Agrin-like Molecules at Synaptic Sites in Normal, Denervated, and Damaged Skeletal Muscles

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Abstract. Several lines of evidence have led to the hypothesis that agrin, a protein extracted from the electric organ of *Torpedo*, is similar to the molecules in the synaptic cleft basal lamina at the neuromuscular junction that direct the formation of acetylcholine receptor and acetylcholinesterase aggregates on regenerating myofibers. One such finding is that monoclonal antibodies against agrin stain molecules concentrated in the synaptic cleft of neuromuscular junctions in rays. In the studies described here we made additional monoclonal antibodies against agrin and used them to extend our knowledge of agrin-like molecules at the neuromuscular junction. We found that anti-agrin antibodies intensely stained the synaptic cleft of frog and chicken as well as that of rays, that denervation of frog muscle resulted in a reduction in staining at the neuromuscular junction, and that the synaptic basal lamina in frog could be stained weeks after degeneration of all cellular components of the neuromuscular junction. We also describe anti-agrin staining in nonjunctional regions of muscle. We conclude the following: (*a*) agrin-like molecules are likely to be common to all vertebrate neuromuscular junctions; (*b*) the long-term maintenance of such molecules at the junction is nerve dependent; (*c*) the molecules are, indeed, a component of the synaptic basal lamina; and (*d*) they, like the molecules that direct the formation of receptor and esterase aggregates on regenerating myofibers, remain associated with the synaptic basal lamina after muscle damage.

THESE experiments were undertaken as part of a project aimed at identifying and characterizing the molecules at normal neuromuscular junctions of the frog that cause the aggregation of acetylcholine receptors (AChRs)[1] and acetylcholinesterase (AChE) on regenerating muscle fibers. The AChR- and AChE-aggregating molecules are components of the portion of the muscle fiber's basal lamina sheath that occupies the synaptic cleft and they remain adherent to the sheath when the myofiber and the axon terminals innervating it degenerate after damage. Accordingly, in regenerating muscles when new myofibers develop within the sheaths of the original muscle fibers and regenerating axons grow to the original synaptic sites on the sheaths (13, 26), the AChR- and AChE-aggregating molecules play a direct role in forming the postsynaptic apparatus on the new muscle fibers (1, 2, 4, 16, 17), and, thus, in restoring neuromuscular transmission. Such synaptic organizing molecules are of interest not only because they are involved in the regeneration of the neuromuscular junction, but also because they may be similar to the molecules that mediate the formation of AChR and AChE aggregates on myotubes during synaptogenesis in the embryo. They may also be similar to the molecules that maintain these cell surface specializations at neuromuscular junctions in the adult.

Previous studies conducted in this laboratory revealed that agrin, a protein found in basal lamina-containing extracts of the electric organ of the marine ray, *Torpedo californica*, causes the formation of patches on cultured myotubes at which three components of the neuromuscular postsynaptic apparatus are aggregated: AChRs, AChE, and butyrylcholinesterase (8, 18, 25, 30, 31). Molecules antigenically similar to agrin which cause AChR aggregation on cultured myotubes have also been extracted from *Torpedo* muscle, although in much smaller amounts than from electric organ (8). Moreover, mAbs directed against agrin stain molecules in the synaptic cleft of *Torpedo* neuromuscular junctions (6). Together, these findings have given rise to the "agrin hypothesis": the AChR- and AChE-aggregating molecules in the synaptic basal lamina at the neuromuscular junction are similar to agrin (6, 12, 31).

We have now raised additional mAbs against agrin. As presented in the accompanying report (18), we have used anti-agrin antibodies to purify and characterize agrin from electric organ extracts. The aims of the study described here were as follows: (*a*) to characterize the library of mAbs; (*b*) to determine by immunohistochemistry whether or not agrin-like molecules are concentrated at neuromuscular junctions of animals other than rays; (*c*) to gain insight into how the agrin-like molecules at the neuromuscular junction are regulated; and (*d*) to learn whether agrin-like molecules are stable components of the neuromuscular junction's synaptic

1. *Abbreviations used in this paper*: AChE, acetylcholinesterase; AChR, acetylcholine receptor.

basal lamina. The findings help establish a basis from which to test the agrin hypothesis. Brief accounts of some of these studies have appeared elsewhere (6, 12, 22, 28).

Materials and Methods

Monoclonal Antibodies

Female BALB/c mice were given an intraperitoneal injection of 20 μg (230 U) of partially purified agrin (ion exchange pool; reference 19) emulsified in Freund's complete adjuvant. A similar injection but with Freund's incomplete adjuvant was given 4 wk later. After an additional 4 wk, 300 μg of ion exchange pool without adjuvant was injected beneath the capsule of the spleen. Spleens were removed 3 d later and the cells were fused with a myeloma cell line, P3x63-Ag8.653, according to the procedure of Oi and Herzenberg (20).

Hybridoma supernatants from our first fusion were screened initially for their ability to stain the innervated surface of electrocytes (the site of the synaptic basal lamina) in frozen sections of *Torpedo* electric organ (Fig. 1). They were subsequently tested for their ability to immunoprecipitate agrin from electric organ extracts. Our method of immunoprecipitation has been described elsewhere (6). In brief, 100 μl of hybridoma supernatant were mixed with 1.5 U of electric organ extract for 1–2 h at 37°C. 20 μl of goat anti-mouse IgG Sepharose beads were added and the suspension was mixed at room temperature for 2–3 h. The suspension was then spun at 1,000 *g* for 1 min to remove the beads, bearing both immune complexes and free mouse IgG, from the supernatant. The supernatant was added to chick myotube cultures and assayed for AChR-aggregating activity (8). Hybridoma supernatants from our second fusion were screened initially for their ability to immunoprecipitate agrin.

mAbs were classified using a Screen Type mouse Ig subtyping kit (Boehringer Mannheim, Indianapolis, IN). The IgG concentration of the hybridoma supernatants that recognize seven different epitopes (see Results) was determined by a standard ELISA (29) and ranged from 15 to 50 μg/ml. The maximal dilution of these supernatants for staining neuromuscular junctions in frozen sections of *Torpedo* muscle ranged from 1:400 to 1:1,600.

Muscles

We examined muscles from two rays (*Torpedo californica*: back and tail muscles; *Dyscopyge ommata*: fin muscles), frog (*Rana pipiens*: anterior tibialis and cutaneous pectoris muscles), chicken (White Leghorn: anterior and posterior latissimus dorsi, pectoralis major, and scalp muscles), and rat (Sprague–Dawley: hindlimb and forelimb muscles). All muscles were pinned out and dissected in Sylgard-coated petri dishes containing Ringer's solution (ray, 27; frog, 16; chicken, 7; and rat, 10). Muscles from the rays and chicken included twitch and slow muscle fibers. For rays the slow fibers were readily distinguished by their relatively small diameter, their reddish color, and their peripheral position in the muscles. The anterior latissimus dorsi muscle of chicken is composed entirely of slow fibers while the posterior latissimus dorsi and pectoralis major muscles are entirely twitch. Scalp muscles of chicken were used only for electron microscopy; they contained both slow and twitch fibers which were distinguished by fine structural criteria established by Page (21). The muscles from frog and rat contained only twitch fibers.

Staining

We routinely stained muscles for agrin-like molecules in two ways. One method was to make frozen cross sections and treat the sections with an anti-agrin mAb followed by a fluorescein-labeled second antibody. The synaptic sites in the same sections were stained with rhodamine-α-bungarotoxin which specifically binds to AChRs. Thus we could relate anti-agrin staining to synaptic sites by fluorescence microscopy. We could not tell from these preparations, however, whether the stain was external or internal to the plasma membrane of cells nor could we always be certain of the identity of the nonjunctional structures that stained. Therefore, the second staining method we used was to bathe whole muscles in the anti-agrin mAbs. Because the plasma membrane remains intact under such conditions, only the external surfaces of cells and extracellular matrix were exposed to the antibodies. To determine the distribution of mAb binding we used fluorescein-labeled secondary antiserum for fluorescence microscopy or biotinylated secondary antiserum followed by an avidin-biotin-horseradish peroxidase complex for brightfield light microscopy and electron microscopy.

