Structure and Transmembrane Nature of the Acetylcholine Receptor in Amphibian Skeletal Muscle as Revealed by Cross-reacting Monoclonal Antibodies

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ABSTRACT   A collection of 126 monoclonal antibodies (mAbs) made against acetylcholine receptors (AChRs) from the electric organs of *Torpedo californica* or *Electrophorus electricus* was tested for cross-reactivity with AChRs in cryostat sections of skeletal muscle from *Rana pipiens* and *Xenopus laevis* by indirect immunofluorescence. 49 mAbs (39%) cross-reacted with AChRs from *Rana*, and 25 mAbs (20%) cross-reacted with AChRs from *Xenopus*. mAbs specific for each of the four subunits of electric organ AChR (α, β, γ, δ) cross-reacted with AChRs from each amphibian species. mAbs cross-reacting with *Xenopus* AChRs were, with one exception, a subset of the mAbs cross-reacting with *Rana* AChRs. The major difference detected between the two species was in binding by mAbs specific for the main immunogenic region (MIR) of the α-subunit. Whereas 22 of 33 anti-MIR mAbs tested cross-reacted with *Rana* AChRs, only one of these mAbs cross-reacted with *Xenopus* AChRs.

Some (32) of the cross-reacting mAbs were tested for binding to AChRs in intact muscle. 21 of these mAbs bound to AChRs only when membranes were made permeable with saponin. Electron microscopy using immunoperoxidase or colloidal gold techniques revealed that these mAbs recognize cytoplasmic determinants and that mAbs that do not require saponin in order to bind AChRs in intact muscle recognize extracellular determinants.

These results suggest that AChRs in skeletal muscle of *Rana* and *Xenopus* are composed of subunits corresponding to the α-, β-, γ-, and δ-subunits of AChRs from fish electric organs. The subunit specificity of mAbs whose binding was examined by electron microscopy suggests that parts of each subunit (α, β, γ, δ) are exposed on the cytoplasmic surface and that, as in AChRs from fish electric organs and mammalian muscle, the MIR on α-subunits of *Rana* AChRs is exposed on the extracellular surface.

The nicotinic acetylcholine receptor (AChR)[1] is the best characterized transmembrane channel that is gated by small molecules. From studies on AChRs from mammalian and amphibian skeletal muscle, a considerable amount has been learned about the biophysical properties of AChRs (reviewed in references 5, 7, 11, 24, 39, and 44). Unfortunately, skeletal

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[1] *Abbreviations used in this paper:* AChR, nicotinic acetylcholine receptor; HRP, horseradish peroxidase; mAb, monoclonal antibody; MIR, main immunogenic region; RG, frog Ringer with 2% normal goat serum; RGS, RG with 0.025% saponin.

muscle is not a rich source of AChRs, and many of the biochemical and structural analyses on AChRs have been done instead on receptors purified from electric organs of the marine ray (*Torpedo california*) and the electric eel (*Electrophorus electricus*). AChRs in electric organs are found on cells that are modified muscle cells in the sense that their origin and early developmental program resembles that of skeletal myofibers (35).

The AChR from the electric tissue of both *Torpedo* and *Electrophorus* is a complex of four subunits in the molar ratio $\alpha_2\beta\gamma\delta$ (10, 32, 40, 41). The complete amino acid sequence of

all four *Torpedo* subunits has been deduced from their complementary DNAs (8, 13, 36–38), and their calculated peptide molecular weights are 50,000 (α), 54,000 (β), 57,000 (γ), and 58,000 (δ). The apparent molecular weights of the subunits of receptor from *Electrophorus* electric organ (30) and bovine skeletal muscle (14) are similar, but not identical, to the *Torpedo* subunits as determined by SDS PAGE. There is extensive homology between the AChRs of these species in subunit amino acid sequence (9, 10), immunological cross-reactivity (14, 17, 30), and the size and shape of the purified AChRs as determined by electron microscopy (14). Using antisera and monoclonal antibodies to subunits of AChR from *Torpedo*, antigenic determinants coresponding to the α-, β-, γ-, and δ-subunits have been detected in AChRs from *Electrophorus* electric organ (30), *Electrophorus* muscle (29), rat muscle (33), human muscle (31), bovine muscle (33), and chicken brain (46). In instances where AChRs have been subsequently purified, subunits corresponding to all four sets of antigenic determinants were detected, including *Electrophorus* electric organ (30), *Electrophorus* muscle (29), and rat and bovine muscle (14). The observation that these four kinds of subunits exist both in elasmobranchs and bony fish and that the subunit stoichiometry is $\alpha_2\beta\gamma\delta$ in both cases indicates that the subunit structure of nicotinic AChRs was established some 400 million years ago in a primitive vertebrate whose evolution ultimately produced both *Torpedo* and *Electrophorus* (10). Therefore, it is not surprising that the subunit structure of AChRs from the higher vertebrates thus far examined appears to be $\alpha_2\beta\gamma\delta$.

In the present study we have analyzed the structure of AChRs in amphibian skeletal muscle using cross-reacting monoclonal antibodies (mAbs) prepared using electric organ AChRs. We found that AChRs in intact muscle of both *Rana pipiens* and *Xenopus laevis* have antigenic determinants recognized by mAbs specific for each of the four electric organ receptor subunits (α, β, γ, δ). This suggests that amphibian AChRs have four subunits homologous to those in electric organs. Biochemical studies of AChR-containing membranes from *Torpedo* electric organ indicate that the most immunogenic part of the intact AChR, the main immunogenic region (MIR), is located on the extracellular surface of the molecule (18, 47, 48) and that the most immunogenic parts of its denatured subunits are located on the cytoplasmic surface (15, 16). We have confirmed and extended these studies by visualizing mAbs bound to the postsynaptic membrane in intact muscle using electron microscopy.

MATERIALS AND METHODS

Reagents: mAbs were obtained from hybridomas prepared from rats immunized with AChRs purified from the electric organs of *Torpedo* or *Electrophorus* as previously described (mAbs 1–19, reference 48; mAbs 21–61, reference 47; mAbs 91–188, reference 18 and footnote 2). mAb titer is expressed in moles of ^{125}I-α-bungarotoxin–labeled *Torpedo* AChR bound per liter of mAb. Affinity-purified goat antibodies to rat IgG were reacted with fluorescein isothiocyanate (Sigma Chemical Co., St. Louis, MO) to produce a conjugate containing 4–5 moles of fluorescein per mole of IgG. Biotinylated rabbit anti-rat IgG, avidin DH, and biotinylated horseradish peroxidase H (HRP) were purchased in kit form from Vector Laboratories (Burlingame, CA). Rhodamine-conjugated α-bungarotoxin was the generous gift of Drs. Earl Godfrey and U. J. McMahan (Stanford University). Colloidal gold–coated protein A (3–4 nm) was kindly provided by Dr. Ruud Brands (Stanford University).

