Articles

Species Specificity of Anti-Acetylcholine Receptor Antibodies Elicited by Synthetic Peptides†

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ABSTRACT: Two peptides corresponding to amino acid residues 351–368 of the α-subunits of *Torpedo* and human acetylcholine receptor (AChR) were synthesized. These peptides contain a segment (residues 355–364) which displays the greatest variability in amino acid sequence between the two species. Antibodies elicited against the two peptides cross-reacted with the respective *native* AChRs and were shown to be species specific by radioimmunoassay, immunoblotting, and immunofluorescence microscopy. Thus, antibodies against the *Torpedo* peptide cross-reacted with *Torpedo* AChR but did not bind to mammalian or chicken AChR. Antibodies against the human peptide proved to be specific probes for mammalian muscle AChR. They cross-reacted with mammalian AChR (human, calf, mouse, and rat) but not with *Torpedo* or chicken AChR. These antibodies were also shown to react preferentially with the the extrajunctional form of muscle AChR, as compared to their reactivity with junctional muscle AChR. In immunofluorescence experiments, the anti-human peptide antibody stained AChR aggregates in sectioned or ethanol-permeabilized rat and mouse myotubes grown in culture but did not stain living myotubes. This indicates that the sequence 351–368 of the α-subunit of *mammalian* AChR is on the cytoplasmic face of *muscle* cell membranes, as predicted theoretically.

The nicotinic acetylcholine receptor (AChR)[1] is probably the best characterized neurotransmitter receptor and transmembrane ionic channel studied thus far. It is composed of five subunits present in a stoichiometry of $\alpha_2\beta\gamma\delta$ (Karlin, 1980; Conti-Tronconi & Raftery, 1982; Changeux et al., 1984). The AChR from electric fish has been isolated, and the amino acid sequences of the subunits have been deduced by recombinant DNA technology (Noda et al., 1982, 1983a,b; Claudio et al., 1983; Devillers-Thiery et al., 1983). The strong homology between the *Torpedo* AChR and AChR from mammalian skeletal muscle made it possible to clone and deduce the sequence of the receptor from muscle tissue, where its amount is 3–4 orders of magnitude lower than that of the *Torpedo* electric organ (Tanabe et al., 1984; Takai et al., 1984; Nef et al., 1984; Lapolla et al., 1984; Boulter et al., 1985). Our interest has been directed mainly to the α-subunit of the AChR as it has been shown to contain essential elements of the ligand binding site (Haggerty & Froehner, 1981; Karlin et al., 1983; Gershoni et al., 1983; Tzartos & Changeux, 1983; Wilson et al., 1984, 1985; Neumann et al., 1985, 1986) as well as highly immunogenic domains which may be involved in the autoimmune disease myasthenia gravis (Tzartos & Lindstrom, 1980; Souroujon et al., 1986). The amino acid sequences of the α-subunits of human muscle and *Torpedo* electric organ AChR display greater than 80% homology (Noda et al., 1983c). However, in spite of the considerable conservation of receptor structure during evolution and the partial immunological cross-reactivity between AChRs from *Torpedo* and skeletal muscle, polyclonal and/or monoclonal anti-*Torpedo* AChR antibodies are not optimal reagents for probing AChR from other sources.

In an attempt to elicit antibodies specific for mammalian AChR by immunization with synthetic peptides, we searched for sequences in the α-subunit that are least conserved between *Torpedo* and human AChR, assuming that such regions might be highly immunogenic. The region which encompasses the greatest variability between these two species corresponds to residues 355–364 of the α-subunit, with only one identical amino acid residue. We have shown previously that in the *Torpedo* AChR this sequence is indeed localized within a

