

# The Relationship of the Postsynaptic 43K Protein to Acetylcholine Receptors in Receptor Clusters Isolated from Cultured Rat Myotubes

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**Abstract.** We have examined the relationship of acetylcholine receptors (AChR) to the *M*<sub>2</sub> 43,000 receptor-associated protein (43K) in the AChR clusters of cultured rat myotubes. Indirect immunofluorescence revealed that the 43K protein was concentrated at the AChR domains of the receptor clusters in intact rat myotubes, in myotube fragments, and in clusters that had been purified ~100-fold by extraction with saponin. The association of the 43K protein with clustered AChR was not affected by buffers of high or low ionic strength, by alkaline pHs up to 10, or by chymotrypsin at 10 µg/ml. However, the 43K protein was removed from clusters with lithium diiodosalicylate or at alkaline pH (>10).

Upon extraction of 43K, several changes were observed in the AChR population. (a) Receptors redistributed in the plane of the muscle membrane in alkali-extracted samples. (b) The number of binding sites accessible to an anti-AChR monoclonal antibody directed against cytoplasmic epitopes (88B) doubled. (c) Receptors became more susceptible to digestion by chymotrypsin, which destroyed the binding sites for the 88B antibody only after 43K was extracted. These results suggest that in isolated AChR clusters the 43K protein covers part of the cytoplasmic domain of AChR and may contribute to the unique distribution of this membrane protein.

IN vertebrate skeletal muscle, nicotinic acetylcholine receptors (AChR)<sup>1</sup> are concentrated in the postsynaptic region in the membrane immediately underlying the motor nerve terminal (13, 14, 27; for review, see reference 12). Although the mechanism by which receptors become postsynaptically localized is not yet understood, a great deal of attention has been focused on the structural, or cytoskeletal, proteins that are concentrated on the cytoplasmic side of the postsynaptic membrane (for reviews, see references 16 and 38). These proteins include actin (20),  $\alpha$ -actinin, filamin, vinculin (6), talin (34), a 51-kD protein resembling tonofilament protein (7), and a 43,000-D protein distinct from actin (8, 15, 18, 19, 28, 32, 33, 35). We focus our attention in this paper on the latter protein, termed 43K (29) or  $\nu_1$  (19).

The 43K protein (43K) has received a great deal of attention because of its close association to the AChR. During purification of AChR from *Torpedo* electroplax, the 43K protein copurifies with receptor-rich membrane fragments (11, 19, 36). Immunocytochemical studies of such preparations at the ultrastructural level have shown that 43K is very close to the membrane bilayer (24, 35) and is coextensive

with AChR (35). The 43K protein is found only in tissues that have nicotinic AChR where the concentrations of the two proteins appear to be equimolar (10, 21, 36). The attachment of 43K to the postsynaptic membrane is very stable, and may involve a hydrophobic interaction with the lipid bilayer (29). Its removal can only be effected by detergents, chaotropic agents, or at alkaline pH (11, 23). Biochemical and biophysical studies have shown that removal of the 43K protein from *Torpedo* postsynaptic membrane changes many of the properties of the membrane-bound receptor. These changes include increased susceptibility to heat denaturation and proteolytic degradation, and an increased rate of rotational and lateral diffusion (1, 10, 22, 31, 39). These observations suggest that AChR and 43K can interact with one another in a mature postsynaptic structure. In agreement with this possibility, Burden and his colleagues have recently reported that the 43K protein can be chemically cross-linked to the beta subunit of the AChR (9).

In contrast to our considerable knowledge of the postsynaptic structure in the electric organ of *Torpedo*, we know little of the relationship between 43K and AChR in vertebrate skeletal muscle. A protein antigenically related to 43K is present at the postsynaptic membrane of mammalian muscle (15, 18), and coaggregates with AChR in cultures of *Xenopus* muscle cells (8, 26). Little is known about the role of this 43K-like protein in the initial concentration of AChR at the developing neuromuscular junction, or whether it interacts

1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; F, fluorescein; FGAM, fluoresceinated goat anti-mouse Ig antibody; LIS, lithium diiodosalicylate; R, tetramethylrhodamine; R-BT, monotetramethylrhodamine  $\alpha$ -bungarotoxin.

with AChR in vertebrate systems, as it appears to do in isolated *Torpedo* membrane fragments.

We have addressed these questions by studying the AChR clusters present in cultures of rat myotubes. These clusters resemble in nearly every way the AChR clusters that form at the earliest stages of synapse formation in the rat (38). They are organized into distinct, interdigitating membrane regions (5), termed AChR and contact domains. AChR domains are rich in receptors; contact domains are poor in AChR but are located closer to the tissue culture substrate (5). By virtue of their stability in the presence of the cholesterol-specific detergent, saponin, they can be isolated from intact myotubes without undergoing significant morphological changes (3). The isolated clusters can then be manipulated in various ways to study the proteins associated with the cytoplasmic face of the membrane. The effects of these manipulations can be monitored with semiquantitative fluorescence microscopy (4) by normalizing the signal arising from the presence of a particular protein to the amount of AChR present in the same patch of membrane.

In the present communication, we make use of monotetramethylrhodamine  $\alpha$ -bungarotoxin (R-BT) and highly specific monoclonal antibodies to the AChR (17) and to the *Torpedo* 43K protein (15) to study the AChR clusters present in intact myotubes and in preparations isolated with saponin. We recognize that the protein in rat myotubes that cross-reacts with antibodies to the *Torpedo* 43K protein has not been biochemically characterized. Nevertheless, we prefer to avoid using another name for this protein, and so refer to it simply as 43K. We report here that the relationship between AChR and 43K in receptor clusters isolated from cultured rat myotubes resembles that found in *Torpedo* electric organ in at least four ways: (a) the distribution of these two proteins is very similar; (b) extraction of the 43K protein is coincident with a redistribution of the AChR; (c) extraction of the 43K protein renders particular sites on the AChR susceptible to proteolytic degradation; (d) extraction of the 43K protein makes the cytoplasmic domain of the AChR more accessible to a monoclonal antibody. Our results suggest that one 43K protein is present per AChR molecule and that it lies in close proximity to cytoplasmic projections of the AChR.

## Methods

Cultures of rat myotubes were prepared as described elsewhere (2, 5). Briefly, muscle tissue from neonatal rat hindlimb was dissociated using collagenase and subjected to mild trituration. After brief centrifugation, dissociated cells were suspended in medium to a final density of  $10^6$  cells/ml. Medium consisted of Dulbecco-Vogt modified Eagle's medium (DME) supplemented with 10% calf serum and 5% fetal calf serum. Small aliquots (0.4 ml) of this cell suspension were plated onto glass coverslips (25 mm diameter, No. 1 thickness; VWR Scientific, San Francisco, CA). Cultures were supplemented with 1.5 ml medium the following day. 4 d after plating, the medium was changed to DME plus 10% fetal calf serum and 20  $\mu$ M cytosine arabinoside, to kill dividing cells. Cultures were used 6–8 d after initial plating.