Immunofluorescence: Cross-reactivity of mAbs with AChRs in am-

phibian skeletal muscle was assayed using cryostat sections of the peroneus muscle from *Rana* and the extensor cruris brevis muscle from *Xenopus*. Animals were killed by decapitation and pithing, and muscles were removed and pinned in a Sylgard-lined dish containing frog Ringer [114 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 2 mM HEPES, pH 7.4]. The Ringer solution was then replaced with liquid nitrogen, and pieces of frozen tissue were embedded in Tissue-Tek freezing medium. Transverse sections 8–16 μm thick were cut in a International-Harris cryostat (International Equipment Co., Boston, MA) and placed on dry slides previously dipped in 0.5% (wt/vol) gelatin. The sections were incubated for 10 min with Ringer containing 2% normal goat serum (RG), for 30 min with mAb diluted in RG (final titer = 20–1000 nM), for 10 min in RG, and for 30 min with fluorescein-conjugated goat anti-rat IgG (2.7 × 10⁻⁷ M in RG). After a final wash in RG the sections were mounted in a mixture of 90% glycerol/10% Ringer (vol/vol) and were examined using a Zeiss standard microscope equipped with an epifluorescence attachment and a fluorescein filter cube. The location of end-plates in adjacent cryostat sections was determined using either rhodamine-α-bungarotoxin or cholinesterase staining (25). In some instances end-plates were visualized with fluorescent toxin in the same sections as used for mAb binding (as in Fig. 1). None of the mAbs used in this study are directed against the bungarotoxin binding site of the AChR (47, 48).²

For testing mAbs on intact muscle preparations, the cutaneous pectoris muscle of *Rana* or the dorsalis scapulae muscle of *Xenopus* was fixed for 30 min with 0.25% formaldehyde in 0.12 M sodium phosphate, pH 7.2. Muscles were preincubated for 10 min in RG or Ringer plus 2% goat serum plus 0.025% (wt/vol) saponin (RGS) and incubated for 1 h with mAbs diluted in RG or RGS (final titer: 20–1000 nM). The muscles were washed for 30 min in RG or RGS and incubated for 1 h with fluorescein-conjugated goat anti-rat IgG (2.7 × 10⁻⁷ M) in RG or RGS. A final 20-min wash in Ringer preceded examination by fluorescence microscopy.

Fine Structural Localization of Antibody Binding: Muscles were prepared for electron microscopy by using initially the same protocol as above for intact muscle, but by following the mAb incubation and wash with 0.015 mg/ml biotinylated rabbit anti-rat IgG in RG or RGS for 1 h. The tissue was washed for 30 min with RG or RGS and incubated with avidin and biotinylated HRP in RG or RGS for 1 h according to the method of Hsu et al. (21). After a 30-min wash in Ringer the muscle was fixed for 1 h in 1% glutaraldehyde (wt/vol) in 0.06 M sodium phosphate, pH 7.2. The muscle was then washed in Ringer for 10 min and incubated in 0.05 M Tris, pH 7.6, for 10 min, in Tris containing diaminobenzidine (0.36 mg/ml) for 20 min, and in Tris containing diaminobenzidine and H₂O₂ (0.01% vol/vol) for 1 h. In some experiments the incubation with second antibody (biotinylated rabbit anti-rat IgG) was followed by incubation for 1 h with colloidal gold–coated protein A (particle size = 3–4 nm). The muscles were then washed in Ringer for 30 min and fixed in 1% OsO₄ (wt/vol) in 0.09 M sodium phosphate, pH 7.2, for 1 h. After another 10-min wash in Ringer the muscles were dehydrated in an ethanol series and embedded in Epon-Araldite. Ultra-thin sections of innervated regions of the muscle were cut on an AO Ultracut ultramicrotome and examined with a Philips 201 or Philips 400 electron microscope. Sections were not grid-stained.

In one experiment we fixed a *Rana* cutaneous pectoris muscle in phosphate-buffered 0.25% formaldehyde and cut it through the central region of innervation in order to expose the cytoplasm to antibodies. Incubations with the cut muscle preparation were done as with intact muscle, except that the duration of each step was increased by two- to threefold to account for slow diffusion of reagents longitudinally within the myofibers.

RESULTS

Cross-reactivity between Species

A collection of 126 mAbs raised against AChRs purified from the electric organs of *Torpedo* or *Electrophorus* were assayed for cross-reactivity with AChRs of skeletal muscle from *Rana* or *Xenopus* on cryostat sections using indirect immunofluorescence techniques. End-plates were labeled simultaneously in the same section with rhodamine-α-bungarotoxin or in adjacent sections by either cholinesterase staining or toxin binding. Those antibodies producing patches of stain that corresponded in position to end-plates were presumed to bind to AChRs (Fig. 1). Normal rat serum used in place of the mAb failed to produce end-plate staining. Table I lists all the mAbs tested, their cross-reactivity (+ or −) with each species of frog, and their subunit specificity (references 18,

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² Tzartos, S. J., S. Hochschwender, L. K. Langeberg, and J. M. Lindstrom, manuscript in preparation.
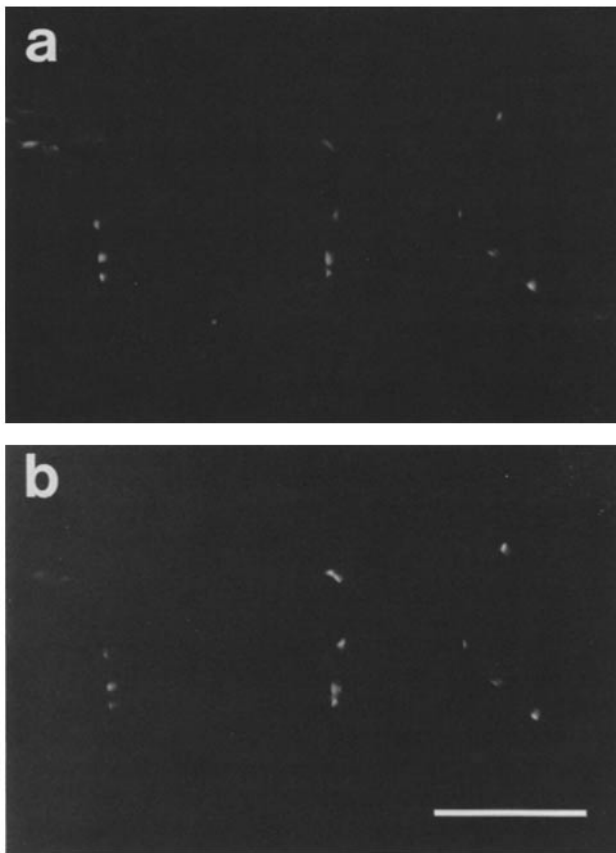
FIGURE 1 Fluorescence micrographs of antibody (a) and toxin (b) binding to a transverse section of *Xenopus* skeletal muscle. The same cryostat section was incubated with mAb 166 followed by fluorescein-conjugated goat anti-rat IgG and with rhodamine-conjugated α-bungarotoxin. Photography was done using a Zeiss fluorescence microscope with a fluorescein (a) or rhodamine (b) filter cube. No signal was observed using the rhodamine filter cube when the toxin was omitted from the incubation. The correspondence between patterns of stain in a and b demonstrates that mAb 166 binds to synaptic sites. Bar, 50 μm. × 400.