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[1] Abbreviations: AChR, acetylcholine receptor; α-BTX, α-bungarotoxin; DMEM, Dulbecco's-modified Eagle's medium; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline [10 mM sodium phosphate buffer (pH 7.2)/140 mM NaCl]; H-peptide (H-pep), synthetic peptide corresponding to amino acid residues 351–368 of the α-subunit of human AChR; T-peptide (T-pep), synthetic peptide corresponding to amino acid residues 351–368 of the α-subunit of *Torpedo* AChR; TMR, tetramethylrhodamine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; KLH, keyhole limpet hemocyanin; PAGE, polyacrylamide gel electrophoresis; mcAb, monoclonal antibody; DMM, denervated mouse muscle; NMM, normal mouse muscle; kDa, kilodalton(s); BSA, bovine serum albumin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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highly immunogenic region of the α-subunit (Souroujon et al., 1986). In the human receptor, the segment corresponding to residues 355–364 is exceptionally rich in proline and glycine residues. It was thought that such a highly nonconserved region of unique sequence features might contribute to functional variations in different AChRs and could also play an important immunological and pathological role in the response to AChR.

This study describes the elicitation of species-specific antibody probes for the region corresponding to residues 351–368 of the human and *Torpedo* α-subunits. Antibodies elicited against residues 351–368 of the human α-subunit were shown to be a specific probe for mammalian muscle AChR. Also, by using these antibodies, we could demonstrate that this sequence entity is located on the cytoplasmic face of the muscle cell membrane and that binding to this sequence is increased in the receptors of denervated muscles.

Materials and Methods

Receptor Purification and Peptide Synthesis. *Torpedo* AChR was purified from the electric organ of *Torpedo californica* as described previously (Aharonov et al., 1977). In all experiments, affinity-purified *Torpedo* AChR was employed. Mammalian (fetal calf and denervated and innervated mouse) muscle Triton X-100 detergent extracts were prepared as previously described (Souroujon et al., 1985). The specific α-bungarotoxin (α-BTX) binding activities of the preparations were 550, 640, and 50 fmol of α-BTX/mg of protein for the fetal calf, denervated mouse muscle, and innervated mouse muscle, respectively. Affinity-purified fetal calf receptor was prepared by purifying the receptor on beads of Sepharose–*Naja naja siamensis* toxin. Four 1-mL portions of calf Triton extract (4.6 mg/mL) were incubated consecutively with Sepharose beads (200 mg) (containing 200 μg of covalently bound *Naja naja siamensis* toxin) for 3-h periods. After incubation, the supernatant was discarded, and an additional 1 mL of calf Triton extract was added. Subsequently, the Sepharose beads were washed 3 times in phosphate-buffered saline (PBS: 0.14 M NaCl/0.01 M phosphate buffer, pH 7.2), and receptor was eluted by incubating the beads for 10 min with 200 μL of sample buffer (2% lithium dodecyl sulfate, 0.062 M Tris-HCl, pH 6.8, 10% glycerol w/v, 2% β-mecaptoethanol, and 0.025% bromophenol blue). The sample was then centrifuged, and the supernatant was loaded on the gel (50 μL per slot).

Peptide synthesis was carried out by the solid-phase method of Merrifield (1965), as previously described (Neumann et al., 1984, 1985). The amino acid composition of the synthetic peptides was verified by amino acid analysis. The peptides were conjugated to keyhole limpet hemocyanin (KLH; Calbiochem) by using 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (Muller et al., 1982).

Immunization Procedure. Rabbits were immunized by multisite intradermal injections of 1 mg of conjugate in 0.5 mL of phosphate-buffered saline (PBS) emulsified in 0.75 mL of complete Freund's adjuvant. A booster injection (0.5 mg in complete Freund's adjuvant) was similarly administered 4 weeks later. Further booster injections were given in incomplete Freund's adjuvant. Immunoglobulins were affinity purified on the respective Sepharose–peptide column. Peptides were bound to CNBr-activated Sepharose 4B according to March et al. (1974). The amount of peptide bound ranged between 2 and 4 mg/g of Sepharose. Rabbit antipeptide antisera were absorbed onto the respective Sepharose–peptide columns and subsequently eluted by 0.1 M NH_4OH, followed by dialysis against PBS.