For most of the experiments reported here, cultures were labeled with R-BT (30) before proceeding further. Labeling was performed by incubating cultures with R-BT (5  $\mu$ g/ml) in a buffered DME solution containing 5% serum for 15–30 min at room temperature. In some experiments, cultures were treated briefly with Triton X-100 to render the cells permeable to antibodies and then fixed with ice-cold paraformaldehyde (2% wt/vol) in buffered saline. The detergent solution consisted of Triton X-100 (0.5%), 10 mM Hepes, 5 mM  $MgCl_2$ , 200 mM sucrose, 50 mM NaCl, 1 mM

Table I. Monoclonal Antibodies Used in This Study

Antibody	Type	Specificity*	Reference	Labeling of rat AChR clusters
1234A	IgG <sub>1</sub>	43K protein	26	Good
1579A	IgG <sub>1</sub>	43K protein	Froehner, S. C., unpublished observations	Good
1201C	IgG <sub>1</sub>	43K protein	15	Poor
1210A	IgG <sub>1</sub>	43K protein	26	Poor
88B	IgG <sub>1</sub>	AChR and $\gamma$ - and $\delta$ -chains (cytoplasmic domains)	17	Good

\* Specificity is with respect to components of postsynaptic membrane preparations from *Torpedo* electric organ (see references cited).

EGTA, 0.02% sodium azide, pH 7.0. In other experiments, cells were fixed with paraformaldehyde before treatment with Triton X-100, or were simultaneously fixed and permeabilized using ethanol at  $-20^\circ$ .

For most experiments, however, cultures were subjected to extraction with saponin to isolate AChR clusters, as described (reference 3, method I). Protease inhibitors were generally not used during cluster isolation, although no significant differences were observed when they were included at reported concentrations (3). To obtain clusters that retained more of their rectilinear organization, samples were collected shortly after the mass of the cellular material was shed from the coverslip (4). Samples were either fixed in paraformaldehyde, or incubated once or twice for 5 min at 23–24°C in different solutions, and then fixed.

For indirect immunofluorescence, samples were first incubated in 0.1 M glycine in buffered saline to inactivate any remaining free aldehyde. They were then washed briefly in buffered saline containing 0.1% serum albumin. Purified mouse monoclonal antibodies (15, 17) were diluted in the same solution and incubated with the samples for 30–45 min. Unbound antibodies were removed by washing. Fluoresceinated goat anti-mouse Ig antibody (FGAM; Litton Bionetics, Rockville, MD or Cappel Laboratories, West Chester, PA) was diluted to 10–20  $\mu$ g/ml in buffered saline plus serum albumin and incubated for an additional 30–45 min. Samples were mounted in 90% glycerol, 10% 1 M Tris-HCl, pH 8, for observation and quantitation.

Quantitation of fluorescence was performed as described in detail elsewhere (4). Briefly, samples labeled with both R-BT and antibodies were placed onto the stage of a Zeiss IM35 microscope equipped with a photomultiplier and an I-to-V converter. The diaphragm controlling the fluorescence illumination was stopped down to create an illuminated field  $\sim 100$   $\mu$ m in diameter. Epifluorescence arising from a 5- $\mu$ m diameter region on the coverslip was sampled through a pinhole aperture by the photomultiplier, and the voltage displacement was read off the I-to-V converter. Most measurements presented here were based on the ratio method, reported earlier (4). This method involved normalizing the FGAM fluorescence signal for the R-BT fluorescence arising from the same region of the sample, after correcting for background. For control samples, background was usually <25% of the signal.

Binding of  $^{125}I$ -BT followed the methods described by Patrick et al. (25), as adapted for preparations of isolated AChR clusters (3).

Photomicrographs were taken using HP-5 film (Ilford, Basildon, Essex, Great Britain), processed to an ASA of 3200 using Ilford Microphen developer. Unless otherwise noted, reagents were from Sigma Chemical Co. (St. Louis, Mo.).

## Results

Our purpose in the experiments described below was to investigate the relationship between the 43K protein and AChR in the AChR clusters of cultured rat myotubes. To do so, we made use of a set of monoclonal antibodies directed against the 43K protein and against the cytoplasmic domain of the AChR (15, 17). The properties of the antibodies we studied are presented in Table I. Our approach in these experiments was first to establish the presence of 43K at the AChR

clusters of intact myotubes and large myotube fragments, and then to study the relationship between the 43K protein and AChR in isolated receptor clusters.

### Colocalization of AChR and 43K Protein

To study the distribution of intracellular structural proteins in muscle cells, we either treated cells with Triton X-100 and then fixed them with paraformaldehyde, or simultaneously fixed and permeabilized cells with ethanol at  $-20^{\circ}\text{C}$ . We then labeled the fixed, permeabilized cultures by indirect immunofluorescence. Cultured rat myotubes treated in this way and then labeled with one of two monoclonal antibodies specific for the 43K protein (1234A and 1579A) showed bright labeling in regions of the cell that colabeled with R-BT (Fig. 1, *A-D*). The distinctive domain arrangement of clustered AChR was also visible in the pattern generated by the anti-43K protein antibodies, indicating that this protein was concentrated over the AChR domains (Fig. 1, *A* and *B*, arrowhead), but not the contact domains (Fig. 1, *A* and *B*, double arrowheads) of receptor clusters. In some myotubes, AChR clusters were also observed in areas of the cell distant from the substrate (5), and these too were labeled with mAb 1234A (Fig. 1, *E* and *F*). Fibroblasts and areas of the myotubes poor in AChR showed no staining by the antibody. Thus, an antigen resembling the 43K protein of *Torpedo* was indeed concentrated at the AChR clusters of cultured rat myotubes, where it seemed to be colocalized with AChR.

Not all antibodies to the *Torpedo* 43K protein can be used to study the AChR clusters of rat myotubes, however. Two antibodies we have tried, 1201C and 1210A, which react with high affinity with the *Torpedo* antigen (15), labeled clusters only weakly (not shown, but see Table I). This suggests that the antigen present in cultured rat muscle cells is similar but not identical to its *Torpedo* counterpart. Antibody produced by the parent myeloma cells, MOPC-21, also failed to label AChR clusters. This indicates that the labeling illustrated in Fig. 1 cannot be attributed to nonspecific interactions of antibodies with cluster-associated proteins.