47, 48 and footnote 2). More mAbs cross-react with AChRs from *Rana* (49/126 or 39%) than with AChRs from *Xenopus* (25/126 or 20%). With a single exception (mAb 51), the mAbs cross-reacting with AChRs from *Xenopus* are a subset of those cross-reacting with AChRs from *Rana*.

The subunit composition of amphibian muscle AChRs was inferred from an analysis of the specificities of the mAbs that cross-reacted with amphibian muscle (Table II). At least three and as many as 23 mAbs specific for each of the four subunits from electric organ cross-reacted with receptors from both amphibian species. This strongly suggests that AChRs in amphibian muscles possess determinants corresponding to each of the four subunits of AChRs from fish electric organs.

Two classes of antigenic determinants on α-subunits of the AChR have been distinguished using mAbs: (*1*) the main immunogenic region (MIR) and (*2*) regions distinct from the MIR. The MIR is a highly antigenic region of the α-subunit that is exposed to the outside of the cell. Most of the antibodies in antisera to intact AChRs and most of the autoantibodies to AChRs in patients with myasthenia gravis are directed against the MIR (47–49). A large fraction (67%) of the mAbs that recognize the *Torpedo* or *Electrophorus* MIR cross-react with AChRs from *Rana* (Table II). Whereas 82% of the anti-

MIR mAbs that bind to denatured α-subunits cross-react with *Rana* AChRs, only 36% of the anti-MIR mAbs whose binding is conformation dependent cross-react with *Rana* AChRs (Table II). The degree of cross-reactivity between anti-MIR mAbs and *Rana* AChRs is consistent with the observation that the determinants that define the MIR are highly conserved across species. Corresponding determinants have been identified in *Torpedo* (an elasmobranch), *Electrophorus* (a teleost), chicken, mouse, rat, cow, human, and now, *Rana* (46–49).[2] Surprisingly, whereas 22 of 33 anti-MIR mAbs cross-react with *Rana* muscle, only one of these anti-MIR mAbs cross-reacts with *Xenopus* muscle (Table II). This difference between the two species is not apparent from the pattern of cross-reactivity of mAbs specific for other determinants, where the overlap in cross-reactivity is ~90% (Tables I and II).

Binding of mAbs to AChRs in Intact Muscle

Some of the cross-reacting mAbs were tested for binding to AChRs in intact muscle using immunofluorescence techniques. A staining pattern characteristic of the anuran neuromuscular junction (Fig. 2) was observed with 11 of the 32 cross-reacting mAbs tested (Table III). All 11 of these mAbs were made using native receptor as immunogen and are specific for the MIR. The MIR is expected to be on the extracellular surface from the observation that mAbs to the MIR bind well to native vesicles of AChR-rich membranes from *Torpedo* (19) and the observation that mAbs to the MIR both bind to AChRs on muscle in vivo (12) and passively transfer experimental autoimmune myasthenia gravis (48).[2] The remaining 21 mAbs tested did not produce any staining of end-plates in intact muscle. The determinants recognized by these 21 mAbs might not ordinarily be exposed to the extracellular medium yet might be accessible in cryostat cross-sections of muscle. When intact muscles were fixed and exposed to 0.025% saponin, all 21 of these mAbs bound to AChRs (Table III). Fixation alone was not sufficient to uncover the "hidden" determinants. All 21 mAbs tested that required saponin in order to bind AChRs were made using denatured receptor or receptor subunits as an immunogen. This is consistent with the observation that most antibodies in antisera made to denatured AChR appear to be directed at the cytoplasmic surface of native *Torpedo* AChR (15, 16), and that most mAbs to denatured β- and δ-subunits react with cytoplasmic domains of nascent *Torpedo* AChR subunits (2).

We investigated by electron microscopy the possiblity that mAbs that required saponin to bind to AChRs were in fact binding to determinants exposed on the cytoplasmic side of the postsynaptic membrane. Intact cutaneous pectoris muscles from *Rana* were incubated, with or without saponin, in primary antibody, in secondary antibody (biotinylated rabbit anti-rat IgG), and in avidin-biotinylated HRP complex (21). Muscles were then fixed in glutaraldehyde, reacted for peroxidase, and prepared for electron microscopy. Examination with the electron microscope revealed two very different staining patterns. In muscles incubated with mAbs that do not require saponin to bind AChRs in intact tissue (Table II), peroxidase reaction product was found lining the postsynaptic membrane and often filling the synaptic cleft (Fig. 3 *b*, mAb 6; similar results were obtained with mAbs 22, 41, and 42). Specific reaction product was never found inside the myofiber. The same staining pattern was observed when muscles were incubated with these mAbs in the presence of saponin (Fig.

TABLE I

Cross-reaction of mAbs to Fish Electric Organ AchRs with Amphibian Muscle AChRs*

mAb	Source of immunogen	Subunit specificity	Cross-reactivity Rana pipiens	Cross-reactivity Xenopus laevis	mAb	Source of immunogen	Subunit specificity	Cross-reactivity Rana pipiens	Cross-reactivity Xenopus laevis
4	Torpedo (native)	MIR	+	−	61	Torpedo (denatured)	α	+	+
6	Torpedo (native)	α, MIR	+	−	94	Torpedo (denatured)	β	+	+
7	Torpedo (denatured)	δ, γ*	+	+	110	Torpedo (denatured)	β	+	+
8	Torpedo (denatured)	α	+	−	111	Torpedo (denatured)	β	+	+
11	Torpedo (denatured)	β	+	+	113	Torpedo (denatured)	β	+	+
17	Torpedo and Electrophorus (native)	α, MIR	+	−	118	Torpedo (denatured)	β	+	+
					120	Torpedo (denatured)	β	+	+
21	Electrophorus (native)	α, MIR	+	−	121	Torpedo (denatured)	β	+	+
22	Electrophorus (native)	α, MIR	+	−	123	Torpedo (denatured)	β	+	+
24	Electrophorus (native)	α, MIR	+	−	124	Torpedo (denatured)	β, γ*	+	+
28	Electrophorus (native)	α, MIR	+	−	125	Torpedo (denatured)	β	+	+
31	Electrophorus (native)	α, MIR	+	−	127	Torpedo (denatured)	δ	+	−
35	Electrophorus (native)	α, MIR	+	−	139	Torpedo (denatured)	δ	+	+
36	Electrophorus (native)	α, MIR	+	−	141	Torpedo (denatured)	δ	+	+
37	Electrophorus (native)	α, MIR	+	−	142	Torpedo (denatured)	α, β, γ, δ*	+	−
38	Electrophorus (native)	α, MIR	+	−	147	Torpedo (denatured)	α, β, γ, δ*	+	+
39	Electrophorus (native)	α, MIR	+	−	148	Torpedo (denatured)	β	+	+
41	Electrophorus (native)	α, MIR	+	−	149	Torpedo (denatured)	α	+	+
42	Electrophorus (native)	α, MIR	+	−	151	Torpedo (denatured)	β	+	+
44	Electrophorus (native)	α, MIR	+	−	154	Torpedo (denatured)	γ	+	+
46	Electrophorus (native)	α, MIR	+	−	165	Torpedo (denatured)	γ	+	+
47	Electrophorus (native)	α, MIR	+	−	166	Torpedo (denatured)	δ	+	+
50	Electrophorus (native)	α, MIR	+	−	168	Torpedo (denatured)	γ, β*	+	+
51	Electrophorus (native)	α, MIR	−	+	176	Torpedo (denatured)	MIR	+	−
60	Electrophorus (denatured)	δ	+	+	177	Torpedo (denatured)	MIR	+	−
					188	Torpedo (denatured)	MIR	+	−

