/4/5/6	Tα261–280	Balb/C	NT
PVα261–280/7/8	Tα261–280–KLH	Balb/C	no
PVα261–280/9⇒/35[c]	Tα261–280–KLH	Balb/C	NT
PV291–308/1/2/3/4/5	Tα291–308–KLH	Balb/C	no
PVα420–437/1⇒/11	PVα420–437–KLH	Balb/C	no
PVα420–437/12⇒/19	PVα420–437–KLH	Balb/C	NT
Vα420–437/3[d]	Tα420–437	Balb/C	marginally[d]

[a] These mAbs cross-reacted with all of the synthetic peptides tested [either the complete panel of 32 peptides corresponding to the complete α-subunit sequence or smaller panels (5–10 peptides) of randomly selected α-subunit synthetic sequences]. [b] Unless specified, the peptides were used for immunization without coupling to KLH. [c] These mAbs were obtained in different fusions, using different mice. [d] Also reported in Table I. See footnote *j* of Table I.

helper epitope in C57BL/6 mice (Bellone et al., 1991a,b), coupled, using the procedure described above, to the peptide sequence Tα261–280.

Immunization of Mice. Balb/C (H-2^d) and C57BL/6 (H-2^b) mice (Harlan Sprague Dawley, Indianapolis) were injected with peptide pools or individual peptides. Individual peptides were used either as KLH conjugates or uncoupled. Peptide pools were not conjugated to KLH.

Solutions of peptide(s) or KLH/peptide in BPS were emulsified with an equal volume of Freund's adjuvant [(FA) complete FA for first injection, incomplete FA for the following injections]. Approximately 200 μL containing 50–200 μg of peptide (of each peptide when peptide pools were used) was injected in three or four spots subcutaneously along the back. Mice were immunized three to five times at intervals of 2–4 weeks.

In a first set of experiments we used peptide pools, i.e., equimolar mixtures of different peptides, as follows: (pool 1) Tα230–249, Tα261–280, Tα291–308, Tα304–322, Tα318–336, Tα346–364, Tα360–378, Tα374–394, Tα376–393, Tα390–409; (pool 2) Tα291–308, Tα304–322, Tα318–336; (pool 3) Tα346–364, Tα360–378, Tα390–409. After immunization with a peptide pool, the mAbs obtained were mostly against one peptide (see Results). Therefore, mice were also immunized with the following individual synthetic sequences: Tα1–20, Tα230–249, Tα261–280, Tα291–308, Tα304–322, Tα332–350, Tα346–364, Tα360–378, Tα390–409, Tα420–437. The individiual synthetic sequences were used uncoupled or coupled to KLH or as part of a longer peptide which also included a sequence forming a strong T epitope for Balb/c or C57BL/6 mice (see above). Not all immunized mice yielded anti-peptide mAbs. Tables I and II list the mAbs we obtained and the immunogen used.

Dot Blot Assay of Anti-Peptide Abs in the Mouse Sera. One-microliter aliquots of peptide solutions (1 mg/mL in water) were spotted onto nitrocellulose strips. The strips were soaked in PBS containing 0.01% Tween (PBS-Tween) plus 3% bovine serum albumine (BSA) for 20 min, incubated for 2 h at room temperature with different dilutions of mouse antiserum, washed three times with PBS-Tween, incubated

for 30 min with alkaline phosphatase labeled goat anti-mouse IgG (Sigma, a 1/3000 dilution in PBS), and developed for 5 min with alkaline phosphatase substrate solution (bromochloroindolyl phosphate/nitro blue tetrazolium).

Fusion and Generation of Hybridomas. Spleen cells of mice with high serum Ab titers were used for fusions with NS-1 or SP2/0 Ag14 myeloma cells to generate hybridoma libraries. Screening of the hybridomas for anti-peptide and anti-TAChR Ab secretion was done by ELISA.

ELISA Assay of Anti-Peptide and Anti-TAChR Ab Production. Ninety-six-well plates (Nunc, Karstrup, Denmark) were incubated for 4 h at room temperature with either (i) 100 μL of peptide solution (10 μg/mL in 10 mM potassium phosphate buffer, pH 7.4) or (i) 100 μL of a suspension of TAChR-rich fragments (100 pmol/mL in 20 mM Na_2CO_3/$NaHCO_3$ buffer, pH 9.6). The wells were washed twice with PBS-Tween, incubated for 1 h with PBS-Tween plus 3% BSA, washed once with PBS-Tween, incubated for 2 h with hybridoma supernatant, washed three times with PBS-Tween, incubated for 30 min with peroxidase-labeled goat anti-mouse IgG (Bio-Rad, Richmond, CA; a 1/3000 dilution in PBS), washed three times with PBS-Tween, and developed for 5 min with peroxidase substrate solution (0.01 M *o*-phenylenediamine, pH 5.0, containing 48% 0.1 M citric acid, 52% 0.2 M Na_2HPO_4, and 0.024% H_2O_2). The reaction was stopped by adding 2.5 M H_2SO_4. The optical density was read at 490 nm. Alternatively, after the hybridoma supernatant was washed out with PBS-Tween, rabbit anti-mouse IgG antiserum was added (a 1/3000 dilution in PBS), three washes were done with PBS-Tween, and the bound Ab was revealed by incubation with ^{125}I-labeled protein A (100 000 cpm/well in PBS), radioiodinated using Chloramine T (Lindstrom et al., 1981). After incubation at room temperature for 4 h and three washes with PBS-Tween, the bound radioactivity was stripped with 1% SDS and counted in a Biogamma II counter (Beckman Instruments, Fullerton, CA).

