Acetylcholine Receptor Clusters of Rat Myotubes Have at least Three Domains with Distinctive Cytoskeletal and Membranous Components

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Abstract. Cultured rat myotubes develop high concentrations of acetylcholine receptors (AChR) in specialized areas of attachment to their substrate. We examined the ultrastructure of identified AChR clusters by quick-freeze, deep-etch, rotary replication or by thin sectioning of whole myotubes fixed in the presence of saponin and tannic acid to preserve the cytoskeleton. Our findings show that AChR clusters are composed of at least three distinct domains, differing in their cytoskeletal, intramembrane, and external components.

At contact domains, the myotube's ventral membrane lacked AChR and lay within 10-15 nm of the substrate; electron-dense strands connected the two. The overlying cytoplasm contained bundles of parallel microfilaments passing above and through an irregular network of globular material, resembling the relationship of microfilament bundles to focal contacts already described in fibroblasts.

Coated-membrane domains lay between the microfilament bundles and were overlain by cytoplasmic plaques of a regular network of polygons having associated coated pits. These plaques closely resembled the network of polymerized clathrin described in fibroblasts and macrophages. Coated membrane also lacked AChR and adhered to the substrate by electron-dense strands, but did not anchor microfilament bundles.

The cytoplasm overlying AChR domains contained a complex network composed of at least two layers. The layer closest to the membrane consisted of protrusions from the cytoplasmic surface, some connected by fine filaments <5 nm in diameter. An overlying layer contained larger diameter filaments, some forming an anastomotic network reminiscent of the cortical cytoskeleton of erythrocytes. Longer filaments inserting into this network appeared identical to members of nearby microfilament bundles. The morphology of AChR domains supports the idea that AChR are immobilized by a network containing actin and spectrin.

During the normal development of neuromuscular junctions, acetylcholine receptors (AChRs) become concentrated in the postsynaptic region. Similarly, muscle cells in tissue culture concentrate AChR in restricted membrane regions in the presence or absence of nerve (for reviews, see references 10 and 39, and references therein). In rat myotubes, clusters of AChR are associated with cell-substrate attachment, and are composed of interdigitating domains (6). AChR domains are rich in receptors and contain large intramembrane particles (IMP) previously equated with AChR (14). These particles are spaced evenly, but are not in direct contact with one another (31, 32). The membrane of contact domains lies particularly close to the substrate; these domains lack AChR and have about one fourth the IMP concentration of adjacent AChR domains (31).

Both intracellular and extracellular components have been invoked to explain the stability of AChR within clusters (for review, see 10). To further understand this stabilization, we wished to determine if the heterogeneity seen in the membrane of an AChR cluster extended to regions internal and external to that membrane, to relate the ultrastructure of AChR clusters to that of attachment sites in other cultured cells, and to elucidate ultrastructural features that might participate in the immobilization of AChR.

Materials and Methods

Cultures

Myotube cultures were prepared from dissociated cells of neonatal rat hind-limb muscles as previously described (6, 8). Cells were cultured on glass coverslips in DME supplemented with 10% calf serum. They were incubated at 37°C in 94% air and 6% CO2, and used on days 7-8 after initial
an angle of 20°, and then backed with carbon applied at 90°. The replicated
0.5 mm in diameter containing the identified cluster was scribed on the
back of the glass as a guide. Nearly always, at least a portion of
replica containing the identified cluster was picked up on formvar-coated
slot grids.

Alignment of Light and EM
To determine whether AChR-rich regions of clusters had a distinct morphol-
yogy, the fluorescence image of a cluster was aligned with its corresponding
phase-contrast image. Fluorescence and phase-contrast images were aligned
by superimposing spots of debris in the culture that were fluorescent and
had a sharp phase image. These images were also aligned by superimposing
letters imprinted on the film by a back (Recordata; Olympus Corporation of America, New Hyde Park, NY) for the light
microscope. Fluorescence and phase-contrast micrographs were aligned by
low magnification electron micrographs by superimposing images of nuclei,
nucleoli, and cell boundaries of myotubes and adjacent fibroblasts.