Cross-reactivity of mAbs to fish electric organ AChRs with amphibian muscle AChRs. A total of 126 mAbs were tested on cryostat sections of skeletal muscle from Rana and Xenopus as described in Materials and Methods. Those antibodies that produced detectable patterns of stain at synaptic sites, as illustrated in Fig. 1 for mAb 166, were recorded as "+". Each antibody was tested at least three times and as many as nine times on each species. The same result was obtained in all trials or in all but one trial for each mAb except no. 11 (five successes in nine trials for Rana, six successes in eight trials in Xenopus). Subunit specificity was measured using denatured receptor as described by Tzartos and Lindstrom (49), Tzartos and co-workers (48 and footnote 2), and Gullick and Lindstrom (18). mAbs having the indicated specificity MIR compete for binding with mAbs having the indicated specificity α, MIR but do not bind detectably to denatured receptor. Where several subunits are indicated, the subunit with the greater affinity is listed earlier. Additional properties of the mAbs tested can be found in references 2, 18, 48, 49 and footnote 2.

* The following mAbs did not cross-react with AChRs of either amphibian species, 1–3, 5, 9, 10, 12–14, 16, 19, 23, 25–27, 29, 30, 32–34, 43, 45, 48, 49, 52–57, 59, 91, 92, 95–101, 103, 105, 106, 108, 109, 112, 114, 116, 117, 128, 129, 130–132, 134, 136–138, 140, 145, 146, 150, 152, 153, 155, 157, 158, 162–164, 169, 170, 172, 173, 181, and 187.

* Subunits are listed in order of decreasing affinity for antibody.

3 c, mAb 6). Membranes were often interrupted and terminal morphology was poor, but synapses were nevertheless recognizable. The peroxidase reaction product again was found lining the postsynaptic membrane and extending into the synaptic cleft, but reaction product was not found within the myofiber (Fig. 3 c). This showed that permeabilization with saponin did not result in significant diffusion of HRP reaction product across the postsynaptic membrane. A quite different pattern of stain was found in muscles incubated with saponin and with mAbs that do require saponin in order to bind to AChRs in intact muscle (Fig. 3 d, mAb 154). The synaptic cleft was free of reaction product, but the postsynaptic membrane was heavily stained, and reaction product had accumulated on the internal side of the membrane, within the muscle fiber (Fig. 3 d, similar results were obtained with 10 other mAbs, see Table IV).

We have corroborated the immunoperoxidase results by the use of 3–4 nm colloidal gold–protein A. Experiments with mAbs 6, 22, and 42, which by immunoperoxidase techniques recognize extracellular determinants, revealed gold particles primarily within the synaptic cleft (Fig. 4, a and b). Experi-

ments with mAbs 111, 139, and 154, which by immunoperoxidase techniques recognize cytoplasmic determinants, revealed gold particles primarily within the myofiber's cytoplasm (Fig. 4 c).

Table IV shows the location of determinants recognized by four mAbs made against native AChR and by 11 mAbs made against denatured AChRs. All four anti–native AChR mAbs bind to intact muscle in the absence of saponin and recognize extracellular determinants. All 11 anti–denatured AChR mAbs bind to intact muscle only in the presence of saponin and recognize cytoplasmic determinants. These results suggest that the MIR, as expected, is located on the extracellular surface of the AChR. Furthermore, they suggest that the most immunogenic domains of denatured AChR, by contrast with native AChR, are on the cytoplasmic surface of the AChR molecule and that highly immunogenic cytoplasmic determinants are located on all four subunits in AChRs in intact muscle cells.

Whenever the intracellular pattern of peroxidase staining was obtained, the synaptic cleft was observed to be free of HRP reaction product. Under these conditions we have noted

TABLE II

mAbs Cross-reactive with Amphibian Muscle AChRs Listed by Subunit Specificity

Primary subunit specificity	No. of antibodies tested	No. of cross-reacting antibodies	
		Rana pipiens	Xenopus laevis
α	52	27 (52%)	4 (8%)
α, MIR	22	18 (82%)	1 (5%)
MIR	11	4 (36%)	0 (0%)
α, not MIR	19	5 (26%)	3 (16%)
β	39	13 (33%)	13 (33%)
γ	6	3 (50%)	3 (50%)
δ	19	6 (32%)	5 (25%)
?	10	0 (0%)	0 (0%)
Total	126	49 (39%)	25 (20%)

mAbs cross-reactive with amphibian muscle AChRs listed by subunit specificity. The data of Table I are listed by subunit specificity, or primary subunit specificity for mAbs with detectable binding to more than one subunit. Subunit specificity was measured using denatured receptor as described in references 18, 48, and 49. mAbs having the indicated specificity *MIR* compete for binding with mAbs having the indicated specificity α, *MIR* but do not bind detectably to denatured receptor. mAbs having the indicated specificity "?" are completely conformation dependent.
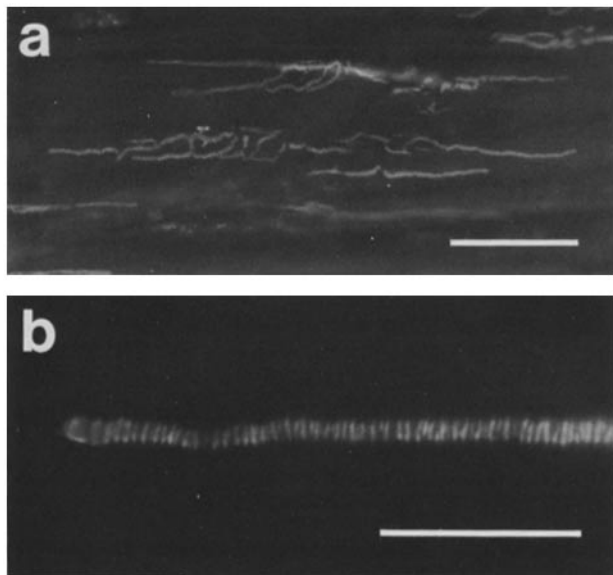

