A Novel 87,000-M_r Protein Associated with Acetylcholine Receptors in *Torpedo* Electric Organ and Vertebrate Skeletal Muscle

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Abstract. To identify proteins associated with nicotinic postsynaptic membranes, mAbs have been prepared to proteins extracted by alkaline pH or lithium diiodosalicylate from acetylcholine receptor-rich (AChR) membranes of *Torpedo* electric organ. Antibodies were obtained that recognized two novel proteins of 87,000 M_r and a 210,000:220,000 doublet as well as previously described proteins of 43,000 M_r, 58,000 (51,000 in our gel system), 270,000, and 37,000 (calelectrin). The 87-kD protein copurified with acetylcholine receptors and with 43- and 51-kD proteins during equilibrium centrifugation on continuous sucrose gradients, whereas a large fraction of the 210/220-kD protein was separated from AChRs. The 87-kD protein remained associated with receptors and 43-kD protein during velocity sedimentation through shallow sucrose gradients, a procedure that separated a significant amount of 51-kD protein from AChRs. The 87- and 270-kD proteins were cleaved by Ca^{++}-activated proteases present in crude preparations and also in highly purified postsynaptic membranes. With the exception of anti-37-kD antibodies, some of the monoclonals raised against *Torpedo* proteins also recognized determinants in frozen sections of chick and/or rat skeletal muscle fibers and in permeabilized chick myotubes grown in vitro. Anti-87-kD sites were concentrated at chick and rat endplates, but the antibodies also recognized determinants present at lower site density in the extrasynaptic membrane. Anti-210:220-kD labeled chick endplates, but studies of neuron-myotube cocultures showed that this antigen was located on neurites rather than the postsynaptic membrane. As reported in other species, 43-kD determinants were restricted to chick endplates and anti-51-kD and anti-270-kD labeled extrasynaptic as well as synaptic membranes. None of the cross reacting antibodies recognized determinants on intact (unpermeabilized) myotubes, so the antigens must be located on the cytoplasmic aspect of the surface membrane. The role that each intracellular determinant plays in AChR immobilization at developing and mature endplates remains to be investigated.

NICOTINIC acetylcholine receptors (AChRs)[1] are concentrated at a surface density of 10,000 AChR/μm^2 in postsynaptic membranes of vertebrate skeletal muscle endplates and in the innervated surface of *Torpedo* electroplaques (for review, see Salpeter and Loring, 1985). EM autoradiography has shown that the region of high receptor density is restricted to the upper third of the post junctional folds (Fertuck and Salpeter, 1975). The receptor density falls abruptly by a factor of 100–1,000 in the lower reaches of the secondary folds and at the margins of the neuromuscular contact. Steep receptor gradients are also characteristic of developing neuromuscular junctions in vivo and in vitro and of receptor clusters found on uninnervated myotubes. These sharp boundaries imply that the mobility of AChRs is restricted at receptor clusters. Indeed, fluorescence photobleaching measurements (Axelrod et al., 1976) have shown that the diffusion coefficient of clustered receptors ($\sim$10^{-12}

cm^2/s) is approximately two orders of magnitude less than the coefficient measured at extracluster regions. It is likely that interactions between AChRs and components of the cytoskeleton restrict receptor mobility. Tank et al. (1982) found that AChRs are 1,000-fold more mobile within membrane blebs induced on isolated rat muscle fibers than are receptors in the adjacent attached plasma membrane. The blebs appeared devoid of organized cytoskeletal components under phase-contrast illumination.

Postsynaptic membranes can be isolated from *Torpedo* electric organs and purified to the extent that AChRs constitute >50% of the protein (for review, see Popot and Changeux, 1984). The rotational and translational mobility of AChRs in these isolated membranes are severely restricted, but the mobility of AChRs is increased after the membranes are exposed to high pH (Cartaud et al., 1981; Rousselet et al., 1981, 1982), a procedure that extracts several peripheral proteins without disrupting AChR ligand binding or ion transport (Neubig et al., 1979). Isolated *Torpedo* postsynaptic membranes are devoid of extracellular matrix and or-

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; LIS, lithium diiodosalicylate; pI, isoelectric point; R-BgTx, rhodamine α bungarotoxin.

ganized cytoskeletal filaments (Bridgman et al., 1987), so interactions between AChRs and peripheral membrane proteins may be sufficient for receptor immobilization.

Considering the many homologies between electroplaques and vertebrate muscle, it seems likely that further studies of *Torpedo* AChR-associated proteins will shed light on mechanisms that regulate receptor distribution at vertebrate endplates. A great deal is already known about the predominant nonreceptor protein of 43,000 M_r (43-kD protein) that is present in highly purified *Torpedo* postsynaptic membranes. It is a peripheral protein that is precisely colocalized with AChRs on the cytoplasmic aspect of the electroplaque membrane (Sealock et al., 1984). An immunologically related determinant has been detected at the light microscopic level at vertebrate motor endplates (Froehner et al., 1981; Froehner, 1984) and at clusters of AChRs on cultured *Xenopus* and rat muscle cells (Peng and Froehner, 1985; Burden et al., 1985; Bloch and Froehner, 1987). The amino acid sequence of *Torpedo* 43-kD protein, determined by direct protein sequencing (Carr et al., 1987) and cDNA cloning (Frail et al., 1987), indicates that it is a novel protein unrelated to known cytoskeletal components or to proteins such as erythrocyte band 4.1 or ankyrin that link cytoskeletal elements to integral membrane proteins. The deduced amino acid sequence of the mouse muscle homologue (Frail et al., 1988) has 70% identity with that of *Torpedo* electric organ.

In addition to the 43-kD protein, less abundant proteins are also removed from isolated *Torpedo* postsynaptic membranes after exposure to alkaline pH or the chaotropic agent lithium diiodosalicylate (LIS). Any or all of these proteins might be required for receptor immobilization. mAbs have been used to demonstrate that a 58,000-M_r protein extracted from *Torpedo* membranes is located on the innervated surface of electrocyte membranes, vertebrate endplates and at receptor clusters on cultured myotubes (Froehner et al., 1987). In addition, antibodies raised against a ∼300-kD protein, that can be extracted from *Torpedo* membranes with LIS, also binds to the cytoplasmic surface of vertebrate endplates (Woodruff et al., 1987). With the aim of studying receptor mobility at adult and embryonic synapses, we raised mAbs to proteins in alkaline and LIS extracts to identify additional receptor associated peripheral proteins. Here, we describe a novel receptor-associated protein of 87,000 M_r. Initial biochemical characterization of this antigen in *Torpedo* membranes as well as immunofluorescence studies of vertebrate muscle are compared with the properties of the previously described receptor-associated proteins.

Materials and Methods

Preparation of Nicotinic Postsynaptic Membranes and Extraction of Peripheral Proteins

AChR-rich membranes were isolated from the electric organs of *T. californica* and purified by discontinuous and linear sucrose gradients according to the procedure of Sobel et al. (1977) with the modifications described by Pedersen et al. (1986). The final membrane suspensions (in 38% sucrose-0.02% NaN₃) were frozen in liquid nitrogen and stored at −80°C under argon. These membranes contained 1-2 nmoles ACh binding sites per mg protein, as measured by a direct [³H]-ACh binding assay (Boyd and Cohen, 1980). Nonreceptor, peripheral membrane proteins were extracted from membranes by exposure to pH 11 (Neubig et al., 1979) or 10 mM LIS (Porter and Froehner, 1983) as described (Carr et al., 1987) except that membranes

brane suspensions contained 7 mg protein/ml. These extracts were used to immunize mice (see below).

In some experiments AChR-rich membranes were further purified by velocity sedimentation according to the protocol of Jeng et al. (1981). To release peripheral proteins that are bound in a Ca⁺⁺-dependent manner, membranes were incubated in 3 mM EGTA, 1 mM EDTA, 10 mM Tris, pH 8.1 for 1 h at 4°C followed by centrifugation. When membranes were isolated afer homogenization of electric organ in a buffer free of divalent cations, the homogenization buffer contained 5 mM EDTA, 5 mM EGTA, 10 mM NaPᵢ, pH 7.5, as well as the protease inhibitors described by Pedersen et al. (1986).