One problem with these results is that treatment with ethanol or with neutral detergent before fixation could be accompanied by a rearrangement of proteins associated with the cell membrane. To test this, we also examined the AChR clusters that were prepared without the use of detergent or ethanol. In this experiment, clusters were obtained from rat myotubes by physically shearing the cultures with a stream of ice-cold, buffered paraformaldehyde. This procedure fixes the samples as it removes the bulk of the cytoplasm and contractile machinery from the AChR clusters, which often remain attached to the glass coverslip (5). Indirect immunofluorescence labeling of these preparations showed that labeling for 43K protein was localized over the AChR domains (Fig. 1, *G* and *H*). This suggests that the observations made in intact, permeabilized cells were not the result of artifactual rearrangement of these proteins.

Upon extraction with the neutral detergent, saponin, myotube cultures shed most (>99%) of their cellular material, but leave most (~85%) of their AChR clusters behind on the coverslip (3). These clusters are not greatly changed from the AChR clusters in intact cells, and they retain their distinctive AChR-rich and AChR-poor membrane domains (3). As shown in Fig. 1, *I* and *J*, the antigenic sites for binding of

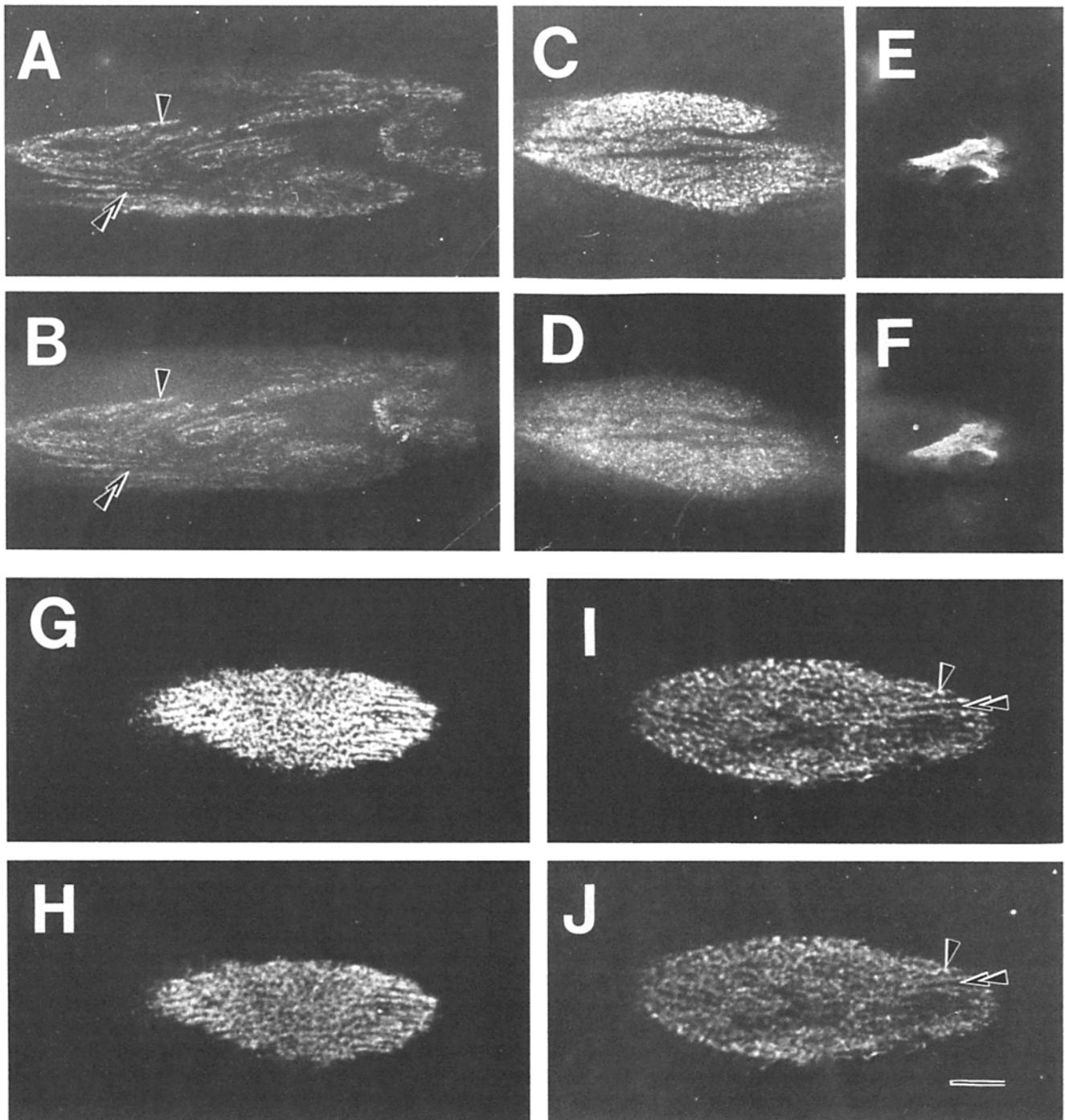
the 1234A anti-43K mAb were retained in isolated AChR clusters. Immunofluorescent labeling was concentrated over the AChR domains and absent over the contact domains, as reported above for permeabilized myotubes. Labeling was not detectable over areas of the myotube membrane poor in AChR, or over fibroblast membrane fragments. Thus, the 43K protein remained associated with clustered AChR after isolation with saponin.

### Antibody Binding to Isolated AChR Clusters

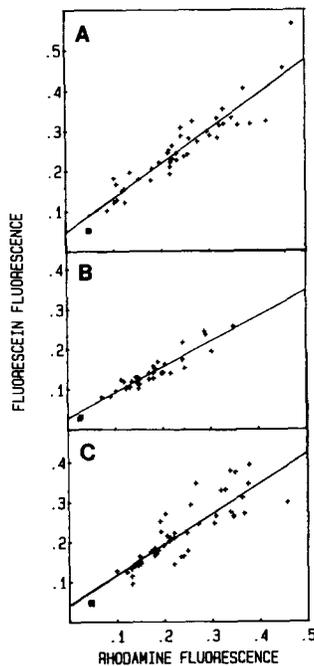
To investigate the relationship between the 43K protein and AChR in isolated clusters, we first had to characterize the binding of the monoclonal anti-43K antibodies to cluster membrane. To do so, we used a semiquantitative fluorescence technique that normalizes the amount of fluorescent second antibody (FGAM) bound within an area of muscle membrane 5  $\mu\text{m}$  in diameter to the amount of R-BT bound to AChR in the same area, as described in Materials and Methods (see reference 4 for more details). As a positive control, and to enable us in later experiments to compare the 43K protein with AChR by immunofluorescence, we also studied a receptor-specific monoclonal antibody, 88B (17; see Table I).

