

# A Postsynaptic $M_r$ 58,000 (58K) Protein Concentrated at Acetylcholine Receptor-Rich Sites in *Torpedo* Electroplaques and Skeletal Muscle

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**Abstract.** In the study of proteins that may participate in the events responsible for organization of macromolecules in the postsynaptic membrane, we have used a mAb to an  $M_r$  58,000 protein (58K protein) found in purified acetylcholine receptor (AChR)-enriched membranes from *Torpedo* electrocytes. Immunogold labeling with the mAb shows that the 58K protein is located on the cytoplasmic side of *Torpedo* postsynaptic membranes and is most concentrated near the crests of the postjunctional folds, i.e., at sites of high AChR concentration. The mAb also recognizes a skeletal muscle protein with biochemical characteristics very similar to the electrocyte 58K protein. In immunofluorescence experiments on adult mammalian skeletal muscle, the 58K protein mAb labels endplates very in-

tensely, but staining of extrasynaptic membrane is also seen. Endplate staining is not due entirely to membrane infoldings since a similar pattern is seen in neonatal rat diaphragm in which postjunctional folds are shallow and rudimentary, and in chicken muscle, which lacks folds entirely. Furthermore, clusters of AChR that occur spontaneously on cultured *Xenopus* myotomal cells and mouse muscle cells of the C2 line are also stained more intensely than the surrounding membrane with the 58K mAb. Denervation of adult rat diaphragm muscle for relatively long times causes a dramatic decrease in the endplate staining intensity. Thus, the concentration of this evolutionarily conserved protein at postsynaptic sites may be regulated by innervation or by muscle activity.

**T**HE neuromuscular junction of skeletal muscle is a highly complex region of cell-cell contact requiring the proper spatial organization of many specializations and structures. These include, among others, precisely localized high concentrations of the nicotinic acetylcholine receptor (AChR)<sup>1</sup> in the postsynaptic membrane (20, 37), accumulations of voltage-sensitive sodium channels in the postsynaptic region (4, 7), and a specific form of acetylcholinesterase in the junctional basal lamina (29, 67). The existence of each of these proteins was originally inferred through its activity in synaptic transmission. In recent years, however, interest in the cell biology of the junction has prompted studies aimed at identification of other junction-associated proteins and antigens not directly involved in transmission. These investigations led to the discovery that the junctional basal lamina is highly specialized with respect to the presence, absence, or concentration of several proteins in addition to acetylcholinesterase (2, 17, 39, 56, 58), and that the cytoplasm just subjacent to the postsynaptic mem-

brane contains high concentrations of several cytoskeletal proteins (10, 12, 30, 61). With the possible exception of the AChR-aggregating factor described by McMahan and colleagues (19, 27, 44, 68), the discovery of these proteins has not led to identification of their functions at the junction. In many cases, however, they are believed to be involved in the cellular mechanisms responsible for the formation and maintenance of junctional specializations.

Since the neuromuscular junction has not been isolated in a form appropriate for biochemical studies, much of the work on junction-associated proteins has depended on tools, such as antibodies, developed in other systems. A more direct approach is afforded by the use of electric organ from electric rays, a tissue that contains high densities of cholinergic synapses closely homologous to the mammalian neuromuscular junction. Highly purified *Torpedo* postsynaptic membranes have been used extensively for study of the acetylcholine receptor protein (18, 42, 64). They also contain other proteins and one can expect that some of these may occur uniquely at synapses. One such component is a cytoplasmic, peripheral membrane protein of  $M_r$  43,000 (the 43K protein) (6, 28, 42, 43, 51), the discovery of which has very much stimulated research in this area (for a review, see refer-

1. *Abbreviations used in this paper:* AChR, nicotinic acetylcholine receptor; 58K protein,  $M_r$  58,000 protein composed of isoelectric variants of  $pI = 6.4-6.7$ ; rhod-toxin, rhodamine-labeled alpha-bungarotoxin.

ence 22). The AChR and the 43K protein share identical tissue distributions (34), occur in near equimolar amounts in electric tissue (34), are coextensively distributed on the postsynaptic membrane (62), and can be chemically cross-linked to each other (14). A mammalian counterpart to the 43K protein occurs at the neuromuscular junction (21, 25) and in clusters of receptors on cultured muscle cells (9, 13, 49). The 43K protein thus appears to be an entirely AChR-specific protein. While its functions have not been established, they may be related to anchoring of the receptor against lateral diffusion since its removal from postsynaptic membranes by alkaline extraction (42) is accompanied by an increase in both lateral and rotational mobility of the receptor (6, 16, 35, 55).

The success of this approach in the case of the 43K protein clearly mandates systematic exploration of all the nonreceptor proteins in isolated AChR-rich membranes. In this paper we examine an *M<sub>r</sub>* 58,000 protein (58K protein) that copurifies with AChR-rich membranes from electric tissue of *Torpedo californica* (21). The protein has the characteristics of a peripheral membrane protein since it can be solubilized from the membranes by alkaline buffers or with low concentrations of lithium diiodosalicylate at neutral pH (21, 51). Using a mAb, we show here that the 58K protein occurs on the cytoplasmic side of the membrane and is concentrated at AChR-rich regions of the postsynaptic membrane in electrocytes and at AChR clusters on cultured muscle. The expression of its counterpart in adult rat muscle, where it is more broadly distributed, may be innervation dependent. The function of this protein remains unknown. However, our results strongly suggest that under at least some circumstances the 58K protein is involved in activities specific to regions of high AChR density.

## Materials and Methods

### Production and Characterization of Monoclonal Antibodies

The procedure for production of mAbs to proteins extracted from *Torpedo* postsynaptic membranes with lithium diiodosalicylate (51) has been described (21, 24). Culture supernatants from hybridomas were assayed first for antibody activity against extracted proteins with a solid phase well assay and selected ones were then tested for reactivity with rat diaphragm muscle by immunofluorescence microscopy (21). Hybridomas secreting antibodies that reacted with muscle endplates were subcloned and retested by both assays. mAbs specific for the 58K protein were identified by one-dimensional and two-dimensional immunoblots (see below). Purification of mAb IgG from ascites fluids was performed as previously described (21).

### Characterization of mAbs by Immunoblot Analysis

Procedures for characterization of mAb reactivity with postsynaptic membrane proteins on one-dimensional and two-dimensional immunoblots have been previously described (21, 24, 51).

### Immunogold Electron Microscopy on *Torpedo* Electrocytes

Live anesthetized *T. californica* were fixed by perfusion with the fixative of McLean and Nakane (38) as modified for elasmobranchs (62). Tissue from well-perfused regions of the electric organs was cut into small pieces and washed and stored in *Torpedo* Ringer's solution at 4°C. Identical results were obtained with tissue used immediately after fixation or stored for at least 2 wk. Large fragments of electroplax split along their central plane to assure access of antibodies to intracellular sites were prepared by chopping fixed tissue finely with a razor blade, homogenizing gently with a

blade-type homogenizer (VirTis Co., Inc., Gardiner, NY), and filtering away remaining large pieces on cheesecloth. Appropriate quantities of the fragments were then centrifuged onto the bottoms of microculture wells (69) and labeled with mAb 1351 or mouse IgG, rabbit anti-mouse IgG, and protein A on 5-nm colloidal gold (41) as previously described for mAbs against AChR and the 43K protein (62, 69). Examination was restricted to fragments or parts thereof in which the cell interior had obviously been well exposed to the labeling reagents.

### Immunofluorescence on Rat Muscle and Cultured Muscle Cells

**Whole Mount Labeling and Epon Sections.** The ankle-most end of the flexor digitorum longus (the anterior half of the flexor digitorum profundus) was found to be suitable for whole mount labeling because of its low content of connective tissue and the clear separations between muscle fascicles. After dissection by a procedure that will be furnished on request and pinning out in Ringer's solution, the muscle was flooded with 1% paraformaldehyde in PBS. During fixation (30 min), razor blade knives were used to cut between fascicles at the ankle end of the muscle to increase penetration of reagents. Mechanical damage to muscle cells was not deleterious, and probably improved subsequent staining. The prepared region of the muscle was cut free, permeabilized in PBS containing 0.5% Triton X-100 for 30 min, and incubated in 10% normal goat serum (the solvent for the staining procedure) for 10 min.

Staining was done with mAb IgG (140 nM) followed in succession by fluorescein-rabbit anti-mouse IgG and fluorescein-goat anti-rabbit IgG (90 min each) with 15-min washes between incubations. The two second antibody solutions were supplemented with 10% normal rat serum and rhodamine-labeled alpha-bungarotoxin (rhod-toxin) (53). After a final wash, the piece was fixed in 5% paraformaldehyde in PBS. All steps were carried out with agitation. Single fibers were then removed manually for individual examination. After infiltration with Epon 812 (Ladd Research Industries, Inc., Burlington, VT) by conventional techniques, the remainder of the muscle was separated into small fascicles. Fascicles were embedded in thin layers of Epon between squares of sturdy aluminum foil (cut from 57-mm aluminum weighing dishes; Fisher Scientific Co., Pittsburgh, PA) or between coverslips that had been treated overnight with Siliclad (Clay Adams Div., Becton, Dickinson & Co., Parsippany, NJ) diluted 1:10 in water, rinsed with water, and dried at 60°C. After polymerization and separation of the Epon wafers from these supports, junction-rich zones were located by epifluorescence under a 10× objective, cut out, remounted on Epon blanks, and sectioned at a nominal thickness of 0.8 μm.

