ANTIBODIES THAT BIND SPECIFICALLY TO SYNAPTIC SITES ON MUSCLE FIBER BASAL LAMINA

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ABSTRACT

Basal lamina (BL) ensheathes each skeletal muscle fiber and passes through the synaptic cleft at the neuromuscular junction. Synaptic portions of the BL are known to play important roles in the formation, function, and maintenance of the neuromuscular junction. Here we demonstrate molecular differences between synaptic and extrasynaptic BL. We obtained antisera to immunogens that might be derived from or share determinants with muscle fiber BL, and used immunohistochemical techniques to study the binding of antibodies to rat skeletal muscle. Four antisera contained antibodies that distinguished synaptic from extrasynaptic portions of the muscle fiber’s surface. They were anti-anterior lens capsule, anti-acetylcholinesterase, anti-lens capsule collagen, and anti-muscle basement membrane collagen; the last two sera were selective only after antibodies binding to extrasynaptic areas had been removed by adsorption with connective tissue from endplate-free regions of muscle. Synaptic antigens revealed by each of the four sera were present on the external cell surface and persisted after removal of nerve terminal, Schwann cell, and postsynaptic plasma membrane. Thus, the antigens are contained in or connected to BL of the synaptic cleft. Details of staining patterns, differential susceptibility of antigens to proteolysis, and adsorption experiments showed that the antibodies define at least three different determinants that are present in synaptic but not extrasynaptic BL.

KEY WORDS immunohistochemistry · collagen · basement membrane · acetylcholinesterase · neuromuscular junction · synaptic cleft

A layer of basal lamina (BL)1 ensheathes each skeletal muscle fiber, and passes between nerve

1 As defined by Sanes et al. (44), the term basal lamina (BL) refers to the 10- to 15-nm thick, electron-dense layer that ensheathes each skeletal muscle fiber, and the term basement membrane refers to the BL plus its coat of collagen fibrils and associated matrix.

and muscle at the neuromuscular junction. BL thus comprises a substantial portion of the synaptic cleft material of the neuromuscular junction, and it plays important roles in the formation, function, and maintenance of this synapse: (a) factors in synaptic BL regulate the differentiation of nerve terminals during reinnervation of muscle, and may account for the topographically precise reinnervation of original synaptic sites (44); (b) clustering of acetylcholine receptors (AChRs) in the postsynaptic membrane may, under some circumstances, be induced or maintained by interactions with...
synaptic BL (8); (c) some of the acetylcholinesterase (ACHE) that terminates the action of neurotransmitter at the neuromuscular junction is contained in or connected to synaptic BL (6, 23, 37); and (d) adhesion of nerve to muscle appears to depend on the integrity of the BL (6).

These studies suggest that the synaptic portion of the BL contains specialized components and differs from the extrasynaptic regions to which it is attached. The aim of the experiments reported here was to obtain evidence for molecular differences between synaptic and extrasynaptic BL. We raised antisera to immunogens that might be derived from or share determinants with muscle fiber BL. Immunohistochemical methods showed that several of these sera contain antibodies that selectively bind to synaptic BL.

MATERIALS AND METHODS

Immunogens and Antisera

LENS CAPSULE: Anterior lens capsules were stripped from bovine lenses (purchased from Pel-Freeze Biologicals Inc., Rogers, Ark.), cleaned as described by Fukushima and Sprio (19), and stored at -20°C. For each immunization, 10-20 mg of lens capsule was dissolved in 1 ml of 0.1 N NaOH by incubation overnight at 37°C. Rabbits were injected three times at 8- to 10-d intervals, boosted after 1 mo, and then bled at 1- to 4-wk intervals, beginning 10 d later. Pertussis vaccine (0.5 ml; Eli Lilly and Co., Indianapolis, Ind.) was injected 3 d before the first immunization to intensify the immune response (26). The first dose of immunogen was neutralized with HCl and emulsified in an equal volume of complete Freund's adjuvant (N. L. Cappel Laboratories Inc., Cochranville, Pa.), to which 5 mg of tubercle bacillus (Difco Laboratories, Detroit, Mich.) was added; subsequent doses were neutralized and emulsified in Freund's incomplete adjuvant. All injections were given subcutaneously at multiple, closely spaced sites along the back and flank.

COLLAGENS TYPES I AND III: Collagen type I was prepared from rat tail tendon and type III from rat skin. Tissues were extracted with 1 mg/ml pepsin (Sigma Chemical Co., St. Louis, Mo.) in 0.5 N acetic acid for 2-3 d at 4°C, and collagens were purified by dialysis and repeated fractional precipitation with NaCl (9, 15, 48). Purity was assessed by SDS-gel electrophoresis (34). Rabbits were immunized on the schedule described above, using 3-6 mg of the appropriate collagen per dose. Antiserum to acid-soluble (nonpepsinized) type I collagen from rat tail tendons was a gift of Heinz Schuenstuhl and Dow Michaeli, University of California, San Francisco.