Immunological Analysis of Antisera. Binding of the antisera to the synthetic peptides was analyzed by solid-phase radioimmunoassay (Mochly-Rosen & Fuchs, 1981). Coating of the plates with the peptides was via glutaraldehyde (Suter, 1982). ^{125}I-Labeled *Staphylococcus aureus* protein A, radioiodinated by the Bolton–Hunter reagent (Bolton & Hunter, 1973), was applied for quantification of binding.

The reactivity of the antisera with *Torpedo* AChR and mammalian muscle (mouse and fetal calf) Triton extracts, prelabeled with ^{125}I-α-BTX, was tested by radioimmunoassay, essentially as previously described (Souroujon et al., 1985). The rabbit antisera at serial dilutions, in a total volume of 100 μL of PBS containing 10% normal rabbit serum, were incubated with 100 μL of the receptor–toxin complex containing 0.1 pmol of α-BTX binding sites. Subsequently, 150 μL of goat anti-rabbit immunoglobulin was added, and the tubes were incubated overnight at 4 °C. The precipitates formed were centrifuged and washed twice, and the precipitated radioactivity was measured in an autogamma counter. The antibody titers were calculated from the precipitation curves obtained, and the titer was expressed as moles of α-BTX binding sites precipitated by 1 L of serum or ascitic fluid.

Gel electrophoresis of the receptor, transfer onto nitrocellulose filters, and antibody overlay were performed as described (Gershoni et al., 1983; Neumann et al., 1985). For the immunoblotting experiments, affinity-purified antibodies (2 μg/mL in PBS containing 1% hemoglobin; Sigma) were employed.

Cell Cultures. Cells of the mouse skeletal muscle cell line C_2 were grown in 35-mm culture dishes in Dulbecco's-modified Eagle's (DMEM; H-21 GIBCO) containing 10% fetal calf serum in a 37 °C incubator with 6% CO_2. When the cells reached confluency (after 3 days), the cultures were fed with 90% DMEM and 10% heat-inactivated horse serum to promote fusion. The cells were used after 3 additional days. Primary cultures of fetal rat muscle cells were prepared and maintained in 35-mm culture dishes as previously described (Schaffner & Daniels, 1982; Olek et al., 1983). Extensive formation of AChR aggregates on these cells was induced by a 5-h incubation with embryonic pig brain extract as described (Olek et al., 1983).

Histochemical Assays. Unifixed C_2 and primary rat muscle cultures and unfixed cryostat sections (15 μm) of *Torpedo californica* electric organ and of rat diaphragm were stained for AChR by incubating them for 1 h with 5×10^{-8} M tetramethylrhodamine-conjugated α-BTX (TMR–α-BTX) prepared according to Ravdin and Axelrod (1977). After TMR–α-BTX staining, some primary rat muscle cultures were scraped from the culture dishes, pelleted by gentle centrifugation, frozen, and cryostat-sectioned at 6 μm. Sections and culture cells were also incubated (1 h at room temperature) with affinity-purified antipeptide antibodies. These were applied at final concentrations of 2 and 3 μg/mL for the anti-human peptide and anti-*Torpedo* peptide antibodies, respectively. Sections and cells were then washed for 30 min and incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (immunoglobulin fraction; Hyland, Costa Mesa, CA) at a final concentration of 10 μg/mL. After an additional 30-min wash, the tissues or cultured cells were fixed with 2% paraformaldehyde in 0.12 M cacodylate buffer (pH 7.2) for 1 h, washed, and mounted in Gelvatol (Monsanto), pH 8.2. Cultures and sections were examined with a Zeiss photomicroscope II equipped with a 100-W mercury arc lamp and epifluorescence optics and a ×25 plan neofluar phase-contrast immersion

	351															368
α-Torpedo	I	S	G	K	Q	V	T	G	E	V	I	F	Q	T	P	L	I	K
α-Human	I	S	G	K	P	G	P	P	P	M	G	F	H	S	P	L	I	K

FIGURE 1: Amino acid sequence of peptides 351–368 of the *Torpedo* and human AChR α-subunit. Note that four amino acids on the carboxy terminus and four on the amino terminus of both peptides are homologous.