Fig. 2 shows that the labeling of muscle membrane with antibodies varied linearly with the labeling of R-BT-AChR complexes. This was true for both of the anti-43K antibodies, 1234A and 1579A, and for the anti-AChR antibody, 88B. The slopes of the plots for all three antibodies usually varied between 0.7 and 1.2 at the concentrations used, which were nearly saturating (see below). (We found in some experiments, performed under similar conditions, that the slopes for the anti-43K mAbs were as low as 0.5. When this occurred, the values obtained for 88B were also lower than normal. The reasons for this occasional variability are not known. The lower than normal values will be apparent in a few of the experiments we report below, but do not contribute significantly to our results). We found, however, that the relationship between fluorescein and rhodamine fluorescence at low levels of staining (*F* or *R* values below 0.1) fell on different regression lines than those shown in the figure. For this reason, we limited our observations to membrane regions that gave fluorescence values  $\geq 0.10$ . At these higher values, the ratio of *F/R*, calculated from determinations on seven or more AChR-rich regions, was constant for any given sample.

We found that the binding of each of the antibodies to isolated cluster membrane saturated as a function of antibody concentration (Fig. 3). The affinities of the antibodies for their respective antigens differed markedly, however, with apparent half-maximal binding of 88B achieved at concentrations 10-fold lower than those needed for half-maximal binding of the anti-43K antibodies. (In experiments not shown here, we confirmed that the concentrations of FGAM we routinely used for these experiments were in excess). The binding parameters obtained from such experiments are summarized in Table II. The values obtained for apparent  $B_{\text{max}}$  suggested that 88B and the anti-43K antibodies bound to clusters in comparable amounts, despite the fact that 88B can in principle bind to two subunits ( $\gamma$  and  $\delta$ ) of the receptor monomer (see Table I). Increasing 88B to levels 100-fold greater than the half-saturating concentration did not further



**Figure 1.** Antibodies to the 43K protein label the AChR domains of receptor clusters. Cultured rat myotubes were labeled with R-BT and treated in several ways to gain access to the cytoplasmic face of the membrane at AChR clusters. After fixation was complete, samples were incubated with monoclonal antibodies to the 43K protein (either 1234A or 1579A, used at 100 nM) followed by FGAM (see Materials and Methods for details). All panels except *E* and *F* depict AChR clusters in substrate-apposed membrane. Photographs were taken under rhodamine (*A*, *C*, *E*, *G*, and *I*) or fluorescein (*B*, *D*, *F*, *H*, and *J*) optics. (*A* and *B*) Fixed and permeabilized in 95% ethanol at  $-20^{\circ}$ ; labeled with 1234A. (*C* and *D*) Permeabilized with Triton X-100 before fixation with paraformaldehyde; labeled with 1579A. Note that this treatment results in a higher background and poorer preservation of structure. (*E* and *F*) Treated as in *A* and *B*, but showing a receptor cluster distant from the substrate. (*G* and *H*) Sheared with a stream of ice-cold paraformaldehyde, then fixed further and labeled with 1579A. (*I* and *J*) Isolated by extraction with saponin, fixed in paraformaldehyde, and labeled with 1234A. The figure shows that AChR clusters studied after a variety of fixation and permeabilization procedures all show labeling by anti-43K antibodies at AChR domains (e.g., arrowheads in *A*, *B*, *I*, and *J*) but not at contact domains (e.g., double arrowheads in these panels). Bar, 10  $\mu$ m.



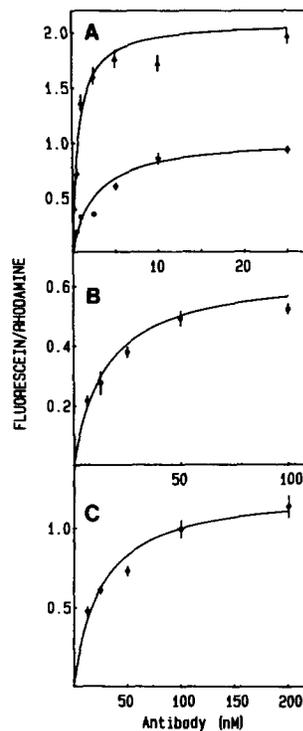
**Figure 2.** Labeling by antibodies is a linear function of R-BT staining intensity. Rat myotubes were labeled with R-BT and extracted with saponin, to isolate AChR clusters. After fixation with paraformaldehyde, samples were labeled with mAb to the AChR (A) or to the 43K protein (B and C). Coverslips were mounted and the intensities of the F and R fluorescence were measured as described in Materials and Methods and, in more detail, in reference 4. The lines through the crosses represent the best fits by regression analysis. Each cross represents an individual measurement of the F and R signals from a receptor cluster. The squares represent the values for F and R obtained from areas of the coverslip free of cellular material. (A) 88B, used at 25 nM; (B) 1234A, used at 100 nM; (C) 1579A, used at 100 nM.

increase labeling by FGAM. In the experiments reported below, binding was done at mAb concentrations that assured ~80% saturation for the anti-43K antibodies, and >90% saturation for the anti-AChR antibody.

We also found that the binding of each of the antibodies to isolated receptor clusters was not significantly reduced by the presence of either of the others (Table III). This suggests that 1579A and 1234A bind to distinct and distant antigenic determinants on the 43K protein of AChR clusters, as also found for *Torpedo* postsynaptic membrane (LaRoche, W. J., and S. C. Froehner, unpublished observations), and that both of these determinants are distant from the binding site on AChR to which 88B binds.

#### Extraction of the 43K Protein

We assayed a number of treatments and reagents to try to remove the 43K protein from isolated AChR clusters, but



**Figure 3.** Labeling by antibodies saturates as a function of antibody concentration. Samples were processed as described in the legend to Fig. 2, but the concentrations of mAb were varied. (A) 88B. Solid circles represent data obtained from control clusters; solid triangles represent data obtained from clusters that had been extracted with 50 mM ethylamine, pH 11, before fixation and immunolabeling. (B) 1234A; (C) 1579A. Curves were fit with rectangular hyperbolas calculated for the following constants for apparent  $K_d$  and  $B_{max}$ , expressed in nM and F/R ratios, respectively: (A, solid circles) 2.7 nM, 1.05; (solid triangles) 0.75 nM, 2.10; (B) 15 nM, 0.65; (C) 25 nM, 1.3. Additional binding parameters are given in Table II.

found the association to be stable to all but the harshest treatments (Table IV). The 43K protein appeared to remain associated with isolated clusters over a wide pH range, at high or very low ionic strength, or after digestion with chymotrypsin. Some loss of 43K occurred between pH 6.5 and pH 5.5, but further decreases in pH had no additional effect. The 43K protein of *Torpedo* electric organ can be removed from the postsynaptic membrane by extraction with lithium diiodosalicylate (LIS) at neutral pH, or at pH 11 (11, 23). We found that these treatments also stripped the 43K protein from isolated AChR clusters, and simultaneously altered the AChR remaining in the membrane.