Production and Screening of mAbs

Balb/C mice were injected intraperitoneally with 80 μg of LIS extract or combination of 50 μg LIS and 70 μg alkaline extracts emulsified in complete Freund's adjuvant (total volume, 0.4 ml). Injections were repeated after 2-3 wk using extract in incomplete Freund's adjuvant and again at 4-5 wk in PBS. At 49 and 50 d after the initial injection, 120 μg of extract were injected in PBS, and the spleens were removed 3 d after the last injection. Spleen cells were fused either with SP2/0 myeloma cells as described (Bridgman et al., 1987) or with Fox-NY cells and then plated in 10 96-well microtiter plates and selected using adenine-aminopterin-thymidine medium (Taggart and Samloff, 1983). Hybridoma supernatants were screened initially by ELISA using alkaline phosphatase-coupled secondary antibodies according to the protocol of Bridgman et al., (1987) except that wells of microtiter plates were coated with 0.4 μg of LIS and/or alkaline extracts. Supernatants from positive wells were further screened by immunoblotting against AChR-rich membranes separated by microslab gel electrophoresis (see below).

The subclass of mAbs was determined by enzyme immunoassay using a double antibody detection system (HyClone Laboratories, Logan, UT). Ascites fluid was produced as previously described (Bridgman et al., 1987).

Gel Electrophoresis and Immunoblotting

For one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the method of Laemmli (1970) was used with modifications (Carr et al., 1987) to optimize resolution of 43-kD protein from AChR α-subunit, creatine kinase, and actin. The proteins used as molecular mass markers were human erythrocyte spectrin (220:240 kD), myosin (205 kD), β-galactosidase (116 kD), phosphorylase b (97 kD), BSA (66 kD), ovalbumin (45 kD) and carbonic anhydrase (29 kD). Two-dimensional gel electrophoresis was performed essentially by the protocol of O'Farrell (1975) with the changes noted (Carr et al., 1987). The gels were stained with Coomassie blue.

In the initial immunoblot experiments, membranes were subjected to one- and two-dimensional gel electrophoresis, transferred to nitrocellulose and blotted with antibodies (Bridgman et al., 1987). In later experiments, proteins were transferred to polyvinylidene difluoride membranes (Immobilon transfer membrane; Millipore Continental Water Systems, Bedford, MA) in 25 mM Tris, 192 mM glycine, 15% methanol. After transfer, the replica was incubated in 5% BSA in blot buffer for 1 h at 37°C to block nonspecific binding of antibody, and the binding of primary antibodies was detected as for transfer to nitrocellulose paper.

Assay for Antigens in Sucrose Gradient Fractions

Antigen distribution was quantified by ELISA. Because antigens were likely to be present within sealed membrane vesicles, fractions (30 μl) were preincubated with 10 mM LIS for 30 min at room temperature and then diluted into 2 ml of carbonate bicarbonate (pH 9.6) coupling buffer. Aliquots of 0.1 ml were then added to the wells of microtiter plates. After overnight incubation, wells were reacted with primary and alkaline phosphatase-conjugated secondary antibodies as described (Bridgman et al., 1987). LIS (10 mM) was chosen on the basis of preliminary experiments where membrane fractions were exposed to varying concentrations of Triton X-100, LIS, SDS, or SDS and LIS before adsorption in microtiter wells. For all antigens, exposure to 10 mM LIS resulted in maximal ELISA signals, and for each antigen conditions were identified that made the assay linear as a function of antigen concentration. Antigen binding to fractions from the continuous sucrose gradient was measured for aliquots of constant volume (7 μl).

Immunofluorescence

Frozen sections were prepared from embryonic day 18 chick anterior latissi-

mus dorsi muscles, from endplate regions of adult rat diaphragms (Sanes and Hall, 1979) and from *Torpedo* electric organ. The muscles were embedded in Tissue-Tek (Miles Scientific Div., Naperville, IL) and frozen in liquid nitrogen. 10-μm sections were cut at $-18°C$, collected on gelatin-coated glass slides and stored at $-20°C$ until used. Dissected columns of *Torpedo* electrocytes were rapidly frozen and sectioned at $-27°C$.

Sections were preincubated for 30 min at room temperature with 10% normal goat serum plus 4% BSA in PBS and washed briefly with PBS before incubation for 2 h with primary antibodies at appropriate dilution in the same buffer. Sections were rinsed three times with PBS before addition of a solution containing fluoresceinated secondary antibody (a mixture of rabbit anti–mouse IgG and IgM, 1:200 dilution; Cappel Laboratories, Malvern, PA). Rhodamine conjugated α bungarotoxin (R-BgTX) was included at this stage to identify motor endplates and electroplacques innervated faces. After 1 h of incubation, sections were washed with PBS, dried, and mounted in 90% glycerol, 10% PBS, containing paraphenylenediamine (1 mg/ml), pH 8.0. Sections were examined with epifluorescence illumination and viewed through a 40× (NA = 1.3) or a 63× (NA = 1.4) objective. Filters (E. Leitz, Rockleigh, NJ) were used to visualize rhodamine (excitation, 530–560; emission >580 nm) and fluorescein (excitation, 450–490 nm; emission: 515–530 nm). Sections were photographed with Tri-X film (Eastman Kodak Co., Rochester, NY).

Myogenic cells were dissected from 11-d-old chick embryo pectoral muscle and grown in culture on collagen-coated glass coverslips as previously described (Fischbach, 1972; Role and Fischbach, 1987). In some experiments, the cultures were seeded with neurons dissociated from E8 or E9 ciliary ganglia. The cells were maintained in MEM supplemented with 10% (vol/vol) horse serum and 2% chick embryo extract. After 7 d in vitro, the cells were labeled with R-BgTx for 1 h, rinsed in serum-free MEM, and then permeabilized by fixation in 95% ethanol at $-20°C$ for 10 min. Primary and secondary antibodies were added as described above for tissue sections, except that blocking buffer contained only 2% BSA and 5% normal goat serum.

Results

Immunoblot Analysis of mAbs

Polypeptides released from *Torpedo* postsynaptic membranes by extraction with 10 mM LIS or alkaline pH range between 37 kD to >205 kD (Fig. 1). The 43-kD protein is the principal band stained by Coomassie blue. In addition, two polypeptides of 49,000 M_r (49 kD) and 51,000 M_r (51 kD) were prominent in both LIS and alkaline extracts, while a polypeptide of 37,000 M_r (37 kD) was released by treatment with alkaline pH but not by 10 mM LIS.

Mice were immunized with a mixture of alkaline and LIS extracts, and hybridoma supernatants that were positive by ELISA were then screened by immunoblot of postsynaptic membranes. The following results are based on eight fusions. As judged by immunoblot, hybridomas were isolated that secreted antibodies directed against six different antigens (Fig. 2). Monoclonal 19F4a,[2] an IgG$_{2a}$, is representative of 11 hybridomas that recognize the 43-kD protein based on one dimensional (Fig. 2, lane C) and two dimensional immunoblot (Fig. 5). Monoclonal 20G4, an IgG$_1$, (Fig. 2, lane D) is one of 12 hybridomas from 5 fusions that recognize a polypeptide of 51,000 M_r. An antibody (mAb 1351E) obtained from Dr. S. Froehner (Dartmouth Medical School) that recognized a 58-kD protein in his gel system recognized the same band as mAb 20G4. Based on the isoelectric properties of the antigen (see Fig. 5), we conclude that mAbs 20G4 and 1351E recognize the same antigen. Monoclonal 20H2, an IgG$_1$, is one of three hybridomas from three fusions that recognized polypeptides of 87, 74, and 65 kD (Fig.

2. In our nomenclature, the first number refers to the animal, the letter is a noncommittal designation of the antigen defined by immunoblot, the following digit refers to the clone, and the last letter indicates the subclone.
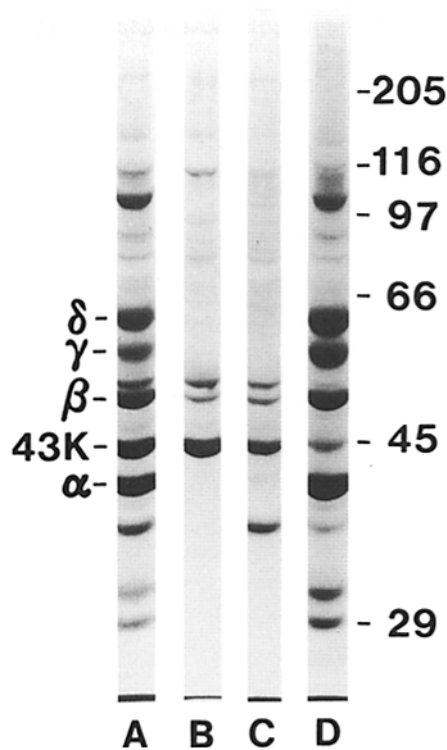

Figure 1. Polypeptides of *Torpedo* postsynaptic membranes as determined by SDS-PAGE (Coomassie blue stain). (Lane *A*) AChR-rich membranes (18 μg) containing 1.9 nmoles [^{3}H]AChR sites/g protein; (lane *B*) LIS extract; (lane *C*) alkaline extract of membranes previously extracted with 10 mM LIS; (lane *D*) membranes after sequential extraction by LIS and alkaline pH. Molecular mass markers are indicated on the right; AChR subunits and 43-kD protein are identified on the left.