Preparation of Solubilized TAChR. TAChR from *Torpedo californica* electric tissue was extracted in Triton X-100 as follows. Fifty grams of frozen electric organ was sliced in 0.5-in. cubes and homogenized in a Virtis 45 homogenizer three times for 30 s at 30 000 rpm with intervals of 3 min, using 50 mL of 500 mM sodium phosphate buffer containing 400 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.8 (homogenization buffer). The homogenate was centrifuged at 25000*g* for 120 min, the pellet was collected and resuspended in a Virtis 45 homogenizer at 20 000 rpm for 20 s three times in 10 mL of homogenization buffer, and 1% Triton X-100 was added. After 1 h of incubation at 4 °C with stirring, the extract was centrifuged at 25000*g* for 1 h and the supernatant was collected. All procedures were carried out at 4 °C.

Immunoprecipitation of Native TAChR by MAbs. Triton X-100 solubilized TAChR ($\sim 10^{-9}$ M in PBS containing 1% Triton X-100) was labeled with 3-fold excess of radiolabeled α-bungarotoxin ([^{125}I]-α-BGT) overnight at 4 °C. To 1-mL aliquots of labeled TAChR was added 10–500 μL of hybridoma supernatant (or supernatant of NS-1 lymphoma cultures as a negative control), the volume was adjusted to 1.5 mL with PBS containing 0.1% Triton X-100, and the mixture was incubated for 4 h at room temperature. An optimal precipitating amount of rabbit anti-mouse IgG antiserum was added and incubated for 2 h at room temperature. The precipitate was pelleted at 15000*g* for 10 min, washed three times by centrifugation in PBS containing 0.1% of Triton X-100, and counted in a Biogamma II counter (Beckman Instruments).

Preparation of TAChR-Rich Postsynaptic Membrane Fragments. TAChR was purified from *T. californica* electric organ as alkali-stripped TAChR-rich membrane fragments (Elliot et al., 1979). TAChR concentration was determined as α-BGT binding sites (Schmidt & Raftery, 1973) and protein concentration by the Lowry assay (Lowry et al., 1951). The specific activity of the TAChR preparations used in this study was 2–6 nmol of α-BGT binding sites/mg of protein (specific activity of pure TAChR: 7.2 nmol/mg of proteins). Protein composition of purified alkali-stripped TAChR-rich membrane fragments, analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Laemmli, 1970), showed only four major protein bands of the molecular weight expected for TAChR subunits. Minute amounts of low molecular weight components, possibly degradation products of TAChR subunits, were frequently present.

Western Blots. Alkali-stripped TAChR-rich membrane fragments were resuspended in 10 mM sodium phosphate buffer, pH 7.4, and electrophoresed (20–40 μg of protein/3 mm of gel) on a 8.75% polyacrylamide slab gel with 4% stacking gel (Laemmli, 1970), and Western blots were carried out as described previously (Nelson & Conti-Tronconi, 1990). Briefly, the electrophoresed protein bands were transferred onto a 0.45-μm nitrocellulose sheet (Towbin et al., 1979). The nitrocellulose sheet was cut into 3-mm strips, blocked with 1 mg/mL BSA in 10 mM Tris and 140 mM NaCl, pH 7.4, containing 0.1% Tween 20 (TBS-T), and incubated for 2 h at room temperature with the test antibody, diluted as needed in TBS-T and washed twice in TBS-T. The strips were further incubated for 2 h with rabbit anti-mouse IgG antibody (Sigma), diluted 1/1000 in TBS-T, and then washed as above. Radioiodinated protein A (1×10^6 cpm in 1 mL of BSA/TBS-T) was added and incubated for 1–2 h at room temperature. After three washes in TBS-T, the strips were dried and autoradiographed for 2–4 days at −70 °C using a Quanta III intensifying screen and Kodak RP film.

Immunoelectron Microscopy. The transmembrane orientation of the TAChR epitopes recognized by sequence-specific mAbs was determined by a modification of the methods of LaRochelle et al. (1985), using TAChR-rich postsynaptic membrane fragments adsorbed onto the bottom of polylysine-coated wells of microtiter plates (Falcon) by centrifugation at 3000 rpm for 20 min in a Beckman GPR centrifuge. The membranes were fixed with 0.2% gluteraldehyde (electron microscopy grade, Electron Microscopy Sciences, Redding, CA) for 20 min, treated with $NaBH_4$ (1 mg/mL, 20 min), incubated with the test mAb supernatant for 2 h at room temperature, rinsed three times with 10 mM sodium phosphate buffer, pH 7.4, fixed and reduced again as above, and incubated for 1 h with goat anti-mouse-colloidal gold (5 nm, Ted Pella, Inc., Redding, CA). A third fixation (1% gluteraldehyde) and reduction followed. The vesicles were then incubated with the control mAb 35 [kindly provided by Dr. Jon Lindstrom; mAb 35 recognizes the main immunogenic region, on the extracellular surface of the TAChR (Tzartos et al., 1981)] for 1 h, and a fixation and a reduction step were carried out as described above, followed by addition of goat anti-rat-colloidal gold (10 nm, Ted Pella). The membranes were fixed first with 2% gluteraldehyde–tannic acid at pH 7.4 for 30 min and then with 1% osmium for 30 min and prepared for electron microscopy as described by LaRochelle et al. (1985). For competition experiments, the mAb solutions were incubated overnight at 4 °C with 250 μg/mL of the relevant peptide before incubation with the TAChR-rich membrane fragments.