**Frozen Sections.** Semi-thin frozen sections (nominal thickness, 0.3–0.5 μm) were cut with a cryoultramicrotome essentially according to Tokuyasu and Singer (66). Small pieces cut from chick anterior latissimus dorsi muscle or from the endplate zones of rat flexor digitorum longus (fixed as above) were infiltrated with 2 M sucrose/0.1 M sodium phosphate, pH 7.4, for 2 h at room temperature. On the freezing stubs of the microtome, the pieces were stood on end in a supporting notch cut in a small square of agar (also infiltrated with sucrose/phosphate solution), then frozen in pentane slush. The pieces were then sectioned transversely approximately perpendicular to the long axis of the muscle fibers. The sections were mounted on untreated glass slides, incubated with sodium borohydride (1 mg/ml in PBS) for 30 min and Triton X-100 (1% in PBS) for 20 min, and then labeled with FITC-alpha-bungarotoxin (53), FITC-labeled affinity-purified anti-alpha-bungarotoxin (labeled according to reference 1), mAb 1351, and rhodamine-labeled second antibodies (goat anti-mouse IgG and rabbit anti-goat IgG; Cooper Biomedical, Inc., Malvern, PA).

Procedures for preparation and staining of 6-μm cryostat sections of embryonic, adult, and denervated adult rat diaphragm have been described previously (25).

**Cultured Muscle Cells.** *Xenopus* myotomal cells were isolated from *Xenopus laevis* embryos at stage 20–22 and cultured on cover glass squares according to published methods (50) with one modification: the sterile coverslips (18 × 18 mm) were treated with 10 μg/ml poly-D-lysine (No. P1149; Sigma Chemical Co., St. Louis, MO) for 15 min and then rinsed three times with 10% culture medium before use. This step was added to facilitate cell adhesion to the substrate. Live cells were labeled with rhodotoxin (49) and then dipped successively in the following solutions for 10 min each: (a) 70% culture medium and 30% fixative solution (fixative is 0.25% paraformaldehyde in 80 mM lysine, pH 7.2); (b) 30% culture medium and 70% fixative; (c) undiluted fixative; (d) 1% paraformaldehyde in PBS, pH 7.2. The cells were then permeabilized with 1% Triton in 100 mM glycine solution, pH 7.2. After two washes in PBS, the cultures were incubated with mAb 1351 IgG (7 nM) or control mouse IgG (70 nM) for 1 h and then were

washed with PBS several times for a total of 20 min. The cells were then incubated with FITC-conjugated goat anti-mouse IgG at 600 nM for 45 min, washed, mounted, and viewed with a Leitz Orthoplan microscope. All antibody solutions were diluted in PBS containing 10% sheep serum.

C2 mouse muscle cells cultured on glass coverslips (63) were labeled with rhod-toxin for 45 min, rinsed with medium, and fixed for 20 min with PBS containing 1% paraformaldehyde, 0.2% saponin. After treatment with 0.1 M glycine/PBS for 30 min, the cells were permeabilized by incubation with 1% Triton/PBS for 20 min and then incubated in PBS containing 10% calf serum and 4% BSA for 30 min to prevent nonspecific binding of antibodies. Incubations with mAb IgG (20 nM) diluted in the same buffer were carried out for 18 h at room temperature. Subsequent incubations with FITC-goat anti-mouse IgG and FITC-sheep anti-goat IgG, both diluted in PBS/10% calf serum/4% BSA, were each carried out for 1.5 h separated by two 10-min washes with PBS.

All samples were mounted in 5% n-propylgallate (26) in glycerol containing 1:9 volume of 2× PBS and were photographed with Kodak Tri-X film developed in Diafine (exposure index 1,600; Acufine, Inc., Chicago, IL) or Ilford HP-5 developed in full strength Ilford Microphen (Ilford Ltd., Essex, England) for 18 min at 20°C (exposure index 3,200).

### Immunoprecipitation of [<sup>35</sup>S]methionine-labeled Proteins from Cultured Muscle Cells

The procedure described by Rotundo (54) was used with modifications. Muscle cells of the C2 line cultured in 100-mm culture dishes (63) were washed twice with DME lacking methionine and then incubated with 1.5 ml of the same medium containing 0.69 μCi of L-[<sup>35</sup>S]methionine and 20 μM L-methionine for 30 min. This radioactive medium was transferred to another plate of cells and reused in two subsequent incubations under identical conditions. All subsequent steps were performed at 4°C. The cells were washed twice with PBS, scraped from the dish in 20 mM borate buffer, pH 9.0, 5 mM EDTA, 1 mM EGTA, 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM NEM, 1 mM sodium tetrathionate, 5 mg/ml BSA (buffer A), and centrifuged for 10 min at 12,000 g (10,000 rpm in a JA 20 rotor; Beckman Instruments, Inc., Palo Alto, CA). The pellets from 12 dishes were extracted for 20 min on ice with 2.4 ml of buffer A containing 1% Triton, centrifuged for 30 min at 40,000 g (15,000 rpm in an SW27 rotor; Beckman Instruments, Inc.), and the supernatants were used for immunoprecipitation.

Aliquots (0.2 ml) of the supernatant were made 50 nM mAb IgG and incubated for 3 h at 4°C. 90-μl of rabbit anti-mouse IgG/IgG-sorb complex were added and the incubation was continued for 1 h. (The complex was prepared by incubating 0.15 ml rabbit anti-mouse IgG serum with 1 ml 10% IgG-sorb for 30 min at 37°C, washing three times with buffer A + 1% Triton, and resuspending in 1 ml of the same solution). Each reaction was then diluted to 1 ml with buffer A + 1% Triton. This was underlaid with 0.1 ml 60% sucrose in buffer A + 0.5% Triton and then centrifuged for 5 min in a microfuge (Eppendorf centrifuge 5413; Brinkmann Instruments, Westbury, NY). The pellets were washed three times with 20 mM borate buffer, pH 9.0, 5 mM EDTA, 1 mM EGTA, 0.5 M NaCl, 0.5% Triton, and resuspended in 30 μl 1% SDS, 0.5 M dithiothreitol. The proteins were then eluted by boiling for 2 min and insoluble material was removed by centrifugation in a microfuge for 5 min at 4°. The samples were then analyzed by two-dimensional gel electrophoresis (51) and fluorography (11).

## Results

### Immunoblot Analysis of Monoclonal Antibody for 58K Protein

As previously described (21), the 58K protein is clearly displayed on two-dimensional gels of isolated *Torpedo* postsynaptic membranes as a group of charge variants with isoelectric points of 6.4–6.7 (Fig. 1 A). mAb 1351 reacts with all of the variants, but with no other proteins, on nitrocellulose replicas of such gels (Fig. 1 B). Since the variants were also recognized by another anti-58K mAb described previously (mAb 1127A; reference 21), they are probably all derived from a single protein. Two other minor proteins also recognized by mAb 1127A, at *M<sub>r</sub>* 75,000 and 150,000, were not recognized by mAb 1351 and may not be related to the 58K protein. Thus, mAb 1351 reacts specifically with the

58K protein of *Torpedo* postsynaptic membranes and was used in all experiments described below.