2, lane *E*). Monoclonal 19J1, an IgG$_{2b}$, is one of three hybridomas from two fusions that secreted antibodies against polypeptides of ~195 and 130 kD (Fig. 2, lane *F*). Monoclonal 13K2, an IgG$_1$, is one of 10 hybridomas that recognized a broad band of ~210,000 M_r (Fig. 2, lane *G*) that in some experiments appeared as a doublet of 210 and 220 kD. (In this immunoblot mAb 13K2 also recognized material at the top of the gel. However, this result was atypical and in most experiments including those using gels of 6% rather than 8% acrylamide, no such staining was observed [Fig. 3]). Monoclonal IID2 provided by S. Burden (Massachusetts Institute of Technology) against a 230:250-kD doublet described as a Schwann cell antigen (Woodruff et al., 1987) labeled the same bands as mAb 13K2 (not shown). Monoclonal 14L1 is one of two monoclonals that recognized a polypeptide of 37,000 M_r (Fig. 2, lane *H*).

Thus, antibodies were raised against three of the four abundant proteins in the extracts; the 49-kD polypeptide was the notable exception. In addition, antibodies were raised against distinctly less abundant proteins that were not readily identifiable by Coomassie blue stain.

The multiple bands recognized by monoclonals such as 20H2 and 19J1 probably resulted from proteolytic degradation. Addition of 5 mM EGTA and 5 mM EDTA to the protease inhibitors already present in the homogenization buffer (see Materials and Methods) prevented degradation of the antigens (Fig. 3). The polypeptide composition of mem-
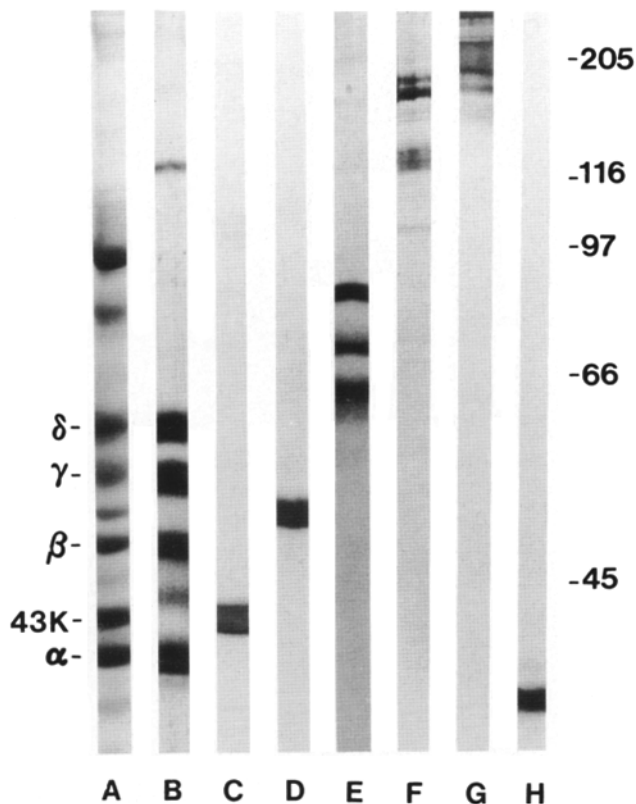
Figure 2. Immunoblots of antigens recognized by mAbs raised against alkaline plus LIS extracts of *Torpedo* postsynaptic membranes. AChR-rich membranes were resolved by SDS-PAGE and stained with Coomassie blue (lane *A*) or transferred to Immobilon (lanes *B–H*). Immobilon replicas were probed at the indicated dilutions of ascites fluid and binding of antibodies was detected by use of a horseradish peroxidase–coupled, secondary antibody; (lane *B*) anti-AChR mAb A12 (1:200); (lane *C*) mAb 19F4a (1:1,000); (lane *D*) mAb 20G4 (1:1,000); (lane *E*) mAb 20H2 (1:83); (lane *F*) mAb 19J1 (1:100); (lane *G*) mAb 13K2 (1:670); and (lane *H*) mAb 14L1 (1:500).

branes isolated in the presence of EGTA and EDTA was not identical to that of membranes prepared in the absence of chelators. The most notable difference is the absence of the 37-kD protein (Fig. 3, lanes *1* and *2*). In addition, several bands >250 kD were prominent. The increase in Coomassie-stained bands at ~85 kD was not observed consistently. The binding of mAb 19F4a (43 kD, lane *3*), 13G2 (51 kD, lane *4*) and 13K2 (210:220 kD, lane *7*) was unaltered, but mAb 20H2 now recognized a single band of 87,000 M_r (lane *5*). The 87-kD band recognized by mAb 20H2 was clearly distinct from the band of ~98,000 M_r that was intensely stained by Coomassie blue and that has been shown to be the α subunit of the Na$^+$/K$^+$-ATPase by amino acid sequence analysis (White and Cohen, 1988). With this preparation mAb 19J1 now labeled a band of 270,000 M_r intensely with weaker labeling of bands at ~210,000 and 145,000 M_r (lane *6*). A supernatant provided by Dr. S. Burden containing mAb 602 that recognizes an ~300-kD *Torpedo* antigen (Woodruff et al., 1987) labeled the same bands as did mAb 19J1 (not shown).

The 87-kD protein as well as the 270-kD protein were each extracted efficiently from *Torpedo* membranes by treatment at pH 11 (Fig. 4). Extraction of the 87-kD antigen was nearly
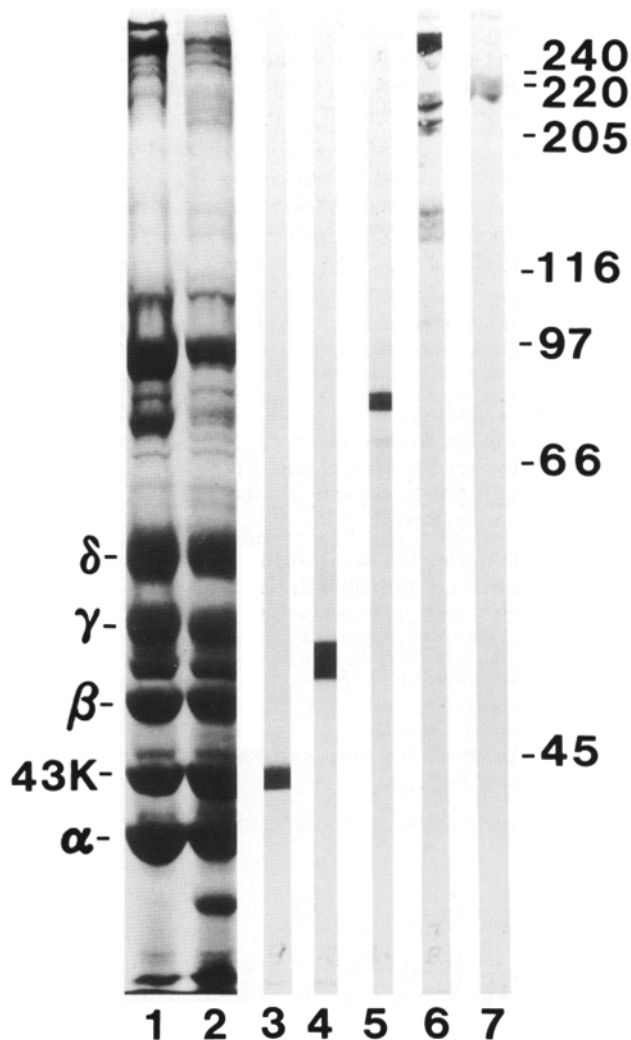

Figure 3. Immunoblots of antigens in AChR-rich membranes prepared in the presence of EGTA/EDTA. Polypeptide composition (Coomassie blue stain) of aliquots of AChR-rich membranes prepared in the presence (lane *1*) or absence (lane *2*) of EDTA/EGTA. Lanes contained 28 µg protein with specific activities (nmoles [³H]ACh binding sites/mg protein) of 1.5 and 1.7, respectively. Membranes prepared in the presence of EDTA/EGTA were transferred to Immobilon and probed with mAbs at the indicated dilutions of ascites fluid: (lane *3*) mAb 19F4a (1:1,000); (lane *4*) mAb 13G2 (1:500); (lane *5*) mAb 20H2 (1:80); (lane *6*) mAb 19J1 (1:100); and (lane *7*) mAb 13K2 (1:1,400).

complete, while the 270-kD antigen, as well as its fragmentation polypeptides, were fully extracted. Thus, the 87- as well as the 270-kD antigen have properties expected of peripheral membrane proteins, even though the extracts used for immunization of the mice may have contained only proteolytic fragments of the antigens.