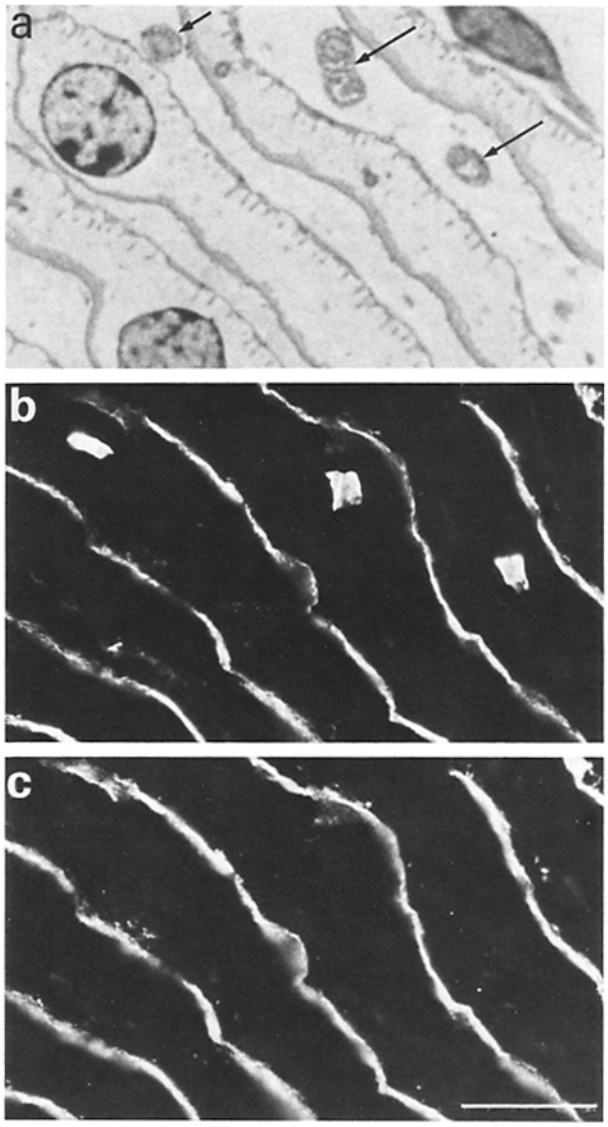


Figure 1. Anti-agrin mAbs stain the innervated surface of electrocytes and preterminal nerve fibers in *Torpedo* electric organ. (*a*) 1-μm-thick cross section through a portion of the electric organ showing electrocytes and preterminal nerve fibers (*arrows*). The tissue had been fixed and embedded in Epon/Araldite; the section was stained with toluidine blue and viewed with brightfield optics. (*b* and *c*) 10-μm frozen section of electric organ viewed under fluorescein optics (*b*) to show 6D4 binding sites and under rhodamine optics (*c*) to reveal rhodamine-α-bungarotoxin-labeled AChRs, which identify the innervated surface of electrocytes. Bar: (*a*) 10 μm; (*b* and *c*) 50 μm.

Immunofluorescence Histochemistry. FROZEN CROSS SECTIONS: 8–10-μm-thick sections were routinely fixed for 5 min in 1% formalin in PBS (137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8 mM Na_2HPO_4). For some experiments, sections were fixed in paraformaldehyde freshly prepared in 115 mM NaH_2PO_4, pH 7.2; the duration of fixation was varied between 5 and 10 min and the concentration of paraformaldehyde was varied between 1 and 10%. The sections were then washed for 5 min in 0.1% Triton X-100 in PBS (PBS-T) and incubated overnight at 4°C in hybridoma supernatant. All supernatants were tested undiluted on all muscles examined, but they were routinely diluted 1:50–1:100 in PBS containing 20% normal goat serum and 0.5% Triton X-100 (PBS-ST). The sections were washed for 3 h in PBS-T and incubated for 1 h in PBS-ST containing both fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) to label bound mAbs and rhodamine-α-bungarotoxin to stain AChR ag-

gregates at neuromuscular junctions. For some experiments, 3.4×10^{-7} M 4,6-diamidino-2-phenylindole 2HCl (DAPI; Sigma Chemical Co., St. Louis, MO), a fluorescent DNA stain, was added to this mixture to label nuclei. Sections were washed 30 min in PBS-T and then coverslips were mounted with Citifluor mountant medium AF1 (City University, London).

WHOLE MUSCLES: pinned out muscles were incubated for 3 h in a 1:10–1:50 dilution of hybridoma supernatant in Ringer's solution containing 5% normal goat serum (dilution medium). They were washed for 30 min in Ringer's and incubated for 1 h in dilution medium containing fluorescein-labeled goat anti-mouse IgG (Cappel Laboratories) and rhodamine-α-bungarotoxin. Muscles were washed 30 min in Ringer's and then mounted between coverslips in Citifluor AF1. *Torpedo* and chicken muscles were dissected down to a single layer of myofibers before exposure to antibodies.

Immunoperoxidase Histochemistry. Whole muscles were incubated for 3 h in a 1:10–1:50 dilution of hybridoma supernatant in dilution medium. They were washed for 30 min in Ringer's solution and incubated for 30 min in dilution medium containing biotinylated horse anti-mouse IgG (ABC kit; Vector Laboratories, Inc., Burlingame, CA). After a 15-min wash in Ringer's, the preparations were incubated 30 min in PBS (for whole muscles of rays, PBS contained 250 mM NaCl) containing an avidin–biotin–horseradish peroxidase complex (ABC kit; Vector Laboratories, Inc), washed 15 min in PBS, fixed 5 min in 0.1% glutaraldehyde in PBS, and washed 15 min in PBS. Muscles were then washed briefly in 180 mM cacodylate buffer (300 mM for whole muscles of rays) and incubated between 30 min and 2 h in 0.05% 3,3-diaminobenzidine (Polysciences, Inc., Warrington, PA) and 0.02% H_2O_2 in cacodylate buffer. After a brief wash, they were fixed in 1% osmium tetroxide in phosphate buffer, dehydrated, and embedded in a mixture of Epon and Araldite. Reagents used for immunoperoxidase histochemistry penetrate whole muscles very poorly. Accordingly, we used this procedure primarily on thin muscles: fin muscles of *Dyscopyge*, cutaneous pectoris muscle of frog, and scalp muscles of chicken. Findings from *Dyscopyge* were checked on superficial fibers in abdominal muscles of *Torpedo*.

The electric organ of *Torpedo* was examined by immunofluorescence histochemistry as described above and brightfield light microscopy. For brightfield microscopy, a piece of electric organ having several columns of electrocytes was pinned out in a petri dish containing periodate–lysine–paraformaldehyde fixative (reference 15) in 100 mM NaCl. The central columns of cells were dissected down to 5–10 layers of cells and fixation in periodate–lysine–paraformaldehyde was continued for a total of 4 h. The tissue was then washed in phosphate buffer (300 mM $NaHPO_4$, pH 7.1), fixed in 2% glutaraldehyde in phosphate buffer containing 0.3% tannic acid for 30 min, washed briefly, fixed 1 h in 1% OsO_4, and dehydrated in ethanol. The remaining layers of cells in the central columns were then cut out and embedded in Epon/Araldite.

Operations

Frogs (2 in, nose to rump) were anesthetized by immersion in 1% tricaine methane sulfonate (Sigma Chemical Co.) in water. They were kept on ice during surgery to reduce bleeding and prolong anesthesia. Before and after surgery they were maintained at 19–22°C and fed crickets thrice weekly.

Denervation. The tibialis anterior muscle was denervated by making a 5-mm-long skin incision on the lateral surface of the thigh and tying a suture around the sciatic nerve near the pelvis. An ≅1-cm stretch of the nerve distal to the suture was then avulsed. Muscles were removed for histochemical staining at ≅2, 3, and 4 wk after denervation. At 4 wk we detected no connection between the central and peripheral nerve stumps in the dissecting microscope (×50) and the tibialis anterior muscle did not twitch in response to mechanical stimulation of the peripheral nerve stump, indicating that regenerating axons had not innervated the muscles. The cutaneous pectoris muscle in frog was denervated by resecting 0.5–1 cm of the nerve at its lateral border. 1–2 wk later, a 1-cm length of the second spinal nerve was removed near the vertebral column to prevent reinnervation.

Crush Damage. Our procedure has been described in detail elsewhere (16). In brief, the paired cutaneous pectoris muscles were removed from the thorax and were pinned out in a petri dish containing Ringer's solution. Each muscle was then crushed throughout its length three times with the fire-polished edge of a glass slide and replaced in its bed in the thorax. To prevent muscle fiber regeneration, frogs were x-irradiated on each of the first 3 d after the operation (irradiation procedure: Philips 250 kV 15 mA x-ray unit, 0.35-mm Cu filter; total dose per day = 2,300 rads). Reinnervation of the damaged muscle was prevented as described above.