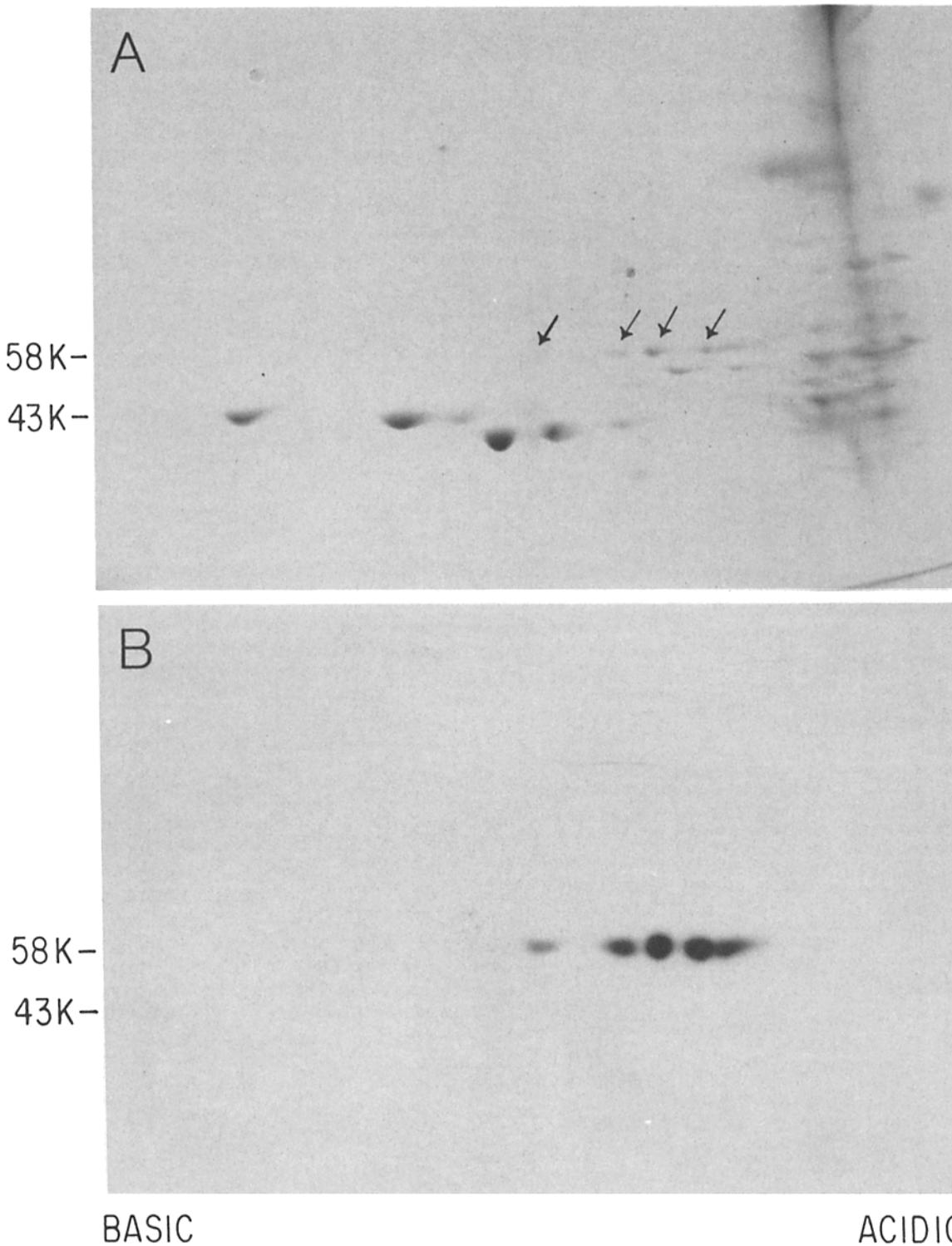
In other immunoblotting experiments, the 58K protein did not bind antibodies specific for tubulin or cytokeratins, nor did it bind a pan-specific mAb (52) that recognizes all classes of intermediate filament proteins (data not shown). It is also different in molecular weight, isoelectric point, and detergent solubility from the 51-kD intermediate filament protein in electroplax described by Burden (12). Since the 58K protein can be solubilized from the membranes by alkaline pH buffers or with low concentrations of lithium diiodosalicylate at neutral pH (21, 51), it is probably a peripheral membrane protein.

### Immunocytochemical Localization of 58K Protein in Electrocytes

The 58K protein was localized in perfusion-fixed electrocytes (62) by the microculture well version of the immunogold method (69). Gold particles were most concentrated on the postsynaptic membrane where they were found in close apposition to the cytoplasmic surface (Fig. 2, A–C). The postsynaptic membrane was not labeled when nonspecific mouse IgG was substituted for mAb 1351 (Fig. 2 E). With mAb 1351, particles were also present on filaments in the electroplax cytoplasm, on the noninnervated face of the cell, and in the nerve terminals but at low levels not noticeably higher than those observed with control mouse IgG (compare Fig. 2, A–D with E and F). Thus, by the criterion of qualitative immunocytochemistry, the electrocyte 58K protein is a postsynaptic protein associated with the cytoplasmic side of the membrane. In the absence of a quantitative analysis and without the use of additional antibodies, we cannot exclude the possibility that significant amounts of the 58K protein may occur elsewhere in the tissue.

Along the postsynaptic membrane, labeling was strongest in the most superficial regions, i.e., those nearest the nerve terminals and on the upper sides of the postsynaptic invaginations (Fig. 2, A–C). This is the region of the membrane known to contain high densities of AChR (60) and the 43K protein (33, 43, 62). The contrast between the amounts of gold particles in the upper and deeper regions of the invaginations was less striking with mAb 1351 than in our previous study (62) with anti-43K mAbs, however. With the anti-58K mAb, membrane in the deeper regions often bore small amounts of diffusely distributed particles (Fig. 2, A–C) or small clumps of particles (Fig. 2, A and B). Label also tended to be present at the bottoms of the invaginations. Clumps of gold particles sometimes were paired on opposite sides of invaginations (Fig. 2 B). When the membrane in upper regions was seen in *en face* views, the distributions of particles had a tendency to occur in rows or bands (Fig. 2 B). Similar, though stronger, tendencies have been repeatedly observed after labeling of electroplax with anti-AChR or anti-43K antibodies (Sealock, R., unpublished observations; Cartaud, J., unpublished observations). The possibility that this reflects a common organizational scheme for all of these proteins (presumably based on the four-abreast rows of AChR known to exist in electroplax [31]) clearly should be tested, in part by a statistical analysis of gold particle distributions.

In summary, immunogold labeling with mAb 1351 suggests that the 58K protein is preferentially located on the cy-

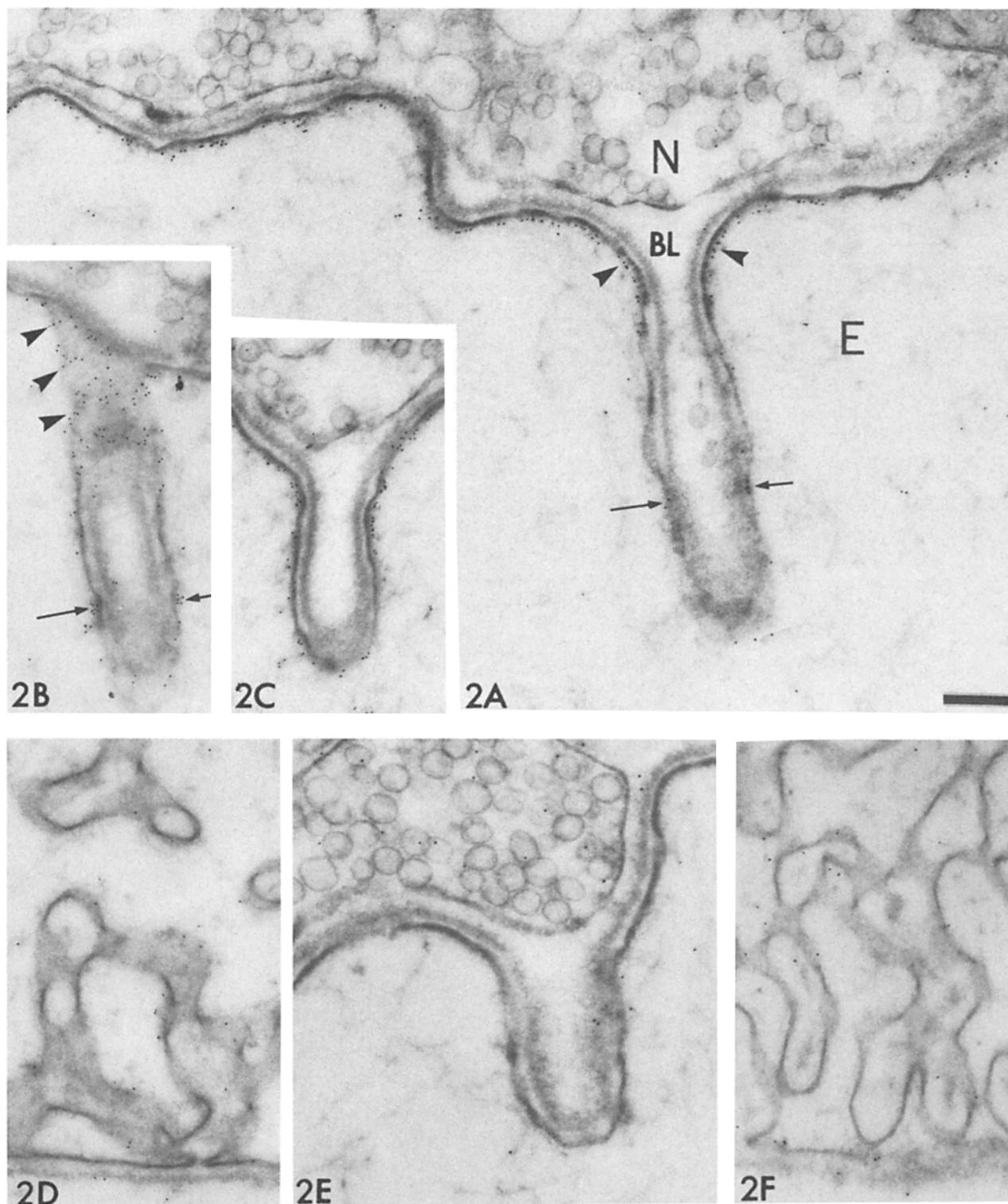


**Figure 1.** Immunoblot analyses of mAb 1351. (A) Proteins in AChR-enriched membranes were separated by two-dimensional gel electrophoresis and stained for protein with Coomassie Blue. Arrows denote the four charge variants of 58K protein. (B) Autoradiograph of a nitrocellulose replica of a companion gel reacted with anti-58K mAb 1351, showing that the mAb reacts with, and only with, all charge variants of the 58K protein. The migration distances of proteins of  $M_r$  43,000 and 58,000 determined by comparison with standard proteins and the orientation of the pH gradient are indicated.