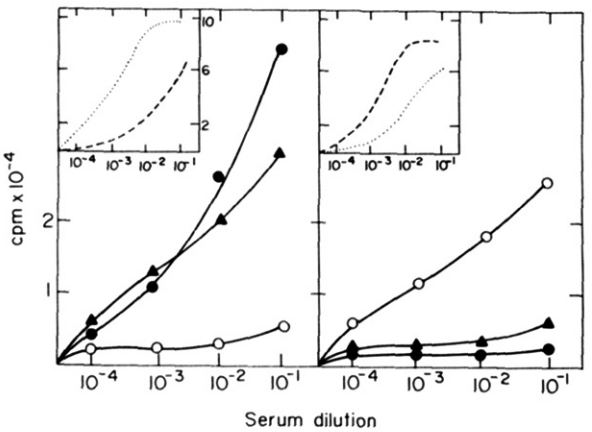

FIGURE 2: Binding of calf AChR (●), mouse AChR (▲), and *Torpedo* AChR (○) complexed with ^{125}I-labeled α-BTX to antipeptide 351–368 antibodies from human (left) and from *Torpedo* (right). The binding of the human (···) and *Torpedo* (– – –) peptides to both antisera, as detected by solid-phase radioimmunoassay, is depicted in the inserts.

objective (N.A 0.8). For rhodamine, a Bp 546/12 excitation filter and an Lp 590 barrier filter were used; for fluorescein, a Bp 485/20 excitation filter and an Lp 515-556 barrier filter were used. With these filters, no fluorescein fluorescence was detected in the rhodamine range and vice versa. Fluorescence photomicrographs were taken at exposures of 8 or 20 s for rhodamine and 15–30 s for fluorescein on Kodak Tri X 400 ASA film processed to ASA 1600, or Ilford HP5 film processed to ASA 800.

RESULTS

Two peptides corresponding to residues 351–368 of the *Torpedo* and human AChR α-subunit were synthesized. The sequences of both peptides are depicted in Figure 1.

The synthetic peptides were conjugated to keyhole limpet hemocyanin (KLH) as a carrier protein, and the conjugates were injected into rabbits in order to elicit antipeptide antibodies. Antibodies against the *Torpedo* peptide (T-pep) cross-reacted partially (about 10%) with the human peptide, and vice versa; i.e., antibodies against human peptide (H-pep) cross-reacted to about 10% with the *Torpedo* peptide (Figure 2, insert). However, the reactivity of these antipeptide antibodies with AChR was shown to be species specific. Thus, antiserum against the H-pep cross-reacted with mammalian AChR (mouse and calf) but not with *Torpedo* AChR (Figure 2, left panel). On the other hand, antiserum against the T-pep cross-reacted with *Torpedo* AChR, whereas it bound only slightly to mammalian AChR (Figure 2, right panel). It should be noted that these antisera recognize the respective receptors in their native conformation, as they precipitated the respective AChR–α-BTX complex.

In order to assess the subunit specificity of both antipeptide antibodies, the immunoblotting method was employed. Affinity-purified receptors from both *Torpedo* electric organ and fetal calf muscle were resolved into their subunits by polyacrylamide gel electrophoresis (PAGE) and subsequently blotted onto nitrocellulose filters. These filters were then

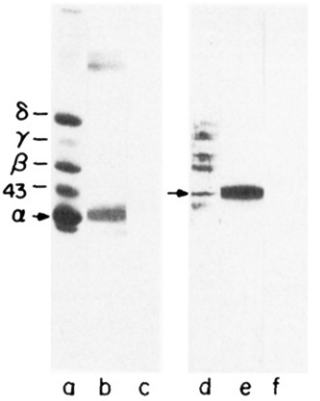

FIGURE 3: Blot analysis of *Torpedo* AChR and fetal calf muscle AChR with antipeptide antibodies. Protein blots of affinity-purified AChR from *Torpedo* (lanes b and f) and from calf muscle (lanes c and e) were overlayed with affinity-purified antibodies against the *Torpedo* peptide (2 μg/mL; lanes b and c) and with affinity-purified antibodies against the human peptide (2 μg/mL, lanes e and f). Quantification of the binding was obtained by incubation with ^{125}I-labeled protein A (500 000 cpm/mL). Lanes a and d demonstrate the protein stain of the *Torpedo* AChR and calf AChR, respectively; the arrows point to the *Torpedo* and calf AChR α-subunits.