Results

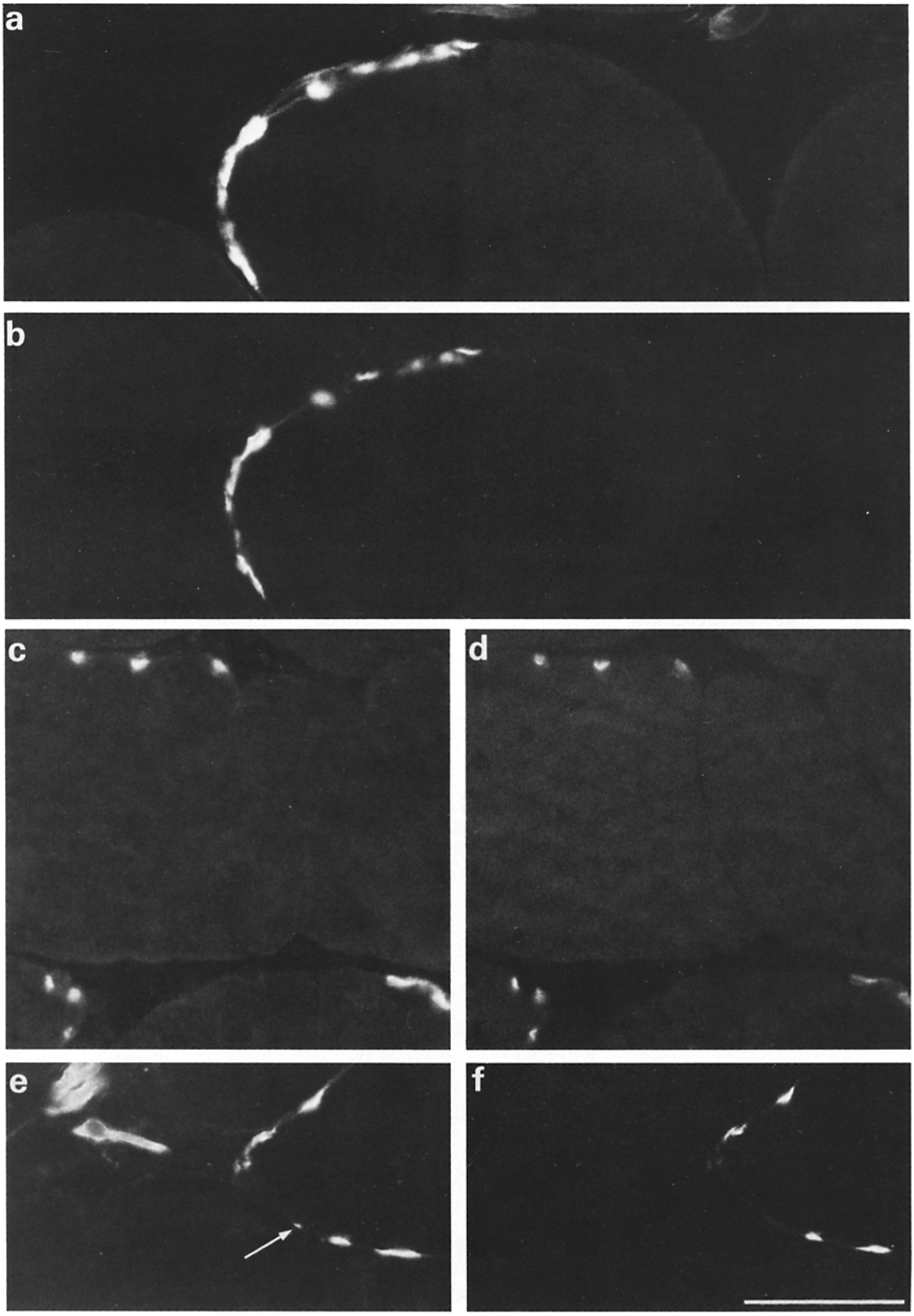
Monoclonal Antibodies

Characteristics of 13 anti-agrin mAbs are presented in Table I. All immunoprecipitated greater than 60% of AChR-aggregating activity from electric organ extracts. Twelve were classified as IgG_1; one (13C5) was classified as an IgG_{2a}. All but one (18D3) stained neuromuscular junctions in frozen sections of *Torpedo* muscle (see below). Of the mAbs that stained *Torpedo* neuromuscular junctions, one (3B5) also stained neuromuscular junctions in frog, two (4B1 and 11D2) stained junctions in chicken, and one (5B1) stained junctions in frog and chicken. All but three (2F6, 10B3, and 15D2) of the mAbs that stained *Torpedo* neuromuscular junctions did so after frozen sections of muscle had been fixed with paraformaldehyde for 5 min. Only one of the mAbs (11D2) stained denatured agrin in immunoblots from SDS gels (18). The differences between mAbs in their ability to stain neu-

Table I. Monoclonal Antibodies that Immunoprecipitate Agrin

mAb	Percent precipitated AChR-aggregating activity*	Neuromuscular junction staining	Formaldehyde sensitivity‡	Blotting ability§	Ig type
2A5	94 ± 2 (6)	Ray	−	−	G_1
2F6‖	77 ± 5 (3)	Ray	+	−	G_1
3B5‖	95 ± 3 (16)	Ray, frog	−	−	G_1
4B1‖	92 ± 3 (6)	Ray, chicken	−	−	G_1
5B1‖	91 ± 3 (8)	Ray, chicken, frog	−	−	G_1
6D4‖	105 ± 2 (3)	Ray	−	−	G_1
10B3	70 ± 5 (11)	Ray	+	−	G_1
11D2‖	89 ± 3 (10)	Ray, chicken	−	+	G_1
13C5	85 ± 3 (8)	Ray	−	−	G_{2a}
15D2	86 ± 3 (29)	Ray	+	−	G_1
17A6	90 ± 3 (10)	Ray	−	−	G_1
18D3‖	61 ± 2 (67)	−	−	−	G_1
19B6	87 ± 5 (4)	Ray	−	−	G_1

* ± SEM; number in parentheses is number of cultures.
‡ +, Fixation for 5 min in 1% paraformaldehyde blocks staining; −, staining unaffected by 10% paraformaldehyde fixation for 10 min.
§ Binds to agrin on immunoblots (see 18).
‖ mAbs that are likely to be against different agrin epitopes.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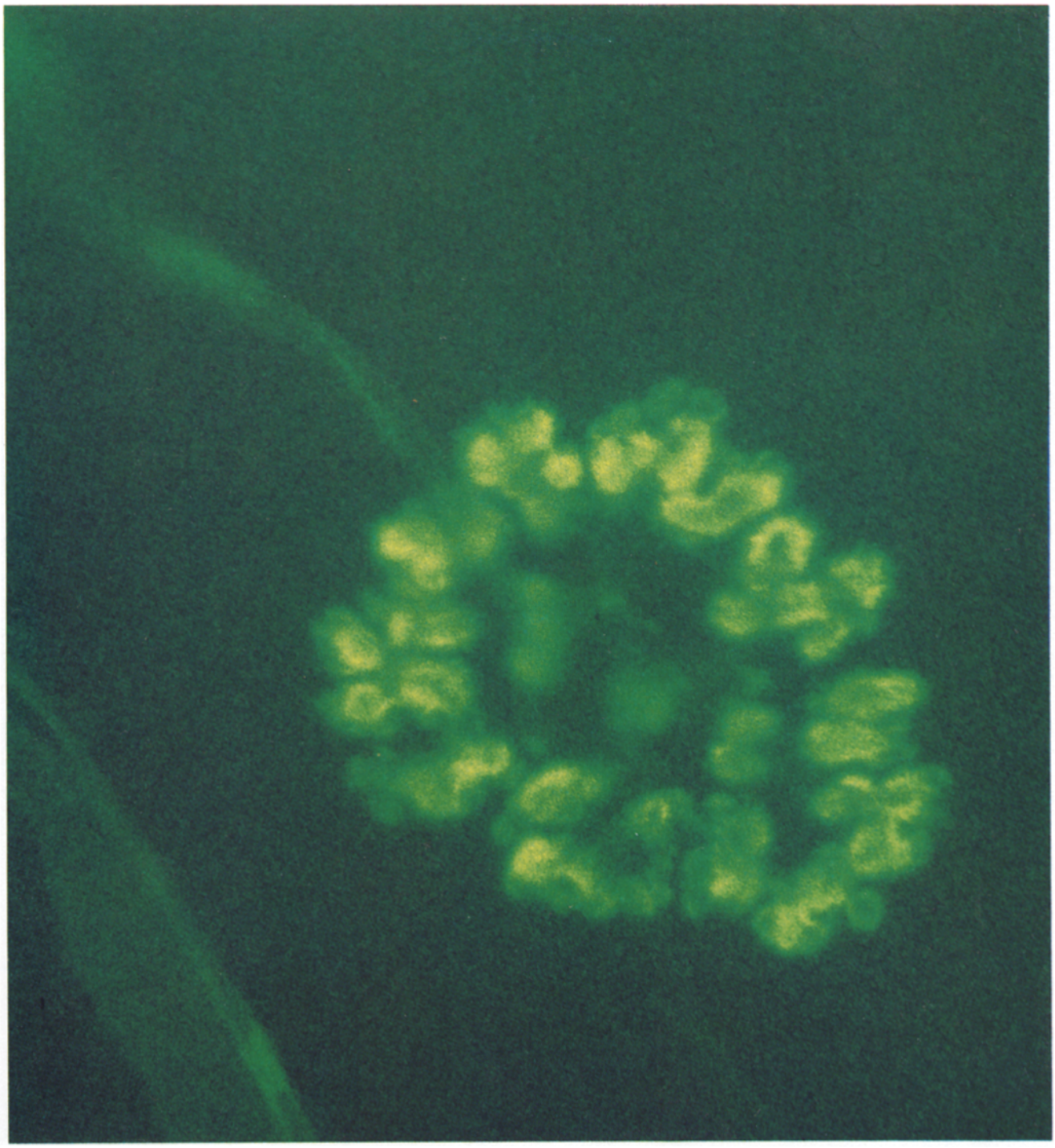

