Binding of Monoclonal Antibodies against the Carboxyl Terminal Segment of the Nicotinic Receptor δ Subunit Suggests an Unusual Transmembrane Disposition of This Sequence Region†

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ABSTRACT: Monoclonal antibodies (mAbs) specific for the carboxyl terminal region of the δ subunit of *Torpedo* nicotinic acetylcholine receptor (AChR), derived from mice immunized with AChR or a synthetic carboxyl terminal sequence of the δ subunit (Cδ-mAbs), were used to determine the transmembrane disposition of their epitope(s) by immunoelectron microscopy, using AChR-rich postsynaptic membrane fragments from *Torpedo* electroplax. Some Cδ-mAbs recognized only the cytoplasmic side of the membranes, some both sides to a similar extent, and others bound mostly, but not exclusively, to the cytoplasmic side. Binding of Cδ-mAbs to the membranes was specifically blocked by synthetic peptides containing the carboxyl terminal region of the δ subunit. Control anti-AChR mAbs specific for the α or the δ subunits, whose epitopes have known transmembrane topology, uniquely recognized the expected side of the postsynaptic membrane. Residues involved in Cδ-mAb binding were identified using single residue substituted peptide analogues of the sequence δ481−501. All Cδ-mAbs recognized epitopes within the same sequence segment, δ485−493, at the carboxyl terminal of the AChR δ subunit. These results suggest that the δ subunit of the AChR might have alternative conformations, leading to exposure of the same sequence region on the extracellular or the cytoplasmic surface. Several Pro residues are present in this region. The alternative *cis* or *trans* conformation of one or more of them might result in different folding patterns of the carboxyl terminal sequence of the δ subunit, as described for a viral protein [Liddington, R. C., Yan, Y., Moulai, J., Sahli, R., Benjamin, T. L., & Harrison, S. C. (1991) *Nature 354*, 278−284].

All nicotinic acetylcholine receptor (AChR)[1] subunits form both extracellular and cytoplasmic domains (Strader et al., 1979). Given their strong sequence similarity, they should have similar transmembrane folding. Since AChR-rich postsynaptic membrane fragments are easily purified from *Torpedo* electric tissue, elucidation of the transmembrane topology of *Torpedo* AChR subunits has been the goal of intense experimental efforts, which yielded conflicting results [reviewed in Maelicke (1988), Claudio (1989), Stroud et al. (1990), Betz (1990a,b), Galzi et al. (1991), and Conti-Tronconi et al. (1994)].

Hydrophobicity analysis of a "typical" AChR subunit [reviewed in Claudio (1989) and Stroud et al. (1990)] identifies a long amino-terminal sequence region of ~200 amino acids rich in hydrophilic residues which could form an extracellular domain, followed by four hydrophobic segments ~20 amino acid long (referred to as M1 to M4), which could form membrane-spanning regions (Finer-Moore & Stroud, 1984; Guy, 1983). The putative transmembrane segments M1 and M2 are likely to be exposed on the lining of the ion channel contained within the AChR molecule [reviewed in Karlin et al. (1986), Claudio (1989), Stroud et al. (1990), Galzi et al. (1991), and Conti-Tronconi et al. (1994)]. Between M3 and M4 there is a long sequence segment, most diverged in the different AChR subunits. M4 is followed by a carboxyl terminal region which is very short in the α subunit and increasingly longer in the β, γ, and δ subunits [reviewed in Karlin et al. (1986), Claudio (1989), Stroud et al. (1990), Galzi et al. (1991), and Conti-Tronconi et al. (1994)].

The amino terminus of the AChR subunits is extracellular because (i) the *Torpedo* δ subunit contains a processed signal peptide (Anderson et al., 1982), indicating that the mature amino terminus has an extracellular location, and (ii) the amino termini of AChR subunits expressed *in vitro* are translocated into the lumen of microsomal vesicles (topologically equivalent to the extracellular space), as are the amino termini of native AChR subunits (Anderson & Blobel, 1981; Anderson et al., 1983; Chavez & Hall, 1991).

All or most the sequence region between M3 and M4 might form a cytoplasmic domain, since sequence-specific antibodies (Abs) recognizing different parts of this region,

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[1] Abbreviations: Abs, antibodies; mAbs, monoclonal antibodies; AChR, nicotinic acetylcholine receptor; Cδ-mAbs, mAbs specific for the carboxyl terminal region of the δ subunit of *Torpedo* AChR; α-BGT, α-bungarotoxin; [^{125}I]α-BGT, α-BGT radiolabeled with ^{125}I; MIR, main immunogenic region; PBS, 10 mM Na phosphate buffer, pH 7.4, 140 mM NaCl; PBS-T, PBS containing 0.1% Tween 20; TBS-T, 10 mM Tris, 140 mM NaCl, pH 7.4, containing 0.1% Tween 20; BSA, bovine serum albumin; BSA/TBS-T, TBS-T containing 1 mg/mL BSA; FA, Freund's adjuvant; RIPA, radio immunoprecipitation assay; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid homogenization buffer, 400 mM NaCl, 5 mM EDTA, 1 mM PMSF, pH 7.8; SV40, simian virus 40.

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in both *Torpedo* and vertebrate muscle AChRs, consistently bound to the cytoplasmic surface (Ratnam et al., 1986a,b; Young et al., 1985; LaRochelle et al., 1985; Kordossi & Tzartos, 1987; Lei et al., 1992). A cytoplasmic disposition of this sequence region is supported by results of studies utilizing proteolysis protection assays of fusion proteins containing a reporter group fused after the nucleic acid sequence encoding each putative transmembrane domain (Chavez & Hall, 1992). Also, a study on incorporation of pyridoxamine phosphate into membrane bound *Torpedo* AChR in sealed microsacs, in the presence and in the absence of saponin, concluded that residue Lys_{380} of the α subunit, which is immediately amino terminal to M4, has cytoplasmic location (Dwyer, 1991). On the other hand, a study which determined the sequence of AChR fragments released upon brief proteolytic treatment of sealed AChR-rich membrane vesicles concluded that the sequence region between M3 and M4, fragments of which were quickly released by trypsin treatment, is at least partially exposed on the extracellular surface (Moore et al., 1979).

Several different models of the transmembrane topology of the AChR subunits have been proposed, based on experimental data and hydropathy analysis, with four, five, or six transmembrane segments [reviewed in Conti-Tronconi et al. (1994)]. The carboxyl terminus is extracellular in the four and six transmembrane domain models, cytoplasmic in the five transmembrane domain models.

Identification of the transmembrane disposition of the carboxyl terminus is important to construct realistic models of the AChR subunits folding, and it has been the goal of several elegant studies, utilizing immunological, biochemical and genetic approaches, which yielded conflicting results [reviewed in Conti-Tronconi et al. (1994)]. In the present study we sought clues to the structural reasons underlying the disparate results of those experimental efforts. We investigated the binding to AChR-rich postsynaptic membrane fragments from *Torpedo californica* electric tissue, of a panel of mouse monoclonal antibodies (mAbs), specific for the carboxyl terminal region of the *Torpedo* AChR δ subunit (Cδ-mAbs). One Cδ-mAb was obtained by immunization with a synthetic AChR peptide and fully cross-reacted with nondenatured *Torpedo* AChR. The other Cδ-mAbs were derived from mice immunized with nondenatured *Torpedo* AChR. The subunit and sequence localization of their epitopes were identified by Western blot and peptide mapping. The transmembrane localization of the Cδ-mAb epitopes was investigated by immuno electron microscopy, using AChR-rich post synaptic membrane fragments from *Torpedo* electric organ.

MATERIALS AND METHODS

Peptide Synthesis and Characterization. Overlapping peptides 18−21 residues long corresponding to the complete *Torpedo* α- and δ-subunit sequences as reported by Noda et al. (1983) were synthesized (Houghten, 1985). They are indicated with codes that include the symbol α or δ for the corresponding subunit and two numbers indicating the position on the AChR subunit sequences of the first and last residues of the peptide. The peptides corresponded to the following sequence segments: α1−20, α15−33, α30−47, α43−60, α55−74, α63−80, α75−94, α91−110, α106−122, α118−137, α126−145, α134−153, α150−169, α165−184, α181−200, α197−216, α214−234, α230−249, α246−265, α261−280, α276−295, α291−308, α304−322, α318−366, α332−350, α346−364, α360−378, α374−394, α390−409, α406−423, α420−437; δ1−20, δ16−35, δ31−50, δ46−65, δ61−80, δ76−95, δ91−110, δ106−125, δ121−140, δ136−155, δ151−170, δ166−185, δ178−200, δ196−215, δ211−230, δ226−245, δ241−260, δ256−275, δ271−280, δ286−305, δ301−320, δ316−335, δ331−350, δ346−365, δ361−380, δ357−380, δ376−395, δ391−410, δ406−425, δ421−440, δ436−455, δ451−470, δ466−485, δ481−501.

We also synthesized a peptide (α304−322/δ487−499) which included the sequence Tα304−322, which forms an immunodominant T helper epitope in Balb/c mice (Bellone et al., 1991), followed by a sequence corresponding to the carboxyl terminal region of the *Torpedo* AChR δ subunit (residues Tδ487−499: EGDPFDYSSDHPR).

Since the Cδ-mAbs recognized specifically the sequence region δ481−501 (see Results), we synthesized a panel of single-residue substituted analogues of this sequence, where the individual residues were sequentially substituted by an alanine. When alanine was present in the original sequence, it was substituted by glycine. The substituted analogues were used to identify residues involved in formation of Cδ-mAbs epitope(s).

The sequence of most peptides was verified by amino acid composition (Heinrickson & Meredith, 1983), which yielded excellent correspondence between expected and experimental values for all peptides, and, for a few randomly selected peptides, by amino-terminal gas-phase sequencing (Applied Biosystems, Foster City, CA), which consistently yielded only the expected sequence. The characterization of most peptides used in this study had been described in detail previously (Bellone et al., 1991, 1993; Lei & Conti-Tronconi, 1993).