Shearing, Rapid Freezing and Etching of Clusters
Cultures were labeled with R-BT and rinsed in PBS, and then incubated for
2–3 min at room temperature in a buffer containing zinc (1 mM ZnCl2; 5
mM MgCl2; 3 mM EGTA; 100 mM MES, pH 6.0; reference 3). Most of
the cellular material was removed by shearing with a stream of 10–12 ml
of ice-cold “intracellular buffer” (100 mM KCl, 5 mM MgCl2, 3 mM
EGTA, 20 mM Hepes, pH 7.0; reference 1) delivered through an 18-gauge
needle. Sheared cultures were immediately immersed in ice-cold 0.12 M
NaPi, pH 7.2, containing 2% paraformaldehyde and 0.1% glutaraldehyde,
or 2% glutaraldehyde alone. After fixation at 4°C for 15–30 min, they were
postfixed in 0.1% OsO4, in the same buffer at pH 6.0 (26), then dehydrated
in graded ethanol and embedded in araldite. After polymerization, the
coverslip was removed with hydrofluoric acid. For sectioning perpendicular to
the substrate, a thin layer of fresh araldite mixture was added and polymer-
ized in place of the coverslip.

To section a cluster parallel to the substrate, the embedded cell was glued
to an araldite blank and trimmed to an area slightly larger than the cluster
region. The block face was approached carefully with a diamond knife, so
that one face of the initial thin section was the surface originally exposed by
removing the coverslip. Silver-gray sections were picked up on formvar-
coated slot grids.

Preparation of Fluorescent Latex Beads
To determine the accuracy of alignment between light and electron micro-
graphs of some replicated clusters, we used fluorescent latex beads (~0.66
μm in diameter (Polysciences, Inc., Ft. Warrington, PA) as markers having
sharp images in fluorescence and electron micrographs.

To remove excess soluble fluorescent material, the original suspension
of beads was diluted 40-fold with PBS, and then recovered by filtration
through a 0.22-μm filter. A 100-μl aliquot of the washed suspension was
added to a culture and allowed to settle before mounting the culture for
fluorescence microscopy.

Results
Thin Sectioning of AChR Clusters
Fixing intact myotubes with saponin-tannic acid-glutaralde-
hyde improved intracellular staining and preserved micro-
filaments, as reported for fibroblasts (26). The object of
these thin-sectioned studies was to examine the morphology
of AChR clusters fixed in this way, and to relate this mor-
phology to the location of AChR. Fig. 1 shows an embedded
culture sectioned in a plane mutually perpendicular to the
substrate and the length of the muscle cell. Within the clus-
ter, the ventral cell surface contained short regions where the
membrane-to-substrate distance was only 10–15 nm; electron
density was increased both in the subjacent cytoplasm
and extracellular space associated with the closely approach-
ing membrane (Fig. 1 A). AChR was absent or much reduced
in membrane closely approaching the substrate. Areas rich
in AChR interdigitated with areas close to the substrate across
the width of the cluster. This interdigitation confirmed, at
the EM level, similar observations of AChR clusters using in-
terference reflection microscopy as an indication of membrane
to substrate distance (8), and the enhanced electron density
suggested additional differentiation in the regions of close
membrane-substrate approach. Although sections taken per-
pendicular to the long dimension of the myotube clearly
demonstrated interdigitation, they did not reveal many de-
tails of these close approaches. To examine them further,
intact myotubes having clusters with clearly defined longitudi-
nal bands of R-BT fluorescence were sectioned perpendicular
to the substrate and parallel to the bands (Fig. 2). In a
band lacking R-BT fluorescence (Fig. 2 A, rectangular box),
the ventral membrane lay parallel and close to the substrate.
The overlying cytoplasm contained a bundle of filaments 6–8
nm in diameter, whose most-ventral members passed through
a zone of increased electron density. Deeper in the cyto-
plasm, thicker (11-nm) filaments appeared among these thin
filaments. In cross-sections of myotubes (not shown), such
a region looked like a nascent myofibril with a thick myosin
filament surrounded by six thin equally spaced actin fila-
ments. It was very difficult to trace individual filaments in
a longitudinal section, even with stereo views. Nonetheless,
only those filaments were...
Figure 1. AChR is absent from cluster membrane lying closest to the substrate. A shows one of a series of thin sections taken vertically through a cluster identified by R-BT binding. Dark lines indicate where the ventral membrane closely approaches the substrate. This section passed through two nuclei (N), one containing a nucleolus (arrowhead). B establishes the location of the section (line between two arrowheads) relative to R-BT fluorescence; outlines of the nuclei (N), nucleoli and cell boundaries (B) were traced from a phase-contrast micrograph and superimposed on the fluorescence image. The angle between the section plane and the axis of the myotube was established by correlating features common to phase-contrast and electron micrographs of this myotube and nearby cells. The position of the section along the myotube was then established by its passage through the nucleolus at the left. Information from the first two pictures is combined in C. The thin section (reduced from A and aligned along the plane of section established in B) shows that regions of close membrane-substrate proximity lack AChR.