Figure 3. A fluorescence micrograph of a neuromuscular junction and preterminal nerve fibers in a whole mount of *Torpedo* muscle stained with 6D4. ×1540.

Figure 2. Agrin-like molecules are concentrated at neuromuscular junctions in skeletal muscles of *Torpedo* (*a* and *b*), frog (*c* and *d*), and chicken (*e* and *f*). Frozen sections of twitch muscle fibers. Distribution of 5B1 staining is shown in *a*, *c*, and *e*, and rhodamine-α-bungarotoxin staining, which marks the synaptic sites in the same sections, is shown in *b*, *d*, and *f*. In addition to staining the neuromuscular junctions, 5B1 stains preterminal nerve fibers. A nonjunctional patch of mAb stain is on the surface of a myofiber in chicken (*e*, *arrow*). Although not always evident in these micrographs, staining of preterminal nerve fibers and nonjunctional patches was less intense than that at neuromuscular junctions (see Table II). Bar: (*a* and *b*) 40 μm; (*c–f*) 45 μm.

romuscular junctions in different species, to stain fixed tissue, and to recognize denatured agrin on immunoblots suggest that at least seven of the mAbs in our library recognize distinct epitopes on agrin (Table I).

We observed no staining with any of our mAbs in rat muscle. However, neuromuscular junctions and certain other structures stained in all muscles from all other animals in our sample—rays (*Torpedo, Dyscopyge*), frog, and chicken. The distribution of the anti-agrin staining in muscles from the different animals is outlined in Table II and is illustrated in Fig. 2–6 and 8 *a*. A detailed account follows.

Neuromuscular Junctions

The distribution of staining at *Torpedo* neuromuscular junctions was the same for all 12 of the mAbs that stained frozen sections of *Torpedo* muscles; there was a dense band of anti-agrin stain coextensive with AChRs in the postsynaptic membrane (Fig. 2, *a* and *b*) and less intense staining of the unmyelinated preterminal nerve fibers leading to the junctions. Neuromuscular junctions in frozen sections of muscles from *Dyscopyge*, frog, and chicken stained in the same way (Fig. 2). Thus, there are molecules concentrated at the neuromuscular junctions of rays, frog, and chicken, that are antigenically similar to agrin. However, since only four of the mAbs that stain neuromuscular junctions in rays also stained junctions in frog or chicken, some of the epitopes on agrin-like molecules in rays either are not shared by those in frog and chicken or are not accessible to the antibodies in these species.

Figs. 3 and 8 *a* show neuromuscular junctions in whole mounts of muscles from *Torpedo* and frog that were exposed to anti-agrin antibodies while plasma membranes were still intact. In both cases preterminal nerve fibers and the junctions themselves are stained. Thus at least some epitopes recognized by anti-agrin mAbs at neuromuscular junctions lie on or are external to the surface of the plasma membranes of the cellular components. More detailed localization of external epitopes with several mAbs was obtained by electron microscopy. As shown in Fig. 4, *a–d*, which shows neuromuscular junctions in *Dyscopyge*, frog, and chicken, horseradish peroxidase reaction product is primarily in the synaptic cleft and junctional folds, in most cases obscuring the myofiber basal lamina in these regions. Thus, in three different species, at least some molecules bearing epitopes recognized by anti-agrin mAbs are concentrated in the synaptic cleft.

Fig. 4, *b–d*, also shows that in frog and chicken anti-agrin mAbs recognize molecules associated with the surface of the Schwann cells that cap the axon terminals. Horseradish peroxidase reaction product is concentrated in the Schwann cell basal lamina and the coat of extracellular material associated with it. The staining differed from that of the synaptic cleft in that it was patchy. The basal lamina-coated surface of Schwann cells that ensheath the preterminal axons in frog and chicken also stained (data not shown). Axons themselves did not stain. Absence of horseradish peroxidase reaction product in the basal lamina of such Schwann cells in our preparations of muscles from rays (Fig. 4 *a*) was probably due to poor penetration of staining reagents; staining of whole mounts with fluorescent antibodies indicates that extracellular agrin epitopes are associated with preterminal nerve fibers in muscles of rays, as in frog and chicken (see Fig. 3).

*Table II. Distribution of Anti-Agrin Staining on Cell Surfaces in Skeletal Muscle**

Structure	Ray	Chicken	Frog
Myofiber			
Synaptic cleft	+++	+++	+++
Extrajunctional—twitch	–	–	–
Extrajunctional—slow	+	+	ND
Satellite cell	–	++	–
Schwann cell			
Neuromuscular junction	++	++	++
Nerve	++	++	–
Preterminal nerve fiber	++	++	++
Node of Ranvier	++	++	++
Blood vessel			
Smooth muscle cells	++	++	–
Elastica interna	++	++	–
Capillary endothelium	–	–	–
Perineurium	–	–	–

* Number of +'s indicates relative intensity of staining, determined by eye.

Horseradish peroxidase stain on the myofiber surface usually extended a short distance beyond the region of apposition between the pre- and postsynaptic membranes (Fig. 4). This narrow region of perijunctional stain was generally greater for chicken muscles than for frog or ray muscles. It may be due to diffusion of the horseradish peroxidase reaction product from the synaptic cleft and/or the Schwann cell surface. Alternatively, the high concentration of agrin-like molecules on the myofiber surface at neuromuscular junctions may not be confined to the synaptic cleft.

Other Structures and Extrajunctional Regions of Muscle Fibers

Basal lamina coats several cells in muscle in addition to muscle fibers and terminal Schwann cells. They include Schwann cells associated with myelinated axons in nerve bundles, perineurial cells which enclose nerve bundles, and endothelial cells and smooth muscle cells of blood vessels. We observed anti-agrin staining associated with basal lamina in the extrajunctional regions of some myofibers and with some, but not all, of the basal laminae of other cells (Table II). Only those antibodies that stained neuromuscular junctions of a specific species stained nonjunctional structures in that species. Furthermore, all of the antibodies that stained in a particular species stained the same nonjunctional structures. Unlike staining at the neuromuscular junction, however, the nonjunctional staining was not the same in muscles of ray, chicken, and frog (Table II).