RESULTS

Production of Anti-Peptide MAbs. Tables I and II summarize the antipeptide mAbs obtained after immunization with different peptide pools or with individual peptides. Table I lists the anti-peptide mAbs which, as described in the next sections, uniquely recognize a sequence region of the TAChR. Table II lists a group of mAbs which, although raised against one peptide sequence, cross-reacted with a large number of unrelated synthetic peptides, as described in the next sections. The sequence-specific mAbs are indicated with codes that include the letter V (versus), the symbol α for α subunit, and numbers indicating the synthetic sequence region of the α subunit used for immunization and recognized by the mAb. The mAbs indicated in Table II, which cross-reacted with synthetic sequences unrelated to those used for immunization, are indicated with a code including the letters PV (putative versus), in addition to the symbol α and the numbers indicating the synthetic sequence used for mouse immunization.

When peptide pools were used for immunization, only one peptide consistently dominated the Ab response. For example, although pool 1 was an equimolar mixture of 10 different peptides, only mAbs against peptide Tα376–393 were obtained. After immunization with pool 2, only mAbs against peptide α291–308 were obtained in C57BL/6 mice, and after immunization with pool 3, only mAbs against peptide Tα390–409 were obtained. The immunodominance of one particular peptide when a pool was used may be related to competition among peptides in the T helper and B compartments, as occurs for immunodominant T and B epitopes on large protein antigens (Berzofsky et al., 1988; Manca et al., 1988).

While several peptides consistently induced formation of Abs which uniquely recognized the corresponding sequences (e.g., Tα1–20, Tα304–322, Tα332–350; Table I), other synthetic sequences preferentially or exclusively induced mAbs that cross-reacted with several unrelated synthetic sequences (Table II). For example, different Balb/C mice injected with peptides Tα230–249 and Tα261–280, either coupled to KLH or uncoupled, only yielded mAbs cross-reactive with many seemingly unrelated synthetic α-subunit sequences. Other peptides, i.e., Tα291–308 and Tα420–437, induced formation both of sequence-specific mAbs and of mAbs cross-reactive with unrelated sequences. Perusal of the sequence of the peptides which induced mAbs cross-reactive with unrelated sequences (Figure 1) reveals that they all contain substantial stretches of hydrophobic residues.

Production of anti-peptide Abs did not necessitate coupling of peptides to a carrier. Therefore, the synthetic sequences we used were long enough (20 residues) to accommodate both a T and a B epitope. Coupling to KLH seemed to correlate with cross-reactivity with unrelated sequences: e.g., sequence-specific mAbs against peptides Tα291–308 and Tα420–437 were obtained when uncoupled peptides were used, while mAbs cross-reactive with unrelated sequences were obtained when we used these peptides conjugated to KLH.

Sequence Specificity of Anti-Peptide MAbs. The sequence specificity of the mAbs produced by hybridomas identified by ELISA screening with the peptides used for the immunization was verified by ELISA using the complete panel of peptides corresponding to the *Torpedo* α-subunit sequence. The mAbs listed in Table I uniquely recognized the sequence regions used for immunization and in some cases, when their epitope was formed by the amino- or carboxyl-terminal regions of the peptide sequence, an overlapping synthetic sequence. The data in Figure 2 report the results obtained with some sequence-specific mAbs (those recognizing sequence regions which

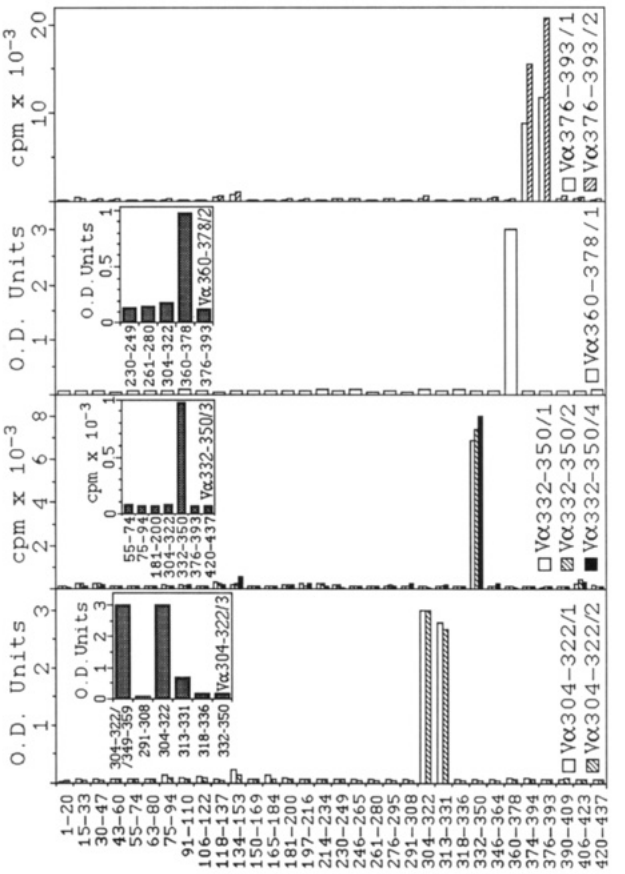


FIGURE 2: Sequence specificity of mAbs cross-reactive with native TAChR. The mAbs were tested in ELISA assays using the complete panel of synthetic peptides screening the TAChR α-subunit sequence or with selected α-subunit synthetic sequences, including the ones used for immunization. Consistently, the mAbs recognized the immunizing peptide and, for some mAbs, one of the overlapping, flanking synthetic sequences (see text for experimental details).