The thin filaments shown in Fig. 2 did not overlie all of the ventral membrane, since few or no filaments were found in views 5-10 serial sections away from the one illustrated, in either direction. To gain further evidence on this point, longitudinal sections covering a wider area were obtained by sectioning clusters nearly parallel to the ventral membrane (Fig. 3). The first serial section had a dark gray area (<35-nm thick) at one edge containing the most ventral material of the myotube superimposed over substrate originally adherent to the coverslip (Fig. 3, B and D). In this thin area of the section, ventral cytoplasm was present only when it was associated with membrane approaching the substrate very closely. This cytoplasm appeared as strips several microns in length, which were aligned with the myotube's long dimension (Fig. 3, B and D). Between these strips, the membrane was slightly further from the substrate, and, as a result, the first section contained only substrate material, appearing as an electron-lucent carpet of globules (Fig. 3 D). Cellular material close to the substrate appeared globular also, but cross-sectioned filaments would have the same appearance. The smallest globules were ~13 nm in diameter (Fig. 3 D). Some were arranged in short strings, but they did not neces-
sarily lie in a single plane. The second serial section contained bundles of microfilaments precisely aligned over strips of material closely approaching the substrate (Fig. 3, C and E), confirming the views obtained by perpendicular sectioning (Fig. 2). Within the bundles, microfilaments were straight and parallel to each other (Fig. 3, C and E). Although some small globules were seen within the bundles of microfilaments, cross-linking structures were difficult to discern.

A second type of close membrane-substrate approach had few or no associated filaments (Fig. 4). Clear examples had regularly spaced projections extending inward from the membrane's cytoplasmic face, as well as between the membrane and substrate. Similar cytoplasmic projections continued over hemispherical invaginations of the membrane 100–150 nm in diameter, resembling coated pits described in other cells (26).

**Quick-freeze, Deep-etch, Rotary-replication (QFDERR)**

We turned to platinum replication for views of identified AChR clusters over a wider area than could be obtained by thin sectioning. These replicas afforded panoramic views of an entire cluster (Fig. 5), and confirmed the impressions obtained from thin sections that the cytoskeleton was not uniform across a cluster. Three types of cytoskeletal organization were apparent, consisting of: (a) bundles of filaments running parallel to the length of the myotube; (b) flat plaques of polygons with a honeycomb appearance attributed to polymerized clathrin; and (c) a loose irregular network of filaments lying close to the membrane with some longer individual filaments attached to it. Each type of cytoskeleton occupied a set of sharply defined nonoverlapping domains. Together, they covered all of the ventral membrane's cytoplasmic face, except for small areas of "open" membrane. The first two cytoskeletal types were also found in fibroblasts occurring in our myotube cultures, as reported previously (21, 27). The third type occurred only in myotubes.