FIGURE 2 Fluorescence micrographs of antibody binding to intact *Rana* skeletal muscle. The cutaneous pectoris muscle was incubated with mAb 42 followed by fluorescein-conjugated goat anti-rat IgG. Photography was done using a Zeiss fluorescence microscope with a fluorescein filter cube. The complete pattern of staining on one myofiber is shown in the center of *a*; the staining is characteristic of amphibian synaptic sites (4). At higher magnification (*b*), the banding of the pattern, due to the presence of receptors in the junctional folds, is apparent. (*a*) Bar, 40 μm. × 420. (*b*) Bar, 10 μm. × 2,700.

the presence of reaction product on the presynaptic membrane (Fig. 3*d*). This presynaptic staining is absent when the mAb is replaced by normal rat serum (Fig. 3*a*). The presynaptic staining may indicate the presence of presynaptic nicotinic AChRs, as proposed by Lentz and Chester (27). Alternatively, the presynaptic staining may result from diffusion of HRP reaction product from the postsynaptic membrane and its selective precipitation on the nerve terminal membrane. This interpretation is supported by our failure to detect

presynaptic AChRs by immuno-gold labeling (Fig. 4*a*). Attempts by other investigators to detect presynaptic nicotinic AChRs using techniques that do not rely on enzyme histochemistry have also failed (23).

One mAb that requires saponin in order to bind to AChRs in intact tissue produced the "intracellular" pattern of staining in the absence of saponin when incubated with cut muscle preparations (mAb 111, data not shown). In this experiment, access to the cytoplasm was provided by cutting lightly fixed muscles through the region of innervation. Thus at least one determinant recognized by a "saponin-requiring" mAb is exposed to the cytoplasm even in the absence of saponin.

DISCUSSION

The experiments reported here utilize a relatively new ap-

TABLE III

Binding of Cross-reactive mAbs to Intact Muscle

mAb	Subunit specificity	Cross-reactivity			
		Rana pipiens		Xenopus laevis	
		Without saponin	With saponin	Without saponin	With saponin
6	α, MIR	+	+		
17	α, MIR	+	+		
21	α, MIR	+	+		
22	α, MIR	+	+		
35	α, MIR	+	+		
38	α, MIR	+	+		
41	α, MIR	+	+		
42	α, MIR	+	+		
46	α, MIR	+	+		
50	α, MIR	+	+		
51	α, MIR			+	+
61	α	−	+	−	+
142	α, β, γ, δ*	−	+		
149	α	−	+		
94	β	−	+	−	+
110	β	−	+		
111	β	−	+	−	+
113	β	−	+		
118	β	−	+		
120	β	−	+		
123	β	−	+		
124	β, γ*	−	+		
148	β	−	+		
151	β	−	+		
154	γ	−	+	−	+
165	γ	−	+		
168	γ, β*	−	+		
7	δ, γ*	−	+		
60	δ	−	+		+
139	δ	−	+	−	
141	δ	−	+		
166	δ	−	+		

Binding of cross-reactive mAbs to intact muscle. A "+" indicates that incubation of intact muscle with a particular mAb followed by fluorescein-conjugated goat anti-rat IgG produced characteristic patterns of fluorescent stain similar to that illustrated in Fig. 2. All 11 mAbs specific for the MIR bound amphibian AChRs in intact muscle in the absence of saponin. The remaining 21 mAbs bound amphibian AChRs in intact muscle only in the presence of saponin.
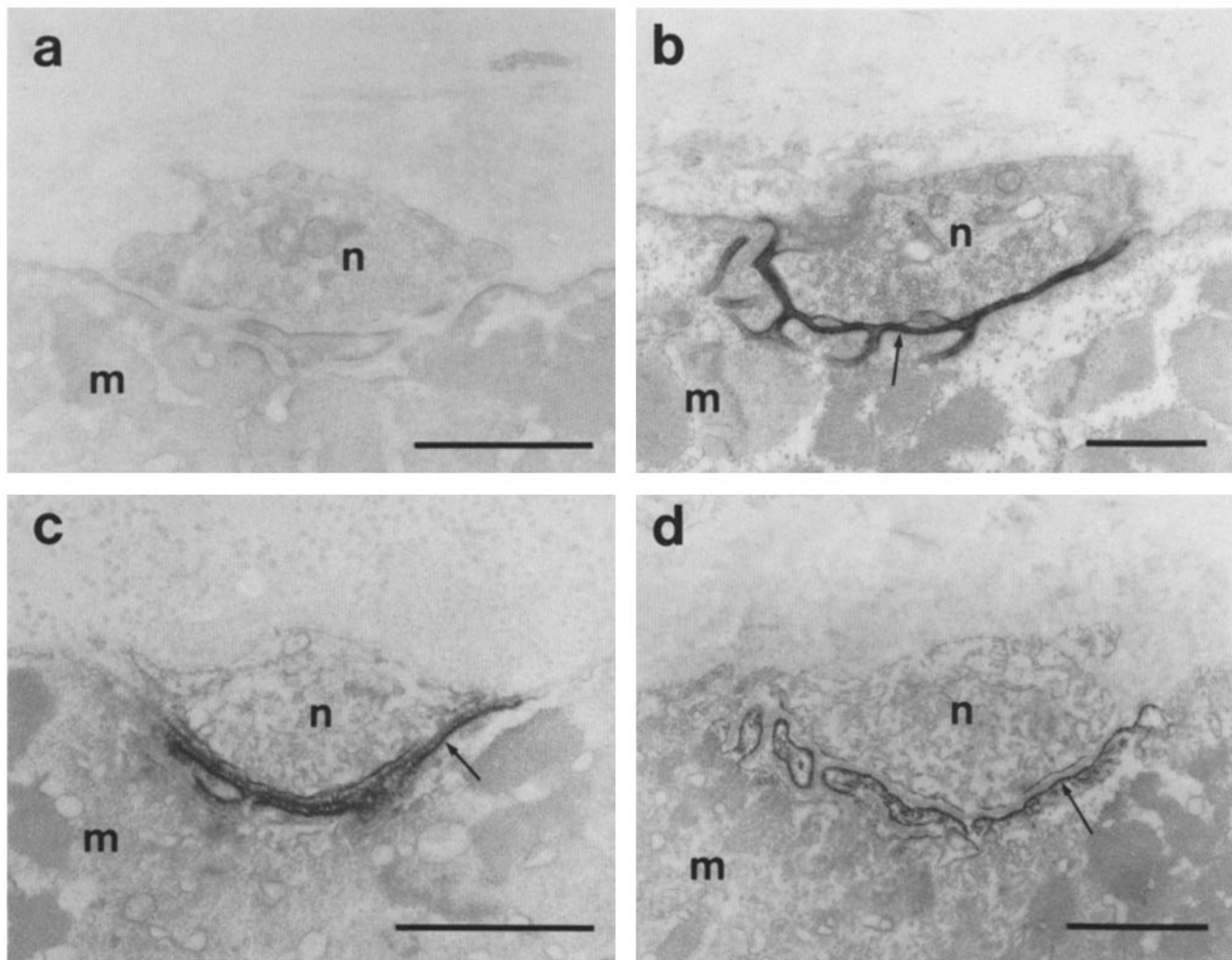* Subunits listed in order of decreasing affinity for antibody.