BASEMENT MEMBRANE COLLAGENS: We modified procedures for preparation of “basement membrane-like” collagens from placenta (9) and bovine muscle (5) to obtain a similar fraction from rat skeletal muscle. Leg muscles were homogenized in 150 mM NaCl, 20 mM Tris, pH 7.4. The residue (~20 g/kg muscle) was suspended in a solution of 1 mg/ml pepsin in 0.5 N acetic acid, at a concentration of 1 g residue/30-40 ml. This suspension was stirred at 4°C for 4 d and then clarified by centrifugation. Collagen was precipitated by the addition of solid NaCl to 1 M, and the precipitate was redisolved in 0.5 M NaCl adjusted to pH 7 with Tris. Interstitial collagens (types I and III) were precipitated by dialysis against 2.6 M NaCl, and putative basement membrane collagen was then precipitated from the remaining solution by dialysis against 4.5 M NaCl. The precipitate was collected by centrifugation, redisolved in 0.5 M NaCl, and subjected to fractional salt precipitation, as described above, several additional times. SDS gel electrophoresis revealed that most of the protein in the original pepsin digest was type I collagen (and pepsin). A small amount of type III collagen was also present, and other proteins comprised only a few percent of the extract. The final precipitate, on the other hand, consisted of polypeptides that migrated on gels in approximately the positions expected for basement membrane collagen chains A and B (5, 9, 42), and were sensitive to hydrolysis by highly purified collagenase from Clostridium (Advance Biofactors, Lynbrook, N. Y.). About 60 mg of these proteins was obtained per kilogram muscle. Rabbits were immunized with 3-mg portions on the schedule described above.

We also raised antiserum to a collagen-rich fraction from lens capsule (29) that contains collagen type IV (28, but see reference 46). Bovine anterior lens capsules (150 mg) were incubated with 10 mg of pepsin for 3 d at 4°C in 15 ml of 0.5 N acetic acid. The resulting solution was clarified by centrifugation and dialyzed against 0.15 M NaCl, 20 mM Tris, pH 7.4. Subsequent dialysis against water precipitated most of the lens capsule proteins but little of the pepsin. The precipitate was dissolved in saline and used to immunize a rabbit (~2 mg protein/dose) on the schedule given above. SDS-gel electrophoresis showed that the precipitate contained several collagenase-sensitive polypeptides, including a major one with a mobility similar to that reported for collagen IV (28).

ACHE: Antisera to highly purified ACH from bovine brain and from Electrophorus electric organ were generous gifts of Doctors Anthony Trevor and Alvin Greenberg, Department of Pharmacology, University of California, San Francisco, who have described the purification of ACH and the properties of its antiserum (12, 20). Anti-electrophorus ACH does not contain antibodies that react detectably with the enzyme's collagenous tail (20).

CONTROL SERA: Serum collected from each rabbit before immunization served as control.

IMMUNOADSORPTION: Antisera that reacted with both synaptic and extrasynaptic BL were adsorbed with an extract that contained extrasynaptic but not synaptic BL. For this purpose, endplate-free regions were cut from rat diaphragms (22), and a connective tissue-rich fraction was prepared from them as described above (see Chemical extraction of myofibers). This fraction, or other tissue to be used as immunoadsorbent, was frozen in liquid N2, pulverized, and washed by centrifugation and resuspension, first in water, then in 150 mM NaCl, 20 mM sodium phosphate, pH 7.4 (PBS), and finally in PBS with 10 mg/ml bovine serum albumin (BSA; Sigma Chemical Co.). The pellet was suspended in a minimal volume of PBS-BSA, a portion of serum was added (5-25 µl serum/ml suspension), and the mixture was incubated at 4°C overnight on a rocker. After centrifugation, antibodies remaining in the supernatant fluid were assayed immunocytochemically.
Histology

**Indirect Immunofluorescence Staining:** The technique of indirect immunofluorescence was used to detect binding of antibodies to unfixed sections of rat skeletal muscle. We used fluorescent α-bungarotoxin to identify neuromuscular junctions in the fluorescence microscope; this protein binds tightly and specifically to AChRs clustered in the postsynaptic membrane (2, 41). The "second" antibody was conjugated to fluorescein (which fluoresces green) and the toxin to rhodamine (which fluoresces red), so that we could distinguish and compare the distribution of the two fluorophores on the same section.

