Association of the Postsynaptic 43K Protein with Newly Formed Acetylcholine Receptor Clusters in Cultured Muscle Cells

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ABSTRACT   The postsynaptic membrane from Torpedo electric organ contains, in addition to the acetylcholine receptor (AChR), a major peripheral membrane protein of ~43,000 mol wt (43K protein). Previous studies have shown that this protein is closely associated with AChR and may be involved in anchoring receptors to the postsynaptic membrane. In this study, binding sites for monoclonal antibodies (mabs) to the 43K protein have been compared to the distribution of AChR in Xenopus laevis muscle cells in culture. In double label immunofluorescence experiments, clusters of AChR that occur spontaneously on these cells were stained with anti-43K mabs. Newly formed receptor clusters induced with positive polypeptide-coated latex beads were also stained with anti-43K mabs as early as 12 h after the application of the beads. Exact correspondence in the distribution of the anti-43K protein binding sites and the AChR was found in both types of clusters. These results suggest that the 43K protein becomes associated with AChR clusters during a period of active postsynaptic membrane differentiation. Thus, this protein may participate in the clustering process.

An early event during the formation of the neuromuscular synapse is the aggregation of acetylcholine receptors (AChR)[1] at the postsynaptic membrane in response to innervation (1, 15, 50). Tissue cultures of skeletal muscle cells are an invaluable tool for the study of this process. In addition to neurally induced receptor aggregates, AChR also form clusters spontaneously (the "hot spots"; 2, 14, 48) or in response to exogenously applied stimuli in tissue culture (11, 23, 31, 34, 42). Although these extrasynaptic AChR clusters have proven to be a convenient system to study, the cellular and molecular mechanisms involved in the clustering process remain largely unknown. Recent studies have indicated that the cytoskeleton may be involved in the aggregation of AChR (12, 28, 38), but the precise nature of the AChR-cytoskeleton interaction remains elusive.

Highly purified postsynaptic membranes from Torpedo electric organ contain, in addition to the ACh receptor, a major peripheral membrane protein of ~43,000 mol wt (43K protein) (13, 19, 26, 36, 46). Exposure to alkaline pH causes dissociation of this protein from the membranes (26) with a concomitant increase in the rotational and translational mobility of the receptor (4, 10, 25, 41). The 43K protein is distinct from actin (36) and creatine kinase (5, 16) and lies on the cytoplasmic side of the membrane (27, 36, 45, 47, 49) in close enough proximity to the receptor so that chemical crosslinking between the two proteins can be induced with a bifunctional reagent (9). Recent results indicate that the 43K protein interacts in a hydrophobic manner with the lipid bilayer (37), and that treatment of purified membranes with copper o-phenanthroline promotes the formation of disulfide-linked homopolymers of this protein (37). Furthermore, immunogold ultrastructural studies with monoclonal antibodies (mabs) have shown that at the Torpedo electrocyte synapse, the distribution of the 43K protein and the receptor are virtually identical; both are restricted to the crests of the folds of the postsynaptic membrane (45). A related protein is present at endplates of mammalian muscle (16, 17). Thus, the 43K protein, which is distinct from the known cytoskeletal

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[1] Abbreviations used in this paper: AChR, acetylcholine receptor(s); FITC, fluorescein isothiocyanate; 43K protein, the major membrane-bound 43,000-mol-wt protein isolated from Torpedo postsynaptic membrane; mab, monoclonal antibody; R-BTX, tetramethyl rhodamine-conjugated α-bungarotoxin.

proteins of other systems, may interact directly with the receptor so as to anchor it beneath the nerve terminal.

In an attempt to understand the involvement of the 43K protein in the formation of AChR clusters, we have studied its distribution at both preexistent and newly formed AChR clusters in *Xenopus* myotomal cell cultures by use of mabs. The studies on newly formed AChR clusters were facilitated by the use of positive polypeptide-coated latex beads as the stimulus for the induction of the clustering process (31, 32). Our results show that within both types of clusters there is an exact correspondence between the receptors and the binding sites for antibodies to the 43K protein. Furthermore, the appearance of 43K protein at receptor clusters at a very early time during their formation suggests that this component may play an important role in the clustering process.

MATERIALS AND METHODS

Cell Cultures and Induction of AChR Clustering: Myotome cells were isolated from stage 20–22 *Xenopus laevis* embryos and cultured on coverglass squares according to published methods (30). To induce the formation of new AChR clusters, we treated the cells with polyornithine-coated latex spheres (4.5 or 10 μm) as previously described (31, 32). The clusters were labeled with tetramethyl rhodamine–conjugated α-bungarotoxin (R-BTX) for fluorescence microscopy (2, 39).