FIGURE 3 Electron micrographs demonstrating immunoperoxidase visualization of antibody binding at the amphibian neuromuscular junction. Intact cutaneous pectoris muscles from *Rana* were incubated with normal rat serum (a), with mAb 6 (b and c), or with mAb 154 (d). The nerve terminal (n) occupies the central portion of each micrograph and appears in cross-section. The nerve terminal is covered by a Schwann cell process and lies over a gutter in the myofiber (m). Primary antibody binding was visualized using the avidin biotinylated-HRP technique as described in Materials and Methods. Saponin was included in the incubations photographed in c and d. In a, no reaction product is evident. In b, a dense band of stain lines the postsynaptic membrane (arrow) and is present throughout the synaptic cleft. A similar pattern is seen when saponin was included in the incubation (c); here the association of the reaction product with the basal lamina is especially evident. Saponin adversely affects nerve terminal morphology (c and d), despite its low concentration (0.025%) and prefixation of the muscle in 0.25% formaldehyde. A pattern of stain quite distinct from that seen in c is observed with mAb 154 (d); here there is once again a dense band of stain lining the postsynaptic membrane (arrow), but the synaptic cleft appears to be free of reaction product. The stain has accumulated instead in the interior of the muscle fiber. The faint stain that appears on that portion of the nerve terminal facing the myofiber does not necessarily reflect the presence of presynaptic AChRs (see text). Sections were not grid stained. Bars, 1 μm. (a) × 25,000; (b) 17,000; (c) 28,000; (d) 20,000.

proach of comparing the structure of macromolecules between species using monoclonal antibodies. For AChRs this technique has the advantage of permitting inferences to be made about the structure of amphibian AChRs, whose biophysical properties have been characterized extensively, by examining the pattern of cross-reactivity with these AChRs of mAbs generated against biochemically characterized AChRs from electric organs.

50 of the 126 mAbs (40%) tested cross-reacted with cryostat sections of skeletal muscle from one or both amphibian species. Since the parent population of mAbs were generated using purified AChRs or AChR subunits and since they all recognize AChR in binding assays, we have assumed that those mAbs that bind to cryostat muscle sections are cross-

reacting with AChRs in the muscle membrane. This assumption is supported by our demonstration that the cross-reacting mAbs bind to discrete patches of the myofiber surface in cryostat sections that correspond in position to end-plates, as determined in the same or in adjacent sections by rhodamine-α-bungarotoxin binding or by cholinesterase staining. The pattern of mAb binding as seen in intact tissue also corresponds to that expected for antibodies with anti-AChR specificity. Patterns of stain (Fig. 2) are observed that display the banded arborization that is characteristic of amphibian synaptic sites (e.g., reference 4). Finally, an electron microscopic immunoperoxidase analysis demonstrates that cross-reacting mAbs bind to the postsynaptic membrane. The peroxidase reaction product is densest in a band that runs along the top

TABLE IV

Transmembrane Orientation of mAb Binding as Determined by
Electron Microscopy

mAb	Immunogen	Subunit specificity	Transmembrane orientation of determinant
6	native	α, MIR	extracellular*
22	native	α, MIR	extracellular*
41	native	α, MIR	extracellular
42	native	α, MIR	extracellular*
142	denatured	α, β, γ, δ†	cytoplasmic
149	denatured	α	cytoplasmic
111	denatured	β	cytoplasmic*
118	denatured	β	cytoplasmic
123	denatured	β	cytoplasmic
124	denatured	β	cytoplasmic
151	denatured	β	cytoplasmic
154	denatured	γ	cytoplasmic*
168	denatured	γ, β†	cytoplasmic
139	denatured	δ	cytoplasmic*
141	denatured	δ	cytoplasmic

Transmembrane orientation of mAb binding as demonstrated by electron microscopy. The transmembrane orientation of antibody binding was determined by immunoperoxidase techniques as described in Materials and Methods and as illustrated in Fig. 3. mAbs listed as "extracellular" produced patterns of stain similar to that illustrated in Fig. 3c (incubations included saponin). mAbs listed as "cytoplasmic" produced patterns of stain similar to that illustrated in Fig. 3d. All mAbs tested that recognized extracellular determinants were made using native AChR as immunogen. All mAbs tested that recognized cytoplasmic determinants were made using denatured AChR or AChR subunits as immunogen.

* Designation of transmembrane orientation confirmed by "immuno-gold" techniques (Fig. 4).

† Subunits listed in order of decreasing affinity for antibody.

of the subsynaptic membrane and part way down the junctional folds (34).

Owing to the qualitative nature of our binding assay, we are unable to say that the 76 mAbs that failed to produce a signal in the cryostat assays in fact have no affinity for amphibian AChRs. It is possible that some of the anti-*Torpedo* mAb samples were tested at too low a titer to have produced detectable cross-reaction. We have found that mAbs that do cross-react with amphibian AChRs are generally effective in cryostat assays when used at a final titer of >2 nM (data not shown). Of the 57 anti-*Torpedo* mAb samples that did not show detectable binding, 15 were measured at <2 nM. Thus the reported number, 50, of the 126 mAbs tested that cross-react with one or both amphibian species should be regarded as a minimum estimate.

The considerable number of electric organ AChR mAbs that cross-react with amphibian AChRs indicates that there is a substantial degree of structural similarity between AChRs from fish electric organs and amphibian muscle. This similarity is consistent with the notion that AChR structure has been conserved through evolution (9, 10, 14). Between 3 and 23 mAbs specific for each of the four electric organ subunits (α, β, γ, δ) cross-react with both *Rana* and *Xenopus* AChRs. This strongly suggests that amphibian AChRs possess subunits that correspond immunochemically to the four subunits recognized in AChRs for *Torpedo* (reviewed in 5, 7, 11, 24), *Electrophorus* (10, 30), and cattle (14). Of course, direct confirmation of our interpretation will entail establishing that

anti-α, -β, -γ, and δ mAbs in fact recognize unique polypeptides in amphibian muscle.