Ray and Chicken. We searched by fluorescence microscopy for staining along the extrajunctional region of twitch muscle fibers, including muscle–tendon junctions where AChR and AChE levels are elevated. We found none except for an occasional small patch at the myofiber surface in chicken muscles (Fig. 2). About 90% (22 of 25; one muscle) of such stained patches observed with the electron micro-

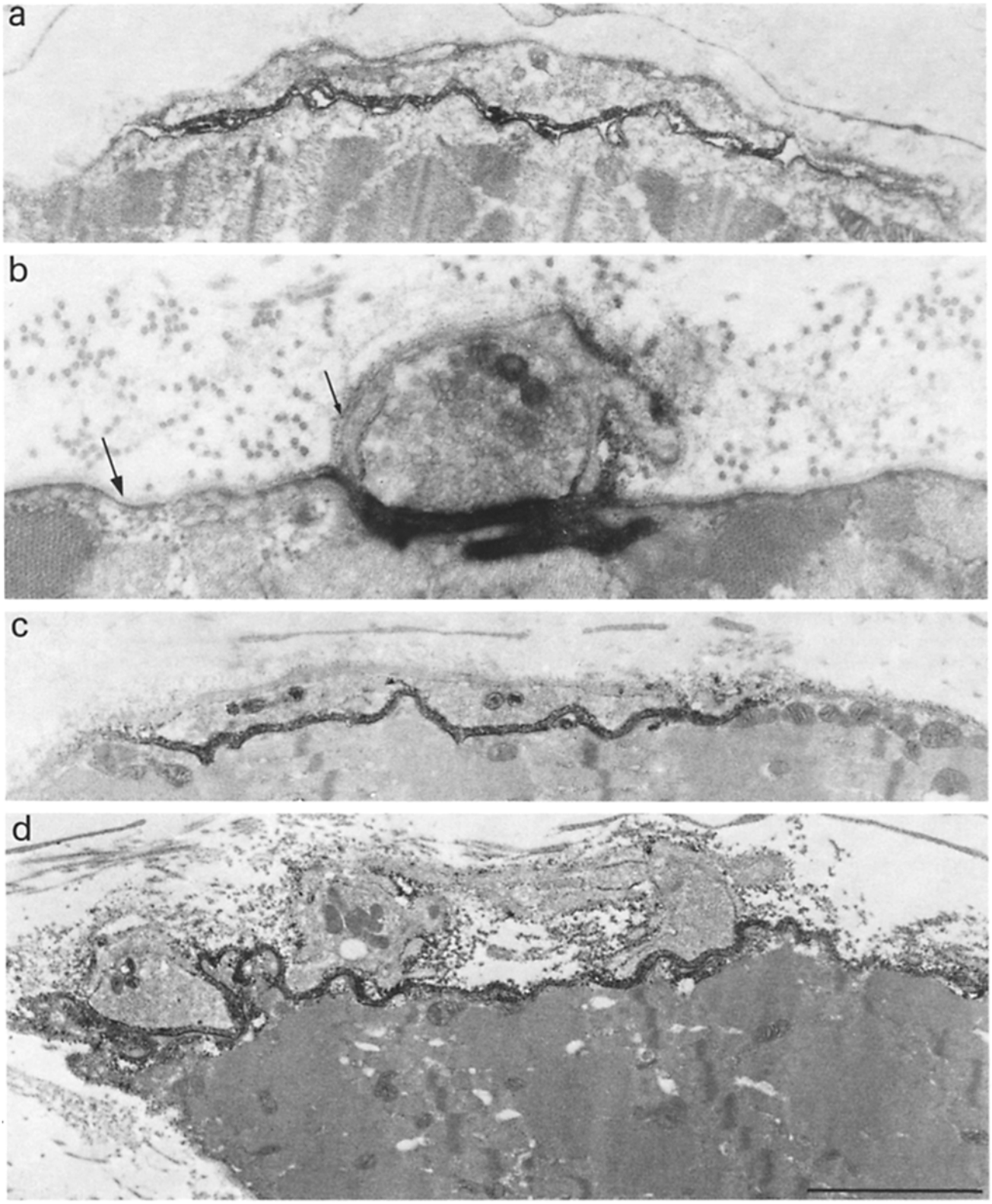

Figure 4. Agrin-like molecules are highly concentrated in the synaptic cleft at neuromuscular junctions of *Dyscopyge* (*a*), frog (*b*), and chicken (*c* and *d*). *Dyscopyge* was stained with 6D4, frog with 5B1, and chicken with a mixture of 5B1 and 11D2. The muscles were intact when treated with the antibodies so that only the external surface of cells and the extracellular matrix were exposed to them. The muscle fiber basal lamina (*large arrow*) and Schwann cell basal lamina (*small arrow*) are indicated in *b*. In all three species, reaction product generated by antibody-bound horseradish peroxidase is concentrated in the synaptic cleft and junctional folds, obscuring the synaptic basal lamina. In *b–d* some stain is associated with the myofiber basal lamina a short distance beyond the edges of the synaptic cleft as well as with the Schwann cell basal lamina and its adjacent extracellular material (*b–d*). Bar: (*a*) 2 μm; (*b*) 1 μm; (*c* and *d*) 2 μm.

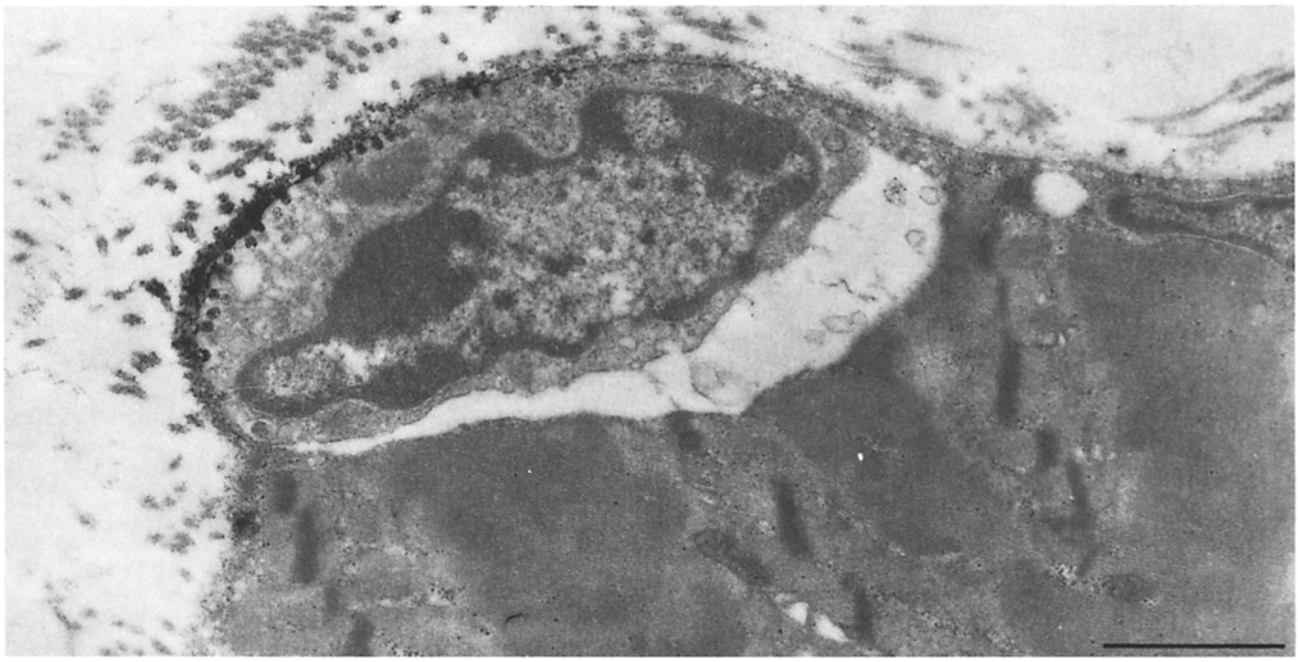

Figure 5. Anti-agrin mAbs stain the basal lamina overlying satellite cells in chicken skeletal muscle. The muscle was stained as in Fig. 4. The staining procedure causes satellite cells to lift off the surface of the myofiber in chicken muscles. Bar, 1 μm.

scope were associated with regions of muscle fiber basal lamina that coated satellite cells (Fig. 5); satellite cells are scattered over the myofiber surface and are characterized by a heterochromatin-rich nucleus surrounded by a thin rim of cytoplasm (e.g., reference 14). The patches of stain that were not associated with muscle satellite cells, as observed by electron microscopy, may have been associated with neuromuscular junctions not included in the plane of section. Indeed, when we cut frozen sections from chicken twitch muscle fibers, which were ≅100 times thicker than the thin sections used for electron microscopy, and stained them with 5B1, rhodamine-α-bungarotoxin, and a fluorescent nuclear stain (DAPI), we found that 98% (98 of 100; one animal) of the extrajunctional anti-agrin-stained patches were within 1 μm of a heterochromatin-rich nucleus.