Strips of muscle from rat diaphragm were frozen in liquid N₂, and 4-μm cross sections were cut in a cryostat. To ensure that the sections we studied would be conveniently rich in neuromuscular junctions, we stacked four strips of diaphragm with their nerves in register, froze them together, and sectioned them as one block: sections were cut from the area just costal to the nerve. Every 200-300 μm, a section was treated with a histochemical stain for cholinesterase (27), which colors motor endplates, to monitor the progress of our sectioning. Sections with neuromuscular junctions were picked up on glass slides, air-dried, and encircled by a line of rubber cement, applied from a syringe. Antiserum was diluted, usually 1:200, in PBS-BSA and applied to the sections in ~50-μl droplets, which were confined by the rubber cement. After a 30- to 60-min incubation at room temperature, the sections were washed with PBS and then incubated for 1 h with a mixture of fluorescein-conjugated goat anti-rabbit IgG (IgG fraction; 1:50 dilution; N. L. Cappel Laboratories, Inc.) and 10 nM rhodamine-α-bungarotoxin (a gift of Doctors Peter Ravdin and Darwin Berg, Department of Biology, University of California, San Diego; see reference 41) in PBS-BSA. Finally, the rubber cement was peeled from the slides, and the sections were washed in PBS, mounted under 90% glycerol-10% PBS, and examined with a Zeiss fluorescence microscope. Fluorescein was excited and viewed selectively with Zeiss 46-77-09 filters (excitation, 540-560 nm; barrier, 520 nm; reflector, 510 nm) and rhodamine with 48-77-15 filters (excitation, 546 nm; barrier, 590 nm; reflector, 580 nm). Fig. 1 shows that these filters allow each fluorophore to be distinguished and photographed with only slight interference from the other.

**Immunoperoxidase Staining:** Antibodies applied to whole, live muscles were located by an indirect method, using horseradish peroxidase-conjugated second antibody. The flexor digitorum superficialis muscle of the forearm was chosen for these experiments because it is rather lightly invested with connective tissue and its distal portion is only a few fibers thick; thus, it permits adequate penetration of immunoreagents. Muscles were pinned by their tendons to Sylgard-coated (DuPont Instruments, Wilmington, Del.) Petri dishes and incubated at room temperature, on a rocker, in an oxygenated atmosphere, with the following series of well oxygenated solutions: mammalian Ringer's solution for 0.5-1.5 h; antiserum diluted in Ringer's containing 10 mg/ml BSA for 2 h; several changes of Ringer's without BSA for 1 h; peroxidase-conjugated goat anti-rabbit IgG (IgG fraction; 1:100 dilution; N. L. Cappel Laboratories, Inc.) in Ringer's solution with BSA for 2 h; several changes of Ringer's first with, and then without BSA for 2 h. After these treatments, the muscles were fixed for 30 min with 1% glutaraldehyde in 90 mM NaCl, 5 mM CaCl₂, 30 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), pH 7.2, washed in Ringer's solution overnight at 4°C, cut into strips 1 mm or less wide, and incubated for 1.5 h at 4°C in the reaction mixture described by Streit and Reubi (47). For light microscopy, single fibers teased from the muscle were dried on slides and mounted under Permount (Fisher Scientific Co., Pittsburgh, Pa.). Material for electron microscopy was rinsed in 60 mM sodium barbital, pH 7.2, fixed in 1% OsO₄ in barbital buffer for 1 h, dehydrated in ethanol, rinsed in propylene oxide, and embedded in Araldite.

**Separation of Plasma Membrane from BL**

To characterize the cell surface components to which antibodies bound, we devised two ways to remove plasma membrane from muscle fibers that preserved BL in a form suitable for morphological study.

**Induced Degeneration of Myofibers:** When muscle is damaged, the myofibers degenerate and are phagocytized, but their sheaths of BL persist (10, 44, 45, 49). As studies have shown, the BL can be identified on the BL after the myofibers' cytoplasm and plasma membrane have been removed (33, 37, 44). We studied binding of antibodies to synaptic BL in damaged rat external intercostal muscles.

The external intercostals are thin, flat muscles that run from rib to rib. A nerve trunk courses along the middle of each muscle, parallel to and midway between the ribs; most of the neuromuscular junctions lie in a narrow band close to the nerve. Rats were anesthetized with pentobarbital (5 mg/100 g injected i.p.), and their intercostals were exposed. Pointed scissors were used to make 2-cm long cuts in the muscle, parallel to and ~1 mm on either side of the nerve trunk. Three adjacent muscles were injured in this way. The body wall was then closed with sutures, and the skin with surgical staples. Later, the damaged external intercostals and the underlying, adherent internal intercostals were cut out together, and frozen, sectioned, and stained as described above.