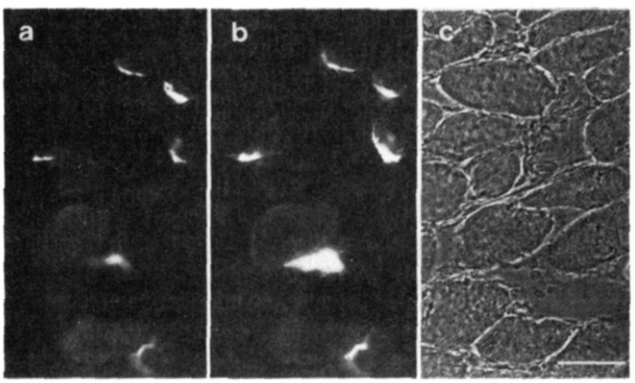

FIGURE 4: Staining of rat diaphragm sections with anti-H-pep antibodies and α-BTX. Unfixed rat diaphragm sections were incubated with affinity-purified anti-H-pep antibodies in PBS containing 1 mg/mL BSA. The sections were washed (30 min) and further incubated with a mixture of TMR–α-BTX and FITC-conjugated goat anti-rabbit IgG. (a) The distribution of α-BTX binding sites. (b) The distribution of anti-H-pep antibody binding sites. (c) The phase-contrast image of the same field. Bar represents 50 μm.

probed with affinity-purified antipeptide antibodies, followed by ^{125}I-labeled *Staphylococcus aureus* protein A. As shown in Figure 3, the antibodies against the T-pep reacted with the α-subunit of *Torpedo* AChR (Figure 3b) and did not recognize the calf receptor (Figure 3f). The anti-H-peptide antibodies bound specifically to the α-subunit of calf AChR (Figure 3e) and did not bind to *Torpedo* AChR (Figure 3c). The slow migration of the α-subunit of calf AChR (Figure 3d,e) is in agreement with a previous report by Conti-Tronconi et al. (1982), who have shown that the α-subunit of calf AChR is of a slightly higher molecular weight (42K) than its *Torpedo* counterpart (40K).

We next visualized the antibody binding to the mammalian receptor by immunofluorescence microscopy. For this purpose, we stained sections from rat diaphragm with affinity-purified antibodies against the H-pep and T-pep, followed by fluorescently labeled goat anti-rabbit immunoglobulins. The pattern of antibody staining was then compared to that obtained with fluorescently-labeled α-BTX, applied to the same sections. Figure 4a shows that the mouse diaphragm sections contain high local concentrations of α-BTX binding sites which correspond to the regions of the neuromuscular junction.