Control mAbs. Controls were anti-AChR mAbs specific for the α, β, or δ subunit, whose epitopes had a transmembrane location well characterized and strictly limited to one side of the membrane. Their salient characteristics are summarized in Table 1. Two mouse mAbs were against native AChR, the other three were against synthetic sequences of the AChR α-subunit: they fully cross-reacted with native AChR and bound exclusively onto the cytoplasmic surface (Lei et al., 1993). The two rat anti-AChR mAbs were generously provided by Dr. Jon Lindstrom (University of Pennsylvania Medical Center, Philadelphia). Of them, mAb 35, recognizes the main immunogenic region (MIR) on the extracellular surface of the AChR (Tzartos et al., 1981), and mAb 141 recognizes the sequence region 318−415 of the δ subunit on the cytoplasmic surface of the AChR (Ratnam et al., 1986).

Preparation of AChR-rich Postsynaptic Membrane Fragments. AChR-rich membrane fragments were purified from *T. californica* electric organ (Pacific Bio-Marine, Venice, CA) by subcellular fractionation on sucrose density gradients as described in detail in Elliott et al. (1986) and alkali-stripped to release extrinsic membrane proteins (Neubig et al., 1979; Elliot et al., 1979). AChR-rich membrane fragments have a propensity to re-seal to form vesicles, which are leaky after alkali stripping. To favor the presence of unsealed vesicles, necessary for the Ab-binding studies, the AChR-rich membrane fragments were broken by pushing them through narrow syringe needles.

Table 1: Control mAbs Used in this Study[a]

mAb	species	immunogen	subunit recognized	epitope specificity	epitope location	reference
6	rat	native TAChR	α	MIR	extracellular	Tzartos et al. (1988)
35	rat	native TAChR	α	MIR	extracellular	Tzartos et al. (1988)
118	rat	native TAChR	β	ND	cytoplasmic	Criado et al. (1985)
141	rat	denatured TAChR	δ	Tδ318−415	cytoplasmic	Ratnam et al. (1986)
7 (CB17)	CB17 mouse	native TAChR	δ	ND	cytoplasmic	this study
16 (CB17)	CB17 mouse	native TAChR	δ	ND	cytoplasmic	this study
7 anti-α	Balb/c mouse	synthetic peptide Tα360−378	α	Tα360−378	cytoplasmic	Lei et al. (1993)
45 anti-α	Balb/c mouse	synthetic peptide Tα332−350	α	Tα332−350	cytoplasmic	Lei et al. (1993)

[a] MAbs 6, 35, 118, and 141 were a generous gift of Dr. Jon Lindstrom. MAbs 7(CB17) and 16 (CB17) were produced during the present study and, although they recognized the δ subunit, they did not recognize detectably any synthetic δ peptide. Therefore they recognize a conformational, "discontinuous" epitope formed by residues contributed by different sequence regions of the δ subunit or by a sequence region poorly represented by our peptide panel. The finding that, while they are unable to bind any of the synthetic δ subunit peptides we used, these two mAb recognize the δ subunit in Western blots is explained by either a partial renaturation of the δ subunit in blot [e.g., see Tzartos and Changeaux (1984)], or by a poor representation of the sequence region forming their epitopes in our peptide panel (e.g., broken between peptides or folded in a strong conformation). MAbs 7 anti-α and 45 anti-α were produced in our laboratory previously. For all these mAbs the transmembrane location of their epitope had been unequivocally characterized and is limited to only one side of the membrane. See also Table 4.

AChR concentration was determined as α-bungarotoxin (Biotoxins Inc., St. Cloud, FL) (α-BGT) binding sites by a DEAE filter assay (Schmidt & Raftery, 1973), protein concentration by the Lowry assay (Lowry et al., 1951). The specific activity of the AChR preparations used in this study was 2−6 nmol of α-BGT binding sites/mg of protein (specific activity of pure AChR: 7.2 nmol/mg of proteins). Protein composition of purified alkali-stripped AChR-rich membrane fragments, analyzed by $NaDodSO_4$−polyacrylamide gel electrophoresis (Laemmli, 1970), showed only four major protein bands of the molecular weight expected for AChR subunits (data not shown). Small amounts of low molecular weight components, possibly degradation products of AChR subunits, were frequently present.

Preparation of Solubilized AChR. Frozen electric organ of *T. californica* (50 grams) was sliced into 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, Sigma Chemical Co., St. Louis, MO), and 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, MO), 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 with stirring, the extract was centrifuged at 25000*g* for 1 h and the supernatant collected. All procedures were carried out at 4 °C.

Immunization of Mice. Balb/c (H-2^d) CB17 (H-2^d), C57B1/6 (H-2^b), and Balb/b (H-2^b) mice (Harlan Sprague Dawley, Indianapolis) were injected with alkali-stripped AChR-rich postsynaptic membrane fragments, solubilized with 1% Triton in 10 mM sodium phosphate buffer, pH 7.4. Twenty micrograms of solubilized AChR [calculated from the protein concentration and the specific activity of the AChR-rich membrane preparation before solubilization in Triton X-100; previous studies indicated that solubilization in Triton X-100 of alkali-stripped AChR-rich membrane preparations such as the ones used in the present study is virtually complete [>90%, e.g., see Hohlfeld et al. (1987)]] in 100 mL was homogenized with 100 μL of Freund's adjuvant (FA; Sigma Chemical Co., St. Louis, MO) (complete FA for the first injection, incomplete FA for the following injections) and injected in 3−4 spots subcutaneously, along the back. Mice were immunized three times, at 3−4 week intervals. Balb/c mice were injected with peptide α304−322/δ487−499, using the same procedure and 50−100 μg of peptide in phosphate buffered saline solution (PBS: 10 mM Na phosphate buffer, pH 7.4, 140 mM NaCl)/injection.

Generation of Hybridomas. Spleen cells of mice with high serum titers of anti-AChR Ab were used for fusions with NS-1 (ATCC) or SP2/0 Ag14 (ATCC) myeloma cells to generate hybridoma libraries. Screening of the hybridomas for anti-peptide and anti-AChR mAb secretion was done by enzyme-linked immunosorbent assay (ELISA). The mAbs obtained and used for the present studies are listed in Table 2.

ELISA of mAb Binding to Peptides and to AChR. Ninety-six well plates (Nunc, Karstrup, Denmark) were incubated for 4 h at room temperature with either 100 μL of peptide solution (10 μg/mL in 10 mM potassium phosphate buffer, pH 7.4) or 100 μL of a suspension of AChR-rich fragments (100 pmol/mL in 20 mM $Na_2CO_3/NaHCO_3$ buffer, pH 9.6). The wells were washed twice with PBS containing 0.1% Tween 20 (PBS-T), incubated for 1 h with PBS-Tween plus 3% bovine serum albumin (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; diluted 1/3000 in PBS), washed three times with PBS-Tween, and developed for 5 min with peroxidase substrate solution (Sigma Chemical Co., St. Louis, MO) (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.

For competition experiments, Cδ-mAb solutions were incubated overnight at 4 °C with 250 μg/mL of relevant peptide before addition of the Cδ-mAb solution to the peptide-coated ELISA plate. Preliminary experiments assessing the dose dependence of the competitive inhibition by the relevant peptides of Cδ-mAb binding to AChR indicated that 250 μg/mL sufficed to inhibit binding of all mAbs.

Table 2: δ Subunit Specific mAbs Used in This Study

mAb	immunogen	strain	reactivity with SAChR	reactivity with MAChR	subunit recognized[a]	sequence specificity: direct binding	sequence specificity: competition assay
1	α304−322/δ487−499	Balb/c	yes	yes	δ	Tδ481−501	Tδ481−501
33	AchR	CB17	yes	yes	δ	Tδ481−501	Tδ481−501
151	AchR	CB17	yes	yes	δ	Tδ481−501	Tδ481−501
46	AchR	CB17	yes	yes	δ	Tδ436−455	Tδ436−455
149	AchR	CB17	yes	yes	δ	Tδ436−455	Tδ436−455
75	AchR	CB17	yes	yes	δ	Tδ436−455	Tδ436−455
2	AchR	C57Bl	yes	yes	δ	Tδ481−501	Tδ481−501
5	AchR	C57Bl	yes	yes	δ	Tδ481−501	Tδ481−501
7	AchR	C57Bl	yes	yes	δ	Tδ481−501	Tδ481−501
10	AchR	C57Bl	yes	yes	δ	Tδ481−501	no detectable inhibition
5	AchR	Balb/b	yes	yes	δ	Tδ481−501	Tδ481−501
8	AchR	Balb/b	yes	yes	δ	Tδ481−501	Tδ481−501
14	AchR	Balb/b	yes	yes	δ	Tδ481−501	Tδ481−501
16	AchR	Balb/b	yes	yes	δ	Tδ481−501	Tδ481−501
25	AchR	Balb/b	yes	yes	δ	Tδ481−501	Tδ481−501
31	AchR	Balb/b	yes	yes	δ	Tδ481−501	Tδ481−501
32	AchR	Balb/b	yes	yes	δ	Tδ481−501	Tδ481−501

[a] Determined in Western blots. See text for experimental details.

Radioimmunoprecipitation Assay (RIPA) of Nondenatured AChR by Cδ-mAbs. Triton X-100 solubilized AChR (~10^{-9} M in PBS containing 1% Triton X-100) was labeled with 3-fold excess of radiolabeled α-BGT ([^{125}I]α-BGT) overnight at 4 °C. Increasing volumes (10−500 μL) of hybridoma supernatant (or supernatant of NS-1 lymphoma cultures as a negative control) were added to 1 mL aliquots of labeled AChR. 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% Triton X-100, and counted in a Biogamma II counter (Beckman Instruments, Arlington Heights, IL).

For competition experiments, mAb supernatants were incubated overnight at 4 °C with 250 μg/mL of relevant peptide before addition of the mAb to the 1 mL aliquots of [^{125}I]α-BGT labeled AChR.

Western Blots. Alkali-stripped AChR-rich membrane fragments resuspended in 10 mM sodium phosphate buffer, pH 7.4, were electrophoresed (20−40 μg of protein/3 mm of gel) on a 8.75% polyacrylamide slab gel with 4% stacking gel (Laemmli, 1970). 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) which 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 (BSA/TBS-T), and incubated for 2 h at room temperature with the test Cδ-mAb [diluted as needed in 10 mM Tris and 140 mM NaCl, pH 7.4, containing 0.1% Tween 20 (TBS-T)] and washed twice in TBS-T. The strips were incubated for 2 h with rabbit anti-mouse IgG Ab (Sigma Chemical Co., St. Louis, MO), diluted 1/1000 in TBS-T, and 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 (Rochester, NY).

For competition experiments, the Cδ-mAb solutions were incubated overnight at 4 °C with 250 μg/mL of relevant peptides before incubation of the Cδ-mAb with the strips. Further incubations and autoradiography were done as described above.