Characterization of Antigens by Two-Dimensional Immunoblot

Two-dimensional gel electrophoresis was used to characterize the isoelectric points (pI) of the antigens in membranes prepared with the EDTA/EGTA homogenization buffer. Separate replicas were prepared for each antibody, but for convenience of presentation, a single replica probed with three

different antibodies is shown in Fig. 5 B. A reference gel stained with Coomassie blue is shown in Fig. 5 A. The 87-kD polypeptide recognized by 20H2 consisted of a streak from pI 6.6 to ∿5.9. As expected for a monoclonal recognizing the 43-kD protein (Froehner, 1984), mAb 19F4a recognized a series of basic isoelectric variants (pI = 7.5–7.0) corresponding to the major spots of 43,000 M_r stained by Coomassie blue. Monoclonal 19G3, which recognized the same 51-kD band as 20G4 on a one-dimensional gel, recognized a group of 5–6 charge variants with isoelectric points ranging from 6.9 to 6.5, as described by Froehner (1984). These polypeptides are visible in the gel stained with Coomassie blue. They are distinct from a series of spots with similar isoelectric points but slightly greater mobility (∿49,000 M_r). For reasons we have yet to determine, repeated attempts to determine the isoelectric points of the 210:220- and 270-kD polypeptides met with failure. When membranes prepared in the absence of EGTA/EDTA were analyzed, the polypeptides of relative molecular masses 110:130:195 kD recognized by mAb 19J1, that are probably derived from 270 kD, focused as a series of acidic spots (pI = 5.5–5.2). The 37-kD antigen (in membranes prepared in the absence of chelators) was characterized on immunoblots by a band, pI = 5.6–5.3 (not shown).

Copurification of Nonreceptor Proteins with AChRs on Sucrose Gradients

We quantitated antibody binding by ELISA to aliquots (2 µg

Figure 5. Characterization of 87-, 43-, and 51-kD peripheral membrane proteins by two-dimensional gel electrophoresis; (*A*) Coomassie blue stain of AChR-rich membranes (40 µg) prepared in the presence of EDTA/EGTA; (*B*) immunoblot of an Immobilon replica of a parallel gel incubated with a mixture of mAbs: anti–43-kD mAb 19F4a (1:200,000); anti–51-kD mAb 19G3 (1:750); anti–87-kD mAb 20H2 (1:80). The antigens recognized were also determined for each antibody individually to verify that mAb 19F4a binds only to 43 kD (pI = 7.5–7.0); mAb 19G3 to 51 kD (pI = 6.9–6.5); and mAb 20H2 to 87 kD (pI = 6.6–5.9).
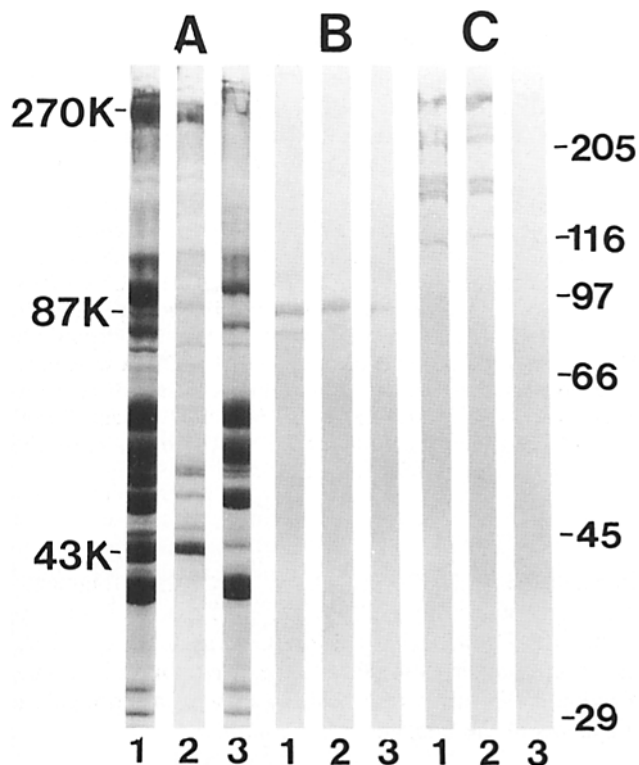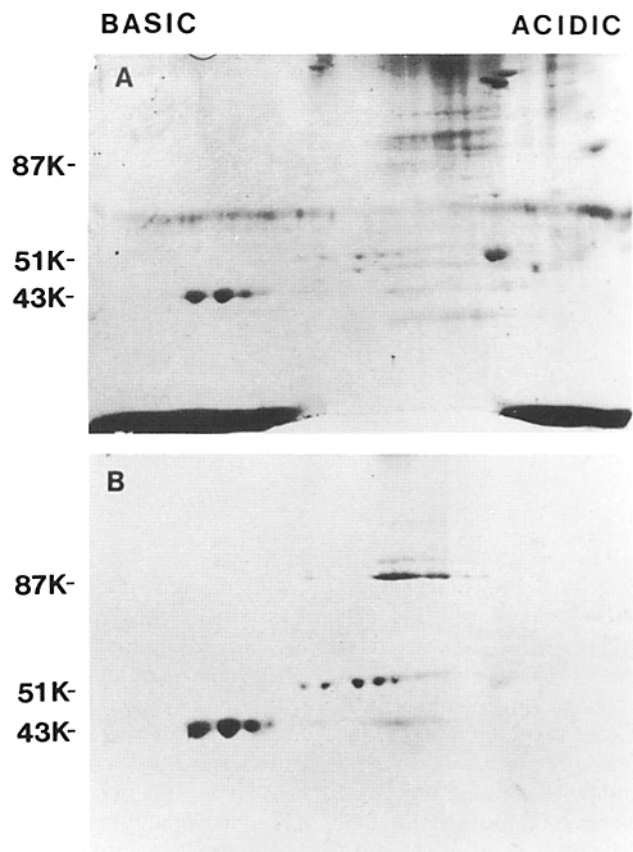

Figure 4. The 87- and 270-kD proteins are released from *Torpedo* AChR-rich membranes following exposure to alkaline pH. AChR-rich membranes prepared in the presence of EDTA/EGTA (lanes *1*); alkaline extract (lanes *2*); and membranes after alkaline extraction (lanes *3*) were resolved by SDS-PAGE and stained with Coomassie blue (*A*) or transferred to Immobilon (*B* and *C*). Immobilon replicas were probed with: (*B*) anti–87-kD mAb 20H2 (1:80 dilution); and (*C*) anti–270-kD mAb 19J1 (1:400 dilution).

protein) of cytosol as well as to aliquots of membranes recovered after fractionation on a discontinuous sucrose gradient (32, 35, 37, 42% wt/wt sucrose). As expected, AChR, 43- and 51-kD proteins were most enriched in membranes recovered at 37% sucrose. This was also true for 87-, 270-, and 210:220-kD antigens. In contrast, the 37-kD antigen was present in similar abundance in all membrane fractions and it was present in higher level in the cytosol fraction.

Membranes used to prepare immunogen in this study were further purified on a linear sucrose gradient from 20–45% sucrose (see Materials and Methods). When antibody binding was determined by ELISA for equal volume aliquots from this gradient, the distribution of 87 kD was similar to that of 43-, 51-, and 270-kD proteins (Fig. 6) and AChR (not shown). In contrast, the 210/220-kD antigen, while present in fractions containing AChR, was also present at similar levels in denser membrane fractions containing very little AChR. The distribution of 37-kD antigen was similar to that of 210:220-kD antigen.

