# Isolation of Acetylcholine Receptor Clusters in Substrate-associated Material from Cultured Rat Myotubes Using Saponin

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ABSTRACT After exposure of rat myotube cultures to saponin, <1% of the cellular protein was found to remain associated with the tissue culture substrate. This substrate-associated material contained ~10% of the acetylcholine receptors (AChRs) and >80% of the large, ventral AChR clusters present in the original culture. The domain structure evident in intact cells was maintained in AChR clusters after isolation using saponin. However, vinculin, present at the clusters of intact cells, was absent from isolated clusters. Dodecyl sulfate PAGE showed that substrate-associated material enriched in AChR clusters contained a distinctive set of polypeptides, the major ones electrophoresing with apparent molecular weights of 43,000 and 49,000. Saponin extraction of cultures of established cell lines also yielded substrate-associated material with characteristics particular to the cell type.

The acetylcholine receptor (AChR)[1] clusters that form in aneural cultures of rat myotubes resemble in many ways the AChR aggregates that form in response to innervation at the developing motor endplate of the rat. AChR clusters formed in vivo and in vitro have similar morphologies (3, 6, 8, 30), similar receptor densities (3, 18, 19, 25, 27), and similar susceptibilities to disruption by treatment with the receptor agonist carbachol, or by exposure to medium depleted of calcium (4, 5, 8). In addition, turnover of the receptors in both structures is similar, occurring with an half-time of ~1 d (25, 27). Because they closely resemble the nerve-induced receptor aggregates formed during synaptogenesis, and also because they are easily studied in cell culture, the AChR clusters of cultured rat myotubes have proved to be a useful model system with which to investigate the early stages of postsynaptic differentiation.

Although they are simpler and more accessible to study than the postsynaptic region of the neuromuscular junction, AChR clusters formed in vitro are highly differentiated structures. They are usually found in close proximity to the tissue culture substrate, where they show distinct lipid domains, an elaborate cytoskeleton associated with the cytoplasmic face of the membrane, and sites involved in attaching the myotube to a solid substrate (6, 21, 22; R. J. Bloch, manuscript in preparation). To understand the molecular basis of AChR clustering, it would be useful to have a purified cluster preparation, free of contaminating myoplasm. Here I describe a method for partially purifying the AChR clusters which relies on the stability of substrate-associated membrane in the presence of saponin. The purification is close to 150-fold, is nearly quantitative, is complete in 30 min, and is essentially nondisruptive to the AChR cluster. The method also seems applicable to the isolation of substrate-associated membrane from a number of other cell types.

[1] *Abbreviations used in this paper.* AChR, acetylcholine receptor; DME, Dulbecco-Vogt modified Eagle's medium; R-BT, monotetramethylrhodamine-α-bungarotoxin; $^{125}$I-BT, iodinated α-bungarotoxin; $C_{18}$-diI, 3,3′-di($C_{18}$,$H_{37}$) indocarbocyanine iodide; saponin-SAM, substrate-associated material isolated using saponin; TCA, trichloroacetic acid.

## MATERIALS AND METHODS

*Cultures:* Rat myotube cultures were prepared as described (4, 6). Medium consisted of Dulbecco-Vogt modified Eagle's medium (DME) supplemented with 10% (vol:vol) cadet calf serum (Biocell Laboratories, Carson, CA). Unless otherwise noted, the substrate consisted of borosilicate glass coverslips (No. 1 thickness, Van Labs, VWR Scientific Inc., San Francisco, CA). In some cases, cells were grown on larger (8 cm diam) coverslips, cut from sheets of coverslip glass (No. 1.5 thickness, Erie Scientific, Portsmouth, NH). Cultures enriched in fibroblasts were prepared from the same cell suspensions as those used for myotube cultures. However, the suspension was exposed to glass coverslips for only 1 h, after which unattached cells were removed by aspiration. Further incubations and medium changes were performed as for myotube cultures. The resulting cultures were enriched in mononucleate cells and



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severely depleted of myotubes. All primary cultures were used 7–8 d after plating.

BC3H-1 and PC12 cells were obtained from Dr. S. Sine (The Salk Institute) and Dr. G. Guroff (National Institutes of Health), respectively. The neuroblastomas, B103 (rat), and NB2A and NB41A3 (both from mouse) were kindly provided by Mr. E. Cimino and Dr. L. Roeder (University of Maryland School of Medicine). These cell lines were maintained in DME supplemented with 20% cadet calf serum (BC3H-1), 10% cadet calf serum, and 5% fetal calf serum (PC12), or 10% fetal calf serum (NB2A, NB41A3, B103). Fetal calf serum was from Gibco Laboratories (Grand Island, NY). Cells were plated onto glass coverslips 2–4 d before extraction.

*Saponin Extraction:* The two procedures used for saponin extraction differed in the precautions taken to avoid contamination of isolated AChR clusters with myoplasm or with serum components from the medium, and in the precautions taken to inhibit proteolysis. The less stringent conditions were used to prepare samples for routine fluorescence microscopic observations. This protocol (method I) consisted of washing cultures at room temperature (22°C) once with PBS (10 mM NaP, 145 mM NaCl, pH 7.4), and once with buffered saline containing 10 mM $MgCl_2$, 1 mM EGTA, and 1% (wt:vol) BSA (Type V, Sigma Chemical Co., St. Louis, MO). Cultures were then placed in this last solution supplemented with 0.2% (wt:vol) saponin (lot No. 20F-0037, from Sigma Chemical Co.). Cultures were shaken for 20 min at room temperature on a Tektator V (American Scientific Products, McGaw Park, IL) rotary shaker at 90–100 rpm. To ensure more complete shedding of materials (see Results), most cultures were shaken further at 140–150 rpm for 5 min. Material remaining bound to coverslips was washed in buffered saline, then fixed for 15 min in 2% (wt:vol) paraformaldehyde, prepared in buffered saline.

More stringent conditions were used when the proteins remaining associated with the coverslip were to be analyzed. In this protocol (method II), coverslips were washed by serial transfer through four beakers containing a large excess of buffered saline and placed in a clean plastic petri dish (Falcon Plastics Labware, Div. of Becton, Dickinson & Co., Oxnard, CA). Cultures were then washed and extracted with saponin as in method I, except solutions generally contained no serum albumin, and were further supplemented with 0.2 U/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 0.25 mM *N*-ethyl maleimide, to inhibit proteases. After extraction, coverslips were washed twice with buffered saline and transferred to a clean petri dish. After excess buffer was drained and removed by aspiration, material remaining associated with the coverslips was dissolved at room temperature in 0.1% SDS, 0.2 U/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 0.25 mM *N*-ethyl maleimide, 0.1 mM Tris-HCl, pH 8. (This solution dissolved most, but not all, substrate-associated material.) Material from several coverslips was pooled, frozen in a dry ice-acetone bath, lyophilized, and stored at −75°C as the dry powder. Material extracted from the coverslips during saponin treatment was also collected, and frozen, lyophilized, and stored similarly.

*Fluorescence Studies:* AChRs were usually labeled with monotetramethylrhodamine-α-bungarotoxin (R-BT), prepared by the method of Ravdin and Axelrod (24), before exposure to saponin solutions. Cells were washed with DME buffered to pH 7.5 with 15 mM HEPES, then incubated for 15 min at room temperature with 5 μg/ml R-BT in HEPES-buffered DME supplemented with 5% cadet calf serum. Extraction with saponin (method I) was as described above. Some AChR clusters were also observed using interference reflection microscopy (12, 16), performed as reported (6).