Immunoelectron Microscopy. The transmembrane orientation of the AChR epitopes recognized by the Cδ-mAbs, as well as by control mAbs, was determined by a modification of the methods of LaRochelle et al. (1985), using AChR-rich postsynaptic membrane fragments absorbed onto the bottom of polylysine-coated wells of 96-well microtiter plates (Falcon, Becton Dickinson, Mountain View, CA) by centrifugation at 3000 rpm for 20 min in a Beckman GPR centrifuge (Beckman Instruments, Arlington Heights, IL). The membranes were fixed with 0.2% glutaraldehyde (electron microscopy grade, Electron Microscopy Sciences, Redding, CA) for 20 min, treated with $NaBH_4$ (1 mg/mL, 20 min), incubated with the test Cδ-mAb supernatant or the control mAb 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-IgG-colloidal gold Ab (5 nm, Ted Pella, Inc., Redding, CA or goat anti-rat-IgG-colloidal gold, 10 nm, Ted Pella, Inc., Redding, CA, as needed). A third fixation (1% glutaraldehyde) and reduction followed. In some experiments the membrane fragments incubated with a Cδ-mAb were further incubated with the control rat mAbs 35 or 141 for 1 h, and a fixation and a reduction step were carried out as described above, followed by addition of goat anti-rat-IgG colloidal gold (10 nm, Ted Pella, Inc., Redding, CA). The membranes were fixed first with 2% glutaraldehyde-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 Cδ-mAb solutions were incubated overnight at 4 °C with 250 μg/mL of the relevant peptide before incubation with the AChR-rich membrane fragments.

RESULTS

Rationale. Several previous studies which utilized immunological, biochemical, and genetic approaches to inves-

tigate the transmembrane location of the carboxyl terminus of AChR subunits yielded conflicting results [reviewed in Conti-Tronconi et al. (1994)].

Studies which utilized biochemical and genetic approaches all concluded that the carboxyl terminus of the AChR subunits should be extracellular. On the other hand, several studies using Abs directed against the carboxyl terminal sequence region of AChR subunits suggested a cytoplasmic location of the carboxyl terminus.

Previous studies using immunological approaches had important caveats. Some studies used Abs obtained from animals immunized with AChR and identified the sequence region forming the Ab epitope using a limited number of synthetic AChR sequences [reviewed in Conti-Tronconi et al. (1994)]. Therefore it cannot be excluded that the Abs may have recognized equally well or even better other sequence regions of the AChR, as it has been described for Abs to certain AChR sequences (Maelicke et al., 1988). Other studies used Abs specific for synthetic AChR sequences [e.g., Young et al. (1985)]. However, the ability of these Abs to fully recognize the native AChR molecule was not investigated. Therefore the Abs might have recognized denatured forms of the AChR, and the transmembrane location of their epitope(s) might not have reflected the location of the same sequence region in the native molecule.

The aim of the present study was to attempt to understand the structural reasons of the conflicting results described above. Toward this goal, we developed a relatively large library of mAbs specific for the carboxyl terminus of the AChR δ subunit. They were obtained in mice strains of different H-2 haplotype, immunized with nondenatured AChR or a synthetic peptide, to optimize the chance of obtaining a library of mAbs against different epitopes within the carboxyl terminal region of the δ subunit. The Cδ-mAbs were carefully characterized for their ability to fully react with nondenatured AChR, and for their sequence specificity.

Having established by Western blots that the mAbs were specific for the δ subunit, identification of the sequence location of their epitope(s) was pursued first by testing the mAb binding to panels of overlapping synthetic AChR peptides, screening the complete sequence of two AChR subunits (the α and the δ subunits), thus reducing the possibility that undesirable cross-reactivity with different AChR sequence regions was present.

Having identified mAbs which specifically recognized carboxyl terminal region of the δ subunit (Cδ-mAbs), the individual residues within this sequence involved in interaction with the different Cδ-mAbs were identified by studying the effect on Cδ-mAb binding of single residue substitutions within this sequence.

The transmembrane location of the epitope recognized by the Cδ-mAbs thus characterized was determined in immunoelectron microscopy experiments using postsynaptic membrane fragments from *Torpedo* electric organ rich in native AChR molecules.

Production of Anti-AChR mAbs and Identification by Western Blot of mAbs Recognizing the AChR δ Subunit. Large libraries of anti-AChR mAbs were derived from Balb/b, CB17, and C57Bl mice immunized with solubilized, nondenatured AChR, as described under Materials and Methods. The hybridomas were qualitatively screened for anti-AChR mAb production by ELISA, using AChR-rich membrane fragments as described under Materials and Methods. The anti-AChR hybridomas thus identified were subcloned, and the presence of anti-AChR mAbs in their supernatants was assessed by ELISA and quantified by RIPA, using AChR solubilized in Triton X-100, as described under Materials and Methods. One mAb, Cδ-mAb 1, was obtained by the same procedure from a Balb/c mouse immunized with peptide α304−322/δ487−499.

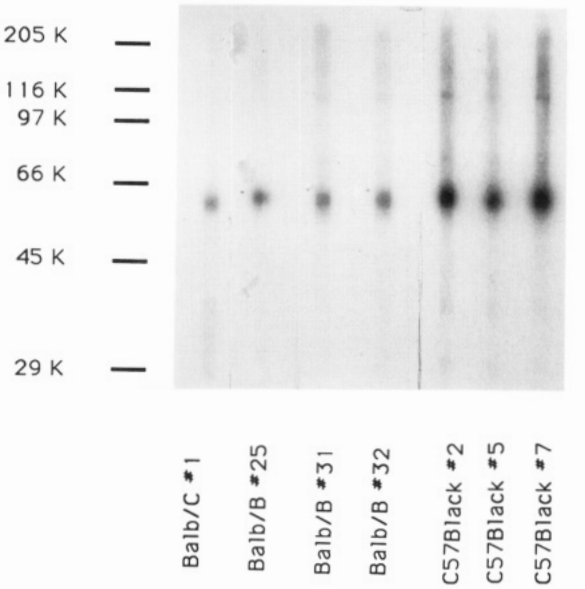

FIGURE 1: Specificity for the AChR δ subunit of some Cδ-mAbs, as indicated, determined in Western blots using *Torpedo* AChR-rich postsynaptic membrane fragments. The results of this experiment are representative of those obtained for all mAbs listed in Table 2. All Cδ-mAbs recognized only one AChR subunit, having the molecular weight expected for the AChR δ subunit. Cδ-mAb 1 (lane "Balb/c #1") was obtained from a Balb/c mouse immunized with peptide α304−322/δ487−499. All other Cδ-mAbs used in this experiment were obtained from mice of different strains, as indicated, immunized with nondenatured *Torpedo* AChR. See text for experimental details.

The subunit specificity of the anti-AChR mAbs was determined in Western blots using *Torpedo* AChR-rich postsynaptic membrane fragments. A few anti-AChR mAbs for each strain uniquely recognized the δ subunit. Figure 1 reports the results of a Western blot experiment, representative of those obtained for all mAbs listed in Table 2.

Cδ-mAb 1 recognized nondenatured AChR both in ELISA (not shown) and RIPA (see below) and specifically recognized only the δ subunit in Western blots (Figure 1). Its binding to AChR was completely inhibited by preincubation of the mAb with the immunizing peptide or with synthetic peptides containing the sequence δ487−499, not by a synthetic peptide containing the T helper epitope sequence α304−322 (Figure 2, bottom panel).

Sequence Regions of the AChR δ Subunit Recognized by Anti-δ mAbs. The sequence specificity of the mAbs which recognized the AChR δ subunit in Western blots [those listed in Table 2, plus mAbs 7 (CB17) and 16 (CB17) listed in Table 1] was investigated by ELISA, using the panel of peptides corresponding to the complete δ subunit sequence. The specificity of mAb recognition was verified by ELISA, using overlapping peptides corresponding to the complete α subunit sequence.

Two mAbs, 7 (CB17) and 16 (CB 17), did not recognize detectably any synthetic AChR peptide (data not shown). Their epitopes therefore are conformation dependent and,