2 d after surgery, the myofibers had degenerated, the muscle was infiltrated with mononucleated cells (Fig. 2c; compare with normal muscle in Fig. 2a), and endplates no longer stained with rhodamine-bungarotoxin (see Results). Staining with antiserum to basement membrane collagen from muscle revealed, however,
FIGURE 2 Basement membrane survives injury-induced degeneration of muscle fibers. (a and b) Cross section of normal intercostal muscle, stained with antisem to muscle basement membrane collagen and fluorescein-second antibody, and photographed with phase (a) or fluorescein (b) optics. (c and d) Cross section of an intercostal muscle that had been injured 2 d earlier, as described in Materials and Methods, stained as in a and b. Myofibers have degenerated (c, phase optics) but sheaths of basement membrane persist (d, fluorescein optics). All parts are at the same magnification; bar, 50 μm.

that the sheaths of basement membrane persisted (Fig. 2d; compare with normal muscle in Fig. 2b). 4 d after surgery, myofibers had regenerated within many of the basement membrane sheaths, and AChRs were once again demonstrable (not shown). Therefore, we froze muscles 2 d after damage for use in subsequent immunohistochemical experiments.

CHEMICAL EXTRACTION OF MYOFIBERS: McCollester (36) devised a procedure for extracting the contractile apparatus of lightly homogenized muscle with low salt solutions, leaving behind short, hollow tubes of sarcolemma—i.e., plasma membrane plus basement membrane. The plasma membrane can then be selectively extracted with detergents (11, 32). Our modification of McCollester's procedure disrupts the tissue minimally, maintains the morphological integrity of the BL, and eliminates steps in which muscle is aged or heated.

Strips of rat diaphragm, 5 mm wide, were frozen on the stage of a Mickle Gel Slicer (Brinkmann Instruments, Inc., Westbury, N. Y.) under dry ice, and cut into 100- to 300-μm thick cross sections. The sections were incubated at room temperature for 30 min in 50 mM CaCl₂ (Fig. 3a), for 1 h in four changes of 25 mM NaCl, 2.5 mM DL-histidine, and for 1 h in distilled water that had been brought to pH 7–8 with NaOH. When the sections were suspended in water, the cytoplasm was expelled from the cut ends of the muscle fiber segments (Fig. 3b). After incubation in water, the sections were shaken to remove adherent cytoplasm. Left behind is a “honeycomb” of sarcolemmal tubes, held together by the largely intact connective tissue framework of the muscle (Fig. 3c). To extract components of the plasma membrane, the honeycombs were incubated for 1 h in detergent solutions—Triton X-100, Lubrol PX (Sigma Chemical Co.), NP40 (Particle Data Inc., Elmhurst, Ill.), or sodium deoxycholate (Sigma Chemical Co.), all 1% in 50 mM NaCl, 1 mM EGTA, 20 mM Tris, pH 7.4—then washed with water and dried on glass slides for immunohistochemical study.

RESULTS
Choice of Immunogens and Production of Antisera

Synaptic BL has not been isolated, and therefore could not be used as immunogen. Instead, we obtained antisera to structures or molecules that could be isolated, and sought antibodies in these sera that would cross-react with synaptic but not extrasynaptic BL. First, we raised antisera to an isolated BL, anterior lens capsule, because there is wide immunological cross-reactivity among BLs (28, 40). Second, because BLs are collagenous (28, 46), we raised antisera to collagens prepared from muscle and other tissues by pepsin treatment and salt fractionation. Finally, we used antisera to AChE because part of the AChE in muscle is associated with synaptic BL (6, 23, 37). AChE has not been purified from muscle, so we used antisera to AChE from bovine brain and Electrophorus electric organ (20).

The preparation of immunogens and of antisera is described in Materials and Methods. Preliminary tests in which a solid-phase radioimmunoassay was used (24) showed that all antisera contained antibodies directed against the appropriate immunogen. Sera were then tested immunohistochemically.
Antiserum to Lens Capsule

Unfixed sections of muscle were incubated with antiserum and then with fluorescein-conjugated second antibody (goat anti-rabbit IgG). To identify endplates, we also incubated each section with rhodamine-α-bungarotoxin which binds specifically to AChRs clustered in the postsynaptic membrane (2, 41). This immunofluorescence technique showed that anti-lens capsule stained neuromuscular junctions (Figs. 4, and 5a and b), but preimmune serum did not (Fig. 5c and d). Anti-lens capsule also stained the walls of large blood vessels (but not capillaries) and the perineurial sheaths that enwrap intramuscular nerve bundles (Fig. 4). However, extrasynaptic portions of the muscle fiber surface stained very lightly and often not at all. Because antiserum to lens capsule bound selectively to synaptic areas of the muscle fibers' surface, we undertook experiments to determine whether the antigens being stained were in fact associated with BL.