After saponin extraction and fixation in paraformaldehyde, coverslips that were to be stained further were washed and incubated in 0.1 M glycine, to inactivate any remaining free aldehyde moieties (1). For labeling with the fluorescent lipid analogue, 3,3′-di($C_{18}$,$H_{37}$)indocarbocyanine iodide ($C_{18}$-dil, kindly provided by Dr. Alan Waggoner, Carnegie-Mellon University) samples were washed several times in buffered saline at 37°C, then placed in 2 ml of buffered saline to which 4 μg of $C_{18}$-dil were added from an ethanolic stock solution (1 mg/ml). Coverslips were incubated for 5–10 min at 37°C, washed several times at 22°C with buffered saline, and mounted in a solution consisting of nine parts glycerol to one part 1 M Tris-HCl, pH 8.0. Fluorescence of $C_{18}$-dil was observed with a Zeiss Plan-Neofluar 25×/NA 0.8 oil immersion objective using the same filter set (catalog No. 487714) used for tetramethylrhodamine.

Immunofluorescent labeling of isolated AChR clusters with antibodies to cytoskeletal proteins was performed as described (1, 6). Affinity-purified antibodies to vinculin, α-actinin, and filamin (7) were used at concentrations of 12–40 μg/ml. All the antibodies were highly specific for their appropriate antigens and showed no cross-reactivity in enzyme-linked immunosorbent assays (7, 13). Counterstaining was with fluoresceinated goat anti-rabbit IgG (Cappel Laboratories, Inc., West Chester, PA), diluted 1:100 in buffered saline containing 1% albumin. Samples were mounted in glycerol (see above) and observed using a Zeiss Planapo 63×/NA 1.4 oil immersion objective.

All light microscopy was performed using a Zeiss IM35 microscope. Photomicrography employed HP-5 film (Ilford, Basildon, Essex, Great Britain). Exposures were for 5–45 s. Film was processed to an ASA of 1200 using the Ilford developer, Microphen.

*Scanning Electron Microscopy:* Myotube cultures treated with saponin for 3 min were fixed in 3% glutaraldehyde (Polysciences, Inc., Warrington, PA) in cacodylate buffer, dehydrated through a graded series of alcohol solutions up to 100%, then critical-point dried with $CO_2$ and shadowed with gold. The samples were observed using an AMR 1000 scanning microscope equipped with an LAB 6 electron gun. Scanning microscopy was performed in collaboration with Dr. Lucy Boone-Barrett (formerly of the Department of Pathology, University of Maryland School of Medicine.).

*Radiolabeling:* For studies of the cellular material left associated with coverslips after saponin extraction, cultures were labeled for several days with [$^{35}$S]methionine (Amersham Corp., Arlington Heights, IL) or with [$^{3}$H]leucine (ICN Pharmaceuticals, Inc., Irvine, CA). These isotopes were usually added on day 4 after plating, when culture medium was replaced with medium containing cytosine arabinoside, to kill dividing cells (14). Cultures used 3–4 d later were thus extensively radiolabeled. After collection of radiolabeled material (method II; see above), precipitation in cold trichloroacetic acid was performed. Precipitates were collected on GC/N glass fiber filters (Schleicher and Schuell, Keene, NH) and counted in Liquiscint (National Diagnostics, Somerville, NJ) or Ready-Solv EP (Beckman Instruments, Inc., Fullerton, CA) using a model 1215 liquid scintillation counter (LKB Instruments, Inc., Gaithersburg, MD).

Radiolabeling of AChRs employed iodinated α-bungarotoxin ($^{125}$I-BT), prepared by the method of Vogel et al. (32), or purchased from New England Nuclear (Boston, MA). Cultures were incubated with 40 nM $^{125}$I-BT, in HEPES-buffered DME containing 2% cadet calf serum, for 1 h at room temperature. Specific binding, defined as the difference in the counts bound in the presence and absence of 0.1 mM D-tubocurarine, was >85% for material extracted into the saponin solution, and >75% for material remaining bound to the coverslip. The incubation conditions used gave >85% saturation of both receptors released from the coverslip by saponin, and of receptors remaining attached to the cover slip (W. G. Resneck and R. J. Bloch, manuscript in preparation). Radiolabeled material was dissolved in 0.1 N NaOH and counted in an LKB model 1270 gamma counter.

*Gel Electrophoresis:* Preparation and electrophoresis of samples were as described by Laemmli (17). Gels were 1.5-mm thick and generally contained 12% acrylamide, 0.32% *bis*-acrylamide (both from Bio-Rad Laboratories, Richmond, CA). Silver staining employed the method of Oakley et al. (20). Molecular weight standards were lysozyme (14,400), carbonic anhydrase (31,000), ovalbumin (45,000), BSA (66,000), phosphorylase *b* (92,000), and β-galactosidase (116,000). The following purified cytoskeletal proteins were also used as standards: rabbit skeletal muscle actin, rat brain tubulin, and tropomyosin, α-actinin, vinculin, myosin, and filamin from chicken gizzard.

*Materials:* In addition to the lot number of saponin used regularly in these experiments (see above), I tested two other lot numbers from Sigma (81F-0133 and 22F-0254). Both gave results qualitatively similar to the ones described below.

The detergents CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) and zwittergent 14 were purchased from Pierce Chemical Co. (Rockford, IL) and Calbiochem-Behring Corp. (La Jolla, CA). Unless otherwise noted, all other materials were from Sigma Chemical Co.

## RESULTS

### *Shedding Induced by Saponin*

Rat myotubes cultured on glass coverslips are normally very stable: very few muscle cells are lost over a period of many hours, even when subjected to potentially disruptive conditions (4, 5). Upon exposure to the cholesterol-specific detergent saponin, about half of the cellular material (in this case labeled with [$^{3}$H]leucine) was lost within 5 min (Fig. 1). Material continued to be extracted for at least the next 20 min, until, at the end of this time, <1% of the original TCA-precipitable label remained associated with the coverslip. Cultures incubated in the absence of saponin released almost no material into solution (Fig. 1, upper curve), suggesting that most of the extraction is caused by the detergent. Similar results were obtained with cultures metabolically radiolabeled with [$^{35}$S]methionine for several days prior to extraction (Table I).

I investigated the saponin-induced shedding of cellular material from the coverslip, to learn if it showed a strong de-

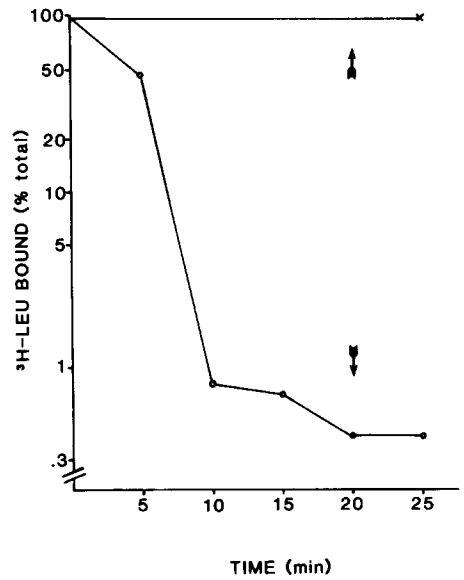


FIGURE 1 Time course of shedding of cellular materials induced by saponin. Rat myotube cultures were labeled for 3 d with [$^3$H]-leucine, then washed and exposed to saponin using method II (see Materials and Methods). As a function of time thereafter, material released from coverslips and material retained on coverslips were collected and TCA-precipitable radioactivity was determined in each. The rotation speed was 90–100 rpm from 0–20 min, and then it was increased to 140–150 rpm (arrows). The ordinate plots the percent of total TCA-precipitable counts remaining associated with the coverslip. Note the logarithmic scale. (O) Extracted with saponin, (×) extracted in buffer lacking saponin.