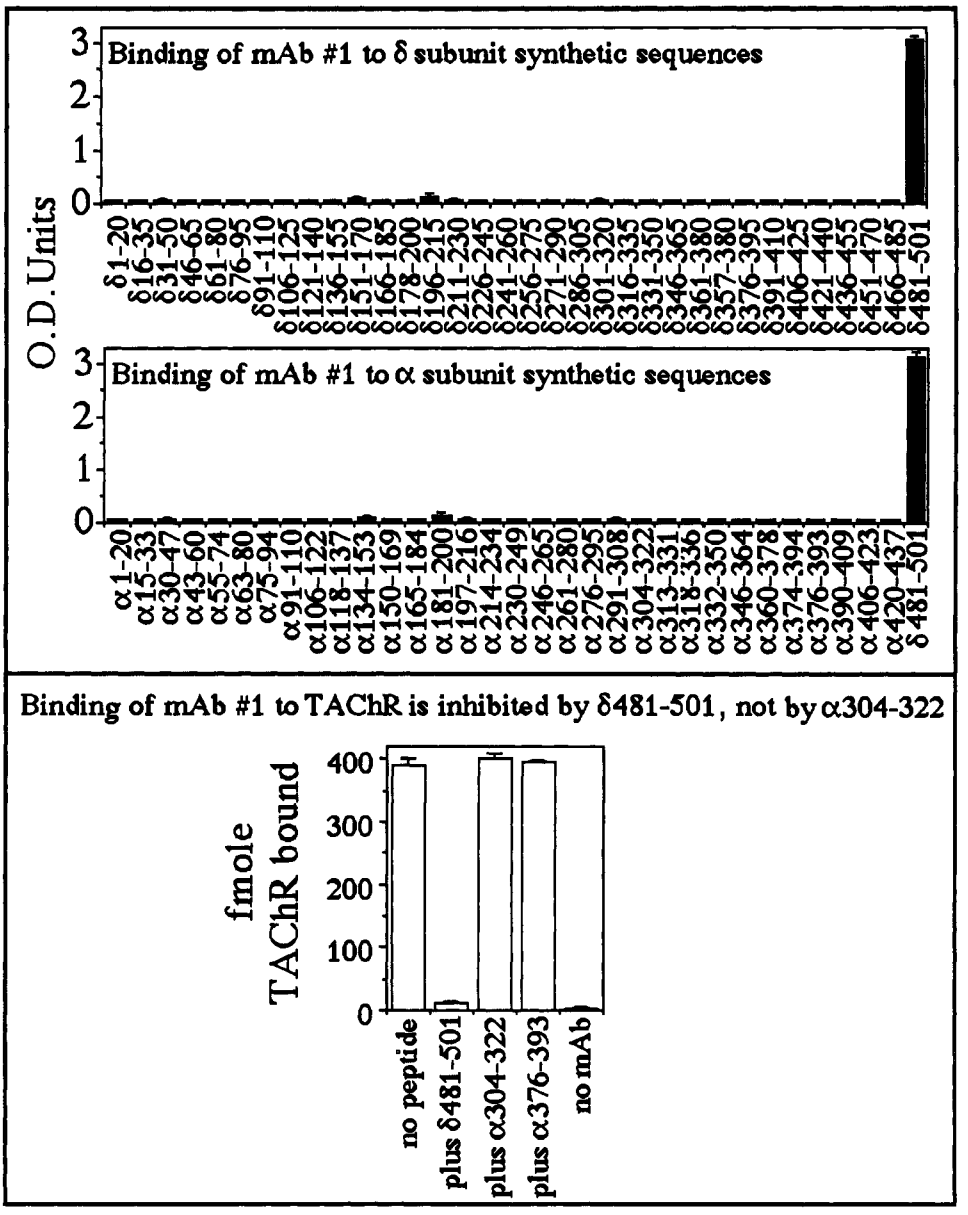

FIGURE 2: (Top panel) Sequence specificity of Cδ-mAb 1, obtained from a Balb/c mouse immunized with peptide α304−322/δ487−499, investigated by ELISA, using overlapping synthetic peptides corresponding to the complete AChR δ subunit sequence (upper graph). The specificity of mAb recognition was verified by ELISA, using overlapping synthetic peptides corresponding to the complete α subunit sequence (lower graph). The peptides corresponded to the sequence regions of the δ and α subunits indicated along the abscissa. Cδ-mAb 1 only bound the synthetic sequence δ481−501, not to any other α or δ subunit peptides, including a synthetic peptide corresponding to the sequence α304−322, which contains a T helper epitope and which was included in the immunizing peptide. See text for experimental details. (Bottom panel) Cδ-mAb 1 recognized nondenatured AChR in RIPA. Its binding to nondenatured AChR was completely inhibited by preincubation of the mAb with the synthetic peptide sequence δ481−501, not by a synthetic peptide containing the T helper epitope sequence α304−322 or by an unrelated AChR peptide sequence (α376−393). See text for experimental details.

while they are still recognizable in Western blots, where the complete δ subunit is present and its partial renaturation may occur (Tzartos & Changeux, 1983, 1984; Haggerty & Froehner, 1981; Wilson & Lentz, 1988), they are lost when short synthetic sequences are used, excised from any other structural element contributing to epitope formation in the native AChR molecule. A similar situation occurs for some anti-MIR mAbs (Tzartos & Changeux, 1984; Tzartos et al., 1988).

Several other anti-δ subunit mAb, listed in Table 2, specifically recognized a well defined sequence region of the δ subunit. Figures 2 and 3 report the results of experiments testing the sequence specificity of the anti-δ mAb 1, and of those obtained from CB17 mice. The data reported in Figure 3 are representative of those obtained for the anti-δ mAbs from CB17 and Balb/B mice, which, therefore, for the sake of brevity, are not shown but simply described in the text. None of them recognized any peptide sequence of the α subunit (data shown only for mAb 1, in Figure 2).

MAb 1, obtained by immunization with peptide α304−322/δ487−499, only bound the synthetic sequence δ481−501, not to any other δ or α subunit peptides including a synthetic peptide corresponding to the sequence α304−322, which was included in the immunizing peptide (Figure 2, top panel). In agreement with the results of the direct binding assay, preincubation of mAb 1 with peptide δ481−501 completely inhibited binding of the mAb to nondenatured,

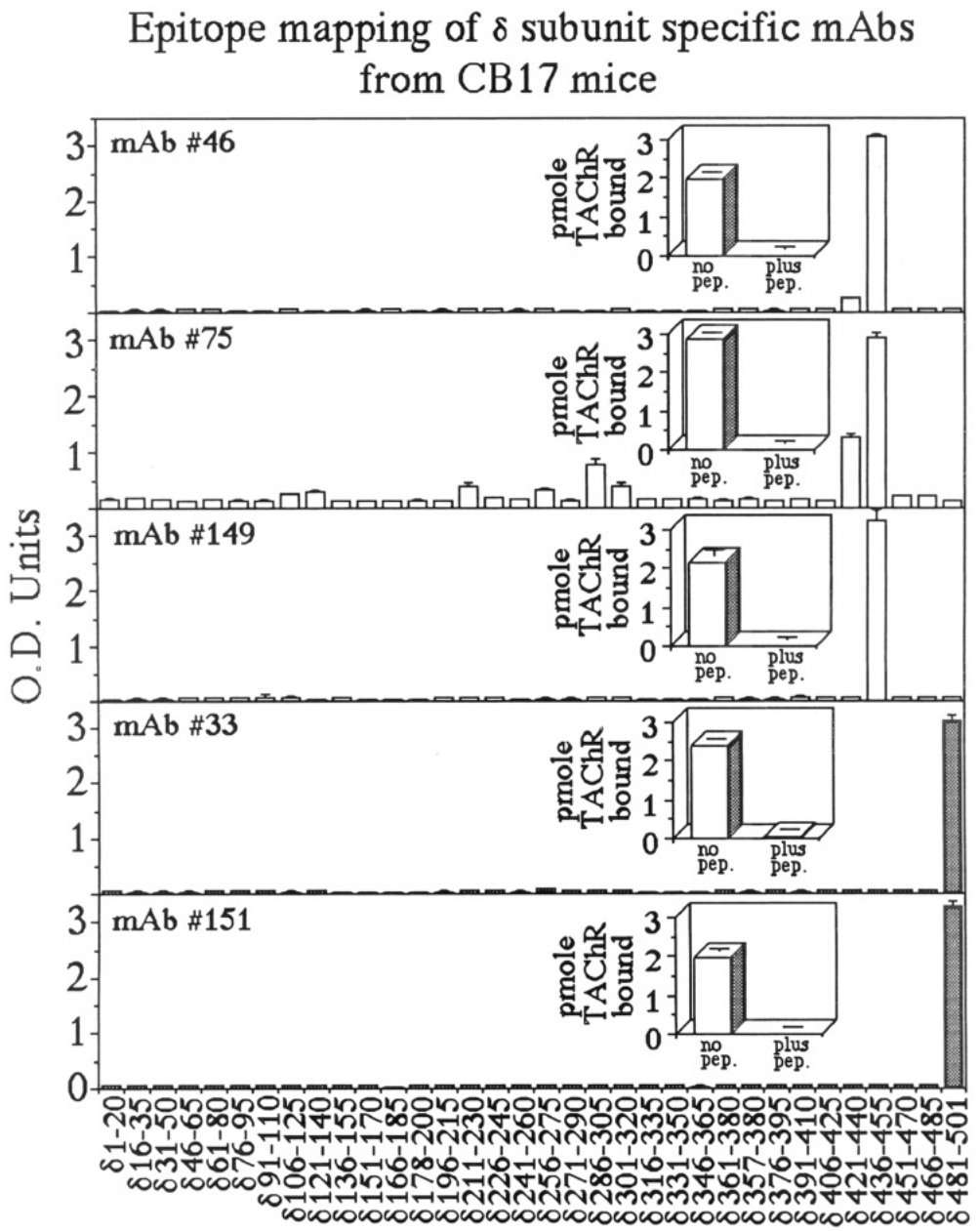

FIGURE 3: Sequence specificity of mAbs specific for the AChR δ subunit (as determined in Western blots) obtained from CB17 mice immunized with nondenatured *Torpedo* AChR, investigated by ELISA, using overlapping synthetic peptides corresponding to the complete δ subunit sequence. The peptides corresponded to the sequence regions of the δ subunit indicated along the abscissa. All mAbs recognized nondenatured AChR solubilized in Triton X-100 (insets). MAbs 33 and 151 uniquely recognized a peptide corresponding to the carboxyl terminal sequence, δ481−501. Incubation of mAbs 33 and 151 with this peptide competitively blocked mAb binding to native AChR in RIPA (inset). MAbs 46, 75, and 149 bound to peptide δ436−455. MAbs 75 and 46 also recognized detectably, although to different extents, the overlapping peptide δ421−440. MAb 75 displayed some binding to many peptides, but inconsistently and to levels much lower than that to the overlapping sequences δ421−440 and δ436−455. Incubation of mAb 46, 75, and 149 with peptide δ436−455 blocked mAb binding to AChR in RIPA (insets). See text for experimental details.

solubilized AChR in RIPA assays, while preincubation of the mAb with peptide α304−322 or with other synthetic α or δ subunit sequences had no effect (Figure 2, bottom panel).

Of the five δ subunit specific mAbs obtained from CB17 mice, two (33 and 151) uniquely recognized a peptide corresponding to the carboxyl terminal sequence, δ481−501 (Figure 3). Incubation of mAbs 33 and 151 with this peptide competitively blocked mAb binding to non-denatured AChR in RIPA (Figure 3, inset). The other three mAbs (46, 75, and 149) bound to peptide δ436−455 (Figure 3). MAbs 75 and 46 also recognized detectably, although to different extents, the overlapping peptide, δ421−440. MAb 75 displayed some binding to many other peptides (Figure 3) but inconsistently and to much lower levels than that to the overlapping sequences δ421−440 and δ436−455. Incubation of mAb 46, 75, and 149 with peptide δ436−455 blocked mAb binding to AChR in RIPA (Figure 3, insets).