Fig. 4 shows the effects of extracting isolated AChR clusters for 5 min at room temperature with 50 mM ethylamine-HCl, pH 11, or with 10–20 mM LIS. Both treatments reduced subsequent labeling by 1234A or 1579A to levels close to background. As these two antibodies bind to different sites

**Table II. Binding Parameters of Monoclonal Antibodies**

Antibody	Controls		After pH 11		After LIS	
	$K_{1/2}$	$B_{max}$	$K_{1/2}$	$B_{max}$	$K_{1/2}$	$B_{max}$
88B	$4.3 \pm 3.5$ (3)	$0.9 \pm 0.3$ (3)	$1.0 \pm 0.4^*$ (3)	$1.9 \pm 0.5^\ddagger$ (3)	$1.6 \pm 0.7^*$ (2)	$1.9 \pm 0.4^\ddagger$ (2)
1234A	$29 \pm 15$ (3)	$1.2 \pm 0.6$ (3)	ND <sup>§</sup>	ND	ND	ND
1579A	$24 \pm 2$ (2)	$1.1 \pm 0.3$ (2)	ND	ND	ND	ND

Binding was determined semiquantitatively using a photomultiplier attached to the fluorescence microscope.  $K_{1/2}$  refers to the concentration (in nanomolars) at which apparent half-maximal binding was observed.  $B_{max}$  is the apparent maximum value of F/R. Both parameters were determined by double reciprocal plots of the data after correction for background. Values are means  $\pm$  SD, followed by the number of determinations, in parentheses. See Materials and Methods for more details.

\* Not significantly different from control values ( $P > 0.2$ ).

† Significantly different from control values ( $P < 0.05$ ).

§ ND, not determined.

**Table III. Independent Binding of Monoclonal Antibodies**

Antibody	Normalized F/R
88B	1.0 (3)
1234A	0.7 ± 0.06 (3)
1579A	0.9 ± 0.08 (3)
1234A + 1579A	1.4 ± 0.1 (3)
1234A + 88B	1.6 ± 0.04 (2)
1579A + 88B	1.6 ± 0.2 (3)

Binding of each of the antibodies, either alone or in pairs, was determined semiquantitatively, as described in Materials and Methods. 88B was used at 25–50 nM; 1234A and 1579A were used at 100 nM each for all experiments. Data were normalized to the value obtained for 88B labeling alone, the F/R value for which was arbitrarily set at 1.0. Normalization was performed to facilitate pooling of results obtained in three separate experiments. The results show that the normalized F/R values obtained for pairs of antibodies are significantly greater than the values for any of the antibodies alone ( $P < 0.01$ ), and are approximately those values expected if each antibody binds independently (i.e., simple sum of values for each antibody alone).

on the 43K protein (see above), this suggests that the protein was removed equally well by these two treatments.

Extraction at alkaline pH or by LIS also had significant effects on the clustered AChR. In the case of extraction at high pH, the AChR remaining in the membrane assumed an almost uniform distribution (Fig. 4 E), indicating that this treatment altered the ability of receptors to remain preferentially in AChR domains. After LIS extraction, however, AChR domains appeared to be preserved (Fig. 4 C). AChR within the receptor domains seemed to be slightly microaggregated, but this effect was subtle. This microaggregation only became obvious and easy to photograph upon longer incubations (e.g., Fig. 4, G and H).

Because its effect on AChR distribution was so different from that of alkali, we considered the possibility that, in addition to extracting 43K, LIS affected the lipid bilayer

**Table IV. Extraction of the 43K Protein from Isolated AChR Clusters**

Treatment	43K Immunolabeling (percent control)
<b>Ineffective</b>	
10 mM NaP, 145 mM NaCl, pH 7.2 (control)	100
+ Chymotrypsin (10 µg/ml)	100 ± 16 (5)
+ 0.6 M KCl	72 ± 9 (2)
2 mM Tris-HCl, 0.2 mM ATP, pH 8.0*	93 ± 10 (7)
10 mM Glycine, 145 mM NaCl, pH 3.5	60 ± 4 (2)
10 mM Imidazole, 145 mM NaCl, pH 4.5	67 ± 8 (2)
pH 5.5	52 ± 3 (2)
pH 6.5	80 ± 11 (2)
50 mM Ethylamine-HCl or 50 mM Tris-HCl pH 9.5	102 ± 14 (3)
<b>Effective</b>	
50 mM Ethylamine-HCl, pH 11	10 ± 3 (7)
LIS, 10 mM	14 ± 4 (6)
20 mM	11 ± 2 (7)

Samples containing isolated AChR clusters were treated for 5 min at room temperature with one of the solutions listed below. Samples were then fixed and immunolabeled with either 1234A or 1579A, at 100 nM, as described earlier. Similar results were obtained with both antibodies, and so were pooled for presentation here. All results, obtained originally as ratios of F/R, are expressed here as percent control. Values are means ± SEM, followed by the number of determinations in parentheses. Only extraction with LIS or with strong alkali effectively removed the 43K protein from isolated AChR clusters. \* Buffer A of reference 37.