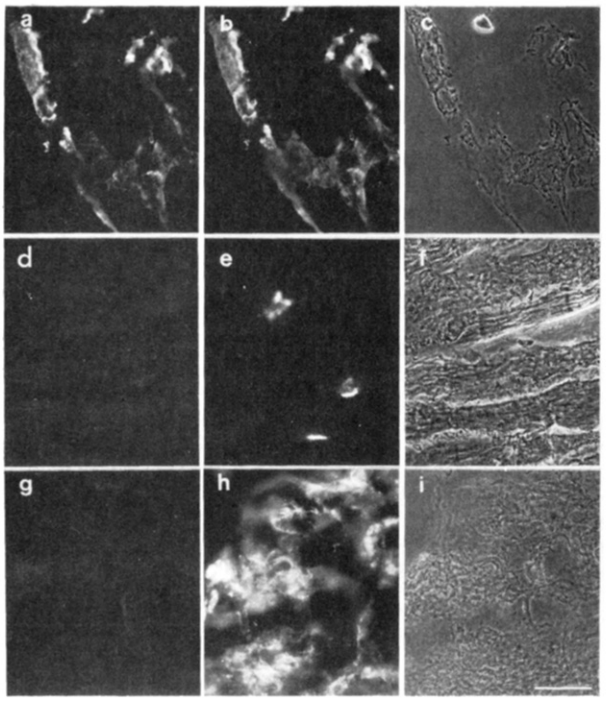

FIGURE 5: Species specificity of antipeptide antibodies as revealed by immunofluorescence. Unfixed sections of *Torpedo californica* electric organ (a–c and g–i) and rat diaphragm (d–f) were incubated with anti-T-pep (a and d) and anti-H-pep (g) antibodies in PBS (rat) or PBS with 0.5 M sucrose (*Torpedo*) containing 1 mg/mL gelatin. The sections were then washed and further incubated in the same buffers containing a mixture of TMR–α-BTX and FITC-conjugated goat anti-rabbit IgG. The left panel shows the binding pattern of anti-T-pep antibodies (a and d) and anti-H-pep antibodies (g) detected with fluorescein fluorescence optics. The middle (b, e, and h) and right (c, f, and i) panels represent the TMR–α-BTX staining and the phase-contrast image of the corresponding fields, respectively. Bar represents 50 μm.

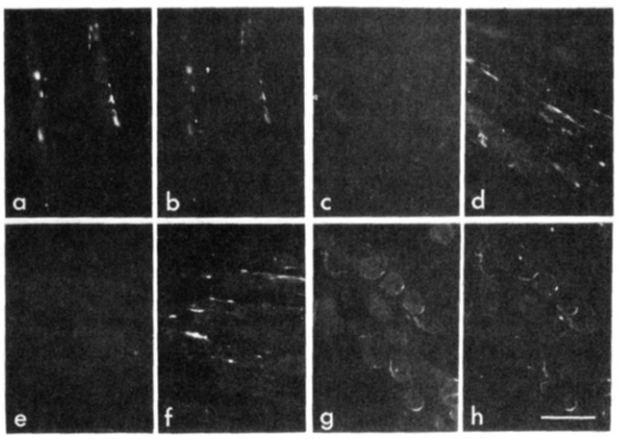

FIGURE 6: Anti-H-pep antibodies bind the cytoplasmic domain of the AChR. C_2 muscle cultures (a–f) were incubated at 37 °C in their growth medium with TMR–α-BTX. The cells were then washed with DMEM containing HEPES. Several cultures were then incubated for 5 min with ethanol (95%) at −20 °C and washed with DMEM–HEPES. Primary cultures of rat myotubes (g and h) were incubated with embryonic brain extract to induce AChR aggregates and stained as above with TMR–α-BTX. After washing, the cells were scraped, pelleted, frozen, and cryostat-sectioned. Cultures or cryostat sections were incubated with anti-H-pep antibodies in DMEM–HEPES containing 1 mg/mL BSA or in PBS containing 4 mg/mL BSA, respectively, and then with FITC-conjugated goat anti-rabbit IgG. Panels a, c, e, and g represent the antibody binding patterns seen with fluorescein fluorescence optics; panels b, d, f, and h represent the corresponding rhodamine fluorescence image showing TMR–α-BTX binding. (A and B) Ethanol-permeabilized cultures; (c and d) untreated cultures; (e and f) permeabilized cultures in the absence of anti-Hhpep antibody; (g and h) cryostat sections. Bar represents 50 μm.

Figure 4b demonstrates that these regions of high α-BTX binding site density were also stained with the anti-H-pep antibodies. Regions which did not react with the fluorescent toxin were also negative with respect to the anti-H-pep antibodies, thereby demonstrating high specificity of the antibodies to sites of receptor location. Figure 5 indicates that the antibodies elicited against the *Torpedo* peptide bound specifically to the *Torpedo* AChR in sections of the electric organ (Figure 5a) but did not recognize the α-BTX binding sites (the AChR) on the rat diaphragm sections (Figure 5d). Moreover, the anti-H-pep antibodies did not label the AChR on sections from *Torpedo* electric organ (Figure 5g). These data further demonstrate the high species specificity of the antibodies to the receptor in the appropriate tissue.