induced formation of Abs cross-reactive with native TAChR, see below). All mAbs listed in Table I yielded results similar to those reported in Figure 2. MAbs Vα304–322/1 and Vα304–322/2 recognized to a similar extent peptide Tα304–322 and the overlapping peptide Tα313–331. Their epitope should comprise the region of overlap of these two synthetic sequences, i.e., Tα313–322. MAb Vα304–322/3, obtained by immunization with peptide Tα304–322/349–359 (see Figure 1), reacted similarly and strongly with the immunizing peptide and with peptide Tα304–322, while it recognized weakly the overlapping peptide Tα313–331. This mAb therefore recognizes an epitope different from that/those recognized by mAbs Vα304–322/1 and Vα304–322/2. In agreement with this conclusion, mAbs Vα304–322/1 and Vα304–322/2 fully cross-react with native TAChR, while mAb Vα304–322/3 does not (see below). MAbs Vα376–393/1 and Vα376–393/2 recognized peptide α376–393 and, to a slightly lesser extent, the largely overlapping peptide Tα374–394. MAbs Vα304–322/3 and Vα360–378/2, which have been raised against synthetic sequences containing two different regions of the α subunit (see Table I), uniquely recognized peptides Tα304–322 (and the overlapping peptide Tα313–331, see above) and Tα360–378, respectively.

The mAbs listed in Table II recognized many seemingly unrelated α-subunit synthetic sequences, suggesting that they recognize a structural feature common to all synthetic peptides (e.g., amino terminus, carboxyl terminus, peptide backbone). MAb Vα420–437/3 recognized many α-subunit sequences when the ELISA was carried out at the normal ionic strength

Table III: Ability of Different Sequence-Specific Anti-Peptide MAbs To Precipitate Native AChR

antibody	experiment 1		experiment 2		experiment 3	
	precipitated AChR (fmol)	%	precipitated AChR (fmol)	%	precipitated AChR (fmol)	%[a]
anti-AChR serum	1011 ± 7.6	100	887 ± 4.7	100	ND[b]	ND
anti-AChR (Balb/b)	582 ± 21	57.6	593 ± 3.3	66.9	644 ± 5	64.5
anti-AChR (CB17)	606 ± 0.5	59.9	658 ± 8.6	74.2	742 ± 9.8	74.2
Vα304–322/1	716 ± 5.7	70.8	780 ± 7.16	87.9	622 ± 4.2	62.2
Vα304–322/2	640 ± 4	63.3	721 ± 7.6	81.3	576 ● 5.8	57.6
Vα304–322/3	159 ± 6	15.7	92 ± 3.4	10.4	ND	ND
Vα332–350/1	754 ± 18	74.6	660 ± 5.9	74.4	903 ± 40	90.3
Vα332–350/2	753 ± 25	74.5	608 ± 10	68.5	977 ± 1.6	97.7
Vα360–378/1	742 ± 3	73.4	605 ± 13	68.2	881 ± 18	88.1
Vα376–393/1	380 ± 20	37.6	348 ± 0.7	39.2	328 ± 4.6	32.8
Vα376–393/2	ND	ND	344 ± 1.5	38.8	322 ± 1.7	32.2

[a] Since the maximum precipitation by anti-AChR polyclonal serum was not determined for this experiment, we assumed 100% to be 1000 fmol, i.e. the nominal amount of Triton-solubilized AChR used for each sample. [b] ND, not determined.

(see Materials and Methods). However, at high ionic strength (PBS plus 0.5 M NaCl) it only recognized peptide Tα406–423, which overlaps the immunizing peptide Tα420–437 by five residues, IIGTV, and—to a low degree—peptide Tα118–137, which contains the sequence IIVT. Furthermore, this mAb recognized nondenatured, Triton X-100 solubilized TAChR inefficiently, while it specifically recognized the denatured α subunits in Western blots (data not shown). All of this suggests that this mAb recognizes a linear, largely hydrophobic epitope which includes residues IIVT.

Anti-Peptide MAbs Can Recognize Native TAChR. All sequence-specific mAbs listed in Table I were tested for cross-reactivity with native Triton X-100 solubilized native TAChR, as described under Materials and Methods. All three mAbs specific for the sequence Tα304–322, two of the four mAbs obtained against the sequence Tα332–350, one of the two mAbs obtained against the sequence Tα360–378, and both mAbs obtained against the sequence Tα376–393 cross-reacted well with solubilized TAChR. One mAb against the carboxyl-terminal region of the α subunit, Vα420–433/3, recognized Triton-solubilized TAChR with a very low apparent titer. Typical titers obtained in the different hybridoma supernatants for those mAbs are reported along the abscissa of Figure 4.

The ability of sequence-specific mAbs to precipitate native TAChR was quantitated in experiments where increasing amounts of mAb were used to precipitate a fixed amount (one nominal picomole) of TAChR. As a control, we used total serum against native TAChR, raised in C57BL/6 mice by immunization with purified membrane-bound native *Torpedo* AChR as described in Bellone et al. (1991a,b), and two mAbs specific for native TAChR derived from Balb/B (H-2^b) and CB17 (H-2^d) mice immunized with native TAChR (Bellone et al., 1991b). We used anti-TAChR mAbs in addition to the polyclonal serum because mAbs are less efficient than polyclonal Abs in precipitation assays, due to their binding to only one epitope on the antigen molecule (two epitopes in the case of TAChR α-subunit (epitopes) and less efficient formation of a tridimensional lattice when the second precipitating Ab is added. Anti-TAChR mAbs from mice of different H-2 haplotype were used because they should recognize different epitopes on the TAChR (Bellone et al., 1991b). The results obtained in three different experiments are reported in Table III. MAbs against peptides Tα304–322, Tα332–350, and Tα360–378 precipitated the majority of the TAChR molecules present, i.e., in amounts comparable to those precipitated by the two native anti-TAChR mAbs. We can therefore exclude the possibility that they recognize a small fraction of partially denatured TAChR molecules.