Immunofluorescence Localization of 43K Protein: After the cultures were labeled with R-BTX, they were fixed with 95% ethanol at −20°C for 5 min, washed with phosphate-buffered saline (PBS), and incubated with mab against 43K protein. The IgG fractions were diluted to a concentration of 100 nM with PBS plus 1–3% bovine serum albumin (BSA). After an incubation period of 1 h, the cultures were washed extensively with PBS and labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti–mouse IgG (Cappel Laboratories, Inc., Cochranville, PA) reconstituted in PBS plus BSA. The cultures were then washed with PBS and mounted with a medium which contained polyvinyl alcohol and glycerol (40). The specimens were examined with a Leitz Orthoplan microscope equipped with rhodamine- and FITC-fluorescence optics (E. Leitz, Inc., Rockleigh, NJ). The images were recorded on Kodak Tri-X film.

In control experiments, we labeled cultures with IgG secreted by the myeloma cell lines MPC-11 and MOPC-21. Neither of these antibodies reacted with adult neuromuscular junction.

Production and Characterization of Mabs to 43K Protein: Membranes enriched in acetylcholine receptors were prepared from *Torpedo nobiliana* electric organ (36), and peripheral proteins extracted from these membranes with lithium diiodosalicylate were used as immunogens (16). Injection of mice, production and maintenance of hybridomas, screening with solid phase assays, and the purification of IgG from ascites fluids were done according to procedures described in Froehner et al. (18) and Froehner (16). Mabs 1210B and 1234A were selected for further characterization because they reacted with endplates of rat diaphragm muscle in immunofluorescence experiments. Both antibodies are the IgG$_1$ isotype.

The procedures for two-dimensional polyacrylamide gel electrophoresis of purified *Torpedo* membranes (36) and for immunoblot analysis of mabs (16, 18) have been described in detail.

RESULTS

Specificity of Anti–43K Protein Mabs

For the preparation of mabs, mice were injected with proteins extracted from purified *Torpedo* postsynaptic membranes with lithium diiodosalicylate. The 43K protein is the major component in this extract, comprising ~70% of the total protein (36). Hybridomas were first screened for reactivity with the antigen by a solid phase assay and then analyzed by one-dimensional immunoblots (18) and immunofluorescence microscopy on cryostat sections of rat muscle. Mabs 1210B and 1234A were selected for further study because they react with a 43,000-mol-wt protein and bind to rat muscle endplates (data not shown).

Because some preparations of *Torpedo* membranes contain several proteins of ~43,000 mol wt (including actin and creatine kinase) (5, 19, 36), we examined the reactivity of the mabs with *Torpedo* membrane proteins separated by two-dimensional gel electrophoresis. As previously described (36),
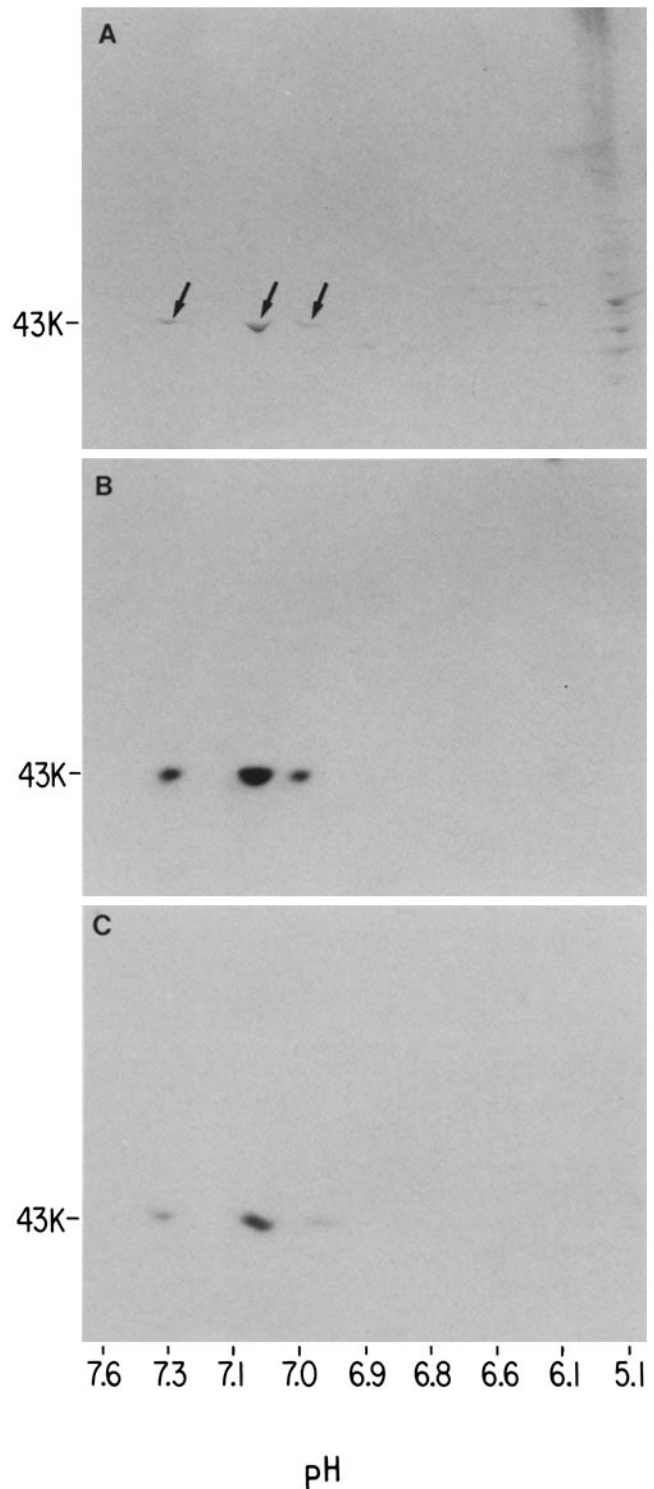