**Mapping of Replicated Clusters**

AChR clusters were previously shown to be composed of interdigitating membrane domains (8, 32). Mapping experiments demonstrated that a given membrane domain was specifically associated with particular cytoskeletal structures. The simplest mapping method took advantage of autofluorescence caused by glutaraldehyde fixation, which weakly illuminated all of the membrane of a sheared cluster without obscuring fluorescence resulting from bound R-BT. We superimposed fluorescence and EM images of the same cluster vertically through nucleoli (1 and 2) and a nucleus (3), as well as through the contact domain in A (rectangular box). The bracketed region of B is enlarged as a stereo pair (C). Ventral membrane lies ~14 nm from the substrate and is associated with some electron-dense wisps projecting to the substrate (horizontal arrowheads). A distinct electron-dense layer lies just deep to the cytoplasmic face of the membrane; a number of fine (8-nm-diam) filaments course through and above this layer. Deeper in the cytoplasm, thicker filaments (11 nm in diameter) appear in the midst of, and are aligned with, the finer filaments (vertical arrowhead). The thin section micrograph shown in Fig. 4 is taken from the region lacking R-BT fluorescence at the tip of the arrowhead in A.
Figure 3. Microfilaments and electron dense material are associated with contact domains of AChR clusters. Serial thin sections were taken parallel to the substrate in the cluster region (box marked by arrowhead, A) located by aligning phase-dense features (outlined) with R-BT fluorescence. The most ventral section is shown in B and D, while C and E show the next section. B and C were aligned with each other by comparing overlying features, while D and E are stereo pairs of the portions of B and C marked by arrowheads. Electron density appears white in D and E (because of photographic reversal) for ease in seeing filaments and for comparison with succeeding views of platinum replicas. Bands of electron-dense material closely approach the substrate (arrowheads, B; double arrowheads, D) and are precisely overlain by bundles of parallel microfilaments (arrowheads, C and E). Additional circular or oval patches of electron-dense material (single arrowheads, D) are composed of polygons (best seen at the arrow pointing downward, D) and have no overlying filaments. The substrate (dark areas, D) appears as a carpet of globules, similar to its appearance in platinum replicas.
to match cytoskeletal features with the location of AChR, as shown in Fig. 5. Areas of the replica containing the loose irregular network colocalized with AChR-rich areas, while plaques of polymerized clathrin were present in areas lacking R-BT fluorescence. However, the relationship between R-BT fluorescence and the bundles of parallel filaments was not always as clear as is shown in Fig. 5, because the increased mass of material in the filaments often gave a higher background fluorescence because of the glutaraldehyde fixation. Since determining the spatial relation between AChR and cytoskeletal domains required superimposing light and electron micrographs, we needed a way to determine how accurately these images could be aligned. To do this, we used fluorescent latex beads as position markers. Beads were allowed to settle onto coverslips bearing sheared clusters, and become attached to the cluster during subsequent fixation. Fluorescence micrographs were taken of a cluster with several attached beads. The boundaries of filament bundles, clathrin plaques, and the attached beads were traced from a montage of electron micrographs of the same cluster after replication. The tracing was photographically reduced and superimposed on the original fluorescence image of the cluster (Fig. 6). When the EM tracing of a given bead was superimposed over the fluorescence image of the same bead, filament bundles were aligned with elongated bands lacking R-BT fluorescence. To check on the accuracy of this alignment, the tracing of the EM image was shifted ~1 μm to superimpose tracings of filament bundles over R-BT-rich areas. After the shift, the EM tracing of a bead no longer coincided with the fluorescence image of the same bead (Fig. 6 B). The minimum detectable misalignment between the fluorescence and EM images of a bead was ~50% of the bead's diameter (0.35 μm), comparable to the resolution of the light microscope. To this degree of resolution, the result confirms that AChR were excluded from membrane overlain by the filament bundles.

An additional finding from the mapping experiments concerned the location of clathrin-coated membrane. When EM tracings were correctly aligned with corresponding fluorescence images (Fig. 7, A and C), clathrin plaques traced from the EM image were consistently at the edges of AChR-rich areas, but did not directly overlie them. Recent label fracture studies support this conclusion (Pumplin and Bloch, manuscript submitted for publication).

Features of the three domains of AChR clusters are presented in Table I and in more detail below.

**Contact Domains Are Overlain by Bundled Microfilaments**

Bundles of parallel filaments overlying contact domains of AChR clusters are shown in Fig. 7. In agreement with stereo views of comparable thin sections (Figs. 2 C and 3 E), the parallel filaments seen after QFDERR formed a round or slightly flattened bundle rather than a flat ribbon (Figs. 7 and 8). These replicated filaments were 6–9 nm in diameter with a 3–3.5-nm platinum coat, and had repeating cross-striations with a periodicity of ~5.5 nm, presumably reflecting both a subunit structure as well as local aggregation of platinum deposition (41). This diameter and cross-striated appearance have been considered diagnostic for actin microfilaments viewed after platinum replication (22). Furthermore, contact domains of AChR clusters are stained by phalloidin (11), which specifically binds to actin filaments.