Portions of three cells—motor neuron, Schwann cell, and muscle fiber—contribute to the neuromuscular junction. The possibility that antigen is confined to nerve terminal or Schwann cell processes was tested by examining denervated muscle. A few days after axotomy, nerve terminals degenerate; during the following month, Schwann cells retract from the endplates, leaving the original synaptic surface "naked" (38). AChRs, however, remain concentrated at original synaptic sites long after denervation (18), so rhodamine-bungarotoxin can be used to identify denervated endplates. Antisera to lens capsule stained endplates in muscles denervated for 7 wk (Fig. 5e and f) with an
FIGURE 4 (a) A frozen section of rat diaphragm, stained with cresyl violet to show cross-sectioned muscle fibers, intramuscular nerve branches (N), and a blood vessel (BV). Cresyl violet fluoresces red, and the micrograph in a was taken with rhodamine optics. The section was also incubated with anti-lens capsule and then with a mixture of fluorescein-second antibody and rhodamine-bungarotoxin. Fluorescein optics (b) shows that anti-lens capsule stains blood vessels, perineurial sheaths, and a few small patches on the muscle fiber surface. These patches can be identified as neuromuscular junctions because they also stain with rhodamine-bungarotoxin (a). However, cresyl violet obscures rhodamine fluorescence (as well as reducing specific and increasing nonspecific immunofluorescence); therefore, it was not used routinely. Micrographs of an endplate stained with anti-lens capsule and rhodamine-bungarotoxin but not cresyl violet are shown in Fig. 5. Bar, 100 µm.
FIGURE 5 Anti-lens capsule but not normal serum stains a synaptic antigen that persists in denervated muscle. Sections incubated with serum, fluorescein-second antibody, and rhodamine-bungarotoxin were photographed with fluorescein (a, c, and e) or rhodamine (b, d, and f) optics. (a and b) Normal muscle (diaphragm) incubated with anti-lens capsule. (c and d) Normal muscle incubated with preimmune serum. (e and f) 7-wk-denervated diaphragm incubated with anti-lens capsule. All micrographs at the same magnification; bar in f, 20 μm.

intensity not detectably less than that at innervated junctions in normal muscle. Thus, most or all of the synaptic antigen to which anti-lens capsule binds is associated with the surface of the muscle fiber.

Next, we tested the possibility that antibody binds exclusively to elements such as the postsynaptic densities (7) that lie beneath the plasma membrane. Anti-lens capsule and peroxidase-conjugated second antibody were applied to intact muscles, so that only external sites would be accessible to immunoreagents; muscles were then washed, fixed, and stained for peroxidase. Stained endplates were visible on fibers teased from the muscle (Fig. 6a), and electron microscopy (Fig. 6b) showed deposits of reaction product in the BL that runs through the synaptic cleft and extends into the junctional folds. From these results, we conclude that at least some antigenic sites are present on the external cell surface.

The presence of reaction product in the BL of the synaptic cleft (Fig. 6b) suggests that the antigen is in the BL, but it does not provide strong evidence on this point. One is limited not only by the possibility that the product may diffuse before it precipitates but also by the molecular dimensions of the immunoreagents. An antibody molecule (IgG) is 7–12 nm in length, and ferritin, the highest resolution electron-dense label available, is 11 nm long (51). The electron-transparent gap between plasma membrane and BL is 10 nm wide at most; therefore, antibodies directed against integral components of the postsynaptic membrane might fail to stain synaptic BL.

To circumvent this limitation, we used two independent methods to physically separate plasma membrane from BL. In the first, we damaged muscle fibers; their cytoplasm and plasma membrane degenerated and were phagocytized, but their sheaths of basement membrane were left behind (see Materials and Methods and Fig. 2). Previous ultrastructural studies have documented the loss of plasma membrane from the sheaths (10, 44, 45, 49). To test for removal of postsynaptic membrane, we incubated damaged muscles with rhodamine-bungarotoxin to label AChRs. The AChR is an integral membrane protein and the major protein of the postsynaptic membrane (16); its loss must therefore indicate removal of the bulk of the postsynaptic membrane.

2 d after muscle damage, most endplates were depleted of histochemically demonstrable AChR,
but they stained with anti-lens capsule as intensely as did normal endplates (Fig. 7). Thus, the synaptic antigen survived removal of a large fraction of the postsynaptic plasma membrane. In a few endplates, patches of AChRs remained, presumably as a result of incomplete phagocytosis. However, there was no tendency for endplates rich in receptors, or receptor-rich patches within endplates, to be stained more intensely by antibody than endplates or patches with no detectable AChRs. The lack of correlation between antibody and toxin staining provides further evidence that the synaptic antigen is not associated with plasma membrane.