The similarity between *Rana* AChRs and biochemically characterized AChRs from other species extends to the MIR, a highly antigenic and highly conserved region of the AChR that is formed by the α-subunit and is exposed to the extracellular space (19, 48, 49). A sizable fraction of the mAbs generated using native *Torpedo* or *Electrophorus* AChR as immunogen cross-reacts generally with AChRs from skeletal muscle of fetal calf, rat, mouse, human, chicken, and now, *Rana*. There are several closely spaced antigenic determinants within the MIR which can be distinguished by the pattern of competitive binding of mAbs directed to them and by the ability of mAbs to some of these determinants to cross-link AChR monomers (12, 28, 49). The MIR, as most strictly defined (28), is typified by mAbs like no. 6 and no. 35, which cross-link AChRs and react with denatured α-subunits (though much less well than with intact AChR). It is anti-MIR mAbs of this type that are most cross-reactive with AChR from *Rana* (Table II). Anti-MIR mAbs that are absolutely conformation dependent appear to bind less often to *Rana* AChRs (Table II). In view of the evolutionarily conserved nature of the MIR, we were surprised to find that only one of 33 anti-MIR mAbs cross-reacted with *Xenopus* AChRs (Table II). Curiously, the one anti-MIR mAb that did cross-react with *Xenopus* AChRs was the single exception to the rule that mAbs cross-reacting with *Xenopus* AChRs were a subset of the mAbs cross-reacting with *Rana* AChRs (Table I). *Xenopus* AChRs may lack an MIR of the sort detected on all other species tested. A comparison between *Rana* and *Xenopus* receptors as regards the MIR may have a parallel in the comparison of snake AChRs and other AChRs as regards the binding of α-bungarotoxin. Some snake AChRs do not bind α-bungarotoxin, yet their antibody binding properties, ligand-binding properties, and functional characteristics are similar to AChRs from other species that do bind α-bungarotoxin (6, 20, 22, 26). Although *Xenopus* AChRs may lack an MIR, they appear to be similar in other respects of AChRs from *Rana*: 26 of 29 mAbs that recognize portions of the AChRs other than the MIR and that cross-react with *Rana* AChRs also cross-react with *Xenopus* AChRs (Table II). These mAbs encompass antibodies specific for each electric organ subunit. Therefore, AChRs from *Rana* and *Xenopus* are likely to be fundamentally similar, with the major distinction being that *Xenopus* AChRs appear to have a substantially altered MIR.

A study of the binding of mAbs to intact muscle revealed (Table III) that only 11 of the 32 mAbs tested bind to AChRs in intact tissue in the absence of saponin. All 11 mAbs are specific for the MIR. Since these mAbs recognize *Rana* AChRs in the absence of a permeabilizing agent, they presumably recognize extracellular determinants. This conclusion was confirmed for four mAbs by visualizing antibody binding using immunoperoxidase and colloidal gold techniques in conjunction with electron microscopy (Fig. 3 and 4; Table IV). Our conclusion that the *Rana* MIR is exposed to the extracellular space is consistent with the previously determined location of the MIR in AChRs from electric organs (19, 49) and skeletal muscle (12, 49).[2] The remaining 21 cross-reacting mAbs tested for their binding to intact muscle bound AChRs only in the presence of saponin. Given saponin's ability to render membranes permeable to macromolecules (e.g., reference 43) we interpreted this to mean that these 21
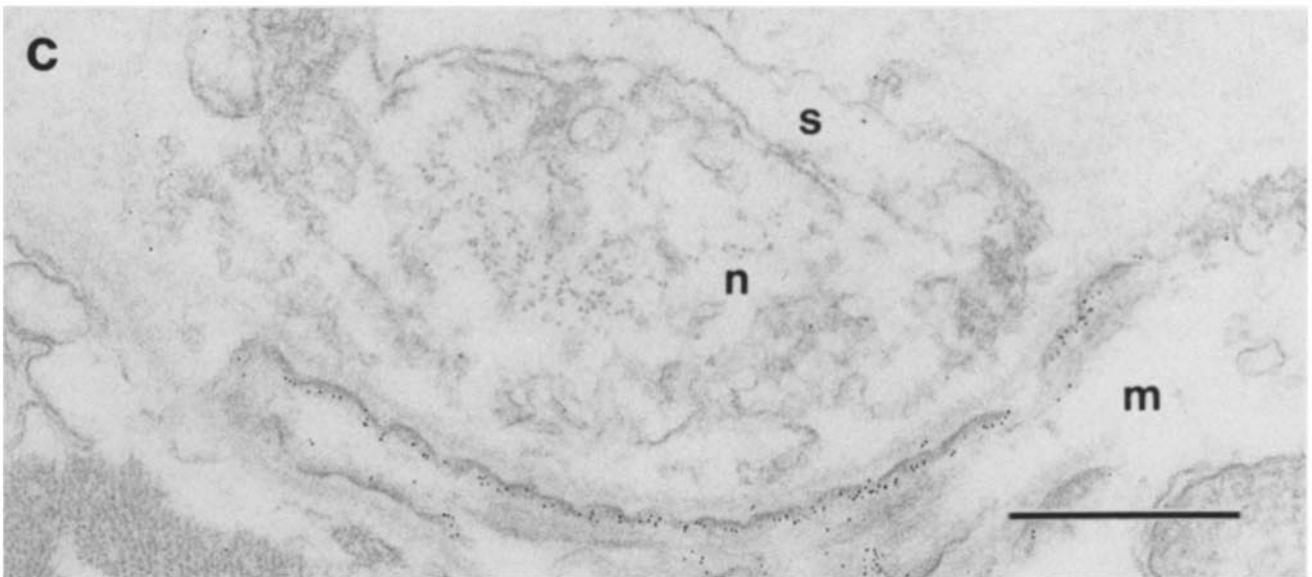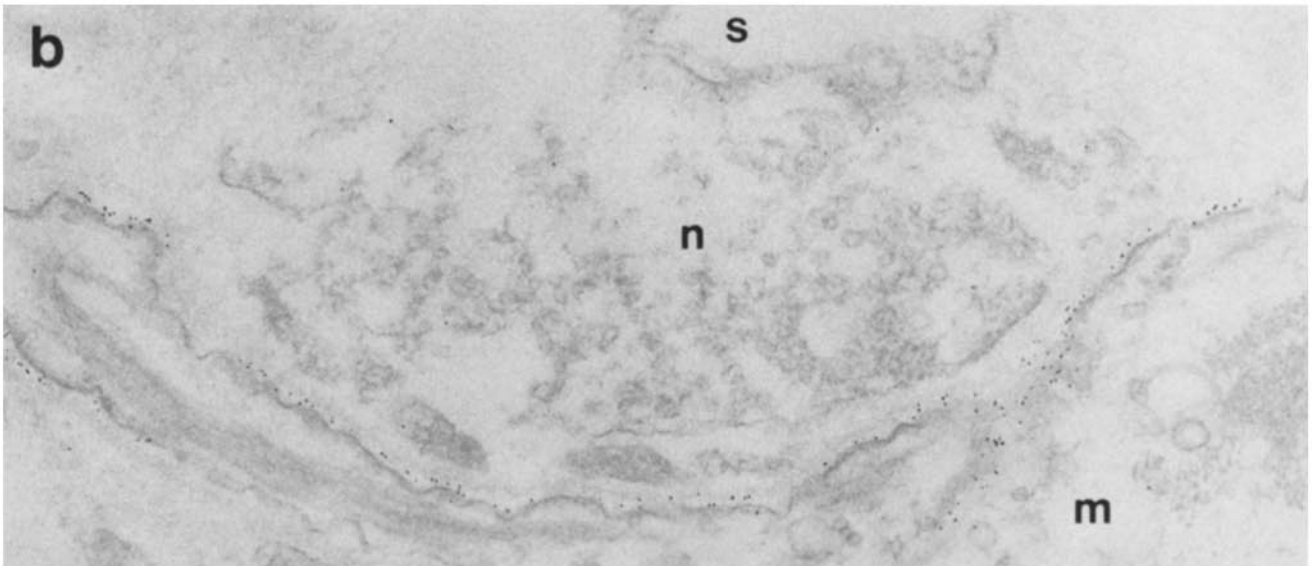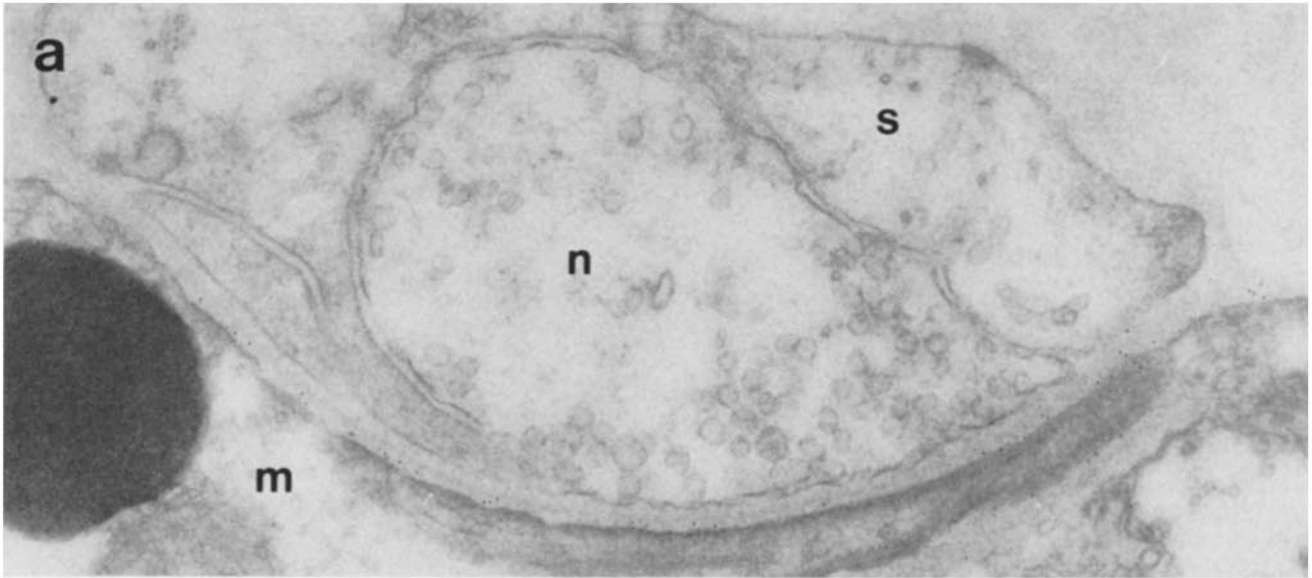
mAbs recognize cytoplasmic determinants. This interpretation was confirmed for 11 of the 21 mAbs by immunoperoxidase techniques and electron microscopy. All 11 mAbs tested produced deposits of reaction product on the cytoplasmic side of the postsynaptic membrane (Fig. 3d; Table IV), and 3 of these 11 mAbs tested using colloidal gold–protein A produced gold particles selectively on the cytoplasmic side of the postsynaptic membrane (Fig. 4c; Table IV). Saponin is not likely to be required specifically in order to produce this pattern of staining, since a similar result was obtained when a fixed muscle that had been cut through its central area of innervation was incubated with a "saponin-requiring" mAb (no. 111) in the absence of saponin. Thus the determinants recognized by these mAbs appear to be exposed to the cytoplasm even in the absence of detergent. The presence of at least three mAbs specific for each electric organ subunit among the 21 "saponin-requiring" mAbs strongly suggests that each subunit (α, β, γ, δ) of the amphibian AChR is exposed to the cytoplasm. The result is consistent with the results of Wennogle and Changeux (51), Strader and Raftery (45), and Froehner (15) on AChR-rich vesicles which suggest that all four Torpedo subunits are exposed to the cytoplasmic side of the postsynaptic membrane. Our results, although indirect, provide the first evidence that all four subunits of the AChR are exposed to the cytoplasm in situ. Since the α-chain, at least, is also exposed to the extracellular space in situ, our results suggest, not surprisingly, that the AChR is transmembrane in situ. Strader and Raftery (45) have found that all four Torpedo subunits in AChR-rich vesicles are exposed to the extracellular space. Since all subunits of Torpedo AChR contain sugar (33, 50), have signal peptides (8, 13, 36–38), and are inserted across the membrane during synthesis (1), they presumably have extracellular domains in situ.