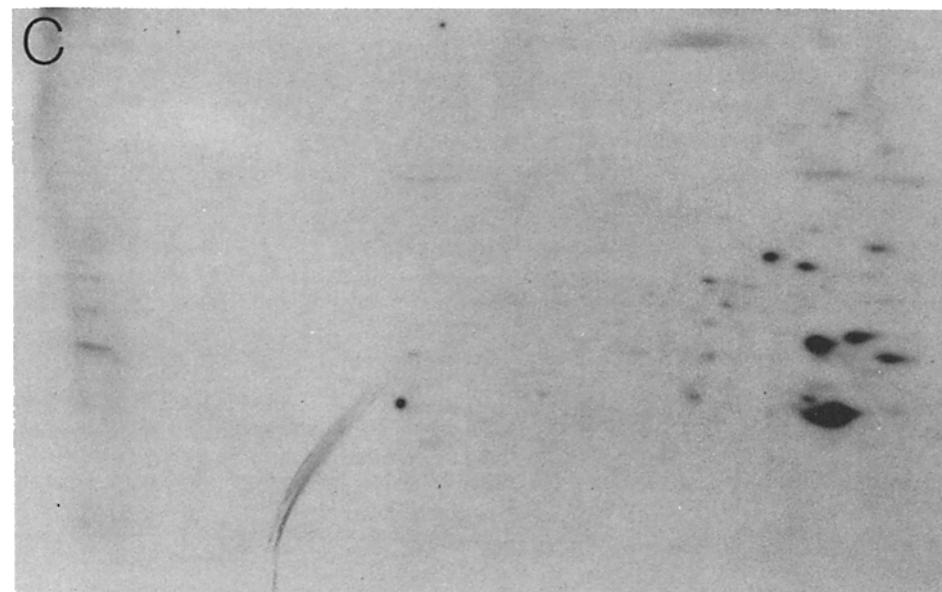
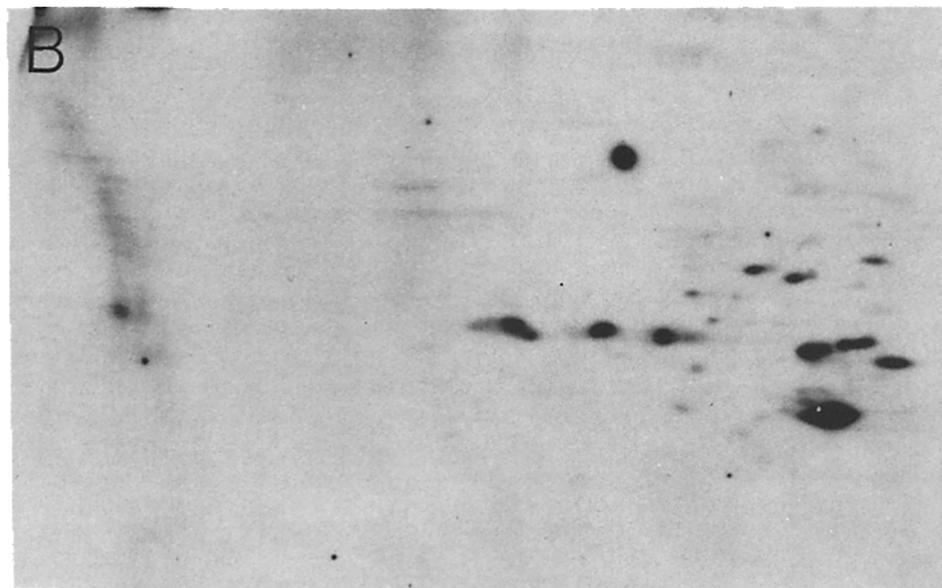
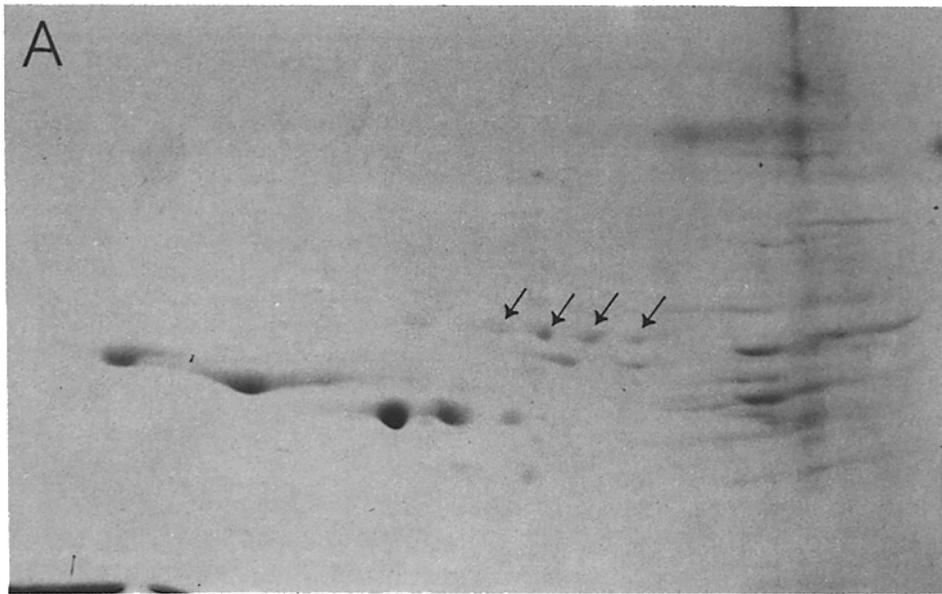
toplasmic side of the receptor-rich postsynaptic membrane, occurs in lower amounts on the receptor-poor regions of the postsynaptic membrane, and is present in still lower quantities, if at all, in other regions of electric tissue.

#### **Identification of the Muscle 58K Protein**

We used immunoprecipitation to search for a protein in skeletal muscle having immunological and biochemical properties similar to those of the *Torpedo* 58K protein. Cultured



**Figure 2.** Immunogold labeling of *Torpedo* electroplax with anti-58K mAb (A–D) or control mouse IgG (E and F). (A) Gold particles occur on the cytoplasmic surface of the postsynaptic membrane nearest nerve terminals (N) and on the upper sides of the postsynaptic invagination (arrowheads) but not on most of the membrane in the deeper portions of the invagination. Arrows indicate two sites of labeling that appear to be paired across the invagination. Basal lamina (BL) and electroplax cytoplasm (E) are indicated. (B and C) Additional invaginations that together with that in A show the approximate range of labeling observed as assessed by inspection. Arrowheads indicate rows and bands of gold particles. Arrows as in A. (D) Canaliculi of the noninnervated face. Occasional gold particles can be seen on membranes. (E and F) No areas of concentrated label were seen with pooled, control mouse IgG. Bar, 1  $\mu$ m.



BASIC

ACIDIC

**Figure 3.** Immunoprecipitation of [<sup>35</sup>S]methionine-labeled proteins from C2 myotubes. (A) Two-dimensional gel of *Torpedo* receptor-rich membranes stained with Coomassie Blue. Arrows denote the 58K protein. (B and C) Fluorograms of proteins immunoprecipitated from Triton extracts of labeled C2 cells with (B) anti-58K mAb IgG or (C) control mAb IgG. A prominent group of spots similar in apparent molecular weight and isoelectric points to *Torpedo* 58K protein are specifically immunoprecipitated by mAb 1351.

muscle cells of the C2 line (63) were pulse labeled with [<sup>35</sup>S]methionine and extracted with Triton X-100. Solubilized proteins were immunoprecipitated with mAb 1351 or with a control mAb, separated by two-dimensional gel electrophoresis, and detected by fluorography (Fig. 3). Several radioactive proteins are nonspecifically precipitated by both mAbs. However, a prominent group of spots similar in apparent molecular weight and isoelectric points to those of the *Torpedo* 58K protein is specifically recognized by mAb 1351. Thus, the muscle antigen recognized by mAb 1351 is biochemically very similar to the *Torpedo* protein.