The other method for separating plasma membrane from BL involves removing the cytoplasm from short muscle fiber segments (see Materials and Methods and Fig. 3), and then treating the remaining sheaths with detergent to remove plasma membrane but not BL (11, 32). Endplates survived removal of the bulk of the contractile apparatus, and they could be detected by immunofluorescence (Fig. 8a), toxin binding (Fig. 8b), or the histochemical stain for cholinesterase (Fig. 3d). Subsequent incubation with the detergent Triton X-100 removed histochemically detectable AChR, but staining by anti-lens capsule was unchanged (Fig. 8c and d). As in damaged muscle, AChR was detectable at some detergent-extracted endplates, but anti-lens capsule serum stained AChR-rich and AChR-poor endplates equally well. Similar results were obtained with each of three other detergents tested, Lubrol, NP-40, and sodium deoxycholate, although extraction of AChR was more complete with deoxycholate than with the others. Finally, in one experiment, we prepared detergent-extracted sarcolemmal tubes from muscles that had been denervated 1 wk earlier. In this preparation, both pre- and postsynaptic membranes were removed, but anti-lens capsule still stained endplates. Thus, both methods for separating plasma membrane from BL led to the same conclusion: whether plasma membrane was removed in vivo after injury, or in vitro with
detergents, the synaptic antigen(s) to which anti-lens capsule binds retained an association with BL.

**Antiserum to Collagens**

We tested antisera to four collagen preparations. Two were basement membrane collagens: a pepsin extract of lens capsule that contains collagen type IV (28, 29, 46), and a collagen from muscle that has been called AB (5) or type V (4). The other two were interstitial collagens: type I from tendon and type III from skin. As noted above, each serum contained antibodies to the immunizing collagen; we therefore refer to the sera as anti-collagens, although they may also contain antibodies to noncollagenous contaminants.

Antiserum to basement membrane collagen from muscle stained both synaptic and extrasynaptic portions of the muscle fibers’ surface, as well as axons, perineurial sheaths, and blood vessels, including capillaries (Figs. 2, and 9a and b). After adsorption of this serum with endplate-free regions of skeletal muscle, however, the staining pattern was dramatically different: staining of nerves, blood vessels, and extrasynaptic regions of muscle fibers was completely abolished, while staining of neuromuscular junctions persisted (Fig. 9c and d). Apparently, the muscle adsorbed antibodies to extrasynaptic and/or common antigens but spared antibodies to synapse-specific antigens.

We used the strategy detailed in the previous section to determine whether the synaptic antigen to which the adsorbed sera bound is associated with BL. Staining persisted in muscles denervated for 7 wk, endplates were stained when antiserum was applied to intact muscles, and staining survived removal of plasma membrane, either in vivo after injury, or in vitro with detergents. In short, the antigen revealed by adsorbed anti-basement membrane collagen serum, like that revealed by anti-lens capsule, is closely associated with synaptic BL.

Antiserum to lens capsule collagen stained muscle in a pattern similar to that seen with anti-basement membrane collagen from muscle: unadsorbed sera stained both synaptic and extrasynaptic portions of the muscle fiber’s surface, as well as nerves and blood vessels, while serum adsorbed with endplate-free regions of muscle stained only neuromuscular junctions. Synapse-specific staining by this adsorbed antiserum survived denervation or removal of plasma membrane, and thus also revealed an antigen associated with synaptic BL.

Antiserum to collagens type I (pepsin- or acid-extracted) and type III did not bind selectively to synaptic BL. Like Duance et al. (14), we found that these sera stained perimysial and epimysial connective tissue intensely. The muscle fiber surface stained lightly. Staining was slightly more intense in extrasynaptic than in synaptic areas, but this result is difficult to interpret: extrasynaptic, but not synaptic BL, is overlaid with a collagen-containing layer of fibrils (44), to which anti-collagens might bind. Adsorption of anti-collagen type I or anti-collagen type III sera with endplate-free segments of muscle reduced staining generally, without revealing any synapse-specific antibodies.

**Antiserum to AChE**

Antiserum to AChE from bovine brain stained the neuromuscular junction (Fig. 10). Endplates
also stained in muscles denervated for 7 wk; although AChE is lost from endplates after denervation, a considerable fraction (21), including some in synaptic BL (37), survives. By the criteria outlined above, at least some of the antigen was associated with synaptic BL: antigenic sites were exposed on the surface of the muscle fiber (Fig. 10c) and persisted after plasma membrane was removed, either in vitro (Fig. 10d) or in vivo. In every instance, the distribution of bound antibody was similar to that of reaction product from a histochemical stain for cholinesterase activity (27) (e.g., compare Figs. 3d and 10d).

An antiserum to AChE from Electrophorus electric organ did not stain rat neuromuscular junctions.

Distinctions among Antigens of Synaptic BL

Results presented so far show that four sera contain antibodies that bind selectively to synaptic BL. Although the sera were prepared against different immunogens, it seemed possible that the synaptic-specific antibodies in each of them recognize the same antigen(s). However, the following observations demonstrate that this is not the case.