Short filaments, apparently of somewhat smaller diameter, made end-to-side connections with those long filaments of

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**Figure 4.** Coated membrane lies close to the substrate and has external material associated with it. This section is perpendicular to the substrate through the region lacking R-BT fluorescence at the tip of the arrowhead in Fig. 2 A. The membrane to substrate distance is ~12 nm, but is obscured by the intense tannic acid staining. Projections extend between the membrane and substrate at regular intervals of ~19 nm, coinciding with similar projections from the cytoplasmic membrane face (arrowhead). The cytoplasmic projections continue over invaginations ~120–140 nm in diameter. No microfilaments are directly associated with the coated membrane, although nascent myofibrils (F) are present deeper in the overlying cytoplasm.

**Figure 5.** The cytoskeleton of an AChR cluster contains distinct domains. This identified AChR cluster was isolated by shearing and viewed by QFDERR. You are looking downward at the inner (cytoplasmic) surface of the ventral membrane, together with those parts of the cytoskeleton that resisted shearing. Features of the replicated cytoskeleton can be related to the location of AChR visualized by fluorescence of R-BT bound to the same cluster (inset). A loose bundle of filaments (F), running parallel to the myotube's length, overlies membrane lacking AChR (region 2 of replica, arrow 2 in inset) and nascent myofibrils (F) are present deeper in the overlying cytoplasm. The cytoskeleton (M) shows occasional particles and the grain of aggregated platinum. AChR is absent from coated membrane and membrane free of cytoskeleton (in replica; arrow 4, inset). Membrane enriched in AChR (1, 3, and 5 of replica; arrows 1, 3, and 5, inset) is covered by a loose irregular network of anastomosing filaments. In J, this network is partially obscured by longer overlying filaments. The network does not appear in membrane areas outside of the cluster.
Figure 6. Mapping cytoskeletal features to the location of AchR with fluorescent latex beads. Beads were allowed to attach to coverslips bearing AchR clusters. After fluorescence microscopy and QFDERR, regions occupied by microfilament bundles (elongated outlines), coated membrane (circular to oval outlines) and beads (circles) were traced onto a transparent overlay from a montage of electron micrographs. A photograph of the tracing was projected onto the fluorescence micrograph of the cluster to superimpose the fluorescent and EM images of the beads (single arrowheads, left). When images of the beads were superimposed, elongated outlines in the tracing were located over areas lacking R-BT fluorescence. When the image of the tracing was shifted laterally to superimpose the elongated outlines of microfilament bundles over fluorescent areas, the fluorescent and EM images of the same bead no longer coincided (arrowheads, right). A few beads were lost or grossly shifted in position after fluorescence micrography, but before replication. Thus, some fluorescent images of beads had no EM counterpart, and vice versa (double arrowheads, left). Note the extensive region of coated membrane at the left-hand edge of the cluster.

The bundle lying closer to the membrane (Fig. 7). The short filaments ended on the cytoplasmic membrane face, but their ends were not obviously specialized. Although connections between the filament network and the membrane were not obvious, the long filaments must be attached to the membrane since they were preserved despite the loss of much of the cytoplasm during shearing. The bundled filaments are apparently maintained under some tension, since they were straight and parallel in thin sections of cells fixed in situ. Shearing breaks the filaments, and their distal ends must have relaxed somewhat before and/or during fixation.

Similar microfilaments were associated with focal contacts of replicated fibroblasts (Fig. 8). Because a close membrane-substrate approach and the presence of vinculin were features shared by contact domains of myotubes and focal contacts of fibroblasts (8), the replicated cytoskeleton in these sites was carefully compared (Figs. 7 and 8). In both types of contact, the long parallel filaments did not have a single attachment to the membrane, but were connected to it by numerous shorter cross-linking filaments. The ends of long filaments were often hard to recognize in the complex networks, but did not appear to insert perpendicularly into the membrane nor end on a particle of larger diameter than the filament itself. Filaments were somewhat more densely packed, and formed a nearly flat ribbon, at focal contacts of fibroblasts (Fig. 8).