When examined by fluorescence microscopy, staining rimmed the entire surface of the extrajunctional region of slow myofibers in ray and chicken (Fig. 6 *a*). However, the intensity of stain was faint compared with that at junctions. Indeed, the concentration of anti-agrin epitopes was so low in extrajunctional regions of slow myofibers that no extrajunctional staining was observed by electron microscopy (except for that associated with satellite cells in chicken).

Capillaries did not stain in ray and chicken. Thus, the basal lamina of the capillary endothelium of muscle has few or no agrin-like antigens associated with it. On the other hand, blood vessels with muscular walls stained intensely. Such staining was observed by fluorescence microscopy in whole mounts as well as in sections, indicating that, as on myofibers, the antigens are on cell surfaces. The stain rimmed the smooth muscle cells, which have a basal lamina, and occupied the elastica interna (Fig. 6, *b* and *c*), which is a narrow connective tissue-filled space bounded by the basal lamina of the smooth muscle cells and endothelial cells. Consistent with the fluorescence microscopy, we found by electron microscopy that stain was associated with both the basal lamina of smooth muscle cells and the elastica interna (not shown).

Cross sections of intramuscular nerve bundles also stained (Fig. 6 *d*). The stain rimmed the Schwann cell–myelin sheaths of axons, indicating that it was associated with the basal lamina–coated Schwann cell surface as at the neuromuscular junction. Perineurium did not stain.

Frog. There was no extrajunctional staining of myofibers in frog (Fig. 2, *c* and *d*; the frog muscles we examined had only twitch fibers), even at the position of muscle satellite cells, nor was there staining of blood vessels or perineurium. Moreover, we only rarely saw staining in cross sections of myelinated axon bundles. We sought to determine what structures in the nerve were recognized by the antibodies by staining longitudinal sections of the sciatic nerve or by teasing ≅1-cm-long stretches of individual axons away from isolated segments of the sciatic nerve and exposing them to mAbs with their plasma membranes intact (except for the cut ends). The distribution of the stain was the same in both sorts of preparations. In most cases, stain was on the axonal surface at nodes of Ranvier (Fig. 6 *e*). In some instances, however, two internodal segments in which the myelin was thick were separated by a segment in which the myelin was thin or absent; in such cases, the thin segment was also rimmed by stain. Thus while myelinated axons in chicken and ray were outlined throughout their entirety by antibody staining, in frog the stain was restricted to specific locations, at which the epitopes were extracellular. Anti-agrin mAbs stained nodes of Ranvier in frozen sections of both dorsal roots and sensory nerves as well as in ventral roots, indicating that agrin-like molecules are concentrated at the nodes of both sensory and motor axons in frog.

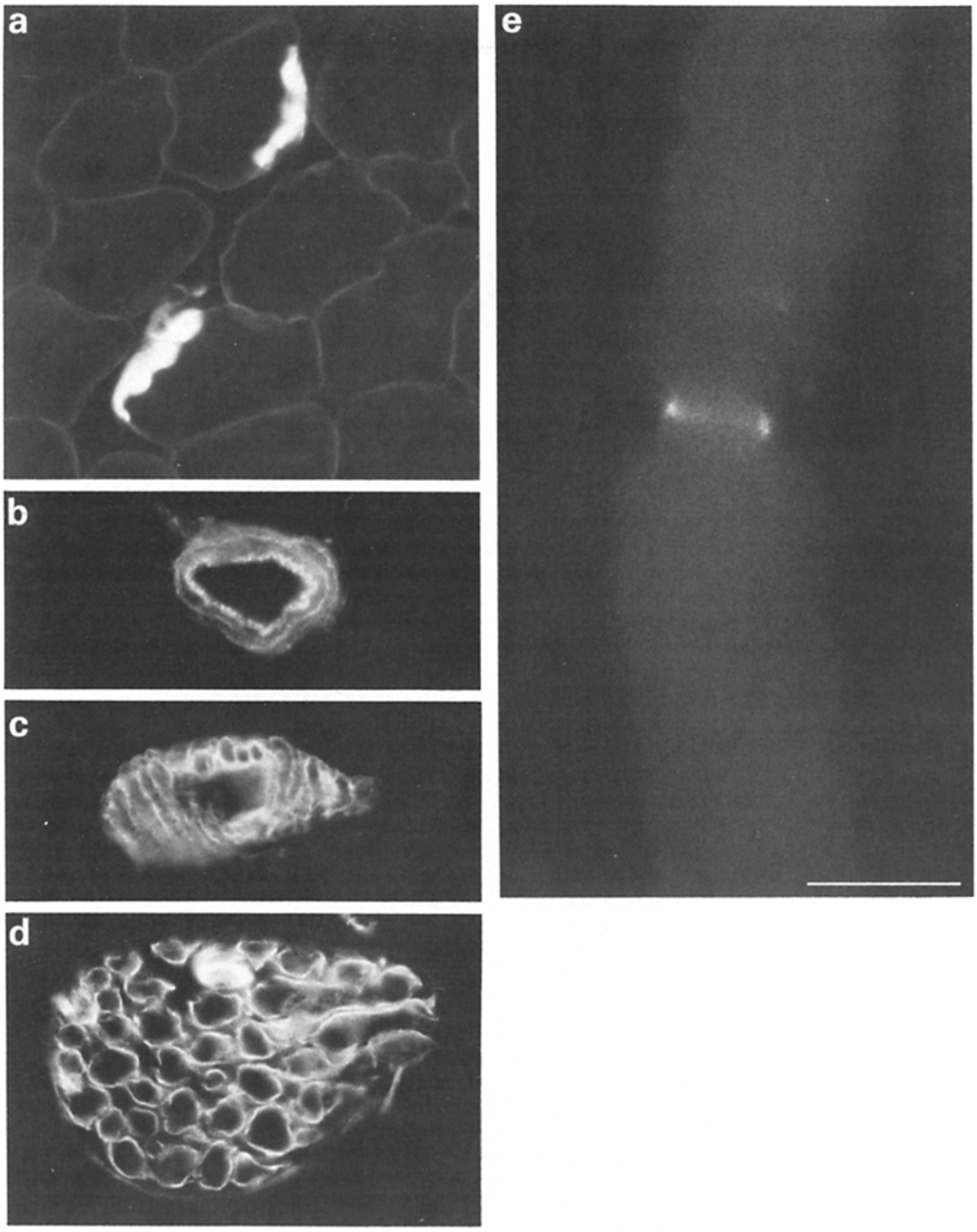

Figure 6. Nonjunctional staining of anti-agrin mAbs in frozen sections of skeletal muscles. (*a*) Cross section through *Torpedo* slow muscle fibers treated with 6D4. The extrajunctional surface of myofibers is lightly stained in comparison with the intense staining of the junctions; the position of junctions was revealed by staining the same section with rhodamine-α-bungarotoxin (not shown). (*b* and *c*) Blood vessels with muscular walls in chicken skeletal muscle stained with 5B1. (*b*) Cross section: the elastica interna (wavy line) and the adjacent layer of smooth muscle cells are stained. (*c*) Glancing longitudinal section: stain rims the smooth muscle cells. (*d*) Cross section of a myelinated nerve from chicken labeled with 5B1. Stain rims the Schwann cell–myelin sheath of axons. (*e*) Whole mount of frog sciatic nerve treated with 3B5 and 5B1. Stain is at a node of Ranvier. Bar: (*a*) 50 μm, (*b–d*) 43 μm; (*e*) 13 μm.