pendence on pH, divalent cation concentration, or ionic strength (Table I). I found little variation in counts bound when the pH was varied from 6.0 to 8.0, when $Mg^{++}$ was varied, or when $Ca^{++}$ was present. Increasing the ionic strength by addition of 0.3 M NaCl had little effect. In contrast, decreasing the ionic strength greatly reduced the amount of radiolabeled material shed into the saponin solution. These results suggest that saponin extraction in the presence of $MgCl_2$ and EGTA, at isotonic salt concentrations, is well suited for the extraction of the bulk of cellular materials in myotube cultures. In other experiments, I studied the detergent requirement by varying the concentration of saponin, or by using other neutral detergents. Saponin at concentrations as low as 0.025% effectively extracted most cellular material, leaving a residual 0.6% bound to the coverslip (not shown). Triton X-100 and β-octylglucoside left similar amounts bound (Table I). However, the material left associated with the coverslip in the presence of either of these detergents was distinct from that left after saponin extraction (see below).

To learn if the material left on the coverslip after treatment with saponin had been associated with the glass before addition of saponin, or became associated after the cellular material and debris were released from lysing cells, I fixed cultures shortly after they were first exposed to detergent and processed them for scanning electron microscopy. Muscle cells, apparently in the process of retracting from the coverslip, are visible in these samples (Fig. 2). In such cases some material seems to be left behind on the glass coverslip (Fig. 2, large arrowhead). It has the shape appropriate to the retracting cell, and

TABLE I

*Recovery of Radiolabeled Protein and AChRs in Substrate-attached Material after Saponin Extraction under Different Conditions*

| Condition | Radiolabel bound to substrate | | |
|---|---|---|---|
| | [$^{35}$S]Met | [$^3$H]Leu | $^{125}$I-BT |
| | | % total | |
| Saponin solution* (pH 7.0) | | | |
| +Mg, +EGTA | 0.54 ± 0.03 (3)‡ | 0.67 ± 0.27 (4) | 10.0 ± 3.5 (10) |
| −Mg, +EGTA | 0.59 | 1.46 | 6.3, 3.7 |
| −Mg, −EGTA | 0.72 | 1.33 | 3.1, 3.9 |
| −Mg, −EGTA, +Ca | 0.80 | 0.98 | 3.7, 3.1 |
| +Mg, −EGTA | 0.57 | 0.83 | 4.9, 5.2 |
| +Mg, −EGTA, +Ca | 0.68 | 0.80 | 6.2, 4.2 |
| +Mg, −EGTA, +NaCl | 0.50 | 0.80 | 9.8, 10.5 |
| Low ionic strength§ | 19.3 | 41.9 | 71.0, 56.4 |
| Vary pH‖ | | | |
| 6.0 | 0.66 | 0.85 | 7.4, 5.6 |
| 6.5 | 0.55 | 0.87 | 9.2, 6.3 |
| 7.5 | 0.46 | 0.83 | 9.7, 6.5 |
| 8.0 | 0.62 | 0.68 | 10.1, 5.7 |
| Other detergents¶ | | | |
| Triton X-100 (0.5%) | 0.75, 0.52 | ND | 0.85, 0.34 |
| β−Octylglucose (1%) | 0.73, 0.79 | ND | 2.0, 2.5 |

Cells were extracted with solutions of different composition of pH, using method II (see Materials and Methods), with two modifications: (*a*) all solutions were supplemented with BSA (1%), and (*b*) the initial wash solution, before addition of detergent, was free of $MgCl_2$ and EGTA. For analysis of cellular material, myotube cultures were metabolically radiolabeled for 3 d with [$^3$H]leucine or [$^{35}$S]methionine, and processed as described in Fig. 1. For analysis of AChRs, cells were labeled with $^{125}$I-BT before extraction. Most of the toxin in the extracted and substrate-bound fractions is bound specifically to AChRs (see Materials and Methods). *ND*, not determined.

* This solution consisted of 0.2% saponin, 10 mg/ml BSA, in 10 mM NaP, 145 mM NaCl, pH 7.0. Mg, when present, was at 10 mM as the chloride salt; EGTA, when present, was at 1 mM; Ca, when present, was at 1 mM as the chloride salt; NaCl, when indicated, was supplemented to a final concentration of 445 mM.

‡ Mean ± SD, followed by the number of determinations, in parentheses. Where only one value is given, those conditions were assayed only once for that particular isotope.

§ Low ionic strength solutions were prepared in two ways. One solution consisted of saponin and albumin, 10 mM NaP, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.0. The other consisted of saponin and albumin in 1 mM NaP, 14.5 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.0.

‖ The solution was 0.2% saponin, 10 mg/ml BSA, 10 mM NaP, 145 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, with the pH adjusted by addition of either NaOH or HCl.

¶ Other detergent solutions were prepared without saponin, with the same salt concentrations as listed in the preceding footnote. The pH was 7.0.