All anti-δ subunit mAbs obtained from C57Bl/6 mice strongly recognized peptide δ481−501. MAbs 2 and 5/C57 had low and sporadic unspecific binding to a few other peptides. The unspecific binding of mAb 7 to other peptide sequences, although lower than that to the sequence δ481−501, was substantial. MAbs 2, 5/C57, and 7 bound well to nondenatured AChR in RIPA, and their binding to AChR in RIPA was completely blocked by preincubation with peptide

Table 3: Ability of Cδ-mAbs To Precipitate Nondenatured TAChR

antibody	experiment 1 precipitated AChR (fmol)	experiment 1 precipitated (%)	experiment 2 precipitated AChR (fmol)	experiment 2 precipitated (%)
anti AChR serum	921 ± 4	100	858 ± 31	100
mAb 1 (Balb/c)	622 ± 8	72.5	577 ± 4	67.2
mAb 33 (CB17)	230 ± 10	26.8	247 ± 2	26.7
mAb 151 (CB17)	547 ± 1	63.8	576 ± 4	62.5
mAb 2 (C57Bl)	706 ± 38	82.3	641 ± 0	69.9
mAb 5 (C57Bl)	636 ± 47	74.1	621 ± 2	67.4
mAb 7 (C57Bl)	764 ± 5	89.0	782 ± 1	84.9
mAb 10 (C57Bl)	94 ± 5	11.0	76 ± 1	8.3
mAb 5 (Balb/b)	616 ± 3	71.8	726 ± 3	78.8
mAb 8 (Balb/b)	622 ± 0	72.5	696 ± 16	75.6
mAb 14 (Balb/b)	500 ± 5	58.3	541 ± 12	58.7
mAb 16 (Balb/b)	637 ± 1	74.2	702 ± 4	71.0
mAb 25(Balb/b)	691 ± 7	80.5	662 ± 4	71.9
mAb 31(Balb/b)	639 ± 0	74.5	728 ± 16	79.0
mAb 32(Balb/b)	592 ± 2	69.0	674 ± 8	70.2

δ481−501. MAb 10 bound to AChR in RIPA poorly (see below), and this marginal binding was not blocked by preincubation with peptide δ481−501, although this peptide sequence was strongly and uniquely recognized in ELISA.

All seven anti-δ mAbs from Balb/b specifically and strongly recognized, in both ELISA and competition RIPA, the sequence δ481−501. Several of them showed in ELISA some low-level, sporadic recognition of a few other peptides.

For all these mAbs, ELISA experiments were carried out using the overlapping peptides screening the α subunit sequence. No binding to any peptide was detected (data shown only for Cδ-mAb 1, in Figure 2).

Cδ-mAbs Recognize Nondenatured AChR in RIPA. MAbs in principle could recognize denatured forms of the antigen (Ag), i.e., epitopes present on a small fraction of the Ag molecules, whose structure is not representative of that the native Ag. This caveat applies even more strongly to mAbs raised using denatured forms of the Ag, such as synthetic peptide sequences as in the case of Cδ-mAb 1, which was derived from a mouse immunized against the synthetic peptide α304−322/δ487−499. It was therefore important to verify that the Cδ-mAbs recognize the native AChR molecule and to quantify their ability to bind nondenatured AChR.

This was accomplished by investigating by quantitative RIPA the ability of Cδ-mAbs to precipitate Triton X-100 solubilized AChR. The results of two representative experiments are reported in Table 3. The ability of the different Cδ-mAbs to precipitate AChR is compared to that of a high affinity anti-*Torpedo* AChR serum obtained from C57Bl mice immunized to *Torpedo* AChR. It should be noted that polyclonal antisera precipitate the relevant Ag better than mAbs because they contain many Abs, recognizing different epitopes. This facilitates formation by the second Ab of a three-dimensional lattice, i.e., of a precipitate. On the other hand, mAbs bind in a stoichiometry of one Ab per Ag molecule, an unfavorable condition for precipitate formation.

As expected, the anti-AChR serum precipitated AChR effectively. In the two experiments reported in Table 3 each sample contained a nominal amount of AChR of 1 pmol: the anti-AChR serum precipitated 90% of the AChR. All Cδ-mAbs precipitated AChR less effectively than the polyclonal serum. However, most of them precipitated the majority of the AChR molecule [70% or more: Cδ-mAbs 1, 2, 5 (C57Bl), 7, 5 (Balb/b), 8, 16, 25, 31, 32]. Two mAbs were somewhat less efficient in precipitating AChR, but still recognized the majority of the molecules (~60%: Cδ-mAb 151 and 14). Two other mAbs (Cδ-mAb 33 and 10) precipitated a minority of AChR molecule (27% and 10%, respectively).

Table 4: Transmembrane Mapping of the Binding of Control mAbs, Assessed by Immunoelectron Microsopy of Leaky AChR-Rich Membrane Vesiscles[a]

mAb	beads counted	% outside	% inside	expected mapping	sequence specificity
6[b]	1300	>99	<1	outside	α67−76 (MIR)[c]
35[b]	435	>99	<1	outside	α67−76 (MIR)[c]
118[b]	411	1	99	inside	β subunit[d]
141[b]	13	0	100	inside	δ318−415[e]
7 anti-δ (CB17)	655	<1	>99	inside	δ subunit
16 (CB17)	98	<1	99	inside	δ subunit
45[f]	398	2.8	97.2	inside	α332−350[f]
7 anti-α (C57/Bl)[f]	283	2	98	inside	α360−378[f]

[a] The salient characteristics of these mAbs are summarized in Table 1. [b] Generously provided by Dr. J. M. Lindstrom. [c] See Tzartos et al. (1988). [d] See Criado et al. (1985). [e] See Ratnam et al. (1986a,b). [f] Described in detail in Lei et al. (1993).

Transmembrane Mapping of the Binding of Control mAbs. To assess the reliability of the mapping of the transmembrane location of the epitopes recognized by the Cδ-mAbs, we investigated the binding to AChR-rich membrane fragments of the control mAbs listed in Table 1. Table 4 summarizes the results obtained in several consistent independent experiments. All mAbs uniquely bound to the expected site of the membrane fragments.

Figure 4A reports representative electron micrographs obtained in experiments investigating the transmembrane location of the binding of some control mAbs. The binding of control mAbs 35 and 141 is visualized using an anti-rat IgG Ab coupled to large gold beads, that of control mAb 7 (CB17) using an anti-mouse IgG Ab coupled to small gold beads. The AChR-rich membrane fragments have a propensity to curve toward formation of vesicles, which, as a result of the alkali treatment and of pushing the membrane suspension through narrow syringe needles, are in most cases unsealed (see Materials and Methods). That most membrane "vesicles" thus obtained were open and well permeable to the mAbs and gold-labeled Abs used in this study was demonstrated by the result obtained in pilot experiments, which investigated the frequency of vesicles labeled with control mAbs (data not shown). For a given preparation, the frequency of membrane fragments labeled with mAbs 35 (extracellular binding) was the same as that of vesicles labeled with mAbs 118 and 141 (cytoplasmic binding). Furthermore, in double-labeling experiments using the rat anti-MIR mAbs 35 or 6 as extracellular markers, and the mouse mAbs 6 (CB17), 16 (CB17), 7, or 45 as cytoplasmic marker, most vesicles decorated with the extracellular anti-MIR rat mAb (visualized by anti-rat IgG Ab coupled to large gold beads) were also labeled by the cytoplasmic mouse mAb (visualized by anti-mouse IgG Ab coupled to small gold beads).

Transmembrane Mapping of the Binding of Cδ-mAbs. Cδ-mAb 1. Cδ-mAb 1 was obtained from a mouse immunized with a synthetic peptide which included the carboxyl terminal sequence of the AChR δ subunit, and it specifically recognizes synthetic peptides which contain this sequence in both ELISA and in solution, as demonstrated by the ability

Table 5: Transmembrane Mapping of the Binding of mAb 1 and of Control mAbs 35 and 141, Assessed by Immunoelectron Microsopy of AChR-Rich Membrane Fragments

	mAb 1			mAb 35 (outside marker)			mAb 141 (inside marker)		
experiment no.	beads counted	% outside	% inside	beads counted	% outside	% inside	beads counted	% outside	% inside
1	153	52	48						
2	334	57	43						
3	622	54	46	127	>99	<1			
4	188	35	65						
5	479	61	39						
6	777	29	71	229	>99	<1	13	0	100
7	187	59	41						
8	75	69	31	18	100	0			
9	636	76	24	61	100	0			
10	488	54	46						
11	316	35	65						
average		52.8± 14.6	47.2 ± 14.6						

of the synthetic peptide δ481−501 to block its binding to native AChR (see above). Furthermore, Cδ-mAb 1 recognized the majority if not all nondenatured AChR molecules (Table 3). It is therefore an ideal tool to investigate the transmembrane location of the COOH terminus of the δ subunit.

The results of 11 independent immunoelectronmicroscopy experiments using Cδ-mAb 1 and AChR-rich membrane fragments are summarized in Table 5. These data, as well as those obtained for the other Cδ mAbs described below and summarized in Table 6, were obtained by counting the transmembrane location of beads in areas of the micrographs where the section of the membrane was clearly visible and orthogonal to the plane of the membrane, as indicated by a clear appearance of the three membrane layers. Representative examples are given in Figure 4B. In some experiments the membrane fragments were double-labeled with both Cδ-mAb 1 and the control anti-MIR mAb 35, which binds to the extracellular surface, or control mAb 141, which binds on the cytoplasmic surface.

Consistently, Cδ-mAb 1 bound to both sides of the membrane. In most experiments the extent of the binding to either side was comparable. In some experiments the binding was higher on the inside (experiments 4 and 6) or on the outside (experiments 5, 8, and 9). As an average, Cδ-mAb 1 bound to a similar extent to either side of the membrane (52.8 ± 14.6 on the outside and 47.2 ± 14.6 on the inside). Binding of Cδ-mAb 1 for both sides of the membrane was abolished by its preincubation with the peptide used for the immunization or with peptide δ481−501, while preincubation of the mAb with peptide α304−322 or with other synthetic α or δ subunit sequences had no effect (data not shown). In double-labeling experiments all control mAbs bound uniquely to the expected side of the membrane.

Transmembrane Mapping of the Binding of Cδ-mAbs. Cδ-mAbs derived from mice immunized with nondenatured AChR. The transmembrane location of the epitopes recognized by all Cδ-mAbs listed in Table 2 was investigated by immunoelectronmicroscopy as described for Cδ-mAb 1. Table 6 summarizes the results obtained in one or more experiments for each Cδ-mAb. Figure 6C,D reports representative electronmicrographs obtained for some Cδ-mAb, used either alone or in combination with a control mAb (rat mAb 6, specific for the MIR on the extracellular surface, and rat mAb 118, which binds to the cytoplasmic surface; they are visualized from the binding of an anti-rat IgG Ab, coupled to large gold beads).