FIGURE 1 Two-dimensional immunoblot analysis of mab specificity. Purified AChR-rich membranes from *Torpedo* electric organ were subjected to two-dimensional gel electrophoresis. (A) Coomasie Blue–stained gel. Arrows indicate three isoelectric variants of the 43K protein. Identical gels were subjected to immunoblot analysis with (B) mab 1210B or (C) mab 1234A.
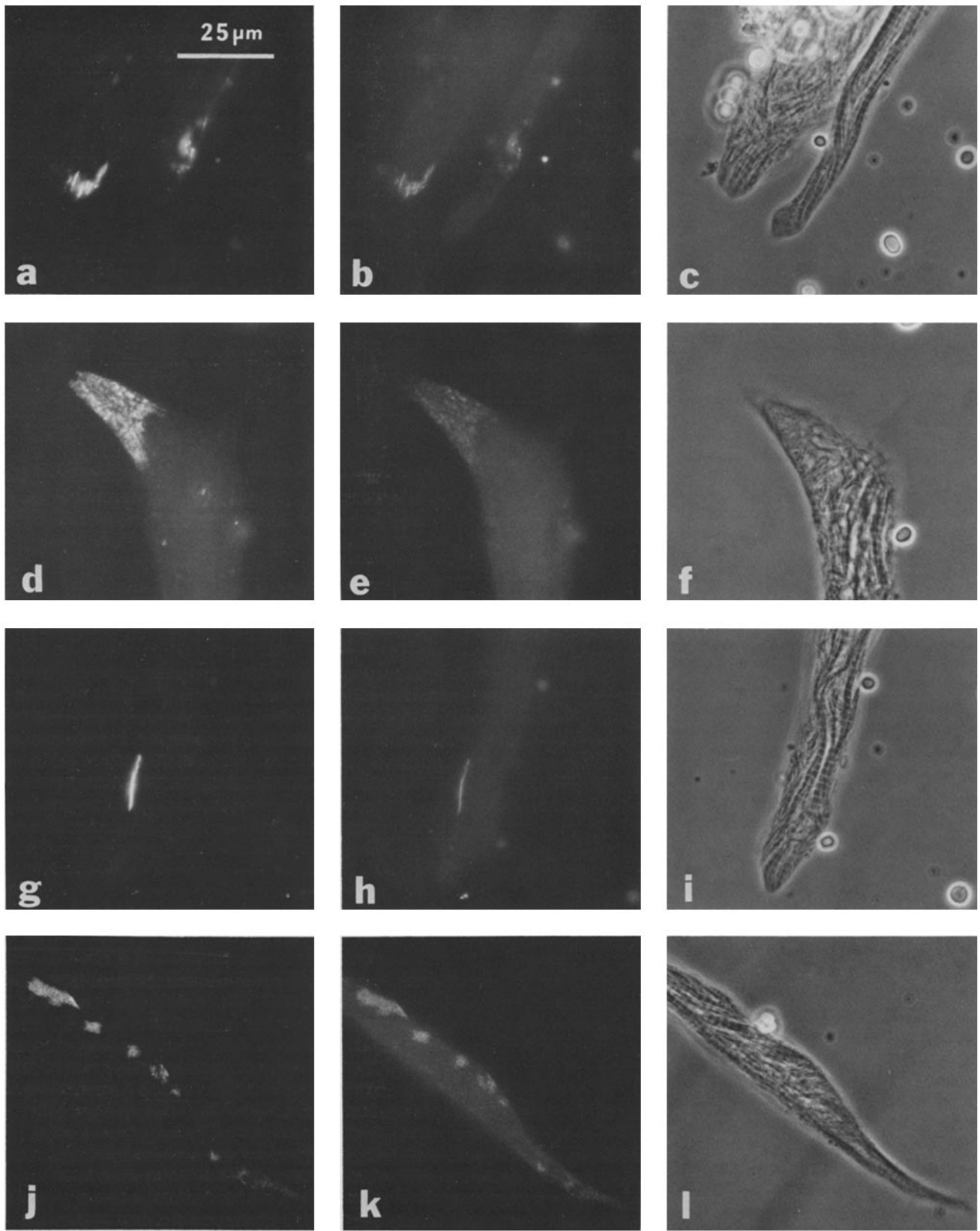
FIGURE 2   Co-localization of AChR clusters and anti–43K mab binding sites. The left column shows R-BTX fluorescence images; the center column shows