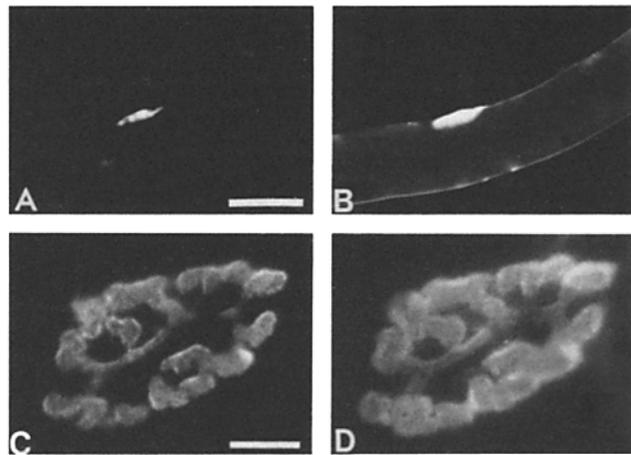
### Localization of the 58K Protein in Muscle

Fixed and permeabilized flexor digitorum longus muscles of adult rats were labeled in a whole mount paradigm with mAb 1351 followed by fluorescein-conjugated goat anti-mouse IgG and rhodamine conjugated alpha bungarotoxin (rhod-toxin) to visualize sites of high AChR concentration (i.e., neuromuscular junctions). If the muscles were permeabilized with Triton X-100 before antibody incubations, the mAb labeled both the junctional and extrajunctional periphery of muscle fibers, but the labeling was particularly intense at the junctions (Fig. 4, *A* and *B*). Without permeabilization, only an occasional junction in each of several fields examined (20–30 junctions/field) was labeled, and then only weakly. Labeling of the extrajunctional periphery was also sporadic and weak except at the cut ends of the muscle fibers, where it was strong. The low levels of labeling in the absence of permeabilization probably reflect mechanical damage to the fibers. By this criterion, the antibody labels intracellular sites in muscle as in electroplax.

In *en face* views the antibody staining readily revealed the general junctional structure, but the images were somewhat larger and less rich in structural detail than those obtained with rhod-toxin (Fig. 4, *C* and *D*). One interpretation of this result, that the mAb binds to the membrane throughout the junctional fold region while the receptor is concentrated at the crests of the folds, was confirmed using Epon sections of muscles stained in whole mount (Fig. 5) or by labeling semi-thin frozen sections of fixed muscle (not shown). In sections, the rhod-toxin label generally appeared as a thin line containing considerable detail (Fig. 5 *A*). In contrast, labeling by mAb 1351 at the junction appeared as a broad belt (Fig. 5 *B*). This pattern persisted through many microns in serial sections. Control mouse IgG applied in the same experiment did not label muscle cells (Fig. 5, *C* and *D*). The contrast between the junctional staining patterns of rhod-toxin and mAb 1351 was most easily seen in sections that barely included receptor-rich regions, or in the spaces between adjacent synaptic gutters (Fig. 5, *E–H*).

Similar images of the junction were obtained when mAb 1351 was replaced by FITC-concanavalin A (FITC-Con A) (assumed to be a marker for the entire muscle cell surface). The lectin also gave a broader fluorescence image than the toxin (Fig. 6, *A–C*), and electron microscopy of an adjacent section (Fig. 6 *D*) showed that the broad band corresponded to the junctional folds. Thus, on the basis of this comparison, the 58K protein in muscle appears to be distributed throughout the junctional fold region in rat muscle, while AChR is concentrated at the crests.

In an attempt to determine whether the 58K protein at the junction might be more concentrated at the crests of the folds



**Figure 4.** Immunofluorescence staining of rat muscle whole mounts with anti-58K mAb. After labeling pieces of intact muscle, single fibers were manually dissected and mounted for conventional fluorescence microscopy. (*A* and *C*) Rhod-toxin staining; (*B* and *D*) fluorescein antibody staining. The antibody-stained images appear slightly larger but with smaller unstained holes and spaces than the toxin-stained areas. Bars: (*A* and *B*) 5  $\mu$ m; (*C* and *D*) 3  $\mu$ m.

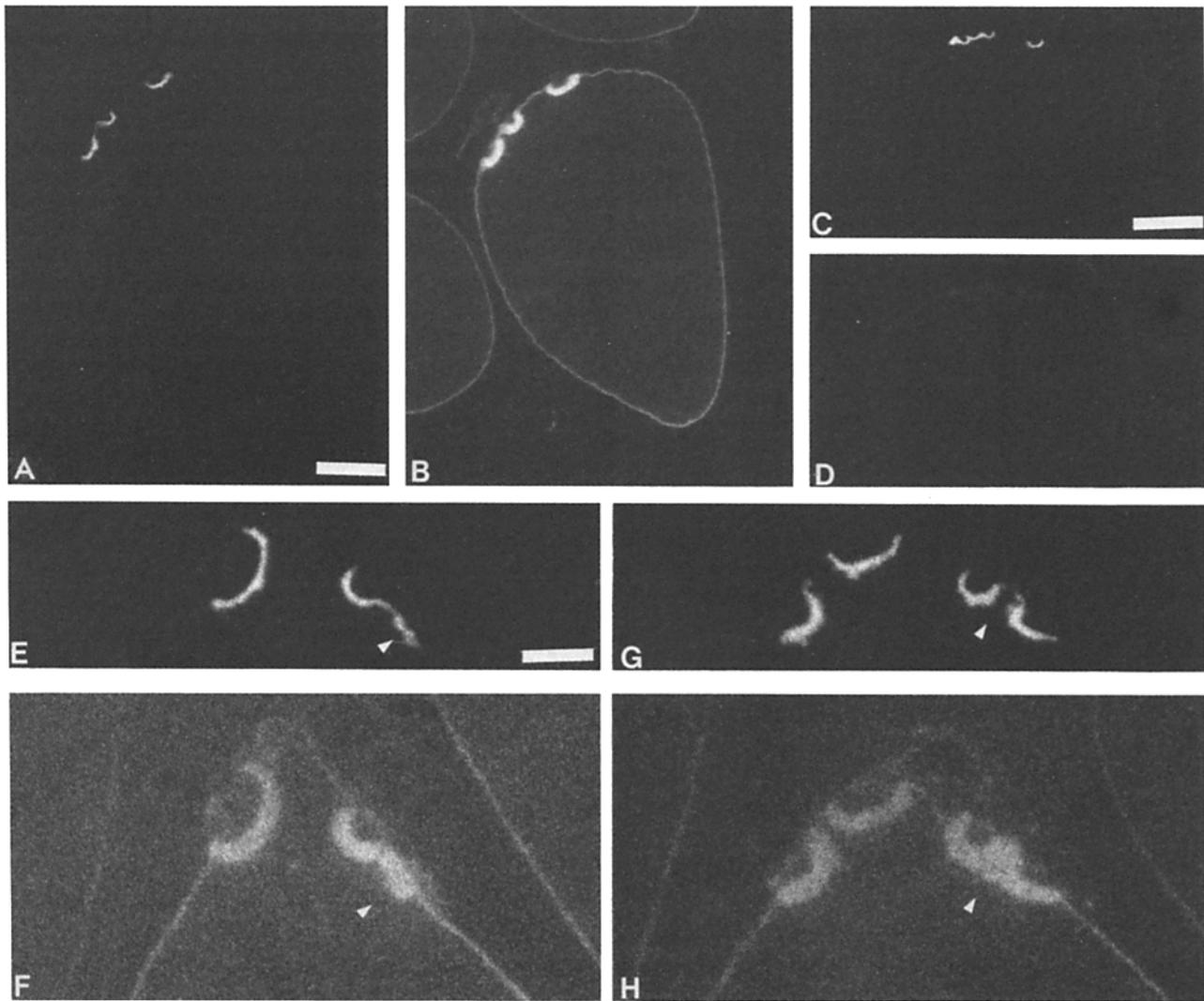
than in the deeper, receptor-poor regions, fluorescence micrographs were examined by inspection and by photographically printing them at increasing exposure times. These procedures failed to reveal any appreciable difference in fluorescence intensity between the two regions, suggesting that the distribution of the 58K protein throughout the folds of the rat junction may be relatively homogeneous, in apparent contrast to the situation in electrocytes.

The multiple foldings of the junctional membrane in adult rat muscle preclude a meaningful comparison of the intensity of labeling on the junctional and extrajunctional membranes. We therefore examined anti-58K staining in neonatal rat diaphragm, in which the junctions have only rudimentary and shallow folds (32), and in chicken anterior latissimus dorsi muscle, in which they lack folds entirely (5). In both muscles, we found moderately greater intensities of anti-58K staining at neuromuscular junctions than on extrasynaptic membrane (Fig. 7). Thus, although binding sites for mAb 1351 are found throughout the sarcolemma in muscle cells of both species, they tend to be more concentrated at AChR-rich membrane regions.

### Localization of the 58K Protein in Cultured Muscle Cells

At even the simplest of neuromuscular junctions there occur several processes with which the 58K protein could be involved. Therefore, in an effort to examine the simplest possible sites of AChR accumulation, we labeled cultured muscle cells of three types with rhod-toxin and, after fixation and permeabilization, with mAb 1351 and FITC-labeled second antibodies.