First, antigens recognized by the antisera were differentially sensitive to collagenase, which digests portions of the synaptic BL but not the postsynaptic membrane (6, 23). After incubation of unfixed sections with collagenase, neuromuscular junctions were stained by anti-lens capsule (and rhodamine-bungarotoxin) but not by anti-AChE, adsorbed (with endplate-free connective tissue from muscle) anti-muscle basement membrane collagen, or adsorbed anti-lens capsule collagen. Thus, anti-lens capsule reveals a determinant that the other sera do not.

The same conclusion was reached from adsorption experiments. Adsorption with kidney, heart, or endplate-free connective tissue from muscle removed synapse-specific antibodies from anti-lens capsule, but not from anti-AChE, anti-muscle basement membrane collagen, or anti-lens capsule collagen.

Finally, although all four sera distinguish synaptic from extrasynaptic BL, their fluorescent staining patterns are not identical. The distribution of synaptic antigens recognized by anti-lens capsule (Fig. 5a and b), anti-AChE (Fig. 11a and b), and anti-lens capsule collagen parallels that of AChRs almost exactly. These antigens are confined to (or highly concentrated in) the synaptic cleft. On the other hand, the synaptic antigens revealed by anti-muscle basement membrane collagen extend between and slightly beyond patches...
Adsorption with connective tissue from endplate-free regions of skeletal muscle reveals the presence of synapse-specific antibodies in anti-basement membrane collagen from muscle. Sections stained with antiserum, fluorescein-second antibody, and rhodamine-bungarotoxin were photographed to show fluorescein (a and c) or rhodamine (b and d). (a and b) Unfractionated serum stains both synaptic and extrasynaptic portions of the muscle fiber's surface, as well as axonal and perineurial sheaths in the nerves (N). (c and d) After adsorption, staining is confined to the neuromuscular junction. All parts at the same magnification. Bar in d, 50 μm.

FIGURE 9

of toxin-stained postsynaptic membrane (Fig. 11 c and d). Thus, antibodies in anti-muscle basement membrane collagen recognize a synaptic determinant that is not recognized by the other sera.

Taken together, these results (summarized in Table I) define at least three different determinants that are present in synaptic but not extrasynaptic BL. Anti-lens capsule stains a determinant that is not recognized by anti-AChE or adsorbed anti-lens capsule collagen, and both (or all) of these determinants are distinguishable from that recognized by adsorbed anti-muscle basement membrane collagen.

DISCUSSION

We have documented the existence of molecular differences between synaptic and extrasynaptic portions of the muscle fiber's BL. This demonstration had two parts. First, we found antibodies that distinguish synaptic regions of the muscle fiber. Then, we showed that these antibodies bound to the BL of the synaptic cleft rather than to other components of the neuromuscular junction.

To obtain synapse-specific antibodies, the most direct procedure would have been to use purified synaptic BL from skeletal muscle as immunogen. Unfortunately, this approach was infeasible. The fraction of muscle fiber BL that is synaptic is extremely small (~0.1%), and muscle fiber BL is itself only a small portion of the muscle's connective tissue. Methods are not available for separating synaptic from extrasynaptic BL, or BL from the bulk of muscle connective tissue. Instead, we used immunogens that we thought might be related to components of synaptic BL. Among the antisera tested, four—anti-lens capsule, anti-lens capsule collagen, anti-muscle basement membrane collagen, and anti-AChE from brain—stained the synapse specifically. Although we cannot rule out the possibility that antigens present along the entire muscle fiber surface are masked by molecules that are present only extrasynaptically, we pre-
FIGURE 10 Anti-AChE stains synaptic BL. (a and b) Section stained with anti-AChE, fluorescein-second antibody, and rhodamine-bungarotoxin, and photographed to show fluorescein (a) or rhodamine (b). Staining is confined to the neuromuscular junction. (c) Endplate from a muscle incubated with anti-AChE and peroxidase-second antibody, then fixed, and stained for peroxidase. (d) Anti-AChE- (and fluorescein-second antibody) stained endplate on a basement membrane sheath prepared by extraction of muscle cytoplasm and detergent extraction of plasma membrane (as described in Materials and Methods). Bar, 50 μm in a and b, 10 μm in c, and 30 μm in d.

Assume that the antibodies recognize synapse-specific antigens.