the corresponding anti–43K mab images (mab 1210B in *b*, *e*, and *h*, and mab 1234A in *k*); the right column shows the phase-contrast images.
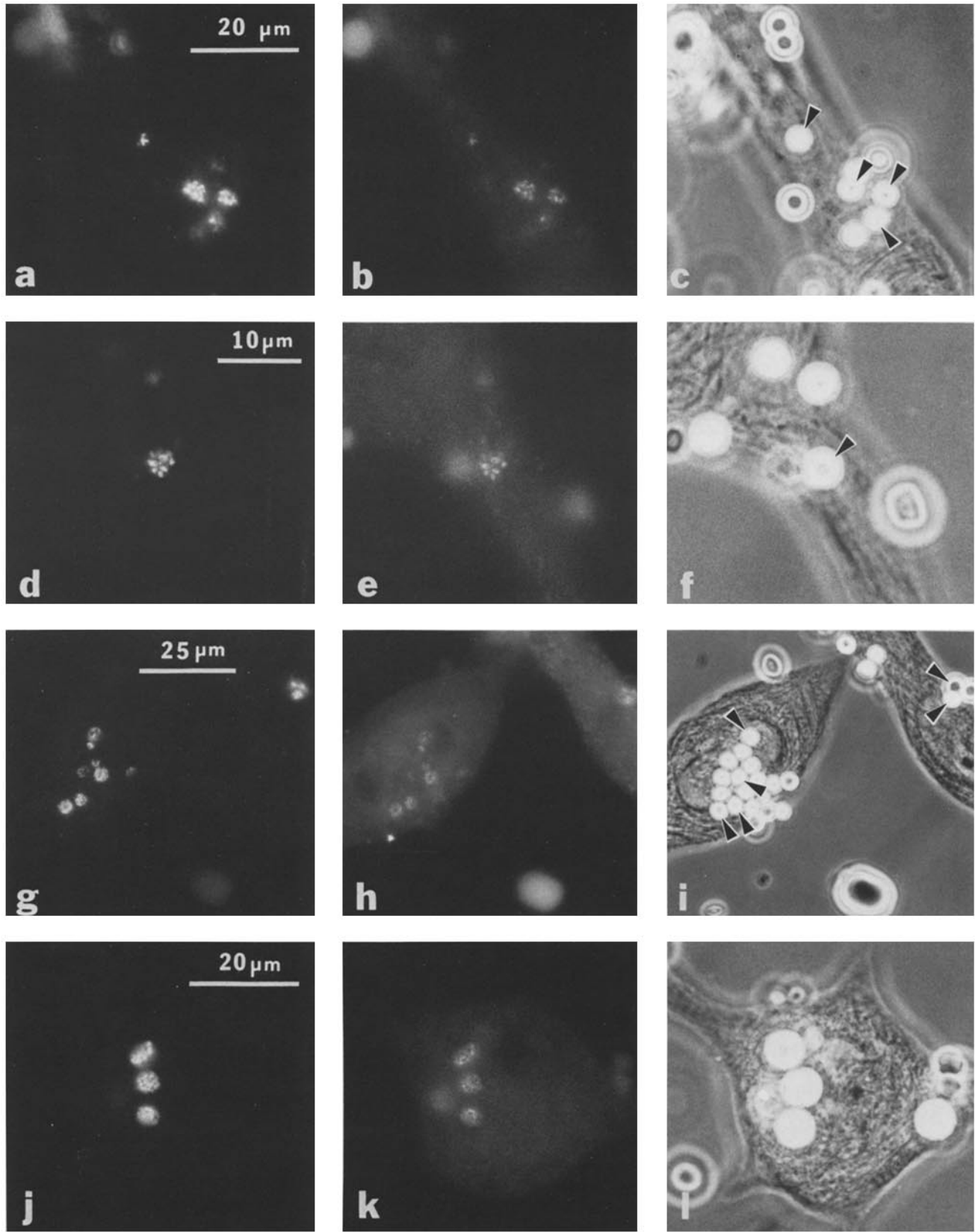
FIGURE 3 Association of 43K protein with newly formed AChR clusters induced by polyornithine-coated latex beads. The arrangement of the columns is similar to that in Fig. 2. The arrowheads in c, f, and i point to examples of bead-muscle contacts that are cluster-positive. The correspondence in the fine structure of the R-BTX and anti–43K mab images can be seen especially