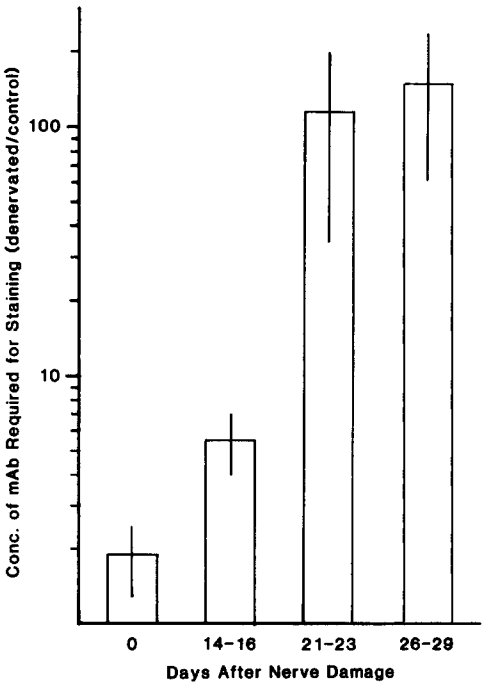

Figure 7. Increasing concentrations of anti-agrin mAbs are necessary to stain synaptic sites in frog muscles after denervation. The right tibialis anterior muscles of frogs were denervated for various lengths of time. Frozen sections of both the right and left muscles were stained with serial dilutions of 3B5. The titer necessary to stain the right (denervated) muscle was normalized to the titer necessary to stain the left (control) muscle in order to compare the results from one animal to that of others. Control, 0 d denervation (n = 5); 14–16 d denervation (n = 4); 21–23 d denervation (n = 6); 26–29 d denervation (n = 5). The increase in concentration of anti-agrin mAbs necessary to stain the synaptic sites after denervation suggests that the concentration of agrin-like molecules decreases. Error bars, SEM.

Denervated Muscles

At ≅2, 3, and 4 wk after denervating the frog's right anterior tibialis muscle, both the right and left muscles were removed and frozen cross sections were made through them. The sections were stained with serial dilutions of 3B5 to determine the minimum titer of anti-agrin antibody required to stain synaptic sites in both the normal and denervated muscles. At all times the stain in denervated muscles was localized to synaptic sites as in normal frog muscles. At 2 wk after denervation, when the axon terminals had been phagocytized (9), the concentration of antibody required to stain the synaptic sites in the denervated muscles was higher than that in normal muscles processed in parallel. As the period of denervation increased the concentration necessary for staining also increased so that by 3 wk the required concentration was 60-fold greater in denervated muscles than in normal ones (Fig. 7). In another experiment, a normal and a 3-wk denervated cutaneous pectoris muscle from the same animal were stained in the same dish with a 50:50 mixture of 3B5 and 5B1 and were processed for immunoperoxidase histochemistry. Horseradish peroxidase reaction product was allowed to accumulate until it was just detectable at some of the normal neuromuscular junctions by light microscopy (×400) and then both muscles were fixed and examined by electron microscopy. Stain was observed in the synaptic cleft at 68% (56 of 82) of cross-sectioned neuromuscular junctions in normal muscles but at only 27% (15 of 56) of the denervated junctional sites, which were identified by the presence of Schwann cells and junctional folds in the myofiber. The findings from both experiments indicate that the concentration of agrin-like molecules at synaptic sites is markedly reduced after denervation.

Damaged Muscles

Our finding that anti-agrin antibodies stain the synaptic cleft at neuromuscular junctions suggests that agrin-like molecules are associated with the basal lamina. An alternative possibility is that such molecules are components of the pre- and/or postsynaptic membrane and are exposed to the extracellular surface but not bound to the basal lamina.

To examine the association of agrin-like molecules with synaptic basal lamina, we crushed the frog's cutaneous pectoris muscle in a way that causes degeneration of all cells at the neuromuscular junction—muscle fiber, axon terminal, and Schwann cell. Regeneration of the myofibers and reinnervation of the muscle were prevented (see Materials and Methods). 3 wk later, when the damaged cells had been phagocytized, the muscles, now consisting of myofiber basal lamina sheaths and scattered mononucleated cells, were removed and stained with anti-agrin antibodies. In whole mounts, the stain was distributed in an arborized pattern similar to that of neuromuscular junctions in normal muscles (Fig. 8). To detect such patterns, however, the damaged muscles had to be stained longer than normal muscles, indicating that crushing muscles, like denervating them, results in a decrease in the concentration of agrin-like molecules at synaptic sites. Cross sections through stained regions examined by electron microscopy showed that the stain was confined to the synaptic basal lamina and Schwann cell basal lamina with its associated extracellular material (Fig. 9). Thus, agrin-like molecules remain adherent to the synaptic and Schwann cell basal laminae for weeks after degeneration of the cellular components of the junction.

Discussion

Our hypothesis that agrin, which causes AChR/AChE aggregation on cultured myotubes, is similar to the AChR- and AChE-aggregating molecules in the synaptic basal lamina of skeletal muscles has been based in part on the observation that mAbs against agrin stain components of the synaptic cleft, the site of synaptic basal lamina, at the neuromuscular junction of rays. To make direct tests of the agrin hypothesis, it will be useful to have markers for such molecules in animals more convenient for experimentation than rays. It will also be important to determine the precise position of agrin-like molecules in muscle and understand how the agrin-like molecules of the neuromuscular junction are regulated.

We find that antibodies against *Torpedo* agrin recognize molecules highly concentrated in the synaptic cleft of neuromuscular junctions in skeletal muscles of frogs and chickens, which are common laboratory animals, as well as rays. The constancy of staining at the neuromuscular junction in widely differing species suggests that agrin-like molecules are components of neuromuscular junctions of all verte-

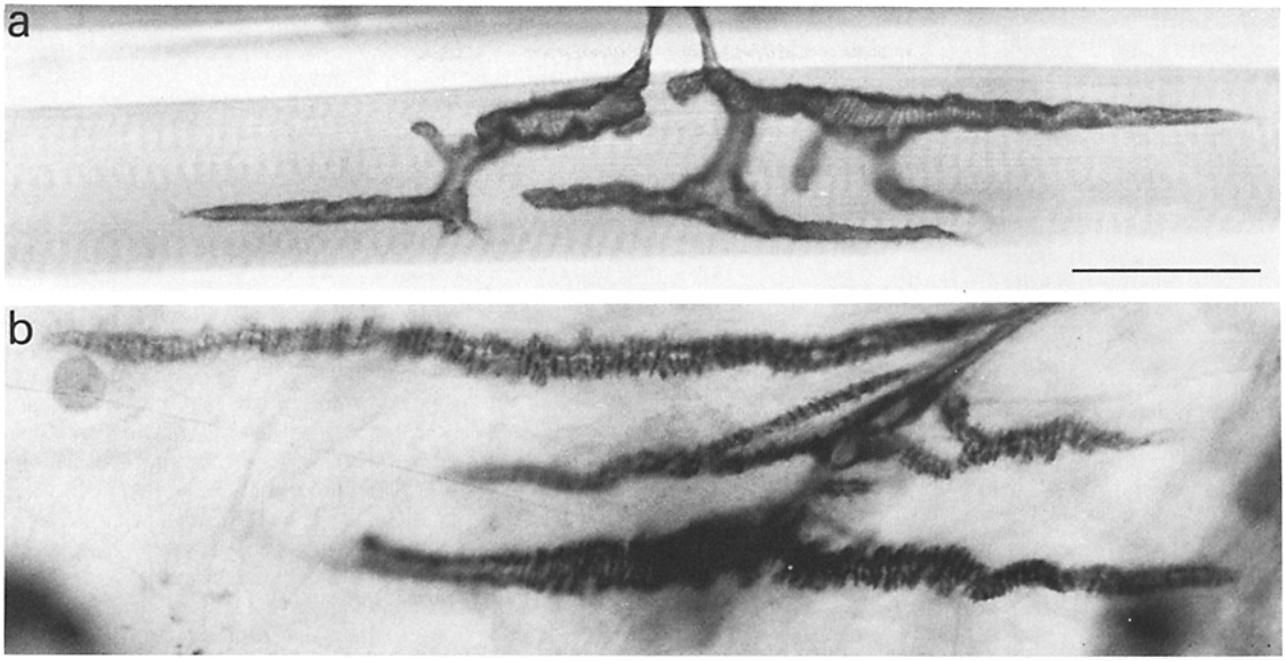

Figure 8. Anti-agrin staining at synaptic sites in normal (*a*) and damaged (*b*) frog muscles. These are whole mounts which were stained as in Fig. 4, except that 3B5 and 5B1 (50:50) were used on the damaged muscle. The damaged muscle was taken 23 d after it had been crushed, which results in degeneration and phagocytosis of muscle fibers, axon terminals, and Schwann cells while the myofiber basal lamina and other connective tissue elements persist. In the normal muscle, horseradish peroxidase reaction product on the myofiber is layed out in an arborized pattern characteristic for neuromuscular junctions. It also coats the short unmyelinated segment of the preterminal axon branches. Stained synaptic sites in the damaged muscle are readily identified by the arborized pattern. Bar, 40 μm.

brates. That our anti-agrin antibodies do not stain neuromuscular junctions in rat may be due to species-specific variation in the molecules or their environment. Indeed, only four of twelve antibodies that stained neuromuscular junctions in *Torpedo* also stained junctions in frog and/or chicken.