When AChR-rich membranes are further purified by velocity sedimentation through shallow sucrose gradients in *Torpedo* physiological saline, ∿50% of AChRs are recovered
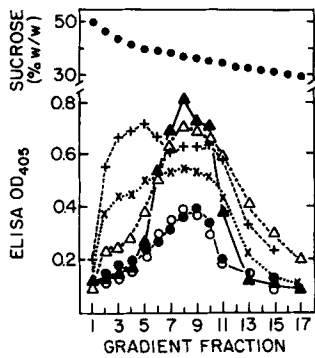
Figure 6. Distribution of non-receptor peripheral membrane proteins in a continuous sucrose gradient. Membranes banding at 37% wt/wt sucrose in a discontinuous sucrose gradient were resuspended in 6% sucrose and centrifuged for 5 h at 100,000 g on a linear (20–45%) sucrose gradient. mAb binding to aliquots of gradient fractions was determined by ELISA for: (×) anti-37-kD mAb 14L1; (△) anti-43-kD mAb 19F4a; (▲) anti-51-kD mAb 20G4; (○) anti-87-kD mAb 20H2; (●) anti-270-kD mAb 19J1; and (+) anti-210:220-kD mAb 13K2. Primary antibodies were used at 1:100 dilution of ascites.

within the gradient in a highly purified population of vesicles that are homogeneous in size, with the remainder of the AChRs recovered in the pellet along with the membranes enriched in Na⁺/K⁺-ATPase (Jeng et al., 1981; Bridgman et al., 1987). In this fractionation, 43-kD protein remains associated with AChRs, but the distributions of the other peripheral proteins have not been previously determined. When analyzed by Coomassie stain neither AChR nor 43-kD protein remains at the top of the gradient (Fig. 7 A, lane 1), a result confirmed by immunoblot using antibodies that recognize AChR α subunit (Fig. 7 B) or 43-kD protein (not shown). In contrast, a significant amount of 51-kD protein (Fig. 7 C) was seen by immunoblot at the top of the gradient. Smaller amounts of 51-kD protein remained associated with AChRs and 43-kD protein. This dissociation was not because of the presence of salts because the same result was obtained

when *Torpedo* saline was omitted from the gradients. The 87-kD protein copurified with AChR (Fig. 7 D). The distribution of this antigen was thus similar to that of 43-kD protein and different from that of 51-kD protein. As expected from the results from the continuous gradient, the 210:220-kD antigen was most enriched in the membranes recovered in the pellet after velocity sedimentation (Fig. 7 F). The analysis of the 270-kD antigen was complicated by severe degradation of the polypeptide under the conditions employed (Fig. 7 E). Although the membranes were isolated from electric organ homogenized in the presence of EGTA and EDTA, a protease must have become active when the membranes were suspended in the salt solution before velocity centrifugation. One interpretation of Fig. 7 E is that intact 270 kD remains associated with AChRs (Fig. 7, lanes 2 and 3) whereas proteolyzed fragments remain at the top of the gradient.

Immunohistochemical Localization of Nonreceptor Proteins

Antibodies directed against the 87-kD antigen stained the innervated face of *Torpedo* electroplaques selectively whereas anti-210:220-kD antibodies labeled noninnervated as well as innervated membrane (not shown). Anti-37-kD antibodies labeled both membranes but the pattern varied in different experiments.

Monoclonals raised against *Torpedo* proteins were assayed on unfixed, frozen sections of chick anterior latissimus dorsi muscle by indirect immunofluorescence. As described in other species, the 43-kD label was restricted to endplates identified with R-BgTx (Fig. 8 a). Anti-51-kD monoclonals labeled endplates intensely with faint but definite labeling of nonjunctional plasma membrane (Fig. 8 b). One of the three anti-87-kD monoclonals (mAb 13H1; Fig. 8 c) labeled chick endplates, and, like anti-51-kD mAbs, it also recognized a
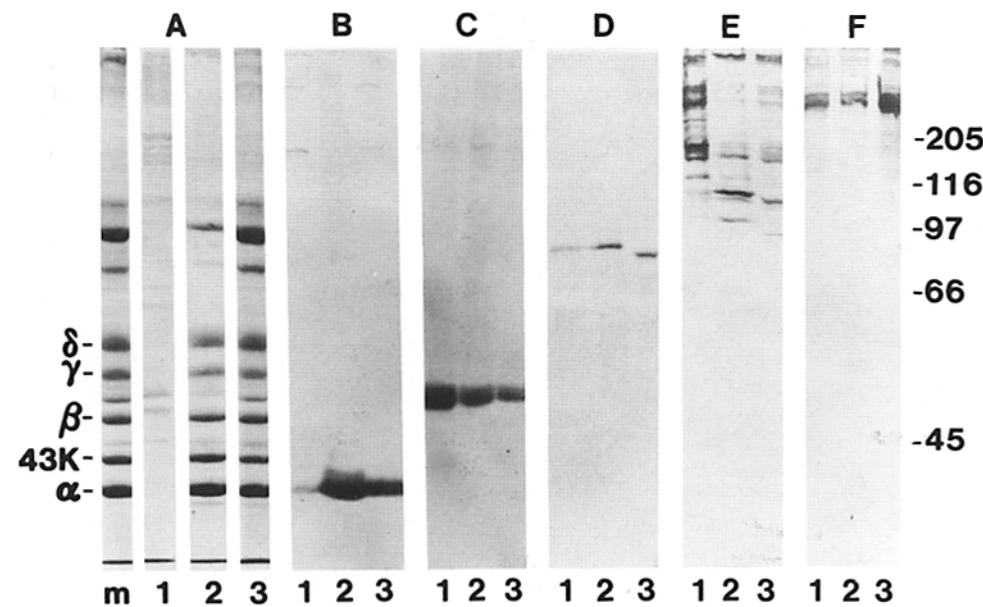

Figure 7. Distribution of AChR and nonreceptor peripheral proteins upon repurification of AChR-rich membranes by velocity sedimentation. AChR-rich membranes prepared with EDTA/EGTA (8.7 mg protein, 1.5 nmole [³H]AChR sites/mg protein) were repurified according to the method of Jeng et al. (1981) on a sucrose gradient containing *Torpedo* physiological saline. An aliquot of 6 µg from the top (lanes 1), aliquots of 11 µg from the middle (lanes 2), and bottom (lanes 3) of the gradient were resolved by SDS-PAGE and stained with Coomassie blue (A) or transferred to Immobilon (B–F). The Immobilon replicas were probed with the following antibodies at the indicated dilution of ascites: (B) anti-α-subunit mAb A18 (1:10,000); (C) anti-51-kD mAb 13G2 (1:50,000); (D) anti-87-kD mAb 20H2 (1:80); (E) anti-270-kD mAb 19J1 (1:100); (F) anti-210:220kD mAb 13K2 (1:666). In A, lane m consists of an 11-µg aliquot of the membranes (containing 1.5 nmoles [³H]AChR sites/mg protein) subjected to velocity fractionation.
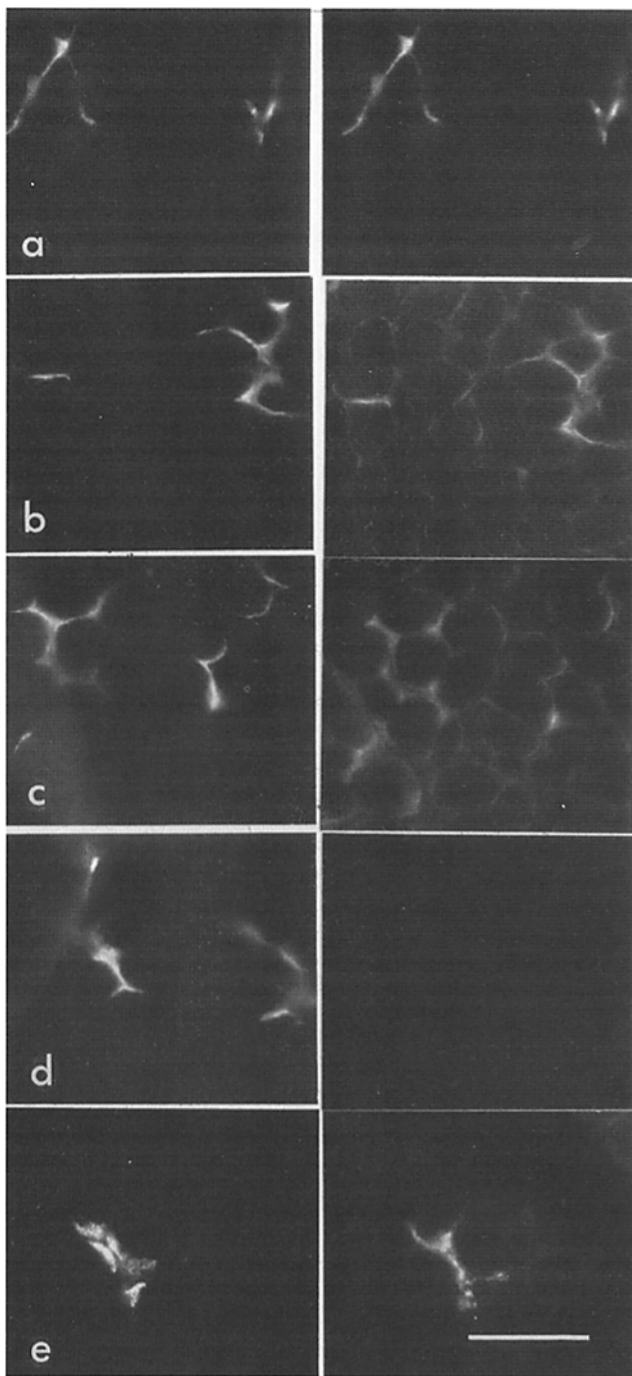
Figure 8. Antibody labeling of chick skeletal muscle. Frozen transverse sections (10 μm) cut from embryonic day 18 anterior latissimus dorsi muscle were labeled with R-BgTx (*left*) to reveal endplates and with the following ascites fluids (*right*). (*a*) anti–43-kD, mAb 19F4a (1:300); (*b*) anti–51-kD mAb 20G4 (1:100); (*c*) anti–87-kD mAb 13H1 (1:100); (*d*) anti–270-kD mAb 22J3 (undiluted supernatant); (*e*) anti–210:220-kD mAb 13K2 (1:300). In each case, the antibody was visualized with fluorescein-conjugated goat anti–mouse IgG plus IgM (1:200). Anti–43-kD is restricted to endplates. Anti–51 kD and anti–87 kD label endplates most intensely but these antigens also are present at extrasynaptic sites around the rim of each muscle fiber. Anti–210:220 kD (*e*) is present at endplates, but it appears to be located in spaces between the R-BgTX streaks. Bar, 20 μm.