well in a and b and d and e. Examples in a–i are from 1-d bead-muscle co-cultures treated with 4.5-μm beads and mab 1210B. The example in j–l is from a 3-d co-culture treated with 10-μm beads and mab 1234A.
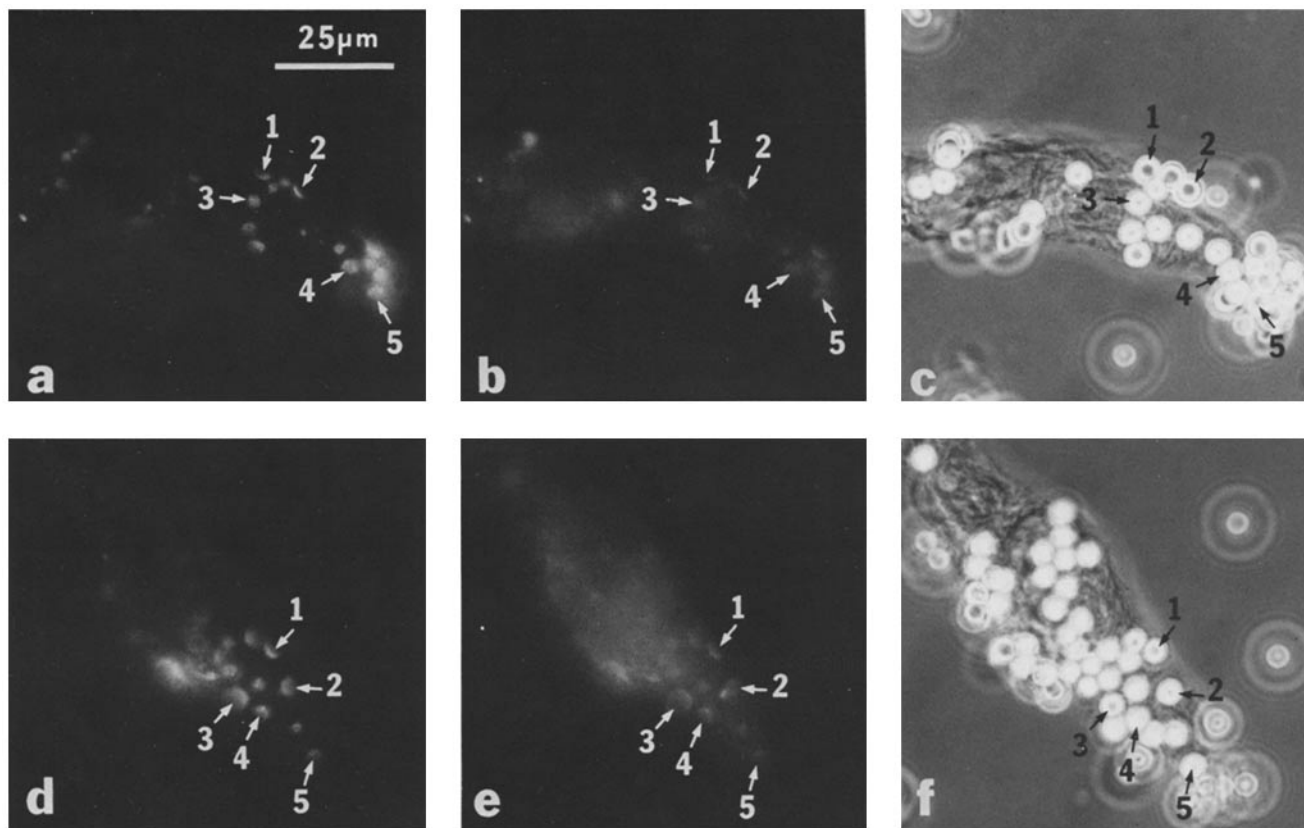
FIGURE 4   Association of 43K protein with bead-induced AChR clusters in 12-h co-cultures. The arrangement of the columns is similar to that in Fig. 2. The numbers indicate the correspondence in the AChR clusters (a and d), 43K mab staining sites (b and e), and the positions of the beads (c and f).