It was of interest to examine the orientation of this sequence entity with respect to the muscle cell membrane. To do this, we first tested alcohol-permeabilized and intact cultured myotubes of the clonal mouse muscle cell line C_2 for their capacity to bind the anti-H-pep antibodies. Fluorescent α-BTX was applied, prior to alcohol treatment, in order to allow the visualization of high receptor densities (receptor clusters) known to reside on these muscle cells (Silberstein et al., 1982). Muscle cultures, labeled with fluorescent α-BTX and permeabilized, were shown to react with the anti-H-pep antibodies (Figure 6a). On the other hand, the receptor clusters in unpermeabilized cells did not stain with the anti-H-pep antibodies (Figure 6c) but were stained with a monoclonal antibody (5.5; Mochly-Rosen & Fuchs, 1981) against a known external AChR epitope under the same conditions (data not shown). A control experiment in which no antipeptide antibodies were added to the permeabilized cells demonstrated that the fluorescent secondary antibodies do not react with intracellular components of similar distribution to that of the receptors (Figure 6e,f). Similar results were obtained with primary cultures of rat muscle cells (not shown). The observation that anti-H-pep antibodies did not bind to AChR on living myotubes but did so in alcohol-permeabilized myotubes suggests that residues 351–368 of the mammalian muscle AChR are located on the cytoplasmic face of the muscle cell membrane. However, it is also possible that residues 351–368 are embedded in the lipid bilayer and that even the brief exposure to ethanol at −20 °C had removed enough lipid to expose the antibody binding sites. In order to rule out this possibility, we used cryostat sections of unfixed, frozen myotubes to expose the cytoplasmic face of the cell membrane without affecting membrane lipids. When these sections were stained with anti-H-pep antibodies, the antibodies colocalized with the α-BTX binding sites at the cell surface (Figure 6g,h), as they had in alcohol-permeabilized cells. We therefore conclude that residues 351–368 of the mammalian muscle AChR are located on the cytoplasmic face of the muscle cell membrane.

The anti-H-peptide antibodies were also tested for their capacity to bind to Triton extracts of denervated mouse muscle (DMM, rich in extrajunctional receptors) as compared to normal mouse muscle (NMM, containing higher amounts of junctional receptors). Such experiments (Table I) indicate that the anti-H-pep antibodies reacted preferentially (by a factor of 1.9) with the extrajunctional form of the muscle receptor (DMM), as compared to their reactivity with junctional muscle AChR (NMM). This pattern is similar to that obtained with a monoclonal antibody (mcAb 5.14), which was previously demonstrated to react better with the extrajunctional form of the AChR (Souroujon et al., 1985), and is

Table I: Binding of Antipeptide (351–368) Antibodies and of Monoclonal Antibodies 5.14 and 1.26 to Triton-Extracted AChR from Normal and Denervated Mouse Muscle

antibody	titer		DMM/NMM ratio
	DMM[b]	NMM[c]	
anti-H-pep	3.3×10^{-7}	1.7×10^{-7}	1.9
mcAb 5.14	1.4×10^{-7}	6.1×10^{-8}	2.3
mcAb 1.26	1.0×10^{-8}	1.0×10^{-8}	1.0

[a] Similar amounts of ^{125}I-labeled α-BTX binding sites from muscle Triton extracts were incubated with serial dilutions of the tested antibody, and the complex of receptor–α-BTX–antibody was then precipitated with goat anti-rabbit immunoglobulins (for the mcAbs). The amount of labeled receptors precipitated was then monitored and expressed as moles of α-BTX binding sites precipitated per liter of antibody. [b] Denervated mouse muscle. [c] Normal mouse muscle.

different from the pattern obtained with another monoclonal antibody (mcAb 1.26) which did not distinguish between the receptors of DMM and NMM (Table I; Souroujon et al., 1985).