determinant in extrasynaptic regions. None of the three anti–270-kD monoclonals labeled chick muscle. Anti–210:220-kD monoclonals labeled chick endplates exclusively (Fig. 8 *e*), but close examination of Fig. 8 *e* shows that the binding of anti–210:220-kD (mAb 13K2) was not exactly coincident with that of R-BgTx. Rather, the fluorescein appears to be located between the AChR-rich regions (see below).

We also examined sections of rat muscle (Fig. 9). The patterns of anti–43-, 51-, and 270-kD labeling have been described, but they are included for comparison (Fig. 9 *a, b,* and *d*). As in the chick, anti–87-kD labeled endplates intensely with faint fluorescence evident around the entire circumference of each fiber (Fig. 9 *c*). No staining was evident in rat muscle sections incubated with anti–210:220-kD (Fig. 9 *e*).

Frozen sections do not reveal whether the antigens are located on the extracellular or cytoplasmic surface of the membrane. This issue was examined by labeling intact and permeabilized chick myotubes grown in vitro. In some experiments, the myotubes were cocultured with ciliary ganglion neurons. Nearly all multinucleated myotubes exhibit several discrete clusters of AChRs that are readily identifiable with R-BgTx. None of the anti–43-, 51-, 87-, 210:220- or 270-kD antibodies labeled receptor clusters on intact myotubes. When the cells were permeabilized by fixation in ethanol at −20°C, anti–43- and 51-kD antibodies labeled determinants at all R-BgTx defined receptor clusters (Fig. 10 *a* and *b*). Labeling with anti–87-kD (mAb 13H1) was inconsistent. In most experiments, no fluorescence above background was detected at receptor clusters (Fig. 10 *c*). This result may be because of the fact that mAb 13H1 (the only anti–87-kD mAb that recognized a chick determinant) is an IgM. Receptor clusters were labeled when mAb 13H1 was microinjected into intact cells (Fig. 11 *E* and *F*). Monoclonal 22J3 (anti–270 kD), which did not label chick endplates in frozen sections, did not label receptor clusters in permeabilized myotubes, (Fig. 10 *d*). Monoclonal 13K2 (anti–210:220 kD) did not label receptor clusters, but it did label the neurites and cell bodies of ciliary ganglion neurons (Fig. 10 *e*). This probably accounts for the unusual distribution of fluorescence observed in frozen sections (Fig. 8 *e*). Clear mAb 13K2 labeling was evident on intact neurons, but the intensity of fluorescence was greater after permeabilization. Thus, this neuronal antigen is probably present intracellularly as well as on the cell surface.

The distribution of 43-, 51-, and 87-kD binding sites with respect to AChR clusters is shown at higher magnification in Fig. 11. While the relation between 43-kD determinants and receptors is precise (Bloch and Froehner, 1987), 51 and 87 kD label clearly extended beyond the region of high receptor density. This is consistent with the extrajunctional staining observed in sections of intact muscle (Figs. 8 and 9).

Discussion

We have raised mAbs directed against proteins extracted with high pH or LIS from AChR-rich *Torpedo* membrane vesicles that were purified by discontinuous and linear sucrose gradient sedimentation. Two of the proteins (87,000 and 210,000: 220,000 M_r) are peripheral membrane proteins that have not been described before. One of them, 87,000 M_r, remained associated with AChRs when the membranes were further
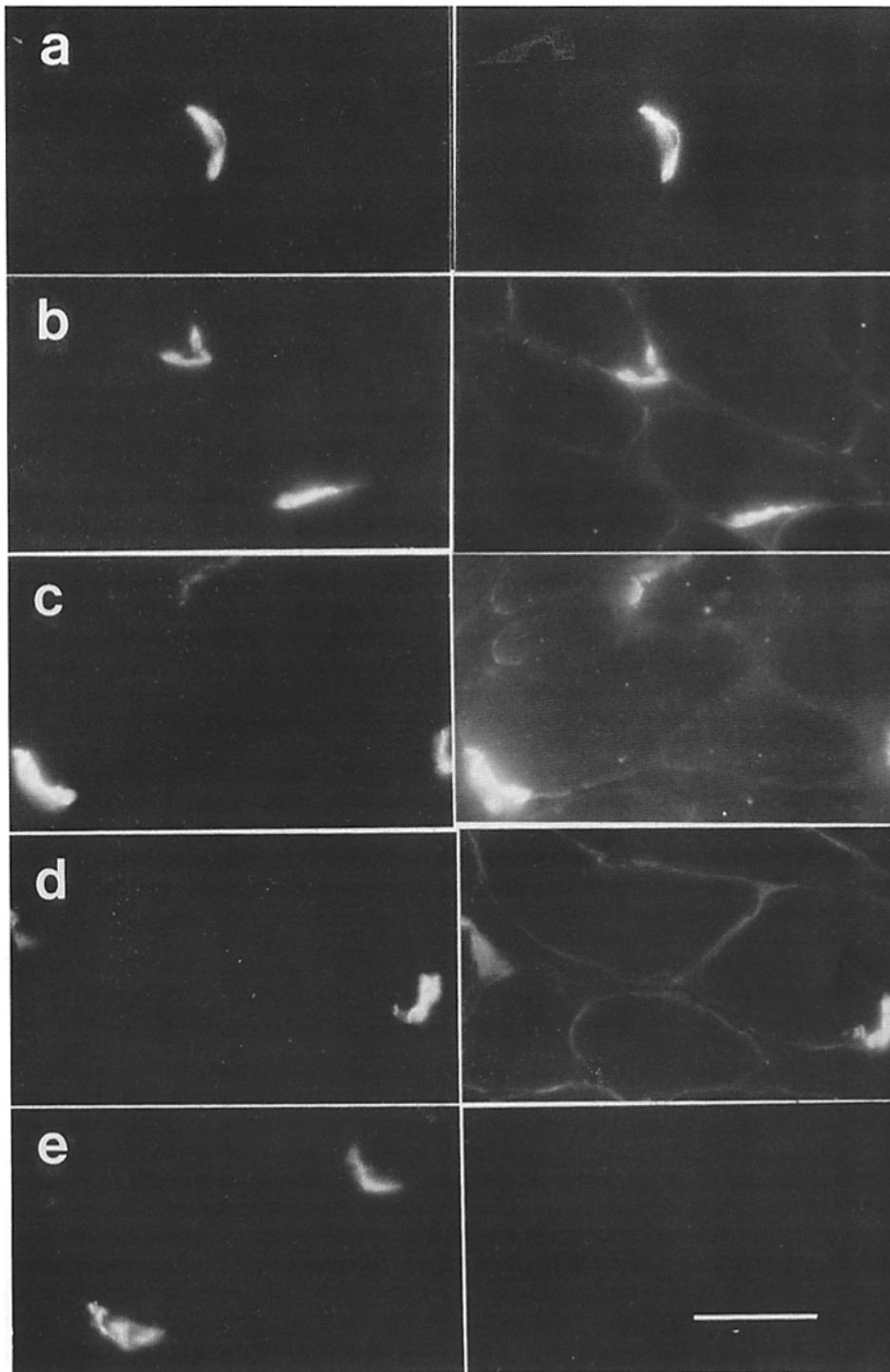
Figure 9. Antibody labeling of rat skeletal muscle. Frozen sections cut from the endplate zone of adult rat diaphragm were labeled with R-BgTX (*left*) and with the following ascites fluids (*right*): (*a*) anti–43-kD mAb 19F4a (1:300); (*b*) anti–51-kD mAb 20G4 (1:100); (*c*) anti–87-kD mAb 13H1 (1:100); (*d*) anti–270-kD mAb 22J3 (1:100); and (*e*) anti–210:220-kD mAb 13K2 (1:100). The antibodies were visualized with fluorescein goat anti–mouse IgG plus IgM (1:200). Bar, 20 μm.

purified by velocity sedimentation in linear sucrose gradients. Anti–87-kD antibodies labeled endplates in sections of chick and rat skeletal muscle. This antigen was also present in the extrasynaptic cell surface.