Three Cδ-mAbs (33, 25, and 10) bound exclusively (>93%) to the cytoplasmic surface. Cδ-mAb 151 seemed to bind only onto the cytoplasmic surface, but the very poor labeling obtained does not allow any firm conclusions.

Several Cδ-mAbs bound to both sides of the membrane. Some of them (5/C57 and 14) bound mostly to the inside (~83%). Still, the binding to the extracellular surface was consistent and much higher than the unspecific binding obtained for control mAbs, which was always <1% (Table 4). Cδ-mAbs 5/Balb/b, 8, and 32 bound two or three times more to the inside than the outside of the membrane. Other Cδ-mAbs bound to a similar extent to both sides (2, 7, 16, 31).

For all Cδ-mAbs, preincubation with peptide δ481−501 abolished their ability to bind to the AChR-rich membrane fragments (data not shown).

Several Cδ-mAbs labeled to the AChR-rich membranes poorly (e.g., 151, 2, 7). They could have been either washed away, given the stringent washing conditions we used (see Materials and Methods), or poorly recognized by the gold-labeled second Ab, resulting in a small number of gold beads decorating the membrane fragments. The relatively small number of gold beads found occasionally for Cδ-mAb 1 and for some control mAbs which are normally good "binders" (see Tables 4 and 5) may be related to the stringent washing conditions we used (see Materials and Methods). Other Cδ-mAbs—some which bound only to the cytoplasmic surface, others which bound to both sides of the membrane—could be visualized well by the gold-labeled second Ab, allowing counting of relatively large numbers of beads.

Identification of Residues Forming the Epitope(s) for Cδ-mAbs. All Cδ-mAbs specifically recognized the synthetic sequence δ481−501, which therefore must contain important structural elements of their epitope. To refine the localization of such elements and to identify binding requirements which might correlate with the differential ability of Cδ-mAbs to bind to the cytoplasmic surface, or to both sides of the membrane, we used a panel of single residue substituted analogues of the sequence δ481−501 to investigate the effect of individual residue substitutions on the binding of Cδ-mAbs to this synthetic sequence. Table 6 summarizes the results of those experiments.

Substitution of Pro_{485} and Phe_{486} strongly reduced or obliterated the binding of two Cδ-mAbs which bound only

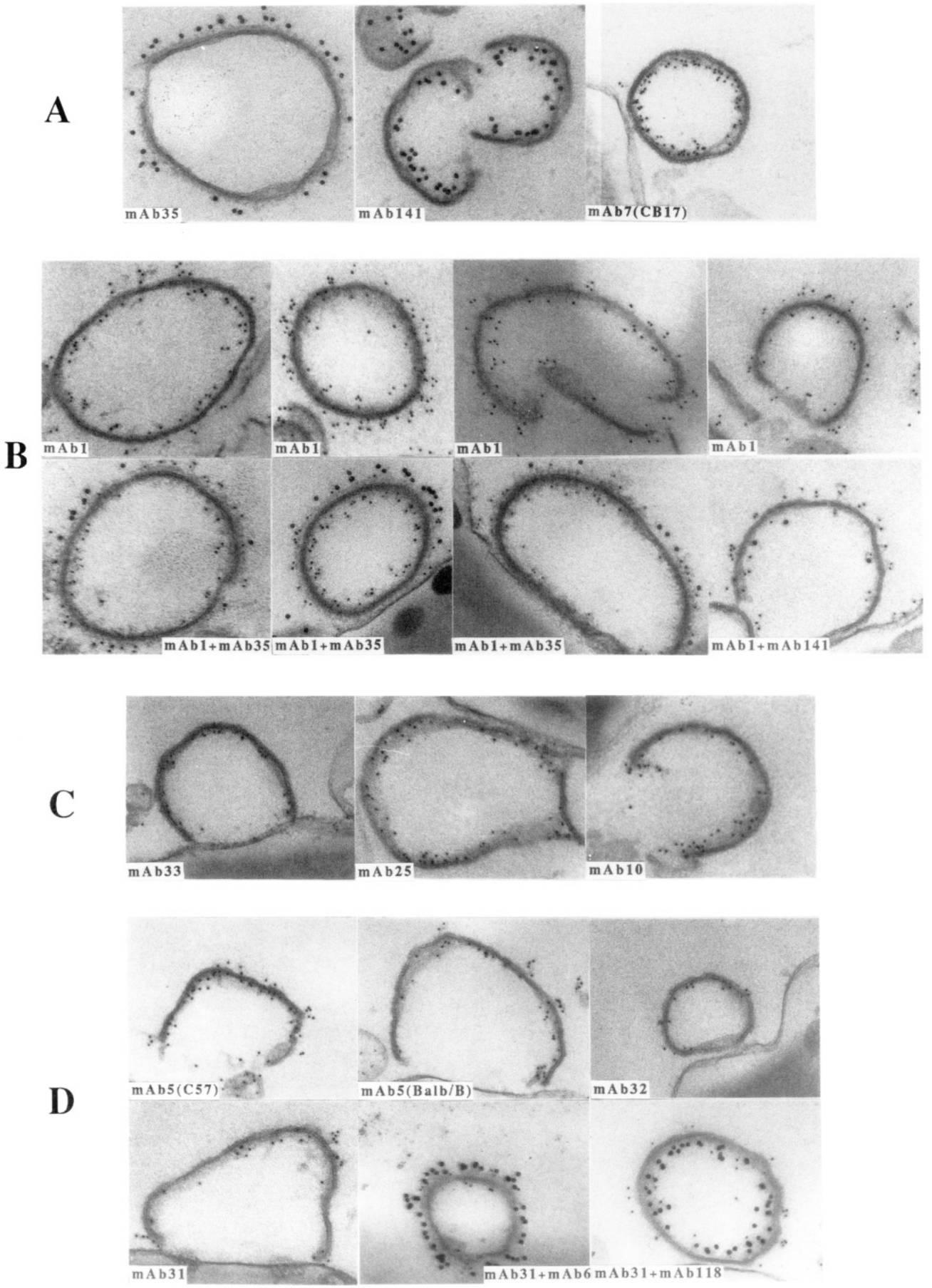

FIGURE 4: Mapping by immunoelectron microscopy of the transmembrane location of the binding of control mAbs and of Cδ-mAbs, using AChR-rich membrane fragments. See text for experimental details. (Panel A) Transmembrane mapping of the binding of control mAbs. The electron micrographs reported here are representative of those obtained in all experiments investigating the transmembrane location of the binding of control mAbs (listed in Table 1). The binding of control mAbs 35 and 141 is visualized using anti-rat IgG Ab coupled to

Table 6: Transmembrane Mapping of the Binding of Cδ-mAbs,[a] and Residues Crucial for or Involved in the Binding of the mAbs to the Synthetic Sequence δ481−501[b]

Cδ-mAb	beads counted	% outside	% inside	residues crucial for or involved in mAb binding[c]
33	230	4	96	P_{485} (81), F_{486} (93), D_{489} (49), P_{490} (48)
151	17	12	88	P_{485} (83), F_{486} (95), D_{489} (66), P_{490} (66)
25	184	7	93	P_{490} (15 ± 4, n = 2), Y_{493} (68 ± 5, n = 2)
10	149	0	100	P_{490} (80 ± 3, n = 3), Y_{493} (90 ± 1.5, n = 3)
2	42	43	57	P_{490} (29 ± 11, n = 2), Y_{493} (61 ± 13, n = 2)
5/C57Bl	285	18	83	P_{490} (30 ± 9, n = 2), Y_{493} (72 ± 3, n = 2)
7	42	50	50	P_{490} (12 ± 8, n = 2), Y_{493} (33 ± 0, n = 2)
5/Balb/b	402	27	73	P_{490} (21 ± 16, n = 2), Y_{493} (39 ± 14, n = 2)
8	141	29	71	P_{490} (25 ± 12, n = 2), Y_{493} (45 ± 8, n = 2)
14	169	17	83	P_{490} (43 ± 5, n = 2), Y_{493} (91 ± 1, n = 2)
16	225	37	63	P_{490} (24 ± 13, n = 2), Y_{493} (50.5 ± 2, n = 2)
31	129	39	61	P_{490} (30 ± 3, n = 2), Y_{493} (37 ± 11, n = 2)
32	235	25	75	P_{490} (36 ± 4, n = 2), Y_{493} (61 ± 2, n = 2)
1[d]	4255	53	47	Y_{193} (70 ± 24, n = 2)

[a] Assessed by immunoelectronmicroscopy of AChR-rich membrane fragments. See text for experimental details. [b] Assessed by ELISA, using the synthetic sequence δ481−501 and synthetic analogues of this sequence, carrying single-residue substitutions of each residue to an alanine. See text for experimental details. [c] The number in parentheses indicates the percent reduction (± standard deviation when more than one experiment was carried out) of the mAb binding to the synthetic analogues of sequence δ481−501, carrying the substitution of that residue to an alanine, as compared to the binding of the mAb to the unsubstituted synthetic sequence δ481−501. In each experiment, each determination of mAb binding to the unsubstituted sequence, and to the substituted analogues, was carried out in triplicate. [d] Data are the sum and averages of the experiments reported in Table 5.

to the cytoplasmic surface. Substitution of Asp_{489} and Pro_{490} reduced their binding moderately. The binding of the two other Cδ-mAbs which recognized only the cytoplasmic surface and of all but one Cδ-mAbs which recognized both sides of the postsynaptic membranes, was affected by substitution of Pro_{490} and Tyr_{493}, although their relative importance was different for each Cδ-mAb. Binding of Cδ-mAb 1 was sensitive only to substitution of Tyr_{493}. Therefore all Cδ-mAbs used here recognized epitopes within the same narrow sequence segment, δ485−493, at the carboxyl terminal of the AChR δ subunit.

DISCUSSION

The results of the present study suggest that the carboxyl terminal of the AChR δ subunit may exist in alternative conformations, having different transmembrane locations. This somewhat unexpected conclusion agrees with and may reconcile those of previous studies on the transmembrane topology of the carboxyl terminus of the AChR subunits [reviewed in Conti-Tronconi et al. (1994)].