the 43K protein migrates as three or four isoelectric variants (pI = 7.0–7.8) that yield identical peptide maps. These components are clearly distinct from actin by their isoelectric points and peptide maps (36). Mabs 1210B and 1234A, like seven other mabs against this protein (16), react with each of the charge variants of the 43K protein (Fig. 1) but not with any other protein in these membranes. A mab with specificity for the same protein cannot bind Torpedo creatine kinase (16). Thus, mabs 1210B and 1234A react specifically with the 43K protein as defined by Porter and Froehner (36). This is probably the same protein that others have termed ν_1 (19).

Association of 43K Protein with Preexistent AChR Clusters

In the absence of innervation or other experimental manipulations, AChR clusters form spontaneously in Xenopus cultures. These preexistent clusters ("hot spots") are found on the top and bottom surfaces or along the edge of the cell. They are usually composed of many sub-clusters and are highly variable in size (Fig. 2). When cultures were double-labeled with both R-BTX and the anti–43K antibodies, striking co-localization of the rhodamine- and FITC-fluorescence patches was observed. Fig. 2, a–f shows two examples of preexistent clusters located on the substrate side of the cell, and g–i shows an example of the edge-situated cluster labeled with mab 1210B. In comparing the fluorescence image pairs, the following points became evident: (a) The location and the sub-structure of the two types of fluorescence patches matched exactly with one another (Fig. 2, a–b, d–e, and g–h). (b) All R-BTX patches that exhibited intense fluorescence were as-

sociated with intense FITC fluorescence. (c) Within the R-BTX patches, areas that lacked rhodamine fluorescence were also deficient in FITC fluorescence. Thus, there is a strong correlation between the AChR clusters and the binding sites for anti–43K antibodies. Similar results were obtained when mab 1234A was used (Fig. 2, j–l).

In addition to the AChR cluster–associated fluorescence, the mab images also showed a diffuse fluorescence throughout the rest of the cell. We were unable to eliminate this background even by exhaustive rinsing steps or by a variation in the concentration of the primary and secondary antibodies. This background was also seen when the muscle cells were labeled with nonspecific IgG (Fig. 6), although the latter staining was less intense. Thus, this staining outside the clusters may contain both a specific and a nonspecific component. Further investigation will be necessary to clarify this point.

Association of 43K Protein with Newly Formed AChR Clusters

Previously we showed that latex beads coated with basic polypeptides can induce the formation of AChR clusters in Xenopus myotomal cell cultures (31, 32). The size of the clusters is proportional to the size of the beads applied. The onset of the clustering process is immediately after the application of the beads. By 24 h of co-culture, most of the clusters are well formed (33). However, the maturation of the clusters as shown by the development of ultrastructural specializations continues throughout the first week of bead-muscle co-culture (H. B. Peng, unpublished results).

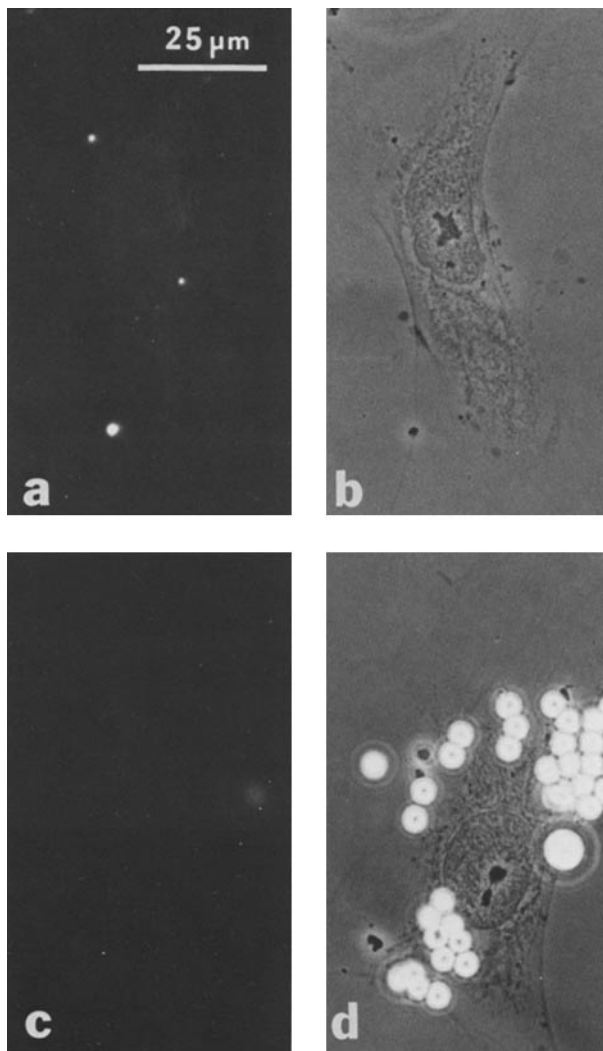When the cultures were double-labeled with R-BTX and

FIGURE 5 Nonmuscle cells treated with anti–43K mab 1210B (a and b) and 1234A (c and d). The mab images are shown on the left, and the corresponding phase images are shown on the right. An absence of mab staining is obvious. The example in c and d was also treated with beads.