We also describe staining of nonjunctional components in muscle with anti-agrin antibodies, which indicates that agrin is antigenically related to molecules that have a broad distribution in skeletal muscle. The pattern of nonjunctional staining was not constant among those species in which the neuromuscular junctions stained, which suggests that agrin may be more closely related to agrin-like molecules in the synapse than to those in other regions of the muscle. As presented in the accompanying paper (18), each of five different anti-agrin antibodies immunoprecipitates four polypep-

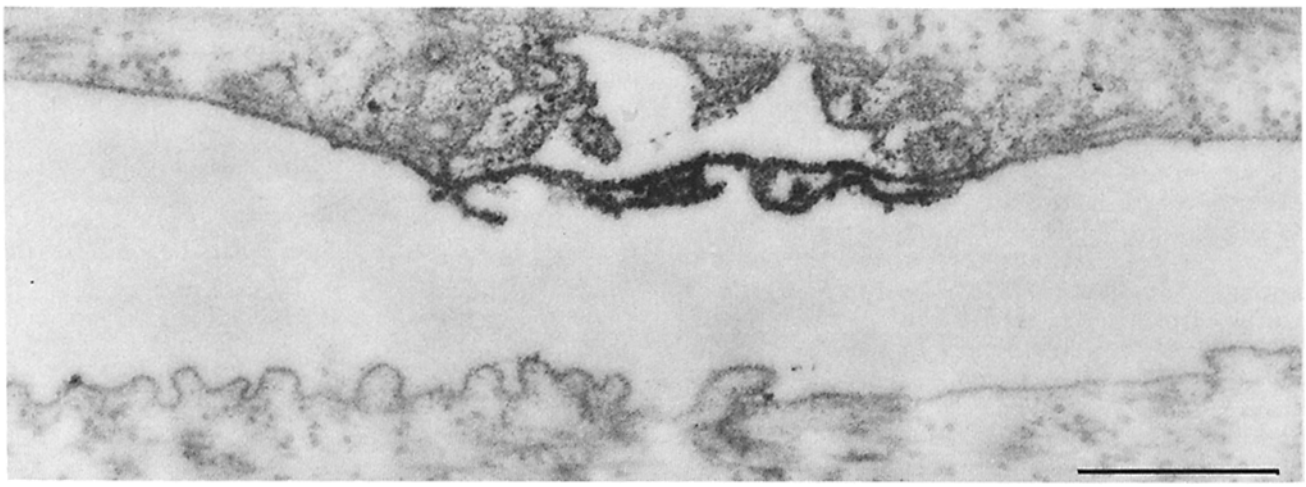

Figure 9. Agrin-like molecules are stably attached to the synaptic portion of the muscle fiber's basal lamina sheath. A cross section through a synaptic site of a 21-d crush-damaged muscle stained with anti-agrin mAbs as in Fig. 8 *b*. Horseradish peroxidase reaction product is concentrated in the basal lamina that formerly occupied the synaptic cleft and the junctional folds. The Schwann cell basal lamina and its associated extracellular material are also stained. The distribution of stain at the synaptic site is the same as at normal neuromuscular junctions; compare with Fig. 4 *b*. Bar, 1 μm.

tides from electric organ extracts: two forms of agrin which cause AChR/AChE aggregation on cultured myofibers, and two proteins which do not. We cannot assess the contribution that agrin and the antigenically related inactive proteins make to either the junctional or nonjunctional staining in the electric organ, nor have we characterized the molecules recognized by our anti-agrin antibodies in muscle. Studies aimed at identifying agrin-like proteins at neuromuscular junctions and determining whether or not they cause AChR/AChE aggregation are under way.

By damaging frog muscles in a way that caused all cellular components of the neuromuscular junction to degenerate and then staining the muscles with anti-agrin antibodies, we show that agrin-like molecules concentrated in the synaptic cleft are associated with the myofiber's sheath of basal lamina. In every case of nonjunctional staining, the stain was on a basal lamina-coated cell surface. Thus it is likely that nonjunctional agrin-like molecules are associated with basal lamina as are agrin-like molecules in the synaptic cleft. Indeed, our experiments on crush-damaged muscles demonstrate this point for agrin-like molecules associated with the surface of terminal Schwann cells. Not all muscle components coated by basal lamina, however, stained with anti-agrin antibodies. Thus it is not likely that agrin-like molecules are common constituents of basal lamina, as are collagen type IV and laminin.

The staining along myelinated axons is of particular interest. In ray and chicken the stain lined the abaxonal surface of the Schwann cells that form the myelin sheath and was at nodes of Ranvier, while in frog stain was localized primarily to the nodes and had a distribution, as observed by fluorescence microscopy, similar to that obtained by staining with anti-N-CAM, anti-Ng-CAM and anti-cytotactin (23). At nodes of Ranvier there is a high concentration of sodium channels in the axonal plasma membrane. It may well be that agrin-like molecules are concentrated at many sites throughout the body where aggregation of plasma membrane proteins is crucial for physiological processes.

It is not yet known whether the concentration of AChR- and AChE-aggregating molecules in the synaptic basal lamina decreases after denervation. However, the decline in staining of synaptic sites by anti-agrin antibodies in denervated frog muscles raises the possibility that it may and indicates further that, like AChE, the maintenance of agrin-like molecules at synaptic sites is neuron dependent. The neuron might play any of the following roles in the maintenance of agrin-like molecules. (*a*) Agrin-like molecules in the synaptic cleft may be synthesized in the cell bodies of motor neurons and released by axon terminals. In fact, antibodies against agrin stain the cytoplasm of cell bodies of motor neurons as they do the synaptic cleft (11, 28). Moreover, extracts from regions of the central nervous system rich in cell bodies of motor neurons contain molecules that cause AChR aggregation on cultured myotubes and such molecules can be immunoprecipitated by anti-agrin antibodies (28). (*b*) Agrin-like molecules in the synaptic cleft may be derived from myofibers but their accumulation is regulated by the nerve terminal; e.g., by electromechanical activity triggered by transmission of nerve impulses. The metabolism of muscle-produced AChE at the neuromuscular junction is regulated in this way (for review see reference 24). That skeletal myofibers can synthesize agrin-like molecules is suggested by the finding that in rays and chickens the surfaces of slow muscle fibers stain faintly with anti-agrin antibodies in their extrajunctional regions. (*c*) Motor neurons may provide synaptic cleft molecules that bind agrin-like molecules. Studies on the electromotor system of *Torpedo* suggest that electromotor neurons synthesize a proteoglycan which is released by their axon terminals at synapses in the electric organ (5). Several components of the synaptic cleft including acetylcholinesterase have a high affinity for heparan sulfate proteoglycan in vitro (3).

Previous studies (2, 16) have shown that in frog muscles that are damaged as were those in this study, but in which myofibers are allowed to regenerate within the basal lamina sheaths of the original myofibers, the new myofibers begin to form ~2 wk after damage and the aggregation of AChRs and AChE induced by the synaptic portion of the basal lamina starts during the following week. Thus, the AChR- and AChE-aggregating molecules persist in the synaptic basal lamina for at least 2–3 wk after damage. The concentration to which AChRs and AChE accumulate under such conditions is usually much less than that at normal neuromuscular junctions (1, 16). Our findings that anti-agrin antibodies bind to synaptic basal lamina 3 wk after degeneration of all cellular components of the neuromuscular junction and that they bind in lower amounts than at the normal neuromuscular junction are consistent with the hypothesis that they are binding to the AChR/AChE-aggregating molecules in the synaptic basal lamina.

Several of our mAbs inhibit the activity of agrin when applied with agrin to myotube cultures. This fact coupled with the observation that anti-agrin antibodies stain molecules stably adherent to the synaptic basal lamina provides encouragement for seeking to determine whether or not agrin antibodies inhibit the ability of synaptic basal lamina to cause AChR and AChE aggregation on regenerating frog myofibers in vivo, which would provide a direct test of the agrin hypothesis.

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