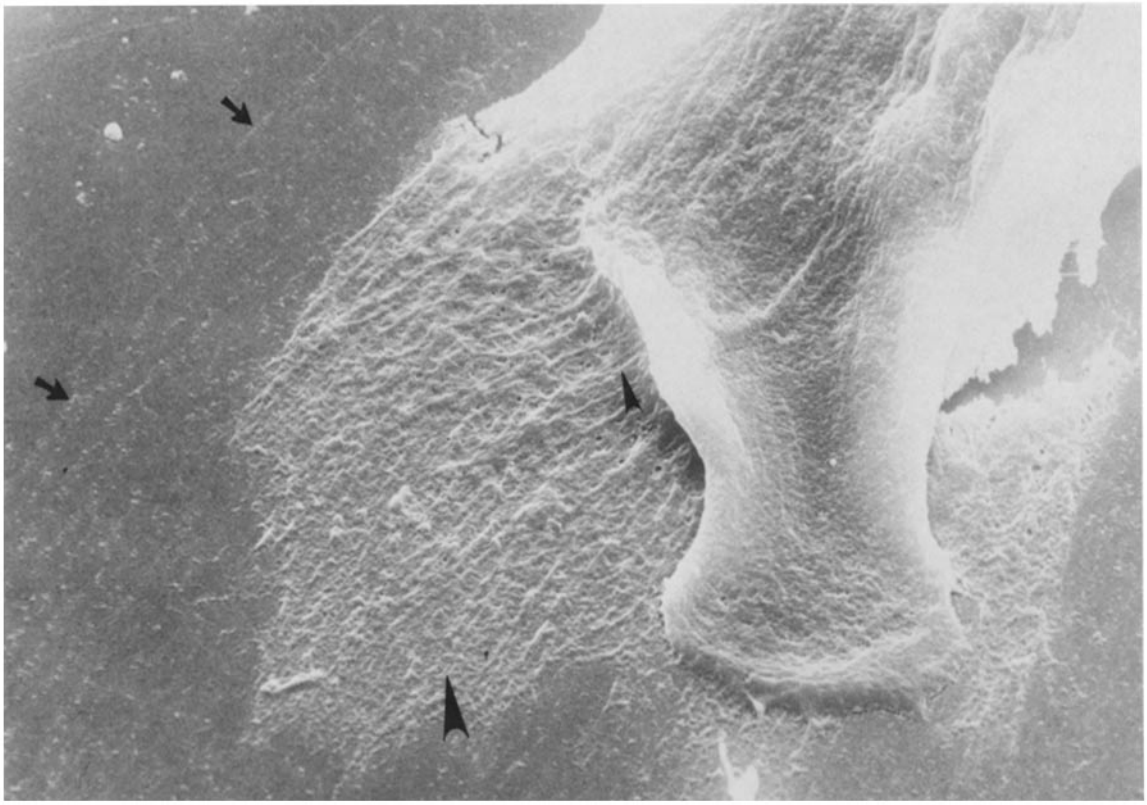

FIGURE 2 Scanning electron microscopy of a myotube shortly after exposure to saponin. Rat myotube cultures were exposed to saponin for 3 min, then fixed in glutaraldehyde and processed for scanning electron microscopy (see Materials and Methods). A myotube is shown that appears to be retracting from the coverslip. A plaque of material remains associated with the substrate as the bulk of the cell retracts (large arrowhead). A "halo" of less dense material surrounds this plaque (arrows). The cell appears to remain associated with the plaque via filamentous structures (small arrowhead). × 6,000.

it seems to be connected to the cell by filamentous structures (Fig. 2, small arrowhead). In addition, there is a thin, irregular network of fibrils and globules forming a "halo" around these structures on the coverslip (Fig. 2, arrows). The latter material may contain small membrane fragments and extracellular material deposited by the cells onto the coverslip during normal cell growth. The former probably represents large pieces of myotubes which remain attached to the glass during treatment with saponin. This material is analogous to so-called "substrate-attached material" or "SAM." SAM is operationally defined as the material left associated with the tissue culture substrate after a particular method is applied to remove the bulk of the cellular protein (for a review, see reference 11). I will refer to the material remaining attached to the substrate after 20–25 min of saponin extraction as "saponin-SAM."

To characterize saponin-SAM further, I used light and fluorescence microscopy. After fixation with paraformaldehyde, little can be seen of this material under phase optics (see below). When fixed and stained with a fluorescent lipid probe, $C_{18}$-diI, saponin-SAM shows label in discrete areas of the coverslip (Fig. 3*A*). These areas can generally be classified into two groups according to their size and shape. The smaller areas vary in size from tiny (1–2 $\mu$m) spots to broad, irregularly shaped structures. The larger areas are oval or elongate, and often have smooth edges. Judging from the shape, and the fact that myotubes seem to leave structures attached to the coverslip (Fig. 2), I conclude that these larger structures arise from myotubes in the original culture. By analogy, the smaller fragments probably arise from fibroblasts or myoblasts. It thus seems likely that the saponin-SAM obtained from rat myotube cultures contains membraneous material derived from the muscle cells and from the contaminating mononucleate cells.

## AChR Clusters in Saponin-SAM

All the large, ventral AChR clusters in rat myotube cultures are found in regions of the myotube membrane that come in close contact with the substrate (2, 6). If the larger structures stained with $C_{18}$-diI do indeed arise from myotubes, they may contain AChR clusters. To test this, I first reacted myotube cultures with R-BT, to label AChRs, and then extracted them with saponin. Large areas of the coverslip were brightly labeled (Fig. 3*B*), but showed no phase-dense material (Fig. 3*C*). If myotubes were incubated with unlabeled $\alpha$-bungarotoxin before exposure to R-BT, or if they were reacted with this label in the presence of $10^{-4}$ M D-tubocurarine, saponin-SAM showed very little R-BT staining (not shown), suggesting that, like the AChR clusters of intact myotubes, the binding of R-BT by these structures is specific.

To determine if such clusters are derived from the AChR clusters of intact myotubes, I performed the following experiment. Cells were labeled with R-BT and photographed under fluorescence optics (Fig. 4*A*). Their grid positions were noted. The culture was then extracted with saponin for 20 min (method I) and fixed with paraformaldehyde. Upon re-examination of material at the same grid positions, I found that the R-BT labeling was essentially unchanged (Fig. 4*B*), although little or no phase-dense material remained (not