Cδ-mAb 1, which labeled strongly and to a similar extent both sides of AChR-rich membrane fragments, was obtained from a mouse immunized with a synthetic peptide containing both the carboxyl terminal δ subunit sequence δ487−499 and residues α304−322 of the TAChR α subunit, which form an immunodominant epitope for T helper cells in Balb/c mice (Bellone et al., 1991) and is exposed on the cytoplasmic surface of the AChR (Lei et al., 1993). Therefore, in principle, Cδ-mAb 1 could recognize an epitope partially formed by the sequence α304−322 and also bind to the cytoplasmic surface. Although this possibility cannot be excluded by the results reported here, it is very unlikely since the synthetic sequence α304−322 does not bind mAb #1 in ELISA and does not inhibit Cδ-mAb 1 binding to solubilized or to membrane-bound AChR, while the synthetic sequence δ481−501 binds to mAb 1 in ELISA and pre-incubation with

large gold beads and that of control mAb 7 (CB17) using anti-mouse IgG Ab coupled to small gold beads. The AChR-rich membrane fragments have a propensity to curve toward formation of vesicles, which, as a result of the alkali treatment and of pushing the membrane suspension through narrow syringe needles, are in most cases unsealed (see Materials and Methods). The transmembrane location of the epitope recognized by the control mAbs had been previously determined unambiguously (see references in Table 1 and Lei and Conti-Tronconi, unpublished results). All mAbs uniquely bound to the expected site of the membrane fragments. The results obtained in several independent experiments are summarized in Table 4. (Panel B) Transmembrane mapping of the binding of Cδ-mAb 1. The transmembrane mapping of the binding of Cδ-mAb 1 was investigated in 11 independent experiments, whose results are summarized in Table 5. The micrographs reported here are representative of those used to collect the data reported in Table 5. In some experiments the membrane fragments were double-labeled with both Cδ-mAb 1 and the control anti-MIR mAb 35, which binds to the extracellular surface, or with both Cδ-mAb 1 and control mAb 141, which binds on the cytoplasmic surface. Binding of Cδ-mAb 1 is visualized by binding of anti-mouse IgG Ab labeled with small gold beads, while the binding of the control mAbs is visualized by binding of anti-rat IgG Ab labeled with large gold beads. Consistently, Cδ-mAb 1 bound to both sides of the membrane. In most cases the extent of the binding to either side was comparable. All control mAbs bound uniquely to the expected side of the membrane. (Panels C and D) Transmembrane mapping of the binding of Cδ-mAbs derived from mice immunized with nondenatured AChR. The transmembrane location of the epitopes recognized by all Cδ-mAbs listed in Table 2 was investigated by immunoelectron microscopy. The results obtained in one or more independent experiments for each Cδ-mAb are summarized in Table 6. The figure reports a sample of electronmicrographs obtained for some of these Cδ-mAbs, used either alone or in combination with a control mAb (rat mAb 6, specific for the MIR on the extracellular surface, and rat mAb 118, which binds to the cytoplasmic surface: see Table 1), which are representative of all those obtained in the experiments summarized in Table 6. Both control mAbs are visualized from the binding of an anti-rat IgG Ab coupled to large gold beads, while the mouse Cδ-mAbs are visualized from the binding of an anti-mouse IgG Ab coupled to small gold beads. Three Cδ-mAbs (33, 25, and 10) bound exclusively (>93%) to the cytoplasmic surface (Panel C). Several Cδ-mAbs bound to both sides of the membrane (Panel D). Cδ-mAb 5/C57 bound mostly to the inside, but the binding to the cytoplasmic surface was consistent and much higher than the unspecific binding obtained for control mAbs. Cδ-mAbs 5/Balb/b and 32 bound two or three times more to the inside than the outside of the membrane. Cδ-mAb 31 bound to a similar extent to both sides of the membrane.

this sequence abolished mAb 1 binding to solubilized nondenatured AChR and to both sides of AChR-rich membrane fragments. In further support of the possibility that labeling by Cδ-mAb 1 of both cytoplasmic and extracellular surfaces of membrane-bound AChR is due to an unusual transmembrane disposition of the carboxyl terminal region of the δ subunit, not to cross-reactivity with a cytoplasmic epitope of the α subunit, are the results obtained with several other Cδ-mAbs, derived from mice immunized with AChR, which also labeled both surfaces of the AChR-rich membrane fragments (Figure 4 and Table 6).

Previous studies using Abs directed against the carboxyl terminal sequence region of AChR subunits all suggested a cytoplasmic location of the carboxyl terminus, because the Abs bound to the cytoplasmic side of the postsynaptic membrane (Young et al., 1985) or, in immunochemical assays, bound only after treatment of AChR-rich sealed microsacs with permeabilizing agents (Ratnam & Lindstrom, 1984; Lindstrom et al., 1984). Although several Cδ-mAbs used in the present study bound to both sides of the membrane, some Cδ-mAbs (33, 151, 25, and 10) bound only to the cytoplasmic surface, similar to the Abs against the carboxyl terminals of AChR subunits used in previous studies (Young et al., 1985; Ratnam & Lindstrom, 1984; Lindstrom et al., 1984).

Studies employing nonimmunological approaches all concluded that the carboxyl terminus of the AChR subunits should be extracellular. In one study residue Lys_{486} of the γ subunit, which is on the carboxyl terminal side of the putative transmembrane domain M4, could be labeled in sealed AChR-rich microsacs from *Torpedo* electric organ with membrane-impermeable reagents, indicating that this residue, and therefore the carboxyl terminus of the γ subunit, may be extracellular (Dwyer, 1991). Another study investigated the location of the carboxyl terminus of the α and δ subunits from mammalian muscle using fusion proteins, with a prolactin reporter sequence attached downstream of the M4 region: the orientation relative to the microsomal membrane of the reporter prolactin domain was determined by a proteolysis protection assay (Chavez & Hall, 1992). That study concluded that the carboxyl termini of the α and δ subunits are located on the lumenal side of the microsomal vesicles—the topological equivalent of the extracellular space.

However, given their qualitative nature of the experimental approaches used, the elegant studies of Chavez and Hall (1992) and Dwyer (1991), while demonstrating that a substantial fraction of the carboxyl termini of different subunits, including the δ subunit, are in the extracellular space or in its topological equivalent, could not exclude the existence of a second population of AChR isomers having the carboxyl termini of one or more constituent subunits exposed on the cytoplasmic surface: the latter would go undetected in those experimental approaches. The conclusions of those studies, therefore, are compatible with that of the present study.

Several studies were based on the existence of *Torpedo* AChR as dimers held together by a disulfide bridge between the second last residue—a cysteine—of the δ subunit of each monomer (DiPaola et al., 1988). Those studies concluded that the disulfide bridges holding together AChR dimers are extracellular, since full conversion of AChR dimers to monomers could be achieved by administering membrane-impermeable reducing agents on the outside of the sealed AChR-rich vesicles (Dunn et al., 1986; McCrea et al., 1987; DiPaola et al., 1988, 1989). Those data can be reconciled with those reported here, and with the seemingly conflicting results of previous immunohistochemical and immunochemical studies, if considering that the great flexibility of the carboxyl terminal region of the AChR δ subunit (see below) implies the ability of this sequence region not only to exist in different conformations, as suggested by the present study, but also to undergo conformational changes when the δ subunits are fully assembled in mature AChR molecules. The different isomeric forms of the carboxyl terminal region of the δ subunit may spontaneously interconvert, thus explaining how exposure to reducing agent on the extracellular surface for relatively long periods of time, as in the studies mentioned above, would result in complete reduction of the disulfide bonds holding two AChR monomers together to form a dimer.

The carboxyl terminal region of the δ subunit is rich in prolyl residues, most of which are highly conserved in the AChRs from different species and tissues (4−6 prolines out of 25 residues in the AChRs from *Torpedo* electric organ and muscle of different species) (Claudio, 1989). A *cys* or *trans* conformation of one more of those Pro residues may well be the structural basis of the different isomeric forms of the carboxyl terminal region of the δ subunit. That AChR subunit isomers different for the isomerization of one or more Pro residue may exist, and that the presence of different isomeric forms is necessary for proper assembly of functional AChR molecules, has been demonstrated for a neuronal AChR which is a homooligomer of five α7 subunits (Halekar et al., 1994). That study, prompted by the observation of a striking abundance of highly conserved prolyl residues in the subunits of the AChR and of other homologous ligand-gated ion channel proteins, demonstrated that the peptidyl−prolyl isomerase cyclophilin is involved in the assembly of functional α7 AChRs and also of the structurally related homooligomeric ionotropic receptor for serotonine (type 3). Block of cyclophilin strongly reduced the expression of the homooligomeric α7 AChRs but did not affect the expression of functional heterooligomeric AChRs of muscle type. Furthermore, in the presence cyclophilin block, expression of functional AChRs containing the α7 subunit could be restored by coexpression of muscle type non-α AChR subunits (β, γ, or δ, whose expression, in the absence of an α-type subunit did not suffice for expression of a functional AChR). Those results suggested the existence of two or more α7 isomers, different for the *cis* or *trans* conformation of one or more prolyl residue, all necessary for assembly of a functional AChR complex, and different in their requirement for cyclophilin activity. The finding that a muscle-type non-α subunit can substitute for the missing $Pro_{cys/trans}$ α7 isomer suggests that muscle-type AChR non-α subunits may fold in different $Pro_{cis/trans}$ isomeric forms without intervention of cyclophilin, and possibly spontaneously. The existence of prolyl isomers has ben directly demonstrated by X-ray crystallography for polypeptides like calbindin-D_{9k} (Svensson et al., 1992) and alamethicin (Fox & Richards, 1982). Staphylococcal nuclease (Fox et al., 1986; Evans et al., 1987) and thioredoxin (Langsetmo et al., 1989) also possess multiple folded or intermediate forms based on prolyl isomerism.

The putative transmembrane segment M4 is very hydrophobic and much less amphipathic than the other putative

transmembrane hydrophobic domains M1, M2, and M3. This very nonpolar nature of M4 could allow formation of a transmembrane domain able to assume different positions relative to the plane of the membrane, thus better accommodating the topographic excursions of the carboxyl terminal "tail" of the δ subunit.

That the carboxyl terminal region of the δ subunit must be able to assume different conformations was implied in the findings of studies which investigated the mobility relative to each other of the two monomers within a *Torpedo* AChR dimer (Fairclough et al., 1983). The translational relation between two monomeric units found in those studies suggested a great degree of mobility, since each could rotate relative to the other up to almost 180°. The conclusion of those findings was that the two carboxyl terminals of the δ subunit must be long and flexible and able to assume more than one conformation. Circumstantial evidence that the carboxyl terminal region of the δ subunit in the native AChR molecule is largely exposed and flexible results also from the present studies, since most anti-δ subunit mAbs obtained by immunization with native AChR, and able to fully recognize the native AChR molecule, bind to the synthetic peptide δ481−501: given their slightly different sensitivity to amino acid substitutions for binding to their synthetic sequence, and the different transmembrane mapping of their binding to AChR-rich membranes, the Cδ-mAbs must recognize different epitopes within, or conformations of, this sequence region.