*Xenopus* myotomal cells in culture form large AChR clusters on their ventral surfaces at sites of cell-substrate attachment and on their exposed (dorsal) surfaces and sides (48). These clusters are often accumulations of submicron clusters of AChR containing considerable structural detail (Fig. 8, *A*, *D*, and *G*). In more than 15 experiments using

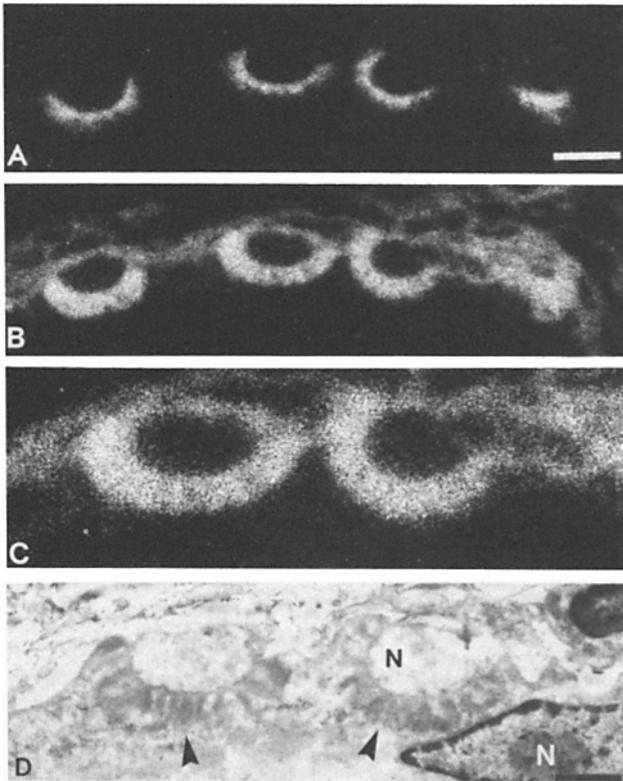


**Figure 5.** Semi-thick Epon sections of rat muscle stained in whole mount with anti-58K mAb. (*A*, *C*, *E*, and *G*) Rhod-toxin staining; (*B*, *D*, *F*, and *H*) fluorescein antibody. All except *C* and *D* were stained with mAb 1351. (*A* and *B*) A single muscle cell. Each of three synaptic gutters is stained in a broad belt by mAb 1351. Extrajunctional staining is also apparent around the entire periphery of the cell. Light staining in the spaces between muscle fibers (*B*) was not studied but would be compatible with the finding that the 58K protein is present in several nonmuscle tissues (see Discussion). (*C* and *D*) A single cell treated as in *A* and *B* but with control mouse IgG in place of mAb 1351. The cell was not stained by the antibodies. The small signal from the rhod-toxin can be seen under the fluorescein optics (*D*). (*E* and *F*) One of a series of sections through a single junction. The receptor-rich area of the gutter on the right (arrowhead in *E*) was just captured in this section but the antibody-stained area remains large and brightly stained (arrowhead in *F*). (*G* and *H*) The spaces between gutters were often open under rhodamine optics but closed under fluorescein optics (arrowheads). Bars: (*A* and *B*) 10  $\mu\text{m}$ ; (*C* and *D*) 14  $\mu\text{m}$ ; (*E*–*H*) 3.6  $\mu\text{m}$ .

cells cultured for periods of 3–8 d, all AChR clusters examined were clearly stained by mAb 1351 (Fig. 8, *B*, *E*, and *H*). The correspondence between the fine structural details seen with the two labels was usually extremely close (compare Fig. 8, *D* and *E* with *G* and *H*). In addition, the cells were stained by mAb 1351 outside the cluster regions at a much lower intensity, with occasional patches of strong staining that were not labeled with rhod-toxin (see Fig. 8, *A* and *B*). Since the plasma membrane at clusters on these cells is not extensively infolded (47), this general staining of the membrane could not have led to an artefactual appearance of strong staining at clusters. None of these types of staining was observed when nonspecific mouse IgG was used in place of mAb 1351 (not shown).

A similar picture was obtained with the C2 line of mouse muscle cells (63). As shown in Fig. 9, anti-58K staining was concentrated at sites of AChR clusters, generally with a distribution very similar to that of the rhod-toxin staining. In addition, however, there was unmistakable although less intense staining of the membrane outside the clusters. Patches enriched in anti-58K staining but without detectable rhod-toxin staining were also present. AChR clusters lacking concentrated anti-58K staining were rare.

The BC<sub>3</sub>H-1 line of mouse muscle cells produces large amounts of AChR (46) and the 43K protein (LaRoche, W. J., and S. C. Froehner, submitted for publication), but does not form AChR clusters spontaneously under our culture conditions. We found these cells to be strongly stained



**Figure 6.** Semi-thick Epon sections of rat muscle stained with FITC-Con A. (A) Rhod-toxin; (B and C) FITC-Con A; (D) electron microscopy of the same junction but from an adjacent ultrathin section. Comparison of C and D shows that Con A labeled virtually the entire junctional fold region. It also labeled extracellular matrix materials on the muscle cell surface and overlying the nerve terminals (B). The fluorescein images contain substructure related to the junctional folds (B and C) or their crests (A). Bars: (A and B) 3.0  $\mu\text{m}$ ; (C and D) 1.5  $\mu\text{m}$ .

in a highly irregular pattern by mAb 1351 (23). Their failure to form clusters cannot therefore be due to a lack of the 58K protein.

#### **Effect of Denervation on 58K Protein Distribution in Adult Muscle**

Denervation is known to affect the concentration and distribution of postsynaptic proteins, including AChR (8), intermediate filament protein (12), and acetylcholine esterase (29). We have also observed changes in the intensity of mAb 1351 staining after relatively long periods of denervation. 9 d after denervation of rat diaphragm by transection of the phrenic nerve, the endplate staining of both rhodamine-toxin and anti-58K mAb were still quite intense (Fig. 10, A–D). However, 6 wk after denervation (Fig. 10, E and F), both junctional and extrajunctional anti-58K labeling was dramatically reduced, even though strong rhod-toxin staining persisted at the junctions, as expected (36).

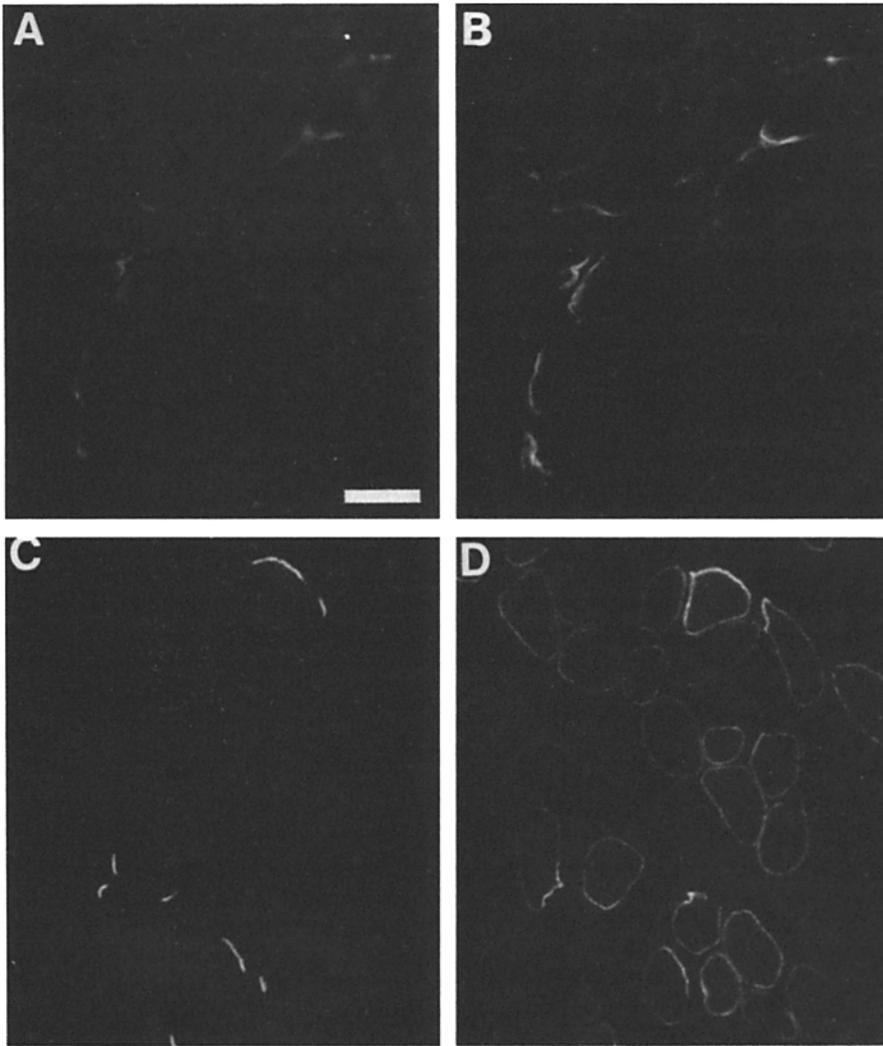
#### **Discussion**

In this paper, we have used a mAb against an *M*, 58,000, peripheral membrane protein of *Torpedo* postsynaptic membranes to show that skeletal muscle contains a very similar