A striking finding of this study is that mAbs generated using denatured AChRs preferentially recognize cytoplasmic determinants. Whereas each of the 11 mAbs identified as recognizing extracellular determinants was made using native AChR as an immunogen, each of the 21 mAbs identified as recognizing cytoplasmic determinants was made using denatured AChR or AChR subunits as immunogen (Tables III and IV). These results suggest that the most immunogenic part of the intact receptor is the partially conformation-dependent MIR on the extracellular surface of α-subunits and that denaturation of AChR using SDS leaves conformation-independent domains on the cytoplasmic surface of the AChR as the most immunogenic domains (native AChR is much more immunogenic than is denatured AChR [31, 32]). This segregation of immunogenicity is especially striking because most of each subunit is probably exposed on the extracellular surface (1,

42). Studies of AChR subunits synthesized in vitro (1, 3) and of the amino acid sequences of AChR subunits (8, 13, 36–38) suggest that the N-terminal two-thirds of each subunit is extracellular. Models proposed on the basis of the hydrophobicity profile of each subunit's amino acid sequence places much of their C-terminal portions on the cytoplasmic side of the plasma membrane (8, 13, 38; see also reference 3). The implication of these models is that the binding sites of most of the mAbs that recognize the cytoplasmic domain of the AChR are directed against the C-terminal portions of its subunits.

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FIGURE 4 Visualization of antibody binding using electron microscopy and colloidal gold. Intact cutaneous pectoris muscles from Rana were incubated with mAb 42 (a and b) or mAb 111 (c), with biotinylated rabbit anti-rat IgG and with colloidal gold-coated protein A (particle size = 3–4 nm). Muscles shown in b and c were treated with 0.025% saponin. All micrographs show cross-sections of a nerve terminal (n) lying within a shallow gutter on a myofiber (m) and covered by a Schwann cell process (s). Gold particles are located preferentially on the extracellular side of the postsynaptic membrane in a and b and on the cytoplasmic side of the postsynaptic membrane in c. mAb 42 thus recognizes an extracellular determinant, while mAb 111 recognizes a cytoplasmic determinant. Gold particle density may be lower in a than in b because of the considerable diffusion barrier encountered by protein A-colloidal gold which in a has access to the second antibody only by diffusing longitudinally through the extracellullar matrix. Saponin (b) presumably provides rapid access to sites within the synaptic cleft via either the presynaptic or the postsynaptic cell. Sections were not grid stained. Bar, 0.5 μm. × 62,000.

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