Coated Membrane Domains

Portions of the cytoplasmic membrane surface were coated with plaques of a regular network of polygons. In fibroblasts, this network was associated with membrane invaginations the size of coated pits, and therefore ascribed to polymerized clathrin (1, 21, 27). In confirmation, antibodies recognizing light chains of clathrin labeled these plaques in fibroblasts (27) and myotubes (Pumplin and Bloch, manuscript submitted for publication).

Clathrin plaques had a relatively constant size, with an approximate diameter of 0.5-1 μm. In regions of clusters lacking elongated contact domains, the clathrin plaques interdigitated with AchR domains of similar size, forming a mosaic (Figs. 6 and 11). Large elongated areas coated with clathrin were often located along the lateral borders of clusters (Figs. 6 and 9). These borders represent a boundary between membrane lying close to the substrate, thus resistant to shearing, the membrane bending away from the substrate to form the sides of the myotube. The existence of clathrin plaques along these borders indicates that they were different
Figure 7. Bundles of microfilaments overlying a contact domain of an AChR cluster run parallel to the ventral membrane. A bundle of filaments (F) at the right becomes flattened and splayed out toward the left. Lateral connections between the parallel filaments occur close to the membrane (arrowheads pointing down). These connections appear to be somewhat smaller in diameter, but this could result from less platinum deposition because of shielding by the long filaments. Filament intersections very close to the membrane suggest attachment points (arrowheads pointing up).

Table I. The Domains of an AChR Cluster

| Domain               | Cytoplasmic                                                                 | Intramembrane (references 31 and 32)                                                                 | Extracellular                                                                 |
|----------------------|----------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|
| AChR                 | Cytoplasmic projections connected by network of short filaments 12-14 and 2-3 nm in diameter. Longer 12-14-nm filaments insert into this network. | Large IMP are evenly spaced at a density of $\sim$1,000/μm².                                        | Membrane $> 15$ nm from substrate. Some electron density.                      |
| Contact              | Bundled microfilaments parallel to membrane, cross-linked by small diameter lateral filaments. | Few IMP, no apparent regular arrangement.                                                            | Membrane $< 15$ nm from substrate. Evenly spaced strands connect membrane to substrate. |
| Coated membrane      | Array of polygons continuous with covering of coated pits.                 | More IMP than in contact domain, no apparent regular arrangement.                                     | Membrane $< 15$ nm from substrate. Evenly spaced strands connect membrane to substrate. |
tation). However, these plaques were present in other areas of myotube ventral membrane (Fig. 11) and on fibroblasts as well.

**AChR Domains**

Platinum replication revealed a distinctive cytoskeleton associated with domains rich in AChR. In AChR domains with a minimal amount of cytoplasm remaining after shearing, individual particles could be seen on the cytoplasmic surface of the membrane. These were similar in size to the cytoplasmic particles revealed on *Torpedo* membrane by a similar replication procedure (12). Some particles were aggregated into small groups or short strings. Nonaggregated particles were connected by an irregular network containing filaments of two different size classes (Fig. 12). Several filaments could end on a single particle (Fig. 12). Including their platinum coat, the filaments had diameters of 9-13 or 3-5 nm. Some of the variability in filament diameters may be because of differences in the amount of deposited platinum, since smaller diameter filaments were found in areas where incoming platinum had been obstructed by adjacent structures. However, filaments of both sizes were encountered quite close to each other in places where platinum deposition should have been similar.

In AChR domains with more cytoplasm remaining after shearing, the protrusions and fine filaments were covered by a more extensive network of the 9-13 nm filaments (Fig. 12 C). Some filaments of this group had clear cross-striations; others did not have these striations and were somewhat smaller in diameter. Additional longer filaments seemed to end on this network (Fig. 10) or passed across the network and were connected to it by short lateral filaments (Fig. 12 C). These arrangements were consistently present, and their prevalence makes it unlikely that long filaments simply fell onto the network during shearing and became fixed there. The diameter and striations of the long filaments were identical to those of actin microfilaments occurring in bundles associated with nearby contact domains. These long filaments generally lay further away from the membrane than the network of shorter filaments described above. The long filaments coursed in all directions with no obvious orientation to the myotube’s length. Occasionally, they left bundles overlying contact domains to insert into the network over an adjacent AChR domain (Fig. 5, area 1).