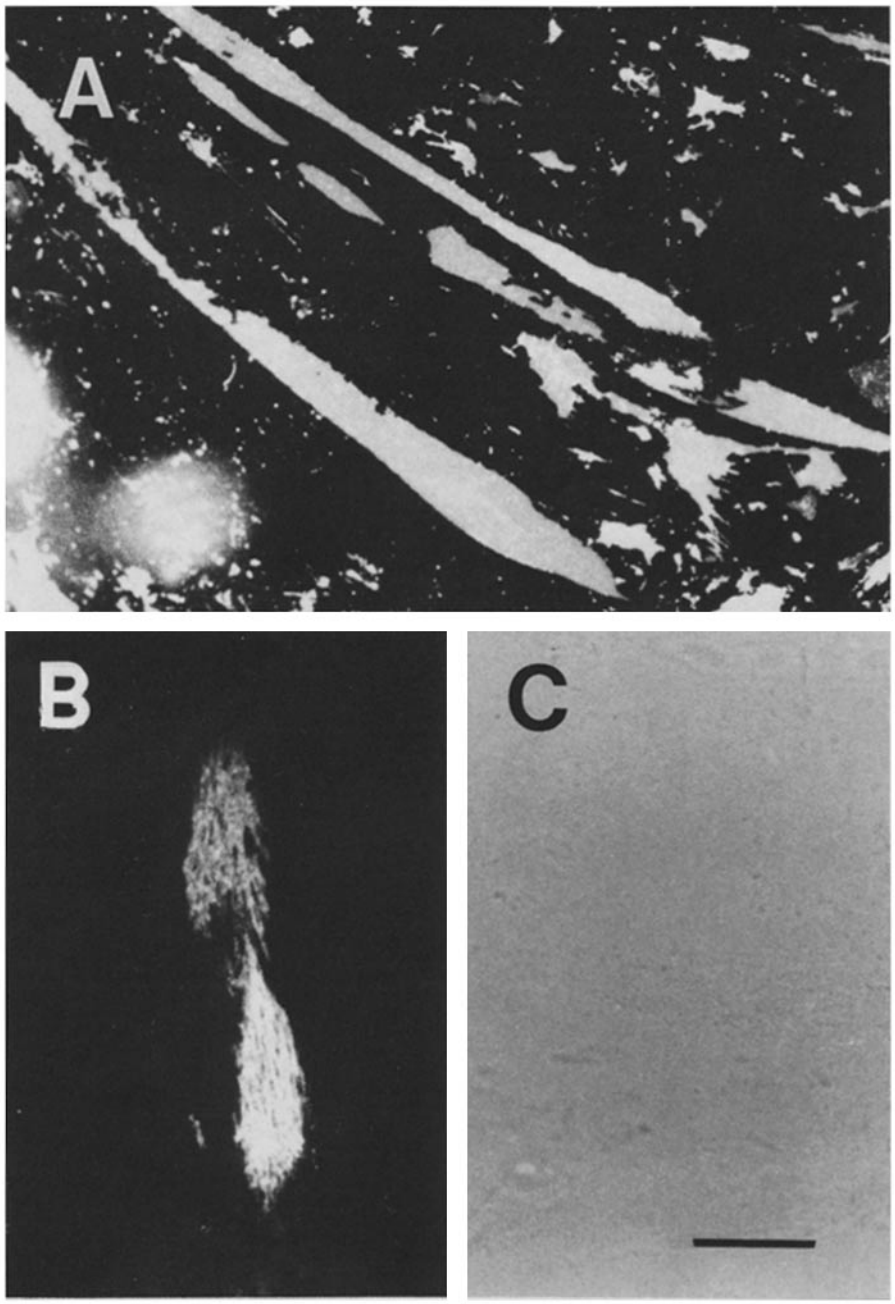


FIGURE 3 Light microscopic visualization of saponin-SAM. Rat myotube cultures were either extracted with saponin (*A*), or first stained with R-BT and then extracted with saponin (*B* and *C*). Samples that contained saponin-SAM were then fixed with paraformaldehyde. *A* shows a view of saponin-SAM which was stained briefly with $C_{18}$-dil. Note the long structures resembling myotubes in shape, and smaller, more irregular structures, which probably arise from the mononucleate cells also present in the culture. *B* shows an AChR cluster found in saponin-SAM, viewed under fluorescence illumination. *C* shows the same field viewed under phase-contrast optics. This particular sample was mounted in buffered saline, to enhance the visibility of any phase-dense material that might be associated with AChR clusters in saponin-SAM. None was seen. Bar 20 μm.

shown). Comparison of the two panels in Fig. 4 shows that the intricate pattern of stained and unstained regions of the intact AChR cluster is almost perfectly preserved after saponin extraction. I did not measure the AChR densities in the clusters of intact cells and in saponin-SAM. Their fluorescence intensities were always similar, however, suggesting that little AChR is lost during the saponin extraction. Close examination of Fig. 4, *A* and *B* reveals two slight differences. (*a*) The hazy "background" staining of the intact myotube has been removed by saponin extraction. This staining can be attributed to AChRs located on the top and sides of the myotube, or to R-BT internalized during the labeling procedure. (*b*) Although the staining pattern is well preserved after extraction, it is slightly (~5%) compressed compared with the original. These results suggest that AChR clusters are recovered in saponin-SAM almost unaltered. To learn how quantitative this recovery from different cells is, I performed the same experiment as illustrated in Fig. 4 on the AChR clusters of 52 cells in two sets of cultures. Of these, 46 (86%) were recovered intact after saponin extraction (method I). This was true even when the AChR density was much lower, or cluster organization much looser, then in the cluster illustrated in Fig. 4. Thus, recovery of intact AChR clusters in saponin-SAM may be nearly quantitative.

However, there are exceptions to this finding. Especially in cultures that are more resistant to saponin extraction, for reasons I do not yet understand,[2] "holes" in the R-BT staining

[2] The isolation procedure may not yet be optimal. For example, rotary shaking subjects the cells on the outer perimeter of the coverslip to more shear than the cells in the center. Greater shear may tear away bits of the cell. A gentler process might allow more gradual retraction from the membrane plaque containing the AChR cluster. Further work using different rocking and shaking regimens may be needed to optimize recovery of intact AChR clusters, without holes.

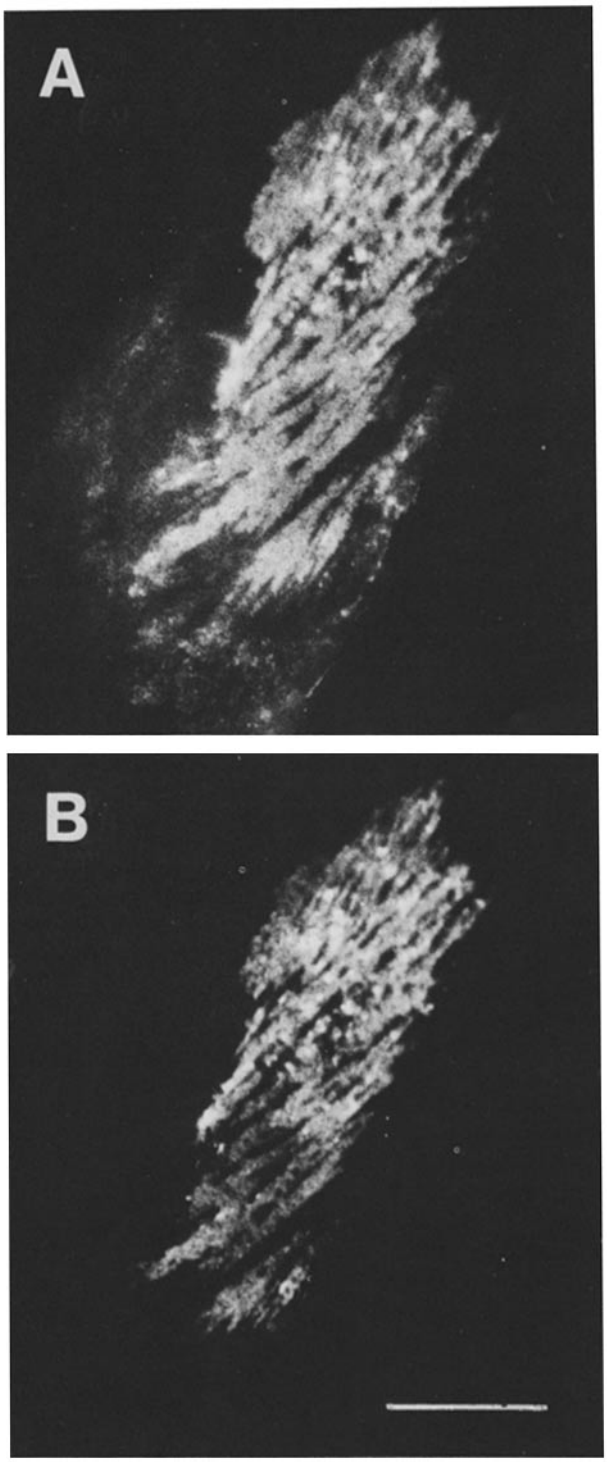


FIGURE 4 Effect of saponin extraction on an identified AChR cluster. A rat myotube culture was labeled with R-BT. A clearly defined AChR cluster was selected and photographed under fluorescence illumination (*A*). Its grid coordinates were noted. The culture was then extracted with saponin for 20 min (method I). After fixation, structures at the same grid coordinates were re-examined. The cluster was recovered essentially intact and unaltered (*B*). Bar, 10 $\mu$m.

pattern appear which are not attributable to the domain structure of AChR clusters (e.g., Fig. 5*A*, arrow). When such samples are reacted with $C_{18}$-diI, these "holes" fail to stain (not shown). Similarly, when such samples are observed by interference reflection microscopy, which visualizes regions of close apposition of membrane to glass (12, 16), no interference signal is obtained from holes (Fig. 5*B*, arrow). Both these observations are consistent with the idea that in some preparations of saponin-SAM, pieces of the cluster-associated membrane have been removed from the cover glass together with the rest of the cell. The qualitative results obtained from such samples always agreed with results obtained from more intact saponin-isolated AChR clusters. Quantitatively, however, fractional recovery of AChRs in clusters, and perhaps other findings, may be affected by such partial loss.