Discussion

We have employed two synthetic peptides in order to obtain highly specific probes for the mammalian and *Torpedo* AChR. Antibodies raised against residues 351–368 of the α-subunits of these receptors were shown to be species specific as determined by radioimmunoassay, immunoblotting, and immunofluorescence microscopy. The domain containing the sequence entity of the mammalian receptor was shown to be cytoplasmic and appears to undergo some alteration in response to denervation.

The experiments of immunofluorescent labeling of sections of muscle and *Torpedo* electric organ demonstrated that each antipeptide antibody reacted only with the AChR on the tissue of the appropriate species (Figures 4 and 5). Furthermore, it seems that the anti-H-pep and anti-T-pep antibodies are indeed species specific and not organ specific, as the anti-T-pep antibodies bound substantially to *Torpedo* muscle, whereas the anti-H-pep antibodies did not (data not shown).

Antibodies against the human peptide bound to human, calf, mouse, and rat AChR but did not bind to chicken (data not shown) or *Torpedo* AChR. On the other hand, antibodies against the *Torpedo* peptide did not bind to any of the mammalian AChRs or to chicken AChR. The amino acid sequence of residues 351–368 has been reported previously to be identical for the human and calf α-subunit (Noda et al., 1983c). From the cross-reactivity of the anti-human peptide with mouse and rat AChR, it could be predicted that the sequence of this region in these two species is also identical with or very similar to that of the human sequence, and different in the chicken. Indeed, the amino acid sequence for the α-subunit of mouse AChR has been deduced recently (Boulter et al., 1985) and was shown to be identical with that of the human and calf, in the sequence of residues 351–368. On the other hand, the amino acid sequence of these residues in the α-subunit of chicken AChR is quite different from both the mammalian and *Torpedo* AChR (Boulter et al., 1985).

From our experiments, using two distinct methods to allow antibody access to the cytoplasm (Figure 6), we concluded that the determinant corresponding to residues 351–368 of the human AChR α-subunit is located in the cytoplasmic domain of the *mammalian* AChR. This is the first demonstration of the orientation of a predefined peptide in *mammalian muscle* AChR. This is in agreement with the various theoretical models (Noda et al., 1983b; Devillers-Thiery et al., 1983; Claudio et al., 1983; Finer-Moore & Stroud, 1984; Guy, 1984) which predict the receptor's transmembrane orientation. Similarly, the orientation of several predefined peptides of the α-, β-, γ-, and δ-subunits of the electric organ of *Torpedo* AChR has been determined experimentally (Criado et al., 1985; Young et al., 1985; Larochelle et al., 1985; Ratnam et al., 1986).

The increased reactivity of the anti-H-pep antibodies with DMM Triton extract as compared to the NMM extract could result from a change in either sequence, tertiary structure, or degree of exposure of this sequence entity in the denervated mammalian muscle cell. In this context, it should be noted that a similar pattern of reactivity with DMM was obtained with five anti-AChR monoclonal antibodies (Souroujon et al., 1985). All these mcAbs reacted preferentially, though not exclusively, with the extrajunctional form of the receptor, whereas one mcAb (1.26) did not discriminate between junctional and extrajunctional receptors. Furthermore, the anti-T-pep (351–368) antipeptides, as well as mcAb 1.34, 1.22, 5.14, and 1.39, all reacted with one 14-kDa proteolytic fragment of the *Torpedo* receptor α-subunit (Souroujon et al., 1986), whereas mcAb 1.26 bound to a distinct 22-kDa fragment of this subunit, not recognized by this group of mcAbs. Hence, this 14-kDa fragment, which is located on the carboxy-terminal portion of the α-subunit, may contain a region which undergoes antigenic developmental changes in the process of synaptogenesis.

Acknowledgments

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Registry No. *Torpedo* AChR (α-subunit), 108711-98-4; human AChR (α-subunit), 108711-99-5.

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