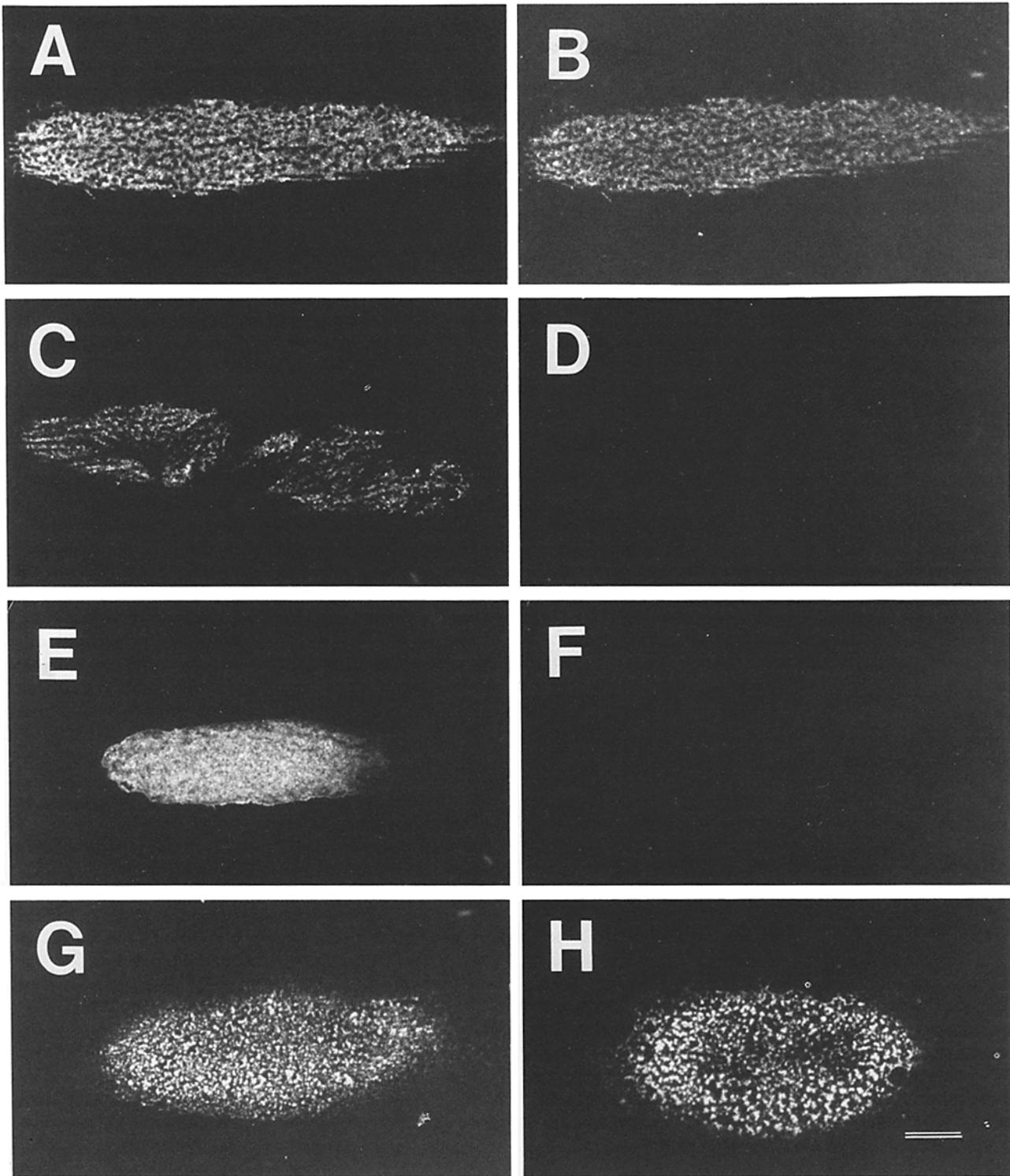
directly. If this were true, then extraction with LIS of membrane preparations that had already been stripped of 43K at high pH might also produce microaggregates of AChR. Also, if the effect of LIS on the bilayer is not easily reversed, then treatment of LIS-extracted membranes at pH 11 should not cause the small AChR aggregates to disassemble. Our observations confirmed both of these predictions, and further indicated that sequential incubations in LIS and in alkali caused additional aggregation of AChR. These results suggest that LIS had effects on AChR clusters in addition to that of removing 43K.

To quantitate some of the effects of LIS and alkali extractions on the stability of the membrane, we measured the amount of <sup>125</sup>I-BT-AChR complexes that they solubilized from the coverslip. We found that, compared with controls, samples extracted at pH 11 lost only ~15% of their specifically bound <sup>125</sup>I-BT, suggesting that high pH was not solubilizing a significant proportion of the receptors in preparations of isolated clusters. In contrast, LIS solutions solubilized ~36% of specifically bound <sup>125</sup>I-BT. This is consistent with the idea that LIS had a significant effect on the stability or organization of isolated muscle membrane fragments.

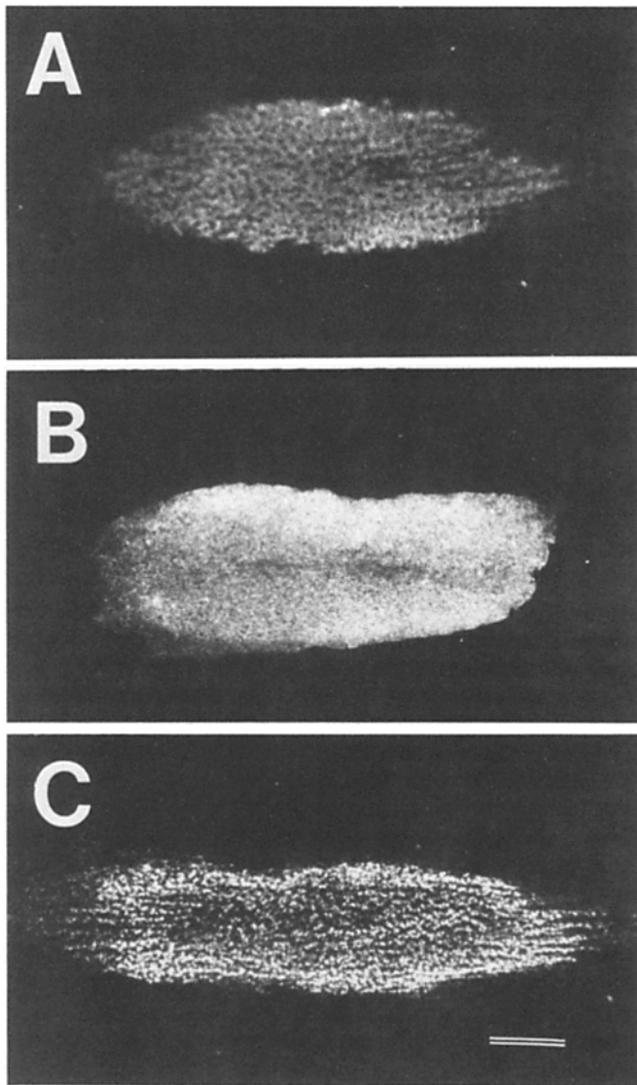
### Relationship to AChR

In clusters from which 43K had been removed by extraction with either LIS or alkaline buffer, subsequent labeling of the AChR by 88B and FGAM increased noticeably compared with unextracted controls (Fig. 5). We measured this increase semiquantitatively and related it to the loss of staining for 43K protein. We found that the 43K protein was resistant to removal from isolated clusters by increases in pH from 7.2 to 10, but was removed nearly completely by increasing the pH from 10 to 11 (Fig. 6, solid circles). This is similar to results obtained with *Torpedo* membrane (11). As labeling for the 43K protein decreased at higher pH, labeling by 88B anti-AChR increased to values approximately twice those observed in controls (Fig. 6, crosses). Similarly, increasing the concentration of LIS from 1 to 10 mM caused a progressive loss of labeling for 43K protein (Fig. 7, solid circles), as also reported for *Torpedo* postsynaptic membranes (11). This was accompanied by an increase in labeling of the AChR by 88B (Fig. 7, crosses). Neither change occurred in samples extracted in buffer alone (10 mM NaP, 1 mM EDTA, 1 mM EGTA, pH 7.4). Quantitative comparisons of the labeling by all three antibodies showed that in samples that were depleted of the 43K protein (<20% of control values), labeling by 88B increased approximately twofold (see also Fig. 3 A and Table II). In samples extracted with ethylamine, pH 11, this increase in B<sub>max</sub> was accompanied by a severalfold decrease in the concentration of 88B needed to achieve apparent half-maximal binding (Table II, Fig. 3 A), although this may not have been statistically significant ( $P > 0.2$ ).