Quantitative estimates of AChRs recovered in saponin-SAM can be obtained using a radioiodinated derivative of $\alpha$-bungarotoxin, $^{125}$I-BT. The results (Table I) show that ~10% of the AChRS of the total in the original culture are recovered in saponin-SAM,[3] giving an estimate of AChRs in substrate-apposed clusters and nearby membrane. This estimate is in approximate agreement with values obtained by autoradiography at the ultrastructural level (18, 27) and by quantitative fluorescence measurements (3). These values can be further compared with recovery of metabolically radiolabeled protein in saponin-SAM. The results (Table I) indicate that enrichment of AChRs in saponin-SAM is about 20-fold compared with intact cultures, while the nearly quantitative yield of AChR clusters in saponin-SAM suggests that saponin extraction gives cluster purification relative to radiolabeled protein of greater than 150-fold. These must be considered upper limits of the purification, however, as materials originating from the tissue culture medium and cell-derived materials deposited on the coverslip before addition of radioactive precursors may also be recovered in saponin-SAM.

The retention of AChRs in saponin-SAM was investigated to learn its dependence on ionic strength, pH, and divalent cation concentration. Only lowering the ionic strength had a large effect on $^{125}$I-BT-AChR complexes retained on the coverslip, in keeping with the much reduced shedding of cellular materials seen in such solutions (Table I). Removing $Mg^{++}$ or adding $Ca^{++}$ to 1 mM seemed to reduce $^{125}$I-BT-AChR in saponin-SAM about twofold. Otherwise, increasing ionic strength or varying pH from 6.0 to 8.0 had no significant effect. These results, and those obtained using metabolically radiolabeled cells (Table I), suggest that the isolation of AChR-rich membrane using saponin under the conditions employed here may be close to optimal.

Other detergents were tested to learn if they would support equally good isolation of AChR clusters. Triton X-100 and $\beta$-octylglucoside removed >97% of the $^{125}$I-BT from the coverslip, suggesting that their ability to maintain intact AChR clusters is considerably less than that of saponin. R-BT-labeled cultures after extraction with these and other neutral (Nonidet P-40; Lubrol PX), anionic (cholate, dodecyl sulfate), or zwitterionic (CHAPS, zwittergent 14) detergents, using the same conditions as used for saponin extraction, showed no AChR clusters remaining associated with the tissue culture substrate.

## *Characterization of Saponin-isolated AChR Clusters*

The AChR clusters isolated in saponin-SAM ("isolated AChR clusters") retain the rectilinear organization of clusters in intact cells, in which strips of receptor-rich membrane

[3] The AChRs removed from the coverslip by saponin probably remain membrane bound. Centrifugation at 100,000 *g* for 1 h of material extracted from myotube cultures by saponin causes all the specifically bound $^{125}$I-BT to appear in the pellet.

(AChR domains) interdigitate with strips of receptor-poor membrane. In intact cells, receptor-poor membrane is closer to the tissue culture substrate than is receptor-rich membrane, and is associated at its intracellular aspect with the cytoskeletal protein vinculin (6). I therefore examined isolated AChR clusters for these same properties. Fig. 5 shows that the strips of receptor-poor membrane are indeed associated with sites of close cell–substrate contact, as visualized using interference reflection optics. To learn if these domains are associated with vinculin, I stained isolated clusters with affinity-purified antibody to vinculin. Unlike the AChR clusters of intact cells (6), isolated AChR clusters display no vinculin. At early times after addition of saponin, before most cellular material is shed from the coverslip (e.g., Fig. 1), vinculin is occasionally still found associated with AChR clusters, and its staining is brighter where R-BT staining is weak (Fig. 6, *A* and *B*).

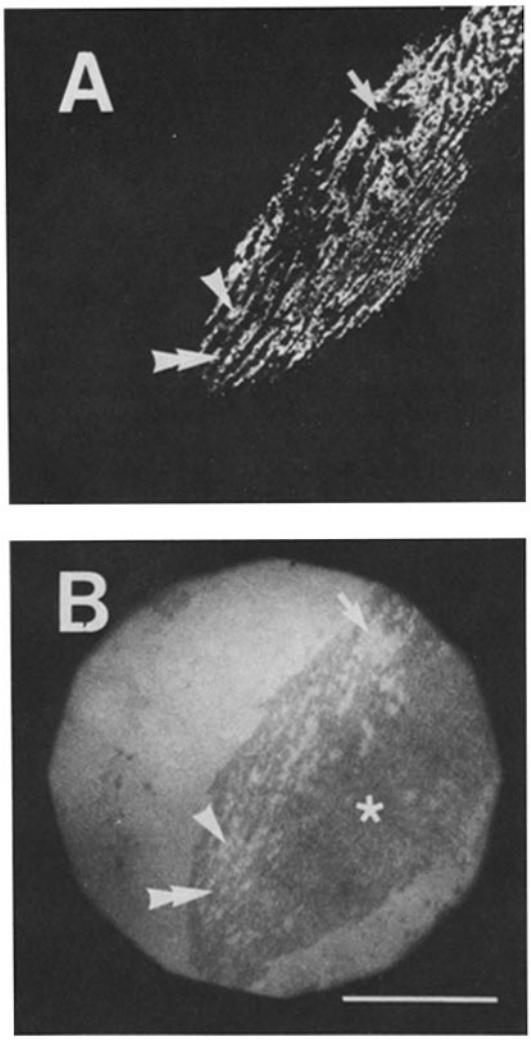


FIGURE 5 AChR-poor membrane domains in isolated AChR clusters are closely apposed to the glass substrate. Isolated AChR clusters labeled with R-BT were viewed under fluorescence (*A*) and interference reflection (*B*) optics, and the images were compared. AChR-rich strips of membrane (*A*, arrowhead) give a light interference color (*B*, arrowhead), while AChR-poor membrane (*A*, double arrowheads) gives a darker interference color (*B*, double arrowheads) typical of close membrane-to-glass apposition (16). Note also that a "hole" in the AChR staining pattern (*A*, arrow) fails to give any interference pattern (*B*, arrow). The area marked by the asterisk in *B* is a tightly organized region, where distinct membrane domains are not visible in the light microscope. Bar, 10 μm.

However, at later times vinculin is no longer detectable in isolated AChR clusters (Fig. 6, *C* and *D*). I conclude that in isolated clusters the interdigitating pattern of AChR-rich and AChR-poor membrane domains is maintained in the apparent absence of vinculin.

To characterize the macromolecular components of isolated AChR clusters and saponin-SAM further, I performed dodecyl sulfate PAGE. As shown in Fig 7, lane *a*, saponin-SAM consists of a number of distinct bands which stain with silver. One major band runs with an apparent molecular weight of 43,000, and co-electrophoreses with purified rabbit skeletal muscle actin. A second major band with an apparent molecular weight of 49,000 is also present. Prominent bands are also found in other regions of the gel. In addition, different saponin preparations contain variable amounts of a large polypeptide that co-electrophoreses with the heavy chain of myosin, and a doublet with the same apparent molecular weight as the tropomyosin subunits. In confirmation of immunofluorescence observations (e.g., Fig. 6), no material electrophoresing with the mobility of vinculin was observed (six preparations).