Table IV: MAbs Vα376–393/1 and Vα376-393/2, Alone or in Association, Precipitate the Same Amount of Native AChR[a]

antibody	precipitated AChR (fmol)	%
anti-AChR serum	469 ± 6.4	100
anti-AChR (Balb/b)	264.5 ± 2.5	56.4
anti-AChR (CB17)	271 ± 15	57.7
Vα376–393/1	157 ± 11	33.5
Vα376–393/2	176 ± 15	37.6
Vα376–393/1 plus Vα376–393/2	151 ± 17	32.3

[a] Maximum amount of native AChR precipitated from identical aliquots of Triton X-100 solubilized AChR, by a polyclonal anti-native AChR antiserum, by two different anti-native AChR mAbs, and by the anti-peptide mAbs Vα376–393/1 and Vα376–393/2, alone or combined. See text for experimental details.

On the other hand, the mAb Vα304–322/3 consistently precipitated a maximum of 10–15% of the TAChR present, suggesting that it indeed recognizes a consistently present small fraction of TAChR molecules which have a special conformation. These may be (i) TAChR molecules denatured during the purification process, (ii) non-fully-assembled TAChR molecules present in the extract of total *Torpedo* electric organ, or (iii) TAChR molecules that underwent a particular, stable conformational change.

Two mAbs specifically recognized the synthetic sequence Tα376–393, which is part of a region of the α subunit that different studies have suggested to be transmembrane and involved in formation of the ion channel (Young et al., 1985), or not (Mishina et al., 1985), or to be exposed on the cytoplasmic surface (Ratnam et al., 1986a,b; Dwyer, 1991; Chavez & Hall, 1992). The two mAbs VαTα376–393/1 and VαTα376–393/2 consistently recognized about one-third of the Triton-solubilized TAChR molecules. They recognize the same or largely overlapping epitopes, or the same configuration of the TAChR, because their ability to precipitate native TAChR is not additive (Table IV). Since the same amount was precipitated by both mAbs in three different experiments done with different TAChR preparations, this suggests that the sequence regions recognized by these Abs can have different conformations in the TAChR, perhaps reflecting different functional states of the TAChR molecule and making it available for Ab binding a constant fraction of the Triton-extracted TAChR molecule.

Sequence-Specific MAbs Recognize the Denatured TAChR α Subunit in Western Blots. The specificity of the anti-peptide mAbs able to cross-react with native TAChR (Table I) was verified by testing their ability to recognize isolated α subunit in Western blots of purified *Torpedo* AChR. All mAbs,

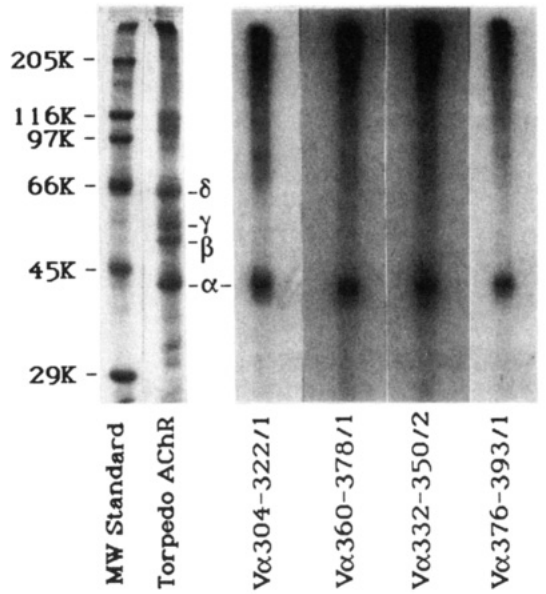


FIGURE 3: Western blots of TAChR probed with four sequence-specific mAbs, as indicated below the blots. On the right side of the figure, Coomassie-stained strips of the corresponding gels are reported, with the molecular weight standards and the TAChR preparation used in the experiment. The four anti-peptide mAbs clearly and specifically recognized the TAChR α subunit (see text for experimental details).

including Vα420–437/3, clearly and specifically recognized the α subunit. The data in Figure 3 report the results of experiments obtained with mAbs Vα304–322/1, Vα332–350/1, Vα360–378/1, and Vα376–393/1, which are representative of all the mAbs tested.

TAChR Recognition by Anti-Peptide MAbs: Comparison of Immunoprecipitation Assay in Solution and Solid-Phase ELISA. It has been reported that anti-peptide Abs able to recognize the cognate protein in solid-phase ELISA assays may be unable to recognize the same protein in solution, this discrepancy being due to partial denaturation of proteins upon adsorption on plastic (Spangler, 1991). We therefore investigated the ability of the sequence-specific mAbs listed in Table I to recognize TAChR adsorbed on 96-well plates, using the ELISA assay described under Materials and Methods. Since several sequence-specific mAbs did not react with solubilized TAChR detectably, we used undiluted hybridoma supernatants for all mAbs, assuming that they have mAb concentrations within the same order of magnitude. The mAbs able to recognize Triton-solubilized TAChR had apparent anti-TAChR titers of 4.3–59 nM precipitable TAChR (measured as α-BGT binding sites). The results of a typical experiment are reported in Figure 4. Along the abscissa we indicated both the mAbs used and, in parenthesis, their ability of recognize native TAChR in radioimmunoassay and the titer against Triton-solubilized TAChR. All antipeptide mAbs which reacted with solubilized TAChR also recognized TAChR adsorbed on a plastic support, although their ability to recognize the TAChR in ELISA and immunoprecipitation assays did not always correlate: for example, mAb Vα304–322/1 recognized the TAChR in ELISA assays about half as much as mAb Vα332–350/2, which has a lower anti-native TAChR titer in immunoprecipitation assays of 40 nM. Most of the antisequence mAbs which did not recognize native TAChR did not recognize the TAChR adsorbed on plastic, with the exception of mAb Vα1–20, directed against the amino terminus of the TAChR α subunit, which moderately but consistently recognized TAChR in ELISA assay.