either of the two anti–43K mabs, we found that all AChR clusters induced by the beads were also associated with fluorescence due to mab labeling. Fig. 3, a–i illustrates three examples with mab 1210B in 1-d co-cultures. Bead-induced clusters stained with R-BTX were often composed of sub-clusters 0.1–0.5 μm wide (Fig. 3, a and d). This intricate fine structure was reproduced in the mab staining patterns (Fig. 3, b and e). Areas within the cluster free of R-BTX labeling also showed a diminished mab staining. Individual clusters were discrete and confined to the area of the bead-muscle contact. Clusters induced by contact of the beads with either the top or the edge of the muscle cells were both associated with mab labeling. Similar results were obtained with mab 1234A (Fig. 3, j–l). In this series, 10-μm beads were used, and thus the resultant clusters were also larger.

We also investigated the association of mab staining with induced clusters at shorter times after bead-muscle co-culture. The density of AChR within these early clusters was relatively low as shown by the dimness of their R-BTX images (Fig. 4). 43K protein antigens could be detected at these developing clusters as early as 12 h of co-culture. Although the mab

images were dimmer than were after 24 h, they clearly stood out against the background. We could not detect 43K antigens with either mab at clusters ≤6 h after bead application. It is possible that they were present but were obscured by the background fluorescence.

Controls

To ascertain the specificity of the anti–43K mab labeling, we performed the following control experiments. First, non-muscle cells were examined after labeling with anti–43K mab. As shown in Fig. 5, a–d, there is a total absence of fluorescence when these cells were labeled with either 1210B or 1234A. Then, we examined muscle cultures labeled with two nonspecific IgG mabs secreted by myeloma cell lines MPC-11 and MOPC-21. As shown in Fig. 6, mabs MPC-11 and MOPC-21 gave the cells a diffuse background staining, but the fluorescence was not intensified at AChR clusters. Furthermore, no mab staining was seen if the cultures were labeled in the living state without permeabilization of the plasma membrane (data not shown). Based on these results, we conclude that the immunofluorescence at AChR clusters as shown in Figs. 2–4 is due to the presence of specific antigenic sites recognized by the anti–43K mabs.

DISCUSSION

In this study, we have demonstrated that a protein recognized by mabs against the postsynaptic 43K protein from *Torpedo* electric tissue is concentrated at both preexistent and newly formed AChR clusters in *Xenopus* myotomal cell cultures. The exact correspondence between the anti–43K mab staining sites and individual aggregates within these AChR clusters indicates that a component related to the 43K protein is present at these clusters in a distribution very similar to that of the receptor. Furthermore, this protein occurs at newly formed AChR clusters induced by positively charged latex beads during a period of active postsynaptic differentiation. This suggests a temporal as well as a spatial correlation between the 43K protein and the AChR clustering process.