The bands seen after silver staining of dodecyl sulfate-polyacrylamide gels of saponin-SAM are distinct from the polypeptides removed from the substrate by saponin. By comparing lanes *a* and *b* in Fig. 7, it is evident that bands at 25,000, 43,000, and 49,000 are enriched in saponin-SAM, while numerous bands are enriched in material released by saponin which are absent from saponin-SAM. I also compared the staining pattern of saponin-SAM with those obtained from cultures that contained mononucleate cells but no myotubes (lane *c*), and from coverslips that were exposed to medium but contained no cells (not shown). The band at 65,000 is present both on cell-free coverslips and in saponin-SAM from mononucleate cells, suggesting that it is derived at least in part from the tissue culture medium. The band at 43,000 is also seen in saponin-SAM from mononucleate cells, but is absent from cell-free coverslips. Numerous other differences are apparent in comparisons of saponin-SAM from myotube cultures and mononucleate cell cultures, suggesting that several polypeptides in saponin-SAM containing AChR clusters originate in myotubes. It should be noted, however, that the AChR polypeptides are not evident in silver staining of isolated AChR cluster preparations.

## *Saponin-SAM from Other Cells*

The results presented above suggest that saponin extraction of myotubes and mononucleate cells can leave cell fragments associated with the glass substrate. To test the generality of this observation, I prepared saponin-SAM from cultures of different cell lines. Cell fragments from such cells can be detected with $C_{18}$-diI in saponin-SAM prepared from BC3H-1 cells, and from two neuroblastoma cell lines (not shown). Dodecyl sulfate-polyacrylamide gel analyses of such preparations and saponin-SAM from PC12 cells (Fig. 8) show that the saponin-SAM fractions are distinct from one another and from the material extracted from the cultures by saponin. As with myotube cultures, saponin-SAM from BC3H-1, a muscle cell line, contains a major band at 43,000. Saponin-SAM preparations from B103 and PC12 do not contain high amounts of this material. Instead, they are enriched in bands at slightly higher molecular weights. The two mouse neuroblastoma cell lines, NB2A and NB41A3, yield saponin-SAM fractions that are virtually identical to each other (not shown).

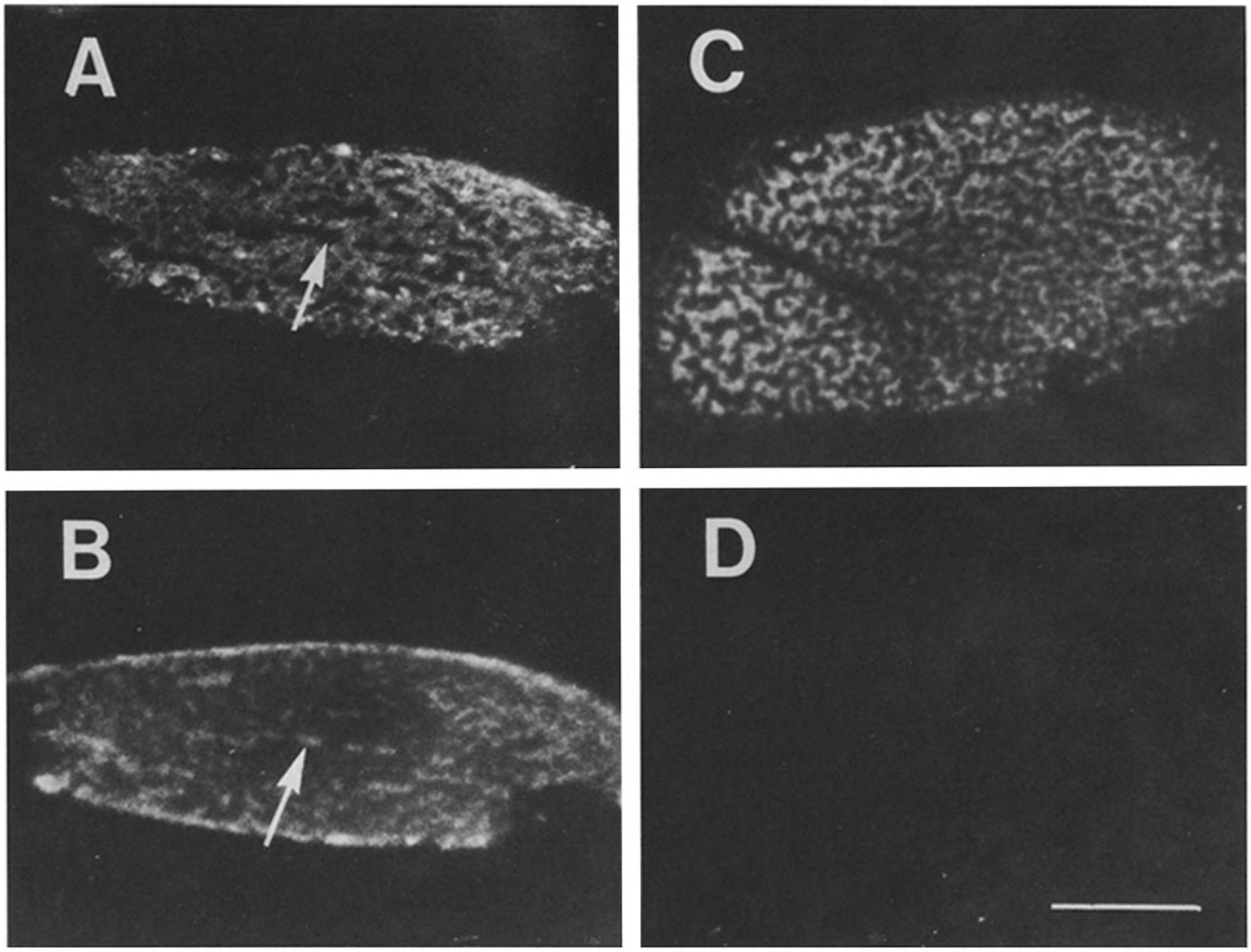


FIGURE 6 Vinculin is lost from AChR clusters during saponin extraction. Rat myotube cultures were stained with R-BT, extracted with saponin (method I) for 2 min (*A* and *B*) or 5 min (*C* and *D*), then fixed with paraformaldehyde, treated with glycine, and stained with affinity-purified antivinculin antibody (12 μg/ml) and fGAR (see Materials and Methods). *A* and *C* show R-BT fluorescence of two AChR clusters; *B* and *D* show fGAR fluorescence in the same two clusters. After 2-min exposure to saponin, vinculin remains associated with some AChR clusters, where it is enriched in AChR-poor regions (*A* and *B*, arrows). At later times, however, no vinculin remains associated with isolated AChR clusters (*C* and *D*). Bar, 10 μm.

## DISCUSSION

The addition of saponin to cultures of rat myotubes causes the shedding of >99% of the cellular material into the detergent solution, and leaves <1% of the cellular material stably associated with the tissue culture substrate. Evidence from scanning electron microscopy and light microscopy supports the idea that during shedding, the cell fragments left associated with the substrate arise from those parts of the cell originally involved in cell–substrate attachment. The regions probably remain intact in the presence of saponin because they contain very little cholesterol. Freeze-fracture studies show that cell–substrate attachment sites react very poorly with the cholesterol-specific probe filipin, whereas surrounding membrane, not involved in attachment, reacts extensively (10, 26). In the case of the AChR clusters of rat myotubes, AChR-rich membrane reacts somewhat more with filipin than does membrane associated with substrate contact, but both these membrane domains react less with filipin than does the myotube membrane outside clusters (22). One would therefore expect that saponin, which reacts preferentially with cholesterol, would destabilize myotube membrane outside AChR clusters and leave clusters relatively unaffected.