The irregular network of particles and filaments colocalized with AChR domains when EM and fluorescence micrographs of the same cluster were aligned (Figs. 5 and 11). This network was readily distinguished from nearby clathrin plaques because it was composed of both large and small diameter filaments that did not form a regular array of polygons. The irregular network was not found on ventral membrane outside the cluster or on fibroblasts. The membrane in these locations was essentially bare of cytoskeleton except for filament bundles and clathrin plaques (Figs. 5 and 10).

**Discussion**

**AChR Clusters Have Two Types of Contact Domains**

Contact domains with a close proximity between membrane and substrate were identified previously by interference reflection microscopy of AChR clusters (8). Two types of contact domains are distinguished in these studies. An elongated contact overlain by a bundle of microfilaments retains the original designation of “contact domain.” The other type, coated-membrane domains, consists of roughly circular
Figure 9. Extensive areas of coated membrane (C) appear at the lateral edges of an AChR cluster, coinciding with the edges of the membrane remaining after shearing. The bracketed area in B is enlarged in C. Networks of polygons extend over shallow coated pits (single arrowheads, A and C) as well as deeper invaginations (double arrowheads, B and C). Since the deep invaginations have survived shearing, they must be attached to the membrane, most likely by a tubular stalk. The loose irregular network characteristic of AChR-rich domains (A) can be readily distinguished from the tight regular polygons of the coated membrane (C). Coated invaginations (double arrowheads) are associated with both coated membrane and AChR-rich domains.
Figure 10. An identified AChR cluster isolated by shearing and subjected to QFDERR. The broken line indicates the border between cluster and noncluster regions. Within the cluster, AChR-rich domains (A) contain projections from the cytoplasmic membrane surface linked by short filaments forming an irregular network. These correspond to the fluorescent area marked / in Fig. 11. This type of cytoskeleton always coincided with AChR and was not present outside of the cluster; e.g., above the broken line in this micrograph. The cytoskeleton of receptor-rich domains is easily distinguished from the plaques of coated membrane (C). Cross-striated filaments (F) with similar diameters (11.5-13 nm, including their platinum coat) have two distinct orientations. Outside the cluster, at the top of the micrograph, the filaments lie parallel to each other and to the myotube's long dimension; within the cluster, the filaments have no preferred direction, branch often, and appear to join end-on to the network of AChR-rich domains (arrowheads).
Figure 11. The irregular cytoplasmic network maps to AChR-rich domains (A) of an AChR cluster, while coated membrane domains (B) interdigitate with AChR-rich domains. Areas of coated membrane and of the irregular network were traced onto separate transparent overlays from an EM montage. Arrowhead 1 indicates the region shown in Fig. 10 while arrowheads 2 and 3 indicate regions shown in Fig. 12. The tracings were photographed and projected onto the R-BT fluorescence image. A mild background fluorescence resulting from glutaraldehyde fixation allowed independent alignment between the EM tracings and the fluorescence micrograph, using the edge of the myotube (shown at the top of both A and B) as well as nearby cells. Areas covered by the irregular cytoplasmic network shown in Figs. 5, 10, 12, and 13 corresponded well to areas rich in R-BT fluorescence (A). However, much of the network was obscured by overlying filaments and could not be traced. This accounts for fluorescent areas that were not outlined. Coated-membrane domains (B) colocalized with areas lacking fluorescence, interdigitating with the AChR-rich domains. Coated-membrane domains appeared in both cluster (bottom) and noncluster (top) regions of the ventral membrane.

patches overlain by a polymerized network of clathrin, but not by cytoplasmic filaments.

Contact Domains of Myotubes Resemble Focal Contacts of Fibroblasts

Focal contacts of cultured fibroblasts are elongated patches of close proximity and adhesion between the ventral membrane and substrate. Extracellular material at these contacts forms periodic membrane-substrate connections (26), while intracellular material consists of bundles of parallel microfilaments (20). Proteins such as vinculin and talin are found at focal contacts of fibroblasts (13, 18) as well as contact domains of AChR clusters (8, 11).