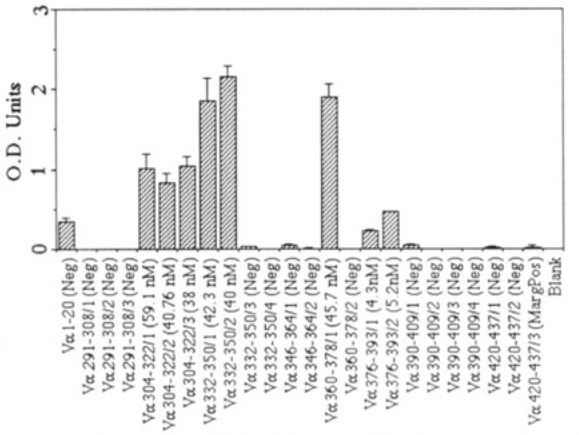


FIGURE 4: Comparison of TAChR recognition by sequence-specific mAbs immunoprecipitation assay in solution and in solid-phase ELISA. The different sequence-specific mAbs obtained in this study were tested in ELISA using TAChR absorbed onto 96-well plates. The mAbs used, their ability to recognize native Triton-solubilized TAChR, and, for the mAbs cross-reactive with native TAChR, the titers of the hybridoma supernatant are indicated along the abscissa (see text for experimental details).

Sequences Tα304–322, Tα332–350, Tα360–378, and Tα376–393 Are Exposed on the Cytoplasmic Surface of the TAChR. The mAbs able to recognize native solubilized TAChR (see Table I) were tested for their ability to recognize native membrane-bound TAChR, using fragments of postsynaptic membranes treated at pH 11 to strip extrinsic membrane proteins. The alkali treatment increases the leakiness of the vesicles formed by the postsynaptic membrane fragments, so that, in the absence of any other permeabilizing treatment, a substantial fraction of the microsacs are permeable to the mAbs and to the gold-conjugated anti-mouse Abs. The sideness of the membrane was verified from the binding of mAb 35 (a generous gift of Dr. Jon Lindstrom), which is a rat mAb raised against native TAChR and specific for the main immunogenic region (MIR), on the extracellular surface of the TAChR (Tzartos et al., 1981). The binding of the anti-MIR mAb was revealed by the use of anti-rat IgG Abs, coupled to large gold beads. The binding of the test anti-peptide mAb was revealed by the use of anti-mouse IgG Abs coupled to small gold beads. Four groups of mAbs were tested, i.e., Vα304–322, Vα332–350, Vα360–378, and Vα376–393. The mAbs against the first three sequence regions clearly and consistently bound to the cytoplasmic surface of the TAChR. Figure 5 reports the results of typical experiments. The binding was fully inhibited by preincubation of the mAb with the relevant synthetic peptide, while it was not affected by preincubation of the mAb with other synthetic α-subunit sequences (not shown). MAbs Vα376–393/1, Vα376–393/2, and mAb Vα420–437/3 did not bind detectably to the membrane bound TAChR.

DISCUSSION

In this paper we demonstrate that synthetic peptides corresponding to three sequence regions of the TAChR α subunit—α304–322, α332–350, and α360–378—can be used to raise mAbs able to fully cross-react with the native TAChR molecule. These sequence segments therefore must be largely exposed on the TAChR surface, and the corresponding synthetic peptides must be able to fold in a conformation reminiscent of that assumed by the same sequence on the surface of the native TAChR.

Our results do not necessitate that the epitopes recognized by the mAbs fully cross-reactive with native TAChR are