protein and to localize these proteins in electroplax and muscle. The *Torpedo* 58K protein has received little attention heretofore, since it is a relatively minor protein in the isolated membranes and is not well resolved from the much more abundant AChR gamma subunit on one-dimensional gels. It is now the second protein, after the 43K protein, of the isolated membranes to be localized in the electroplax and to have led to the identification of a muscle protein. The 43K and 58K proteins are strikingly different in several key aspects. The distribution of the 58K protein in muscle is much broader, the 43K protein being precisely co-distributed with AChR (21, 25, 62). The 58K protein also occurs in several *Torpedo* tissues that contain little or no AChR (heart, liver, pancreas, and brain, with particularly high amounts present in brain) (unpublished results). Nonetheless, our results suggest that the 58K protein is potentially of great interest in studies of postsynaptic development. It is a postsynaptic protein in electroplax and is concentrated at AChR clusters on cultured muscle cells. It also appears to be more concentrated on the postsynaptic membrane of adult neuromuscular junctions than on the surrounding sarcolemma. Furthermore, its expression in adult muscle appears to be innervation dependent. As it is clearly distinct from the other proteins described in these membranes and from major cytoskeletal proteins (see Results), identification of its function(s) in these situations will require further biochemical characterization.

The strongest evidence we have found for an AChR-related role for the 58K protein comes from the very similar, although not always identical, distributions of AChR and the 58K protein in spontaneously formed AChR clusters on cultured muscle. The relative time courses of appearance of these two proteins at such clusters is unknown. However, Tobler et al. (65) have found that the 58K protein accumulates at latex bead-induced clusters with a time course indistinguishable (as judged by immunofluorescence) from that of AChR. This could be explained if AChR existed in a complex with the 58K protein before bead-induced accumulation. However, when diffusely distributed, laterally mobile AChR in  $\text{BC}_3\text{H}_1$  muscle cells were cross-linked by externally applied anti-AChR antibodies, the resulting microclusters of AChR did not contain the 58K protein (23). They did, however, contain the 43K protein, which is believed to exist in a complex with the postsynaptic AChR (14, 62). Apparently, then, the 58K protein is specifically assembled by muscle cells into sites of latex bead-induced AChR accumulation by a pathway separate from that of AChR. The concurrence in time of accumulation of AChR and the 58K protein at these sites could most clearly arise if the 58K protein played a specific role in the process of AChR accumulation.

Several basal lamina antigens present interesting parallels to the 58K protein in that they are not only concentrated at the neuromuscular junction, but also occur on extrajunctional muscle membrane and nonmuscle cells (17, 56–58). In addition, Peng and Cheng have shown that basal lamina, like 58K protein, accumulates at bead-induced clusters (48). The closest parallel involves the heparan sulfate proteoglycan of *Xenopus* muscle basal lamina described by Anderson and colleagues (2, 3). Both proteins show an early concentration at AChR clusters followed by a more general distribution later on, they occur in patches without AChR, and they extend into the junctional folds of the mature neuromuscular



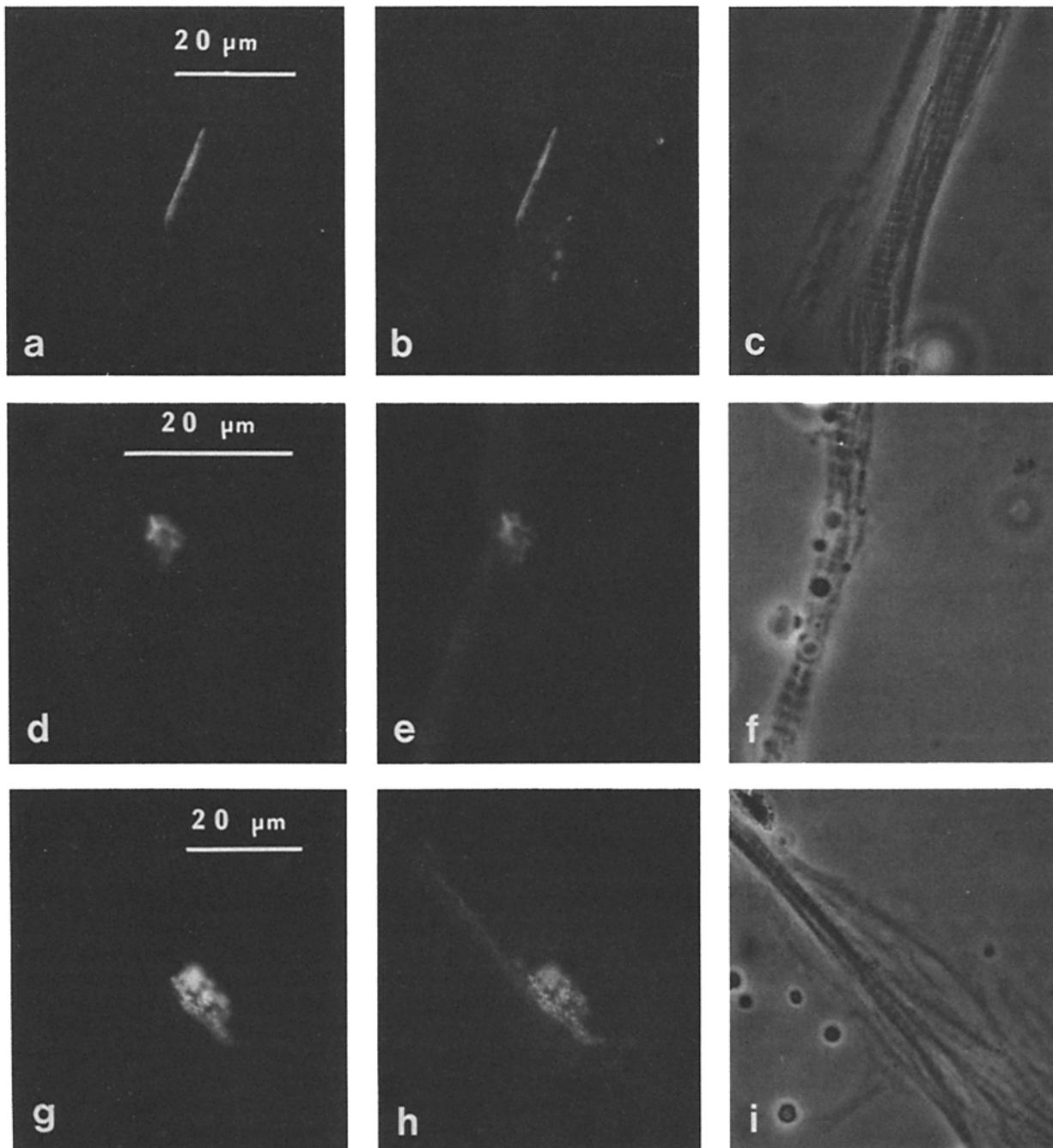
**Figure 7.** Immunofluorescence staining of rat and chicken muscle with anti-58K mAb. (A and C) Rhodotoxin; (B and D) anti-58K mAb. Staining by mAb 1351 occurs around the entire periphery of each muscle cell but is more intense at junctions. (A and B) Cryostat sections of neonatal rat diaphragm muscle. (C and D) Semi-thin frozen sections of chicken anterior latissimus dorsi muscle. The line of extrajunctional staining contains small, periodic interruptions in this muscle but apparently not in any of the others studied. The origin of this feature is unknown. Bar, 10  $\mu$ m.

junction. Very similar observations have been made for laminin on cells in culture (17). These parallels with the 58K protein may be coincidental. However, since neither the transmembrane receptors for specific basal lamina proteins nor the cytoplasmic proteins through which they may exert their effects on AChR distribution are known in this system, further tests of these parallels should be made. For example, it would be interesting to see if the 58K protein, like the proteoglycan (3), remains behind at clusters from which the receptor has been caused to disperse.

The mechanism of association of the 58K protein with isolated membranes is unknown but presumably involves specific interactions with one or more proteins. These proteins are unlikely to include AChR or 43K protein since the 58K protein is associated with membranes which have neither. The 58K protein is also unlikely to be a component of membrane-associated, actin-based cytoskeleton, that might be suspected from its association with AChR clusters since the postsynaptic membrane of the electroplax apparently does not carry such a cytoskeleton (33). Intermediate filaments project to the postsynaptic membrane but also to the noninnervated face membrane. Their involvement with the 58K protein would require differences in these two populations

that have not been recognized so far. All of these considerations, then, suggest that the binding protein(s) may be found among components of isolated membranes that have not yet been described in any detail. It would be particularly interesting if the search for such a protein uncovered another postsynaptic, transmembrane protein (in addition to AChR).