Structures reminiscent of contact domains may exist in muscle cells in vivo. Vinculin is present where the contractile system of adult chicken muscle interacts with the cell surface (28, 36). At the neuromuscular junction, fine extracellular strands connect the postsynaptic membrane to the basal lamina of the synaptic cleft (23), and the postsynaptic cytoplasm contains actin, talin, α actinin, and vinculin (for review, see 10). All these proteins are found in focal contacts of fibroblasts (13). Additionally, clusters of AChR induced by contact between *Xenopus* myocytes and latex beads have associated actin filaments (29).

Bundled microfilaments overlying focal contacts of both myotubes and fibroblasts generally ran parallel to the membrane and were connected to it by small short filaments acting as struts. Actin filaments of cultured cells are polarized such that the end appearing "barbed" with heavy meromyosin decoration is closest to the membrane (38); this is also the preferred end for elongation (42). Elongation would seem to require that the barbed ends be accessible, which is inconsistent with an end-on interaction between the filaments and the membrane.

Myofibrillogenesis

Bundles of actin filaments close to the membrane have long been noted in cultured embryonic muscle cells (for review, see 17). Immature cardiac myocytes were shown to contain continuous bundles staining as a stress fiber distally and as a striated myofibril proximally (15). Thin section observations (these results) of thick filaments lying within a bundle of thin filaments close to the membrane agree with previous fluorescent (15) and EM (30) indications that sarcomeres develop on preexisting stress fibers.

Coated Membrane Domains

Clathrin-containing plaques have been associated with close membrane-to-substrate proximity in normal and virus-transformed fibroblasts (21, 26, 27) as well as in macrophages (1). These plaques have regularly spaced connections to the substrate that presumably mediate adhesion. Although such plaques have not been described in tissues, an electron-dense layer with associated coated pits was found on part of the opposed membranes of synapses developing in culture (35) and in the cerebellum (2). The role of clathrin plaques is not yet clear, although in fibroblasts they appear to be precursors of coated pits (24). Coated pits and vesicles mediate selective endocytosis of membrane proteins (19), but whether these organelles also participate in insertion of new proteins into the surface membrane is being debated (16, 35). The association of clathrin plaques with sites of attachment would make them preferential sites for turnover of membrane proteins, and could thereby direct components necessary for differentiation to sites of cell attachment, as proposed in a model for AChR clustering (10).

AChR Domains

AChR molecules in clusters are immobile in the plane of the
membrane, as judged by fluorescence photobleaching recovery (4). AChR-rich domains of clusters on rat myotubes contain IMP that are evenly spaced in plaques with sharp boundaries, but lie too far apart for direct interactions; this implies that clustered AChR are "locked" into position by binding to a lattice work of other molecules (32). The cytoskeletal network of AChR domains remained attached to the membrane despite shearing, indicating that it is likely to be bound to the IMP. This network has the architecture necessary to maintain receptor molecules in the pattern seen by freeze fracture of rat AChR clusters; i.e., a constant concentration of particles over areas of several square microns (31, 32).

Biochemical (5) and ultrastructural evidence (25, 40) suggests that the cortical cytoskeleton of erythrocytes is a network of spectrin and actin. The association of actin (7) and an isoform of spectrin (9) with AChR domains suggested that the lattice work at these domains might resemble the erythrocyte cytoskeleton. We have identified a network containing large and small diameter filaments that was unique to AChR-rich membrane. Many of the larger filaments at AChR domains are likely to contain actin because they appear identical to members of microfilament bundles, and in preliminary experiments, were not immunogold-labeled with antibodies to beta-spectrin (Pumplin, Strong, and Bloch, manuscript in preparation). These nonbundled actin filaments would account for fluorescence data that actin is present at AChR domains (7), but is not in the form of microfilament bundles staining with phalloidin (11).

Projections from the cytoplasmic membrane face were visible in replicated AChR domains when the overlying network of filaments was removed by shearing. These projections were comparable in size and shape to the particles of Torpedo vesicles (12), which were ascribed to cytoplasmic portions of AChR molecules together with the 43 K protein.
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