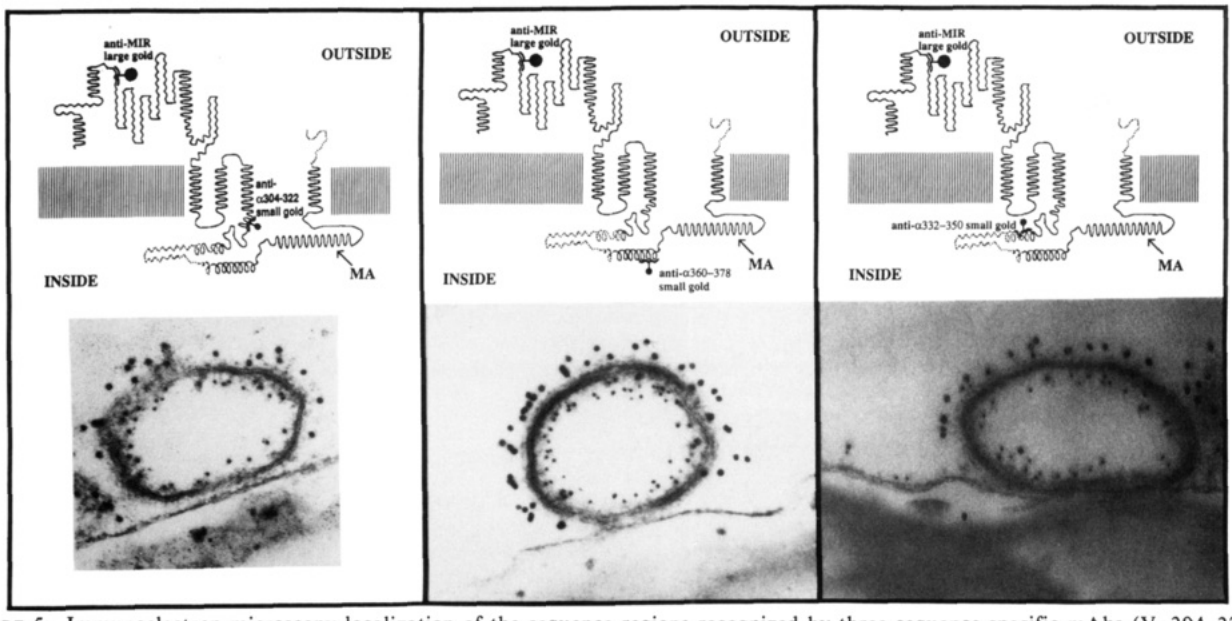


FIGURE 5: Immunoelectron microscopy localization of the sequence regions recognized by three sequence-specific mAbs (Vα304–322/1, Vα360–368/1, and Vα332–350/1). The sequence-specific mAbs are revealed by the use of an anti-mouse IgG antibody coupled to small gold beads, while the extracellular surface of the leaky microsacs formed by postsynaptic membrane fragments used in these experiments is indicated by the binding of a rat anti-MIR mAb, revealed by an anti-rat IgG antibody coupled to large gold beads. In the top part of each panel, a schematic representation of the results is reported, using one of the several models proposed for the transmembrane folding of the TAChR subunit [reviewed in Claudio (1989) and Stroud et al. (1990)].

formed by linear sequences of residues, since it has been shown that anti-peptide mAbs recognize discontinuous linear determinants, i.e., discrete side chains or clusters of side chains along the peptide sequence (Appel et al., 1990). That conclusion was recently verified by a study employing proton NMR spectroscopy of complexes between a high-affinity mAb, raised against a synthetic segment of hen egg lysozyme and able to cross-react with good affinity with native lysozyme, and the peptide antigen, which revealed that the peptide residues strongly immobilized as a result of Ab interaction did not correspond to a contiguous region of the peptide sequence (Cheetham et al., 1991). The immobilized residues, although remote in sequence, are grouped together in the native lysozyme molecule: therefore, the peptide binds to the mAb folded in a conformation very similar to that found for the same sequence in the native protein, which brings together noncontiguous residues (Cheetham et al., 1991). Another study, which investigated the structure of anti-peptide mAbs able to recognize the cognate protein [influenza virus hemagglutinin (IVH)] bound to the peptide immunogen, demonstrated that the dual recognition by the mAbs of both peptide and cognate protein was due both to a folding of the bound peptide in a conformation similar to its cognate sequence in the native IVH and to a conformational change in hypervariable loop of the mAb binding site upon antigen binding, which resulted in a better fit with the peptide (Rini et al., 1992). This flexibility of the Ab binding site, allowing for an induced fit upon antigen binding, may well contribute to the ability of anti-peptide mAbs to fully cross-react with the cognate protein.

While some TAChR synthetic sequences always resulted in sequence-specific mAbs, other sequences resulted frequently or exclusively in induction of mAbs which widely cross-reacted with seemingly unrelated synthetic peptides. All synthetic sequences which induced cross-reactive mAbs had substantial stretches of hydrophobic residues. We do not have at this point any data to speculate about the epitope structure recognized by these mAbs. Still, our data strongly suggest to negatively select peptide sequences containing hydrophobic regions for anti-peptide Abs induction.

Coupling of peptides to KLH did not improve successful mAb induction, and it seemed to correlate with prevalent appearance of mAbs cross-reactive with unrelated peptides (Tables I and II). The ability of carrier-free peptides to induce an Ab response requires that the peptide sequence be long enough to accommodate both a T and a B epitope, which seldom overlap (Maizels et al., 1980; Benjamin et al., 1984) but, even in large protein antigens, can be close together. For example, sequence regions 16–33 residues long of a protein from the malaria parasite *Plasmodium falciparum* each contain immunodominant B and T epitopes (Chougnet et al., 1991), and different conserved sequence regions, 8–34 residues long, of the envelope glycoproteins gp41 and gp120 of the human immunodeficiency virus type 1 each contain both Ab binding sites and T epitopes (Vahlne et al., 1991; Johnson et al., 1992). Our findings that immunization with TAChR synthetic peptides containing an immunodominant T epitope sequence [e.g., Tα304–322 for Balb/C mice, Tα360–378 for C57B1/6 mice; see Bellone et al. (1991b)] plus different TAChR sequence regions consistently induced formation of mAbs which recognized only the T epitope sequence (Table I) support the notion of a close spatial proximity of T and B epitopes on a protein/peptide antigen.

As noted above, our results indicate that 20 residues are enough to accommodate both a T and a B epitope. Other studies found good Ab responses upon immunization with short uncoupled peptides [18 residues in Leonetti et al. (1990), 11–24 residues in Francis et al. (1987), 20 residues in Bellone et al. (1991b, 1992)]. All TAChR peptides that induced sequence-specific mAbs must contain a T epitope. The TAChR α subunit contains only two epitopes immunodominant for Balb/C mice, i.e., the regions α1–20 and α304–322, and three regions immunodominant for C57B1/6 mice, one of which is α360–378 (Bellone et al., 1991a,b). As expected, the synthetic TAChR sequence that corresponded to immunodominant T epitopes induced production of specific mAbs,

even if not coupled to a carrier. Other sequence regions also induced Ab production (α291–308, α332–350, α346–364, α376–393, α390–409, and α420–437). This is probably because they contained cryptic epitopes (i.e., poorly processed/presented; see Gammon and Sercarz (1989)], which could sensitize corresponding T cells because of their being administered in large amounts and in a form (denatured short peptides) more easily processed and presented.