The increased 88B labeling of clusters stripped of the 43K protein could be due to increased accessibility of the cytoplasmic domain of the AChR to antibodies. To learn if other macromolecules also had greater access to AChR, we studied the effect of chymotrypsin on control clusters and on clusters from which 43K had been removed. Binding of 88B was only slightly affected by chymotrypsinization of control clusters, but was drastically reduced in clusters first ex-



**Figure 4.** Effect of extraction with LIS or ethylamine, pH 11, on subsequent labeling for 43K protein, and on the distribution of AChR. Myotube cultures were processed as described in the legends to Figs. 1 and 2. Samples were incubated for 5 min at room temperature in buffered saline, or in solutions known to extract the 43K protein from *Torpedo* postsynaptic membrane. Samples were then fixed and labeled with 1579A (100 nM) and FGAM for subsequent immunofluorescence microscopy. *A*, *C*, *E*, *G*, and *H* were photographed under R optics. *B*, *D*, and *F* were photographed under F optics. (*A* and *B*) Controls, incubated for 5 min in buffered saline. AChR codistributes with the 43K protein and both show distinct membrane domains. (*C* and *D*) Incubated in 10 mM LIS. Labeling for the 43K protein is no longer detectable. AChR seems to be in distinctive domains, but slight microaggregation has occurred. Microaggregates are more apparent in *G* and *H*. (*E* and *F*) Incubated in 50 mM ethylamine, pH 11. Labeling for the 43K protein is not detectable, and AChR appears uniformly distributed in the membrane. Results similar to those shown in *A*–*F* were obtained with samples labeled with 1234A. (*G*) Incubated for 5 min at pH 11, then washed briefly in buffered saline and incubated for 5 min more in 20 mM LIS. R-BT label only shown. (*H*) Incubated for 5 min in 20 mM LIS, then washed briefly in buffered saline and incubated for 5 min more in 50 mM ethylamine, pH 11. R-BT label only shown. Note the extensive microaggregation of AChR in *G* and *H*. Bar, 10  $\mu$ m.

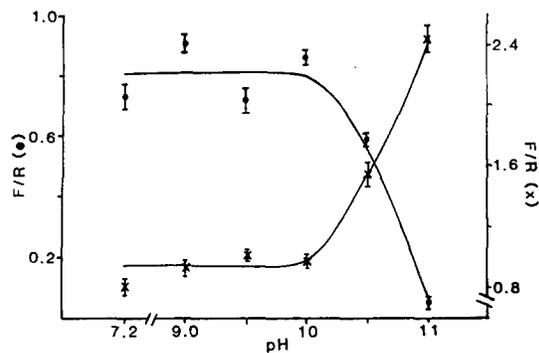


**Figure 5.** Effect of extraction at pH 11 or with LIS on labeling by 88B anti-AChR antibody. Myotube cultures were processed as in the legend to Fig. 4, but immunolabeling employed the 88B anti-AChR antibody (25 nM) followed by FGAM. Only the F signal obtained from bound antibodies is shown. (A) Control, incubated in buffered saline; (B) incubated in 50 mM ethylamine, pH 11; (C) incubated in 20 mM LIS. Pictures of all three clusters were photographed, developed, and printed identically. The intensity of the F fluorescence in B and C is notably greater than that in A. Bar, 10  $\mu$ m.

tracted with LIS or at high pH and then proteolyzed (Fig. 8). These changes were not caused at pH 9 or by the buffer normally used for LIS extraction (not shown). These results suggest that the accessibility of the AChR to chymotrypsin is greatly increased in samples from which 43K has been removed.

## Discussion

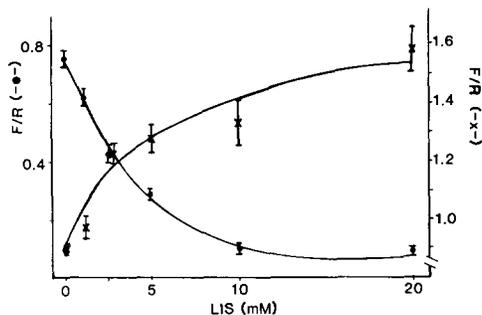
In *Torpedo* electric organ, the 43K protein seems to be closely associated with AChR in the postsynaptic membrane (16). An antigen resembling the 43K protein has also been found at the vertebrate neuromuscular junction (8, 15, 18),



**Figure 6.** AChR and 43K labeling as a function of pH. Myotube cultures were treated as described in the legend to Fig. 4 and 5, except that the pH of the solutions used for the 5-min incubations was varied. The solution used at pH 7.2 was buffered saline. The solutions used at pH 9–11 were 50 mM ethylamine, adjusted with HCl. Samples were labeled with either 1234A anti-43K antibody, or with 88B anti-AChR antibody, followed by FGAM. Fluorescence due to bound antibody was quantitated and normalized to the intensity of R-BT fluorescence arising from the same membrane area, as described in Materials and Methods. The results show that as immunofluorescence labeling with anti-43K antibody (solid circles) is reduced to close to zero, labeling by anti-AChR antibody (crosses) increases approximately twofold. Results are means  $\pm$  SEM.

but the close association of this vertebrate protein with AChR has been difficult to establish. We have used specific monoclonal antibodies to identify a protein resembling the 43K protein of *Torpedo* electric organ at the AChR clusters of cultured rat myotubes, and to study its relationship to clustered receptors. Our results establish many similarities between the 43K protein and AChR in *Torpedo* and mammalian muscle membrane.

