Early Cytoplasmic Specialization at the Presumptive Acetylcholine Receptor Cluster: A Meshwork of Thin Filaments

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ABSTRACT Postsynaptic differentiation can be experimentally induced in cultured *Xenopus* myotomal muscle cells by polyornithine-coated latex beads (Peng, H. B., and P.-C. Cheng, 1982, *J. Neurosci.*, 2:1760–1774). In this study, we examined the time course of this process. Small, punctate acetylcholine receptor (AChR) clusters were detectable as early as 1.5 h after the addition of the beads. Subsequently, both the size and the number of the clusters increased with time until a saturation level was reached between 8–24 h. Because the onset and the site of the AChR clustering could be precisely marked, we were able to examine the early structural specializations associated with presumptive AChR clusters. At 1 h, when <20% bead-muscle contacts displayed AChR clusters, 70% of the contacts already exhibited a meshwork of 5–6-nm filaments, which were of the same size as the thin filaments within the myofibrils and thus may contain actin. A system of cisternae similar to the smooth endoplasmic reticulum was suspended within this meshwork, but other organelles were excluded from it. This meshwork, being the earliest cytoplasmic specialization at the presumptive AChR clusters and appearing before the clusters, may be a mechanism for the clustering process.

During the development of the neuromuscular junction, acetylcholine receptors form clusters at the postsynaptic membrane in response to innervation (1, 9, 25, 33). The mechanism for such receptor clustering is unknown. Besides acetylcholine receptor (AChR)[1] clusters, the neuromuscular junction also has a set of other structural specializations associated with the postsynaptic membrane, including the basement membrane, in-foldings, the postsynaptic density, and a meshwork of cytoplasmic filaments (5, 13). This last specialization has attracted considerable attention recently as perhaps being involved in the formation and/or the maintenance of AChR clusters (10–13, 22, 29). However, the causal relationship between the cluster and this filament meshwork cannot be understood from the study of mature synapses or receptor clusters.

Previously we showed that latex beads coated with positively charged polypeptide molecules can induce the clustering of AChRs in cultured *Xenopus* muscle cells (26, 27). Because the initiation of the clustering process can be controlled by the addition of the beads and the location of the presumptive clusters is marked by the beads with high fidelity, this procedure offers an opportunity to examine the cellular processes involved in the formation of AChR clusters. In this study, we examined the time course of the bead-induced AChR clustering and the early structural specializations associated with this process. Our results have shown that the clusters can be detected as early as 1.5 h after the beads come into contact with the cells. Ultrastructurally, a meshwork of thin filaments marks the bead-muscle contacts at equally early stages.

MATERIALS AND METHODS

Cell Culture and the Induction of AChR Clustering by Latex Beads: Myotomal muscle cells were isolated from *Xenopus laevis* embryos as previously described (14, 24). They were cultured on glass coverslips (for fluorescence microscopy) or in tissue culture dishes (for electron microscopy). 4.5-μm polystyrene latex beads (Polysciences, Warrington, PA) were coated with poly-L-α-ornithine (M_r 30,000; Sigma Chemical Co., St. Louis, MO) according to previous methods (27) and applied to 3–5-d-old muscle cultures. The beads only attached to the top and the sides of the cells and were absent from the cell-substrate interface. The AChR clusters induced by the beads were visualized by first labeling the cultures with tetramethylrhodamine-conjugated α-bungarotoxin (R-BTX [30]) for 30 min. Then the cultures were fixed with 95% ethanol at −20°C and examined with a fluorescence microscope. The position of the AChR clusters in relationship to the bead-muscle contacts was determined by combining the fluorescence with phase-contrast microscopy.

Electron Microscopy: Cultures were fixed with 1% glutaraldehyde in 0.05 M Na-cacodylate buffer, postfixed with 1% OsO_4, en bloc stained with uranyl acetate, dehydrated through an ethanol series, and embedded in Epon. Cells were isolated from the Epon blocks and serially sectioned parallel or perpendicular to the original substrate along the longitudinal axis of the muscle

[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor; EM, electron microscopy; R-BTX, tetramethylrhodamine-conjugated α-bungarotoxin.

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 JULY 1984 344–349

fiber. The sections were picked up on Formvar-coated grids, stained with uranyl acetate and lead citrate, and examined under a Philips EM 300 electron microscope.

RESULTS

To determine the time course of formation of AChR clusters, cultures were treated with latex beads and, at different time intervals, labeled with R-BTX and processed for fluorescence microscopy. The results of two experiments are summarized in Fig. 1. In this figure, the duration of co-culture (abscissa) also included the 30 min during which the culture was incubated with R-BTX. At 1.5 h of bead-muscle co-culture, AChR clusters, as evidenced by R-BTX fluorescence, were already present at 20% of the bead-muscle contacts. The aggregation of AChRs proceeded rapidly during this early period such that by 6–8 h of co-culture, the clustering already reached >90% of the level seen in 1-d co-cultures.

Although the AChR clusters could be detected at the bead-muscle contacts as early as 1.5 h after the beads contacted the muscle cell, these early clusters were much smaller as compared with the clusters seen in 1- and 2-d co-cultures. Examples are shown in Fig. 2. At 1.5 h, the clusters associated with individual beads were 0.5–1 μm in diameter. The size of the clusters increased with time such that in 8-h co-cultures they ranged between 1 to 2.5 μm and in 1-d co-cultures they were 3 to 4 μm in diameter as the size was ultimately limited by the 4.5-μm beads used in this study. Clusters over 1 d old were typically composed of small subclusters as previously reported (27).

Having established the time course for the formation of AChR clusters induced by polyornithine-coated latex beads, we set out to examine the early cytoplasmic specializations at presumptive AChR clusters with thin-section electron microscopy. Cultures grown in tissue culture dishes were fixed at different times after the addition of the beads. The time course of AChR clustering, similar to that shown in Fig. 1, was also determined for each electron microscopy (EM) specimen in sister cultures with fluorescence microscopy.