Based upon its mobility on SDS-polyacrylamide gels, *Torpedo* 87-kD is distinct from α-actinin ($\sim$100,000 M_r in our gel system), an actin binding protein that has been localized by immunofluorescence at vertebrate endplates (Bloch and Hall, 1983). The 87-kD antigen is also clearly different

from the α subunit of the Na$^+$/K$^+$-ATPase, a polypeptide of 98,000 M_r that is present in AChR-rich membrane preparations but not in highly purified postsynaptic membranes isolated by velocity centrifugation. Froehner (1984) has described an antibody that is probably directed against the Na$^+$/K$^+$-ATPase because it recognizes a determinant of $\sim$90,000 M_r that is restricted to the noninnervated surface of *Torpedo* electroplaques. Based upon Coomassie blue stain, the 87-kD protein appears to be present in relatively
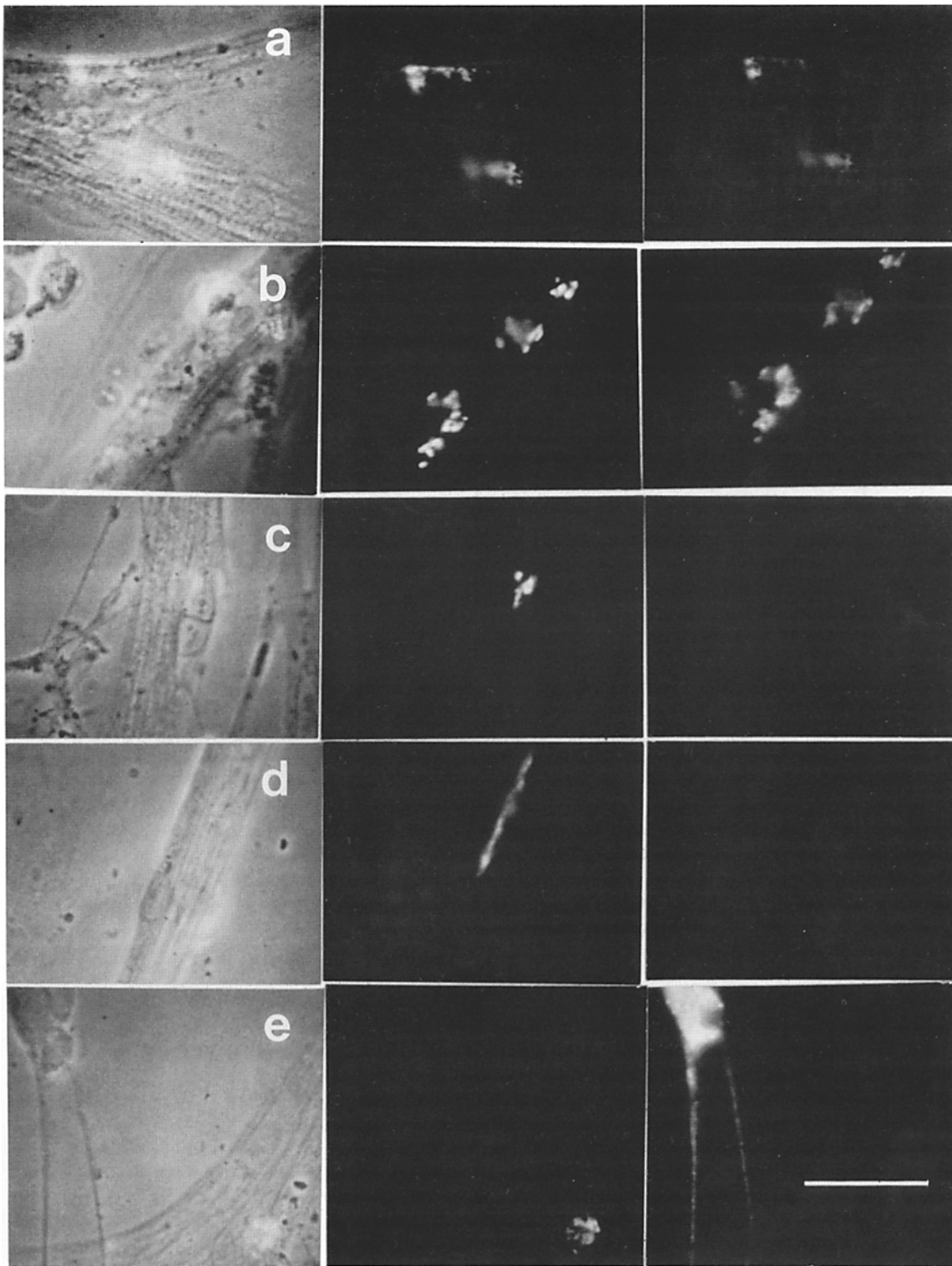
Figure 10. Antibody labeling of permeabilized chick myotube-ciliary ganglion cocultures. The fluorescence micrographs correspond to the cells shown in phase-contrast (*left*). Center: R-BgTx labeling of AChR clusters. On the right, the following antibodies are shown: (*a*) anti–43-kD mAb 19F4a; (*b*) anti–51-kD mAb 20G4; (*c*) anti–87-kD mAb 13H1; (*d*) anti–270-kD mAb 22J3; and (*e*) anti–210:220-kD mAb 13K2. Primary antibodies were used at 1:100 dilution of ascites. Bar, 20 μm.

low concentration in postsynaptic membranes compared to 43-kD protein. However, it remained as closely associated with AChRs as did 43-kD protein throughout each stage of *Torpedo* postsynaptic membrane purification including ve-

locity sedimentation. It is surprising, therefore, that 87-kD protein was detected in extrasynaptic (and extracluster) regions of chick and rat muscle cells.