Immunization with the sequence α318–336 never yielded mAbs. Possible explanations of this failure include lack of binding to MHC molecules and lack of T helper cells recognizing an epitope within this sequence.

The use of sequence-specific mAbs cross-reacting with native membrane-bound TAChR clearly demonstrated that the sequences α304–322, α332–350, and α360–378 are exposed on the cytoplasmic surface, confirming previous studies, carried out with anti-TAChR mAbs of known epitope specificity, which concluded that the neighbor/overlapping or homologous sequence regions α330–346, α339–378, α349–364, α360–378, β350–358, β368–406, and γ360–377 had cytoplasmic location (Ratnam et al., 1986a,b; Kordossi & Tzartos, 1987; LaRochelle et al., 1985). Another study which used incorporation of pyridoxamine phosphate into membrane-bound TAChR in sealed microsacs, in the presence and in the absence of saponin, concluded that residue Lysα380 has cytoplasmic location (Dwyer, 1991).

Our results, on the other hand, are in partial conflict with the conclusions of a study which determined the sequence of TAChR fragments released upon brief proteolytic treatment of sealed TAChR-rich membrane vesicles (Moore et al., 1989). A conclusion of that study was that the sequence region α341–380, fragments of which were quickly released by trypsin treatment, was exposed on the extracellular surface. On the other hand, the easy release from the native TAChR molecule of fragments of this sequence region is in agreement with our conclusions that at least parts of the sequence region between M3 and M4 are largely exposed on the TAChR surface. A similar conclusion was reached by Ratnam et al. (1986a), which identified the sequence region between the putative transmembrane segments M1 and M4 (Claudio, 1989; Stroud et al., 1990) as highly immunogenic when denatured TAChR was used for immunization, and inducing formation of Abs cross-reactive with native TAChR.

MAbs against peptide α376–393, which is part of a sequence region, MA, proposed to be involved in ion channel formation both because of its characteristic of amphiphathic α-helix periodicity (Finer-Moore & Stroud, 1984) and because a synthetic peptide corresponding to the homologous sequence MA of the TAChR β subunit forms ion channels in artificial phospholipid bilayers (Ghosh & Stroud, 1991), consistently recognized one-third of the native TAChR molecules only after solubilization with Triton X-100. Our results therefore do not allow any conclusion about the transmembrane disposition of segment MA, because it is present on the surface of part of the TAChR molecules only after detergent treatment, which could induce structural modifications and exposure of sequence regions not exposed on the surface in the native protein in the membrane-bound state. In other studies, Abs recognizing epitopes within the sequence regions α378–391, α379–385, α395–401, and α389–408 were found to bind to the cytoplasmic surface of membrane-bound TAChR (Ratnam et al., 1986a; Maelicke et al., 1989). Other studies suggested that the sequence MA may not have a transmembrane disposition, on the basis of the disappearance of Ab epitopes comprised in this region upon treatment of native TAChR with trypsin (Roth et al., 1987) and the results of experiments in which the transmembrane disposition of the different putative transmembrane regions M1–M4 and MA was deduced using proteolysis protection assays of fusion proteins containing a reporter group fused after the nucleic acid sequence encoding each putative transmembrane domain (Chavez & Hall, 1992). Further, this segment can be deleted from the *Torpedo* AChR sequence without affecting the formation of the ion channel by subunits expressed in *Xenopus* oocytes (Mishina et al., 1985). Those results supported a model of the TAChR subunit topology in which only the sequence regions M1–M4 form transmembrane domains.

MAb Vα1–20 did not bind to TAChR in immunoelectron microscopy experiments or in RIPA assays, indicating that, in agreement with previous studies (Ratnam & Lindstrom, 1984; Lindstrom et al., 1984), the amino terminus is not exposed in native TAChR. The amino-terminal region of the α subunit, however, can be partially exposed upon partial denaturation of TAChR adsorbed onto a plastic surface, as in the ELISA assay (see Figure 5). The discrepancy of the results obtained by assaying the binding of this mAb by immunoprecipitation of soluble TAChR and in ELISA assays agrees well with a previous study (Spangler, 1991), which demonstrated that mAbs raised against synthetic sequences of different proteins, and able to recognize the cognate protein in solid-phase ELISA assays, failed to recognize the cognate antigen in its true native form in solution. The study concluded that discrepancies between solid- and solution-phase assays result from modifications of the epitope caused by interaction with—and denaturation of—the protein antigen on the solid-phase surface. Our results confirm that binding of anti-peptide Abs to the immobilized cognate protein may not be a reliable method to assess cross-reactivity of Abs with a native protein.

MAbs Vα420–437/1/2/3, directed against the carboxyl-terminal region of the α subunit, did not recognize native membrane-bound TAChR, although one of them weakly recognized Triton X-100 solubilized TAChR (Table I). Although lack of recognition may be simply due to specificity of the mAbs for a conformation of the peptide different from that of the cognate native sequence (Stanfield et al., 1990), it also possible that, at difference with the β and δ subunits, whose longer carboxyl-terminal regions may be exposed and available for Ab binding (Young et al., 1985; Lindstrom et al., 1984), the short carboxyl-terminal region of the α subunit may never be exposed on the TAChR surface for Ab binding.

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