In addition to cholesterol, other factors may also contribute to the stability of AChR clusters in the presence of neutral detergents. For example, Prives et al. (21) have reported that clustered AChRs are more resistant to extraction by Triton X-100 than are the nonclustered receptors, found elsewhere on the muscle cell surface. They suggested that this may be due to interactions of clustered AChRs with the "cytoskeletal framework." It is possible that the detergent resistance they observed was influenced in part by the lipid composition of the cluster membrane. I have also found that under mild conditions and short (2 min) exposures, Triton X-100 leaves AChR clusters intact (6). However, after long (20 min) extractions, Triton X-100 and several other detergents completely disrupt AChR clusters (Table I and text). Unlike saponin, these detergents do not allow significant cluster purification under the conditions described here.

It is not yet clear how destabilization of noncluster membrane by saponin causes shedding of cellular material. Initially, saponin probably renders the plasma membrane permeable to large molecules, thereby permitting the loss of soluble cellular constituents. At later times, however, entire myotubes seem to retract from the coverslip. As they do so, they maintain their attachments to the substrate through cyto-

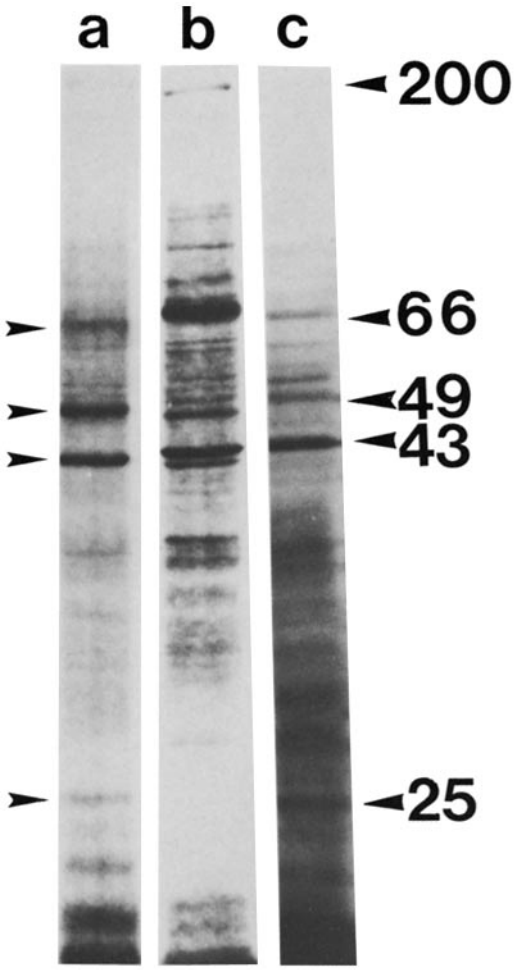


FIGURE 7 Dodecyl sulfate PAGE analysis of saponin-SAM. Saponin-SAM fractions were prepared (method II) from 8-cm coverslips, pooled, frozen, and lyophilized. Total protein in each fraction was determined by the method of Bradford (9). Samples for analysis were prepared and electrophoresed according to Laemmli (17), and stained with silver (20). (lane *a*) Saponin-SAM from rat myotube cultures (2 μg); (lane *b*) material removed from the coverslip during saponin extraction of rat myotube cultures (2 μg); (lane *c*) Saponin-SAM from mononucleate cell cultures prepared from rat hind limb muscle as described in Materials and Methods (5 μg). The arrowheads to the left of lane *a* indicate the positions to which standard proteins of the indicated molecular weights ($\times 10^{-3}$) migrate. Note the prominence of several bands in lane *a* which are less prominent in lanes *b* and *c*.

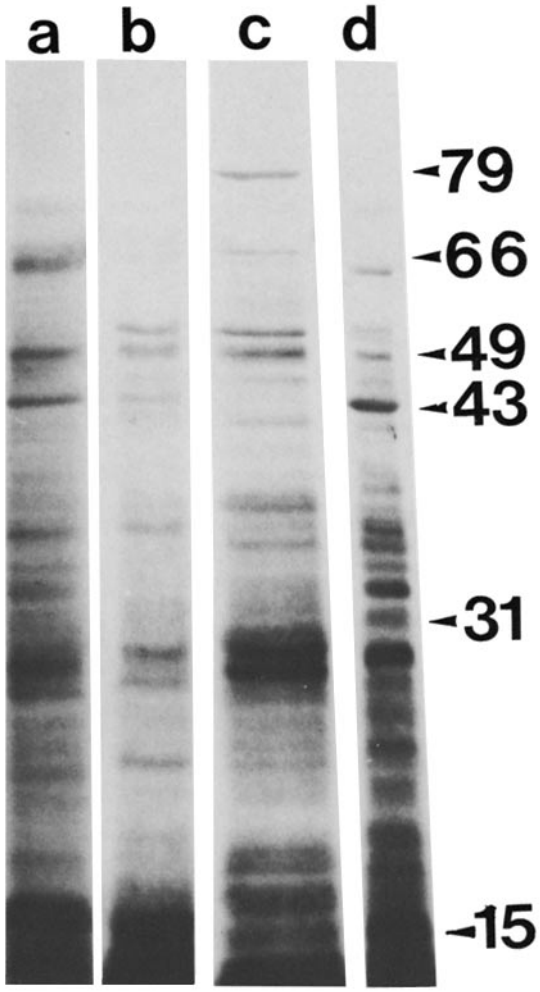


FIGURE 8 Dodecyl sulfate PAGE of saponin-SAM from different cell lines. Cells were grown on 8-cm glass coverslips, then extracted with saponin and analyzed, as described in the legend to Fig. 7. (lane *a*) Saponin-SAM from BC3H-1; (lane *b*) saponin-SAM from B103; (lane *c*) saponin-SAM from PC12; (lane *d*) saponin-SAM from NB2A. Standards are indicated as in Fig. 7.

plasmic filaments (Fig. 2). These filaments may also be visualized using antibodies to cytoskeletal proteins (unpublished observations). As such shedding occurs, the attachment of these filaments to the membrane presumably breaks, and vinculin, as well as much of the cytoskeleton, is released from the substrate. This suggests that the linkage between vinculin and the membrane, believed to be close (15, 28, 31), may easily be severed. Possible exceptions to this are the lacunae seen in isolated AChR clusters, which may result from spots at which the attachment of the membrane to the substrate was less firm that its attachment to vinculin and associated cytoskeletal structures. The easy removal of vinculin from large regions within AChR clusters suggests that the maintenance both of membrane–substrate contact and of the clusters themselves does not require the continued presence of this protein. Because membrane fragments from myotubes and from a number of other cell types remain associated with the tissue culture substrate after saponin extraction, it seems likely that unlike vinculin in myotubes, the molecules involved in maintaining cell–substrate attachment are retained in saponin-SAM. By appropriate radiolabeling and immunological experiments, it may be possible to identify and characterize some of these molecules.

Although AChR clusters remain relatively undisturbed in the presence of saponin, they are altered ultrastructurally in two conspicuous ways. The AChR intramembrane particles, which are quite evenly spaced in intact cells (23), aggregate in the presence of saponin (22). The membrane domains rich in AChRs also deform extensively (22). Thus, some changes in AChR clusters, not evident in light microscopic observations, do indeed occur upon saponin extraction. Nevertheless, the fact that the overall organization of AChRs in clusters is not greatly altered during the isolation procedure suggests that the factors involved in maintaining clusters are retained in isolated preparations. As there are only a few major polypeptides present in isolated AChR cluster preparations, the task of

characterizing them and learning how they participate in clustering now seems feasible.

This paper is dedicated to the memory of Adela Leibman, teacher and friend.

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