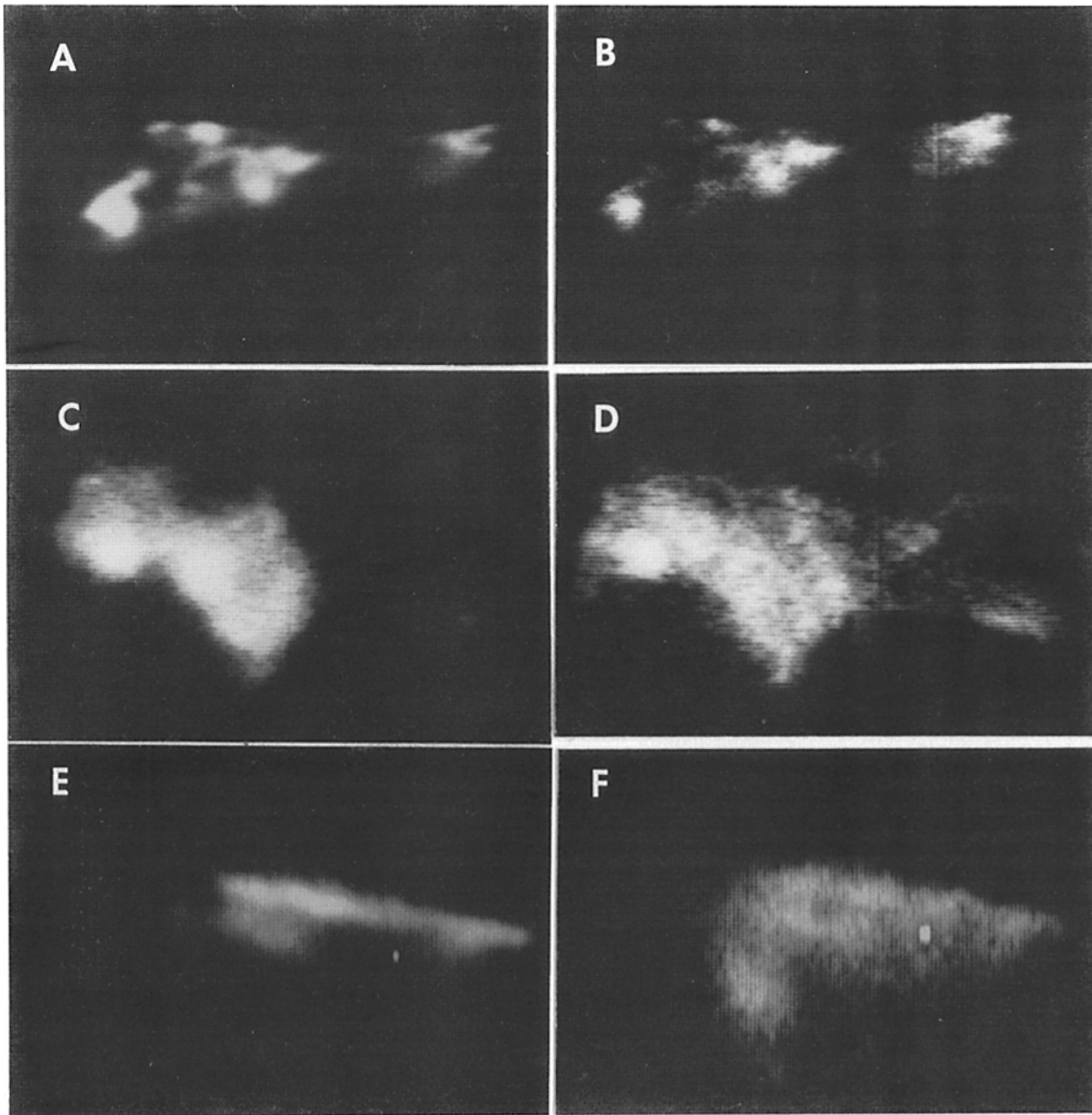The 210:220-kD protein did not remain associated with

Figure 11. High resolution images of AChR clusters on chick myotubes labeled with R-BgTX (*A, C,* and *E*) and mAb 19F4a (*B*), mAb 20G4 (*D*), and mAb 13H1 (*F*). The cells were permeabilized by fixation in ethanol at −20°C. The colocalization of receptors and anti–43 kD is precise (*A* and *B*), whereas clear extracluster labeling is evident with anti–51 kD (*C* and *D*) and 87 kD (*E* and *F*). mAb 13H1 was pressure injected through a fine-tip microelectrode before membrane permeabilization.

AChR-rich membranes on linear sucrose gradients or velocity sedimentation. Antibodies directed against this molecule labeled ciliary ganglion neurites (as well as spinal cord neurites) in vitro, and this probably accounts for the labeling of endplates in sectioned muscle. It is possible that fragments of nerve terminal membranes contaminated the postsynaptic membrane preparation from which the alkaline extract (immunogen) was derived. However, anti–210:220 kD (mAb 13K2) labeled the noninnervated face of the *Torpedo* electrocyte as well as the innervated surface, so this determinant may not be located on neurons exclusively.

The 43-kD protein has been characterized in great detail. Our results extend previous observations to chick endplates and cultured myotubes. The determinant recognized by anti-*Torpedo* 43 kD in transformed mouse and rat muscle cells (LaRochelle and Froehner, 1987; Musil et al., 1988) and in

chick myotubes (Falls, D., D. Harris, and G. D. Fischbach, unpublished observations) is also a polypeptide of 43,000 M_r. Bloch and Froehner (1987) have shown a precise correspondence between 43-kD protein and AChRs at points of attachment between rat myotubes and the glass culture surface. Our results show that the correspondence is also precise on chick myotubes even at AChR clusters that are not at attachment sites; i.e., on the upper surface.

The 51-kD protein, first identified by Froehner (1984; 58 kD in his gel system) is clearly different from the 51 kD protein identified in extracts of *Torpedo* electric organ by Burden (1982, 1985). The latter polypeptide that is recognized by antiintermediate filament antibodies is insoluble in Triton, is extremely sensitive to proteolysis and is characterized by an isoelectric point (pI = 5.6–5.2) more acidic than the 51 kD protein described here (pI = 6.9–6.5).

Although 51-kD protein copurified with AChRs during equilibrium density gradient centrifugation, we show here that a significant amount of this protein was separated from AChRs during velocity sedimentation of *Torpedo* membranes. Thus, 51 kD may be less tightly associated with AChRs in this preparation than are 43- and 87-kD proteins. However, this interpretation is complicated by the fact that 51-kD protein is not precisely colocalized with AChRs in *Torpedo* electroplaques. This antigen, localized at the electron microscope level with antibody–gold conjugates (Froehner et al., 1987), is present in the lower two-thirds of *Torpedo* junctional folds as well as in the AChR-rich upper third. Thus, it may be that the 51-kD protein recovered at the top of the velocity sedimentation gradient was released from non–AChR-rich membranes. This issue could be resolved by antibodies comparing the distribution of anti–51 kD and anti–87 kD with anti–43 kD in electron micrographs of *Torpedo* postsynaptic membranes (Bridgman et al., 1987).

The 270-kD protein comigrated with AChRs at each stage of purification. This protein, previously identified by Woodruff et al. (1987), is extremely susceptible to proteolysis, presumably by a Ca^{++}-sensitive enzyme that is present even in highly purified membrane fractions. Like the 87-kD protein, this determinant was concentrated at rat motor endplates but appeared in extrasynaptic regions as well. Based upon its mobility in SDS-polyacrylamide gels, the 270-kD antigen is distinct from filamin (240,000 M_r in our gel system) and talin (215,000 M_r), two other cytoskeletal elements identified by immunofluorescence at the rat neuromuscular junction (Bloch and Hall, 1983; Sealock et al., 1986).

One extracted protein of 37,000 M_r was present in equal abundance in all *Torpedo* membrane fractions recovered from a discontinuous sucrose gradient, but it was most abundant in the cytosolic fraction. The 37-kD protein was not present in AChR-rich membranes isolated from electric organ homogenized in the presence of EGTA/EDTA when AChR-rich membranes prepared in the absence of EGTA/EDTA were incubated with 3 mM EGTA, 0.1% saponin, 10 mM NaP_i, pH 7, the 37-kD protein was recovered quantitatively in the supernatant (data not shown). Based upon this Ca^{++}-dependent association with membranes and on its molecular weight and isoelectric point, we conclude that 37-kD protein is calelectrin, a Ca^{++}-binding protein widely distributed in *Torpedo* electric organ and electromotor neurons (Walker, 1982; Fiedler and Walker, 1985). This is the first description of the presence of calelectrin in isolated nicotinic postsynaptic membranes, and it is noteworthy since it would have been present in the membrane preparations used in the studies of AChR mobility (Rousselet et al., 1981, 1982).

The function of the postsynaptic peripheral proteins remains to be determined. AChRs are more mobile in alkali extracted membranes than in untreated membranes, and one or more of the proteins described here may be involved. The 43-kD protein is clearly a candidate. It is present in approximately equimolar amounts with AChR in *Torpedo* electric organ (LaRochelle and Froehner, 1986). Moreover, it is the only one of the four that is restricted to receptor clusters. 43-kD protein must be closely associated with AChRs, as cross-linking reagents couple it to the AChR beta subunit (Burden et al., 1983). Although 43-kD protein is unrelated to erythrocyte band 4.1 or to ankyrin, it can bind actin (Walker et al., 1984). Thus, it may serve to link AChRs to actin in the

intact cell. However, other data suggest that 43-kD protein is not sufficient for cluster formation. Although the concentration of 43-kD protein is comparable to that of AChRs in both BC3H1 cells and C2 myotubes (LaRochelle and Froehner, 1987), the latter exhibit receptor clusters whereas the former apparently do not (Froehner and Sealock, 1986). Moreover, 43-kD protein is not present when AChR-rich domains are first detected in developing *Torpedo* electrocytes (Kordeli et al., 1989). Likewise, we have found that 43-kD protein is not present at the first formed clusters on chick myotubes and mononucleated myoblasts (Tsui, H. C., and G. D. Fischbach, unpublished observations). Thus, it appears that more than one peripheral protein is involved in AChR immobilization.

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