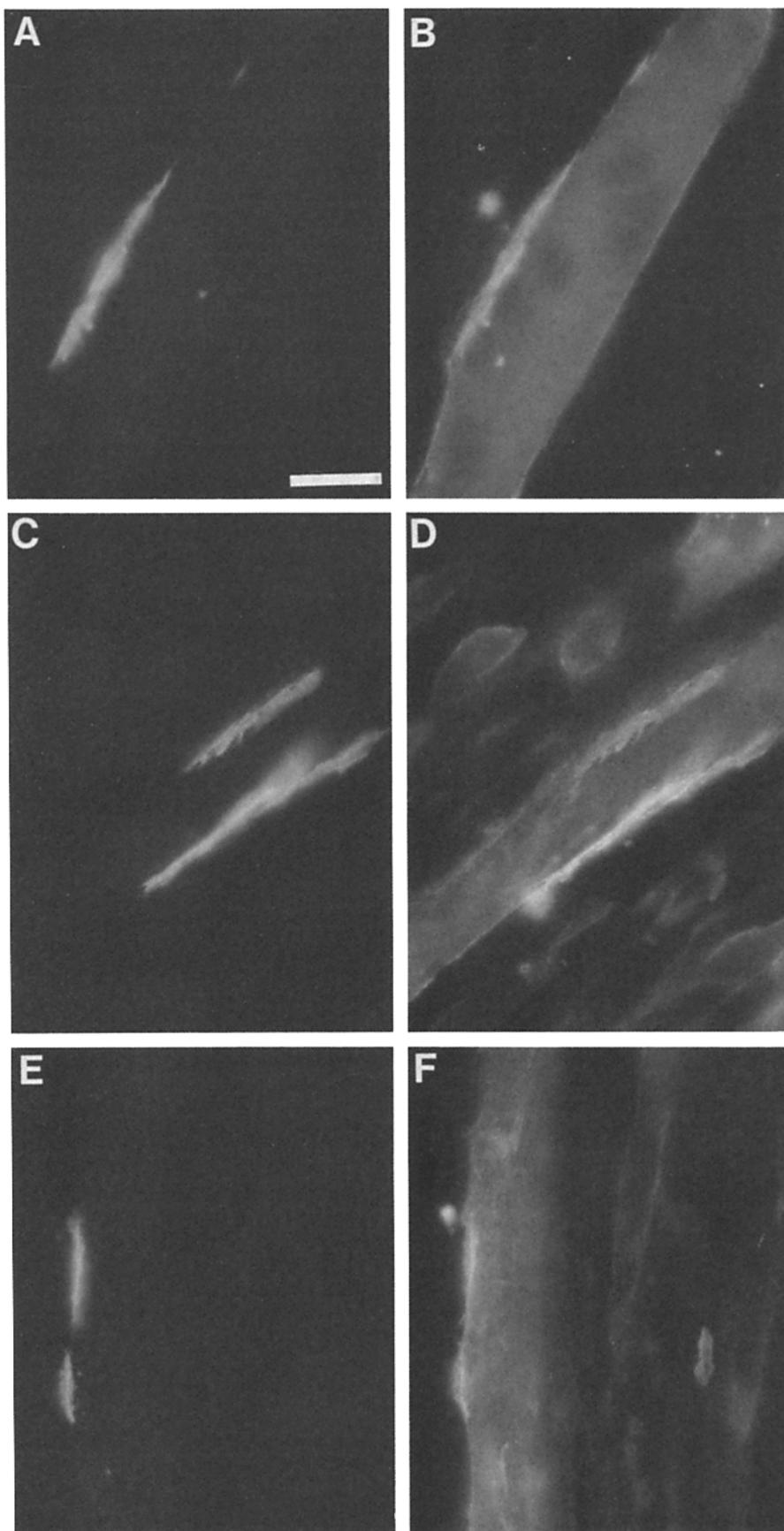
Denervation of adult rat muscle induces several changes in muscle cells. The number of extrajunctional receptors increases dramatically (8), while the half-time for turnover of the junctional receptors decreases (36) (but without change in the junctional receptor site density [36]). These events occur within several days of denervation. Loss of the endplate form of acetylcholinesterase has also been observed after denervation of rat diaphragm (29). After denervation of frog muscle, an intermediate filament protein remains concentrated at endplates, but the extrasynaptic and internal amounts appear to increase (12). In contrast, the concentration of the junctional and extrajunctional 58K protein in rat muscle appears to decrease substantially by 6 wk after nerve transection. At this time period Schwann cells have also withdrawn (40), but this cannot account for the diminution in 58K protein since it is clearly postsynaptic. Although we have assessed the effects of denervation qualitatively (by immu-



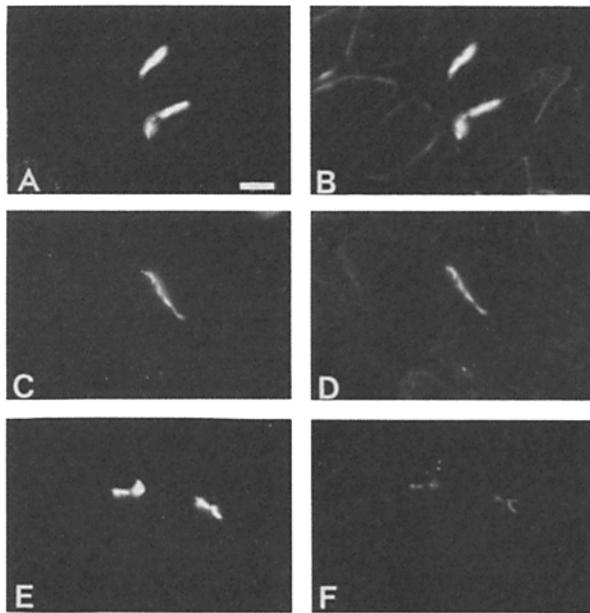
**Figure 8.** Immunofluorescence staining of *Xenopus* myotomal cells with anti-58K mAb. 4-d-old cultures were fixed, permeabilized with Triton, and stained with mAb 1351. (*A*, *D*, and *G*) Rhod-toxin; (*B*, *E*, and *H*) anti-58K mAb; (*C*, *F*, and *I*) phase contrast. (*A* and *B*) AChR cluster along the edge of the cell. (*D* and *E*) A dorsal surface cluster. (*G* and *H*) A cluster on the ventral, substrate-apposed surface. The correspondence between the fluorescein and rhodamine images at clusters was very close. Three small patches of bright mAb staining without corresponding rhod-toxin staining can be seen in *B*. Bars: (*A*–*C*) 20  $\mu$ m; (*D*–*F*) 20  $\mu$ m; (*G*–*I*) 20  $\mu$ m.

nofluorescence) and while we cannot eliminate the possibility that the apparent decrease may reflect changes in the antigenicity of the protein rather than its concentration, the finding nevertheless suggests that the synaptic 58K protein is regulated by innervation. However, the direction of the change in expression as well as the time to onset differs for the receptor and the 58K protein, suggesting that the mode of regulation may be different for these two postsynaptic proteins.

The present data strongly suggest that the 58K protein has specific functions related to AChR. We recognize, however, that they are insufficient to rule out that cellular events that trigger AChR accumulation at a particular site may also trigger other simultaneous, but independent, changes at the same site, and that accumulation of the 58K protein may be instead a part of these other processes. In the same vein, the localization of the 58K protein to the postsynaptic membrane of the electroplax and its absence from the noninnervated



*Figure 9.* Immunofluorescence staining of C2 myotubes with anti-58K mAb. Cultures were fixed, permeabilized with Triton, and labeled with anti-58K mAb and rhod-toxin. (*A*, *C*, and *E*) Rhod-toxin; (*B*, *D*, and *F*) anti-58K mAb. Anti-58K staining was concentrated at AChR clusters, although general, less intense staining of the cell membrane outside of clusters was apparent. Bar, 10  $\mu$ m.



**Figure 10.** Immunofluorescence staining of denervated rat diaphragm muscle. (A, C, and E) Rhod-toxin; (B, D, and F) anti-58K mAb. (A and B) Normal diaphragm. Staining of junctions on two muscle cells was strong; some extrajunctional staining by the antibody can be seen in B. (C and D) 9-d denervated diaphragm. Staining by the antibody remained strong. (E and F) 6-wk denervated diaphragm. In spite of strong staining by rhod-toxin (E), the staining by the antibody was weak. At other junctions and in extrajunctional regions, staining was almost undetectable. Bar, 10  $\mu$ m.

face are particularly intriguing but could result more from unusual properties of the noninnervated face membrane (immaturity?) than from a specific postsynaptic activity of the 58K protein. A similar problem exists with respect to other proteins at the junction such as the cytoskeletal proteins or basal lamina antigens. As more such proteins become known, it will become increasingly important to distinguish among these possibilities. This is clearly a challenging problem likely to call for considerable ingenuity.

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