To establish that the antibodies recognized antigens closely associated with synaptic BL, we showed that antigens were on the external cell surface and that they survived the loss of all components of the neuromuscular junction except BL. Immunohistochemically demonstrable antigen persisted after removal of nerve terminals, Schwann cells (by prolonged denervation), and plasma membrane (either by injury-induced degeneration or by extraction with detergents). In addition, synapse-specific staining by three of the four antisera was abolished by pretreatment of sections with collagenase, an enzyme that removes part of the synaptic BL but spares the postsynaptic membrane. Thus, the synaptic antigens that these antibodies recognize are contained in or connected to the BL of the synaptic cleft. The precise nature of the association remains to be determined, and the possibility that the antigens are attached to plasma membrane as well as to BL is not excluded by our results.

Immunohistochemical staining patterns, differential susceptibility of antigens to collagenase, and adsorption experiments (Table I) showed that the antibodies recognize at least three different determinants in synaptic BL, and that the determinants reside on at least two different molecules. Although rigorous proof is lacking, we presume that anti-brain AChE binds to the catalytic subunit of AChE in synaptic BL; Adamson (1) has shown that AChE from mouse brain and that from mouse muscle are immunologically related. We have, so far, no clues as to the identity of the molecules to which anti-lens capsule, anti-lens capsule collagen, and anti-basement membrane collagen from mus-
TABLE 1
Distinctions among Antigens of Synaptic BL

| Antiserum                          | Adsorbed anti-muscle basement membrane collagen* | Adsorbed anti-lens capsule collagen* | Anti-AChE |
|------------------------------------|----------------------------------------------------|-------------------------------------|-----------|
| Extends into perijunctional region$§ | -                                                  | +                                   | -         |
| After adsorption by heart or kidney| -                                                  | +                                   | +         |
| After treatment of section with collagenase§ | +                                                  | -                                   | -         |

* Sera adsorbed with endplate-free connective tissue from muscle (see Materials and Methods).
$See Fig. 11.
§ Sections were incubated with 0.1 mg/ml collagenase (type CSPLA, Worthington Biochemical Corp., Freehold, N. J.) in 150 mM NaCl, 5 mM CaCl2, 20 mM Tris, pH 7.4, at room temperature for 16 h. Anti-lens capsule also stained neuromuscular junctions in sections that had been incubated with 0.5 mg/ml collagenase for 16 h.

...cle bind. In fact, we have no strong reason to believe that the molecules to which antibodies in the anti-collagen sera bind are actually collagenous; we cannot rule out the presence of small but possibly immunogenic amounts of contaminants.

In addition to the synaptic antigens defined by the antiseras described here, it should be possible to identify antigens that are common to synaptic and extrasynaptic BL. Antisera to collagens type I and III (described here) and to other proteins of the connective tissue matrix (unpublished results) prefer extrasynaptic to synaptic areas but, as we have noted, these results are difficult to interpret because extrasynaptic BL is associated with several connective tissue elements that cannot be resolved in the light microscope and that are excluded from the synaptic cleft. Shared antigens are also difficult to demonstrate, because the rabbit antiseras that we are using contain mixtures of antibodies, not all of which can be resolved by adsorption. This difficulty could be overcome by the use of monoclonal antibodies.

Identification of antigens in synaptic BL, and of molecular differences between synaptic and extrasynaptic BL, represents a first step in understanding the structure, function, and development of synaptic cleft material at the neuromuscular junction. In nonneural systems, cells have been shown to adhere to and interact with both collagenous and noncollagenous components of BLs (e.g., 17, 25, 39). The possibility that the antigens we have identified in synaptic BL are recognized by and/or trigger the differentiation of nerve terminals or muscle cells during regeneration of neuromuscular junctions (8, 44) remains to be tested. Also, we do not yet know which cells produce the synaptic antigens. AChE at the neuromuscular junction is presumed to be synthesized by the muscle fiber (31, 50), but the nerve or Schwann cell may also contribute synaptic enzyme (13, 43). The fact that synaptic but not extrasynaptic regions of the muscle fiber's BL share at least two determinants with an epithelial BL, anterior lens capsule, suggests the possibility that the nerve terminal, an ectodermal derivative, may contribute to the formation of synaptic BL during development. In this context, it is interesting that electron microscopy reveals the presence of electron-dense material in the synaptic cleft of developing neuromuscular junctions, at a time before BL appears over extrasynaptic portions of the developing muscle fibers' surface (3, 30, 33).

We are grateful to Todd Margolis, who collaborated in some of the experiments reported here. We thank Alvin Greenberg, Anthony Trevor, Heinz Schevenstuhl, and Dow Michaeli for generous gifts of antiseras, Peter Ravdin and Darwin Berg for rhodamine-bungarotoxin, and Michael Dennis, Louise Evans, Dan Riley, and Susan Schaeffer for help and advice.

This work was supported by grants from the National Institutes of Health, the Muscular Dystrophy Association, and the National Science Foundation to Z. W. Hall, and a fellowship from the Muscular Dystrophy Association to J. R. Sanes.

Received for publication 4 May 1979, and in revised form 13 July 1979.
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