The specificity of the antibodies we used for these studies has been extensively studied with respect to their *Torpedo* antigens (15, 17; Froehner, S. C., unpublished observations). Because of a shortage of enriched biochemical material, similar assurances of specificity are difficult to obtain with muscle preparations. Several observations suggest, however, that these antibodies do, in fact, react with the appropriate antigens at isolated AChR clusters. (a) The antibodies react only with muscle membrane fragments, and not with fibroblast membrane, suggesting that their respective antigens are present in significant amounts only in muscle. (b) Labeling of muscle membrane with antibodies followed by FGAM reveals a linear dependence of staining intensity on the amount of AChR present, as measured by R-BT fluorescence. This suggests that the binding of the antibodies to muscle membrane is a function of the AChR content. This is expected for the 88B anti-AChR antibody. If a 43K-like protein is closely associated with clustered AChR, it might also be expected for the 1234A and 1579A anti-43K antibodies. A stoichiometric relationship of the two proteins in muscle would be consistent with the near equimolar amounts of 43K and AChR in *Torpedo* electrocytes (21). (c) At least two antibodies that recognize different antigenic sites on the 43K protein of *Torpedo* give identical results in our experiments. Similarly, we have found in preliminary experiments that a second monoclonal antibody, 147A, also specific for the  $\gamma$  and  $\delta$  chains of the AChR (17), gives results similar to those reported here for 88B. These antibodies probably bind to dis-

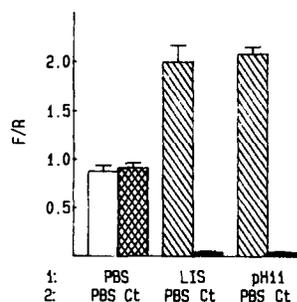


**Figure 7.** AChR and 43K labeling as a function of LIS concentration. Myotube cultures were treated as described in the legend to Fig. 6, except that samples were incubated for 5 min in buffered saline (solid circles) or in 10 mM NaP, 1 mM EDTA, 1 mM EGTA, pH 7.2, containing increasing amounts of LIS. The results show that as immunofluorescence labeling with 1579A anti-43K antibody is reduced (solid circles), labeling by 88B anti-AChR antibody increases approximately twofold (crosses). Results are means  $\pm$  SEM.

tinct but nearby sites on the AChR (17). (d) The anti-AChR and anti-43K antibodies bind to different constituents of isolated AChR clusters, as indicated by the different effects of extraction on their respective antigens. The antigens recognized by these antibodies also behave differently from two other antigens present at isolated AChR clusters, actin (4) and  $\beta$ -spectrin (Bloch, R. J., and J. S. Morrow, manuscript submitted for publication). It therefore seems reasonable to assume that 1234A and 1579A do indeed bind to a protein in isolated AChR clusters that resembles the 43K protein of *Torpedo* postsynaptic membrane, and that 88B binds to the AChR.

If one accepts this assumption, our results suggest that AChR and the 43K protein are closely associated in rat myotubes and in subcellular fractions of rat myotubes. This close association has already been reported in cultured *Xenopus* myocytes (8, 26), and has been inferred from the presence of an antigen related to the 43K protein at the mammalian neuromuscular junction (15, 18). In these systems, however, only a co-distribution of the AChR and 43K proteins has been demonstrated. In our experiments with AChR clusters isolated from rat myotubes, we have shown that clustered AChR are modified in preparations from which the 43K protein has been removed. (a) AChR redistribute in the plane of the muscle membrane. In clusters stripped of the 43K protein at alkaline pH, AChR become more uniformly distributed; in clusters treated with LIS, microaggregation occurs, perhaps as a result of the effects of this reagent on the lipid bilayer (see Results). (b) AChR become more susceptible to proteolysis of its cytoplasmic region. (c) AChR become more accessible to an anti-AChR antibody that binds to the cytoplasmic region. Some of these observations, together with the colocalization of the AChR and 43K proteins, are consistent with experiments performed with isolated preparations of *Torpedo* postsynaptic membrane (1, 10, 39). These results have interesting implications for the organization and possible stoichiometry of receptor clusters.

Our basic observation is that the antibodies to the 43K protein and to AChR bind to approximately equal extents in con-



**Figure 8.** Effect of chymotrypsin on 88B labeling in intact clusters, and clusters lacking 43K. Myotube cultures were treated as described in the legends to Figs. 4 and 5, and were subjected to two sequential treatments. In the first treatment (abscissa, 1) samples were incubated for 5 min either in buffered saline (PBS), in 20 mM LIS, or in 50 mM

ethylamine, pH 11. They were then washed briefly in buffered saline and subjected to a second incubation (abscissa, 2) for an additional 5 min in buffered saline, or in buffered saline containing chymotrypsin at 10  $\mu$ g/ml (Ct). After fixation, samples were labeled with 88B (25 nM) followed by FGAM, and were quantitated as described in Materials and Methods. The results show that 88B labeling is largely resistant to chymotrypsin in controls, but is completely eliminated by chymotrypsin in samples from which the 43K protein has been extracted. Values are means  $\pm$  SEM.

trol clusters, but that, as 43K is removed from the membrane, binding sites for anti-AChR antibodies double in number (e.g., Table II). The simplest model that accounts for this observation would place the 43K protein very close to the cytoplasmic domain of the AChR, where it would block access of the 88B antibody to one of its two potential binding sites on the  $\gamma$  and  $\delta$  subunits. This arrangement, typical of the intact cluster, would allow only one molecule of 88B and one molecule of 1234A or 1579A to bind per receptor. Upon removal of the 43K protein, the second binding site for 88B would become exposed. A similar arrangement could account for the observation that 88B binding sites are not digested by chymotrypsin in intact clusters, but are all completely proteolyzed in clusters from which the 43K protein has been removed. In this case, the simplest model of the intact cluster would have the 43K protein covering both chymotrypsin-sensitive sites on the AChR.

One difficulty with this interpretation is that the methods we use to extract the 43K protein cause other changes in the organization of AChR clusters. As it solubilizes  $\sim$ 35% of the total AChR in preparations of isolated clusters, LIS probably has deleterious effects on the structure and organization of the membrane. This may account in part for the microaggregation of AChR we observed in LIS-extracted samples. Alkaline extraction does not solubilize a significant portion of membrane-bound AChR, but may nevertheless alter the structure of the cytoplasmic domain of the AChR. Such a structural change, rather than the removal of the 43K protein, could account for the increased binding of 88B and for the changes in AChR distribution. Experiments involving reassociation of the 43K protein with muscle membrane may be necessary to settle these issues.

Another possible difficulty with this model is that the 43K protein may not be the only protein that inhibits the access of chymotrypsin and 88B to the cytoplasmic domain of clustered AChR. Other proteins, so far unidentified, may be associated with clustered AChR and may be altered or removed by the same treatments that extract the 43K protein. Preliminary experiments suggest that the removal of actin and

$\beta$ -spectrin from isolated clusters does not affect the stoichiometry of 88B binding (Bloch, R. J., manuscript in preparation). As other proteins associated with clustered AChR are found, it will be important to check their susceptibility to different extraction conditions, and to learn if their selective removal exposes new binding sites on the cytoplasmic face of the AChR.

Despite these difficulties in interpretation, our experiments confirm that the 43K protein of mammalian cells has a relationship to AChR that is similar to that observed in *Torpedo* postsynaptic membrane. It further suggests a particular model for the association between clustered AChR and the 43K protein that was not apparent from experiments with *Torpedo* tissue. This model can be tested by further experiments on isolated AChR clusters, and by similar studies on isolated *Torpedo* postsynaptic membranes.

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