The existence of different foldings of mature proteins having identical primary sequences has been demonstrated in crystallographic studies of the structure of the constituent subunits of the pentameric protein VP1, which forms the outer shell of simian virus 40 (SV40) (Liddington et al., 1991). These pentamers, like the AChR pentameric molecule, may assemble in hexagonal lattices, raising the puzzling problem of fitting pentamers into hexavalent holes, which requires unexpected flexibility and alternative bonding to account for the mismatch of symmetries. Such a situation occurs not only for the AChR and for VP1 of SV40 but also in other polyomaviruses (Rayment et al., 1982; Salunke et al., 1986). In the case of SV40 VP1, this dilemma is solved by assembling pentamers whose subunits have identical conformations except for their carboxyl terminal segment, which may assume alternative stable conformations and take different directions as they emerge from the core of the corresponding subunit, heading toward a neighbor pentamer (Liddington et al., 1991). This accommodates the required variability in the geometry of contacts. That the folding features of the constituent subunits and the packing pattern of mature pentamers might be similar in the AChR and in the SV40 VP1 is further supported by the fact that in both cases the pentamers form disulfide stabilized dimers, which can further pack to form tubular aggregates, although the diameter of such "tubes" is wider for those formed by the AChR than by SV40 VP1 (Brisson & Unwin, 1984; Salunke et al., 1986).

The demonstrated structure of the SV40 shell, formed by VP1 pentamers, and the likely structure of the *Torpedo* postsynaptic membrane, with its tight packing of AChR molecules, seem to have the common characteristic of being formed by dimers linked by flexible C-terminal arms of two neighbors, further stabilized by disulfide bonds and loosely tied together instead of locked in a tight fit between pentameric units. This has been suggested (Liddington et al., 1991) to be *"one general solution to the problem of how to generate a structure with several kinds of contacts among standard building blocks"*. Furthermore, Liddington et al. (1991) suggested *"that subcellular assemblies frequently will be found to exhibit these sorts of linkages. Many such assemblies seem to be flexible yet highly specific. Interaction through arms can ensure specificity without requiring a rigid geometry and without imposing strong restrictions on symmetry."* The case illustrated here for *Torpedo* AChR may be the first demonstration of the truth of their prediction.

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REFERENCES

Anderson, D. J., & Blobel, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5598−5602.
Anderson, D. J., Walter, P., & Blobel, G. (1982) *J. Cell Biol. 93*, 501−506.
Anderson, D. J., Blobel, G., Tzartos, S. J., Gullick, W., & Lindstrom, J. (1983) *J. Neurosci. 3*, 1773−1784.
Bellone, M., Ostlie, N., Lei, S., & Conti-Tronconi, B. M. (1991) *Eur. J. Immunol. 21*, 2203−2310.
Bellone, M., Ostlie, N., Karachunski, P., Manfredi, A. A., & Conti-Tronconi, B. M. (1993) *J. Immunol. 151*, 1025−1030.
Betz, H. (1990a) *Neuron 5*, 383−392.
Betz, H. (1990b) *Biochemistry 29*, 3591−3599.
Brisson, A., & Unwin, P. N. T. (1984) *J. Cell Biol. 99*, 1202−1211.
Chavez, R. A., & Hall, Z. W. (1991) *J. Biol. Chem. 266*, 15532−15538.
Chavez, R. A., & Hall, Z. W. (1992) *J. Cell Biol. 116*, 385−393.
Claudio, T. (1989) in *Frontiers in Molecular Biology* (Glover, D. M., & Hammes, B. D., Eds.) pp 63−142, IRL Press, Oxford.
Conti-Tronconi, B. M., McLane, K. E., Raftery, M. A., Grando, S. A., & Protti, M. P. (1994) *CRC Rev. Biochem. Mol. Biol. 29*, 69−123.
DiPaola, M., Czajkowski, C., Bodkin, M., & Karlin, A. (1988) *Soc. Neurosci. Abs. 14*, 640 (260.5).
DiPaola, M., Czajkowski, C., & Karlin, A. (1989) *J. Biol. Chem. 264*, 15457−15463.
Dunn, S. M. J., Conti-Tronconi, B. M., & Raftery, M. A. (1986) *Biochem. Biophys. Res. Commun. 139*, 830−837.
Dwyer, B. P. (1991) *Biochemistry 30*, 4105−41112.
Elliot, J., Dunn, S. M. J., Blanchard, S. G., & Raftery, M. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2576.
Elliot, J., Blanchard, S. G., Wu, W., Miller, J., Strader, C. D., Hartig, P., Moore, H.-P., Racs, J., & Raftery, M. A. (1980) *Biochem. J. 185*, 667−677.
Evans, P. A., Dobson, C. M., Kautz, R. A., Hatfull, G., & Fox, R. O. (1987) *Nature 329*, 266−268.
Fairclough, R. H., Finer-Moore, J., Love, R. A., Kristofferson, D., Desmeules, P. J., & Stroud, R. M. (1983) *Cold Spring Harbor Symp. Quant. Biol. 48*, 9−20.
Finer-Moore, J., & Stroud, R. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 155−159.
Fox, R. O., Jr., & Richards, F. M. (1982) *Nature 300*, 325−330.
Fox, R. O., Evans, P. A., & Dobson, C. M. (1986) *Nature 320*, 192−194.
Galzi, J.-L., Revah, F., Bessis, A., & Changeux, J.-P. (1991) *Annu. Rev. Pharmacol. 31*, 37−72.
Guy, R. (1983) *Biophys. J. 45*, 249−261.
Haggerty, J. G., & Froehner, S. C. (1981) *J. Biochem. (Tokyo) 256*, 8294−8297.
Heinrickson, S., & Meredith, B. (1983) *Anal. Biochem. 136*, 65−74.
Helekar, S. A., Char, D., Neff, S., & Patrick, J. (1994) *Neuron 12*, 179−189.

Hohlfeld, R., Toyka, K. V., Tzartos, S. J., Carson, W., & Conti-Tronconi, B. M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5379−5382.

Houghten, R. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5131−5135.

Karlin, A., Kao, P. N., & Dipaola, M. (1986) *Trends Pharmacol. Sci. 7*, 304−308.

Kordossi, A. A., & Tzartos, S. J. (1987) *EMBO J. 6*, 1605−1610.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Langsetmo, K., Fuchs, J., & Woodward, C. (1989) *Biochemistry 28*, 3211−3220.

LaRochelle, W. J., Wray, B. E., Sealock, R., & Froehner, S. C. (1985) *J. Cell Biol. 100*, 684−691.

Lei, S., Raftery, M. A., & Conti-Tronconi, B. M. (1993) *Biochemistry 32*, 91−100.

Liddington, R. C., Yan, Y., Moulai, J., Sahli, R., Benjamin, T. L., & Harrison, S. C. (1991) *Nature 354*, 278−284.

Lindstrom, J., Criado, M., Hochschwener, S., Fox, J. L., & Sarin, V. (1984) *Nature 311*, 573−575.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.

Maelicke, M. A. (1988) in *Handbook of Experimental Pharmacology: The Cholinergic Synapse* (Whittaker, V. P., Ed.) pp 267−300, Springer-Verlag, Berlin.

Maelicke, M. A., Plumer-Wilk, R., Fels, G., Spencer, S. R., Engelhard, M., Veltel, D., & Conti-Tronconi, B. M. (1989) *Biochemistry 29*, 1396−1405.

McCrea, P. D., Popot, J.-L., & Engelman, D. M. (1987) *EMBO J. 6*, 3619−3626.

Moore, H., Hsu, H.-P., & Raftery, M. A. (1979) *Biochemistry 10*, 1862−1867.

Nelson, S., & Conti-Tronconi, B. M. (1990) *J. Neuroimmunol. 29*, 81−92.

Neubig, R. R., Krodel, E. K., Boyd, N. D., & Dohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 690−694.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S. Miyata, T., & Numa S. (1983) *Nature 302*, 528−532.

Ratnam, M., & Lindstrom, J. (1984) *Biochem. Biophys. Res. Commun. 12*, 1225−1233.

Ratnam, M., Le Nguyen, D., Rivier, J., Sargent, P. B., & Lindstrom, J. (1986a) *Biochemistry 25*, 2633−2643.

Ratnam, M., Sargent, P. B., Sarin, V., Fox, J. L., Nguyen, D. L., Rivier, J., Criado, M., & Lindstrom, J. M. (1986b) *Biochemistry 25*, 2621−2632.

Rayment, I., Baker, T. S., Caspar, D. L. D., & Murakami, W. T. (1982) *Nature 295*, 110−115.

Salunke, D., Caspar, D. L. D., & Garcea, R. L. (1986) *Cell 46*, 895−904.

Schmidt, J., & Raftery, M. A. (1973) *Anal. Biochem. 52*, 349−354.

Strader, C. B. D., Revel, J.-P., & Raftery, M. A. (1979) *J. Cell Biol. 83*, 499−510.

Stroud, R. M., McCarthy, M. P., & Shuster, M. (1990) *Biochemistry 29*, 11009−11023.

Svensson, L. A., Thulin, E., & Forsen, S. (1992) *J. Mol. Biol. 223*, 601−606.

Towbin, H., Stahelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.

Tzartos, S. J., & Changeux, J.-P. (1983) *EMBO J. 2*, 381−387.

Tzartos, S. J., & Changeux, J.-P. (1984) *J. Biochem. (Tokyo) 259*, 11512−11519.

Tzartos, S. J., Rand, D. E., Einarson, B. E., & Lindstrom, J. M. (1981) *J. Biol. Chem. 256*, 8635−8645.

Tzartos, S. J., Kokla, A., Walgrave, S. L., & Conti-Tronconi, B. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2899−2903.

Wilson, P. T., & Lentz, T. L. (1988) *Biochemistry 27*, 6667−6674.

Young, E. F., Ralston, E., Blake, J., Ramachandran, J., Hall, Z. H., & Stroud, R. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 626−630.

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