Recent studies have shown that a specialized cytoplasmic structure enriched in cytoskeletal elements is associated with AChR clusters (21, 22, 28, 38). This structure may provide resistance of the clustered receptors to detergent extraction (38). In addition to the 43K protein (16, 17, 43), a wealth of cytoskeletal proteins, including actin (20), vinculin (6, 7), α-actinin (7), filamin (7), and a protein of 51,000 mol wt that is probably related to the intermediate filaments (8), are associated with muscle endplates. Although all of these components are concentrated at endplates as shown by immunofluorescence with specific antisera, they are probably not all directly associated with the receptors. In clusters that occur on the ventral surfaces of rat myotubes, for example, vinculin is concentrated in membrane areas that interdigitate with regions of high AChR density (6). On the other hand, there is now biochemical (9) and ultrastructural (44, 45) evidence that the 43K protein is closely associated with and possibly linked noncovalently to AChR in *Torpedo* postsynaptic membranes, an interaction that may restrict the mobility of the receptors (4, 25). Thus, the 43K protein is a good candidate for the mediation of the interaction between the receptors and the cytoskeleton. A more complete characterization of the bio-chemical properties of this protein and its interactions with other synaptic proteins will be necessary to test this proposal.
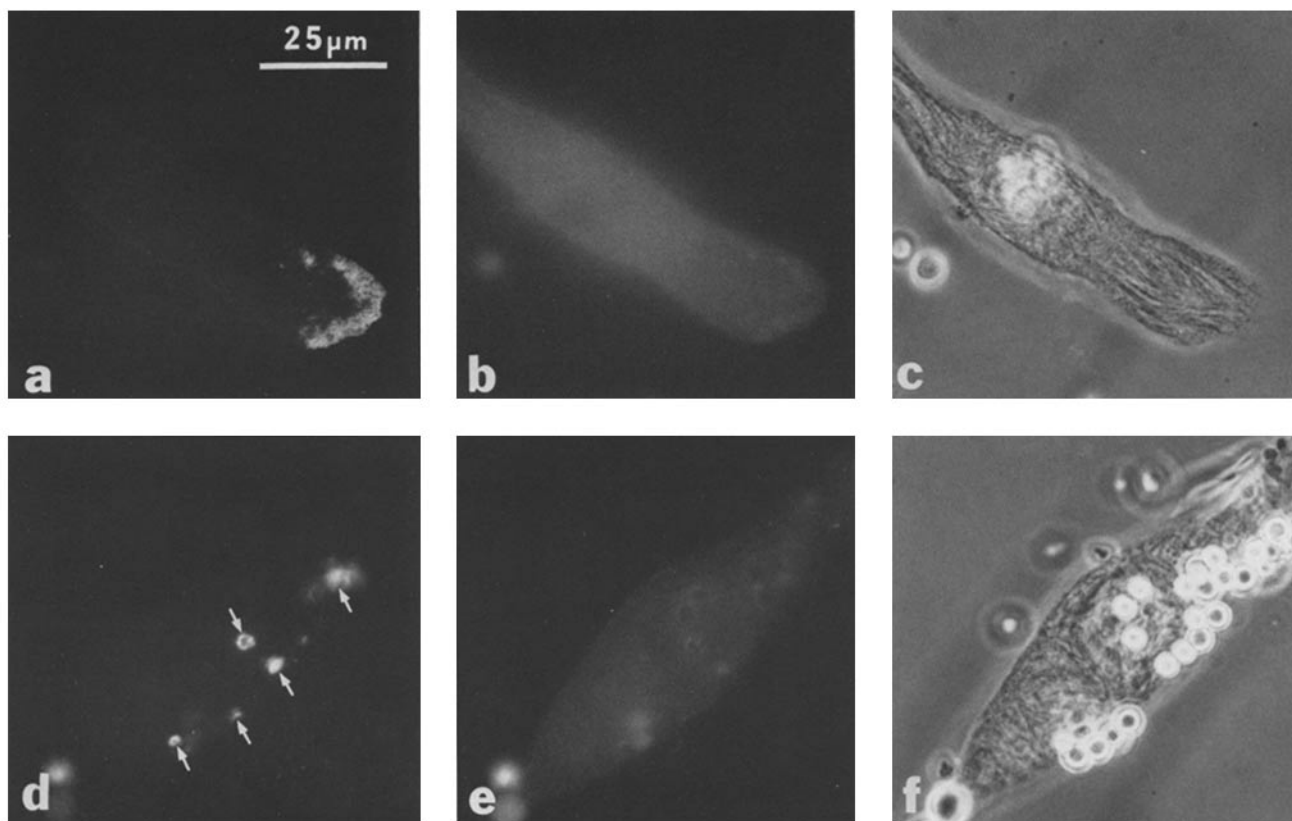
FIGURE 6   Muscle cells treated with control mabs MPC-11 (a–c) and MOPC-21 (d–f). The left column shows the R-BTX images; the center column shows the corresponding mab images; the phase images are shown in the right column. Both the pre-existent (a) and the bead-induced (arrows in d) clusters lack mab staining (b and e).

The precise role of the cytoskeleton in the formation of the AChR clusters is not clear. It is known that diffusely distributed AChR can undergo lateral translocation within the plane of the membrane (3, 35), and that a major part of the receptors within the cluster are derived from these diffuse receptors (1, 24, 32). Thus, the cytoskeleton may organize the surface receptors into clusters. The observation that the 43K protein is present in receptor clusters does not preclude the possibility that diffusely distributed receptors also have this component associated with them. Sensitivity limits of immunofluorescence have so far prevented us from answering this question unambiguously. Thus, the 43K protein may be associated with diffuse receptors and become organized into clusters along with the receptors. Alternatively, diffuse receptors may be devoid of 43K protein and only acquire this component when cluster organization occurs.

Previously we showed that, in addition to the formation of AChR clusters, other postsynaptic specializations, including membrane invagination, the basement membrane, and the postsynaptic density, also develop at the contact between the positively charged latex beads and the *Xenopus* muscle cells (32, 33). Bead-induced clustering of AChR appears to be mediated by calcium and calmodulin (29) and is preceded by the formation of a meshwork of thin, presumably actin, filaments at the site of bead-muscle contact (33). The presence of a postsynaptic-specific protein at the bead-induced AChR clusters as shown in this study further indicates the similarity at the molecular level between this process and the action of the nerve. One can now use this simple system to examine the functions of the 43K protein in the differentiation of the

postsynaptic membrane. The availability of mabs to this protein has enabled us to demonstrate its presence at newly formed AChR clusters. Future studies should test the feasibility of using these antibodies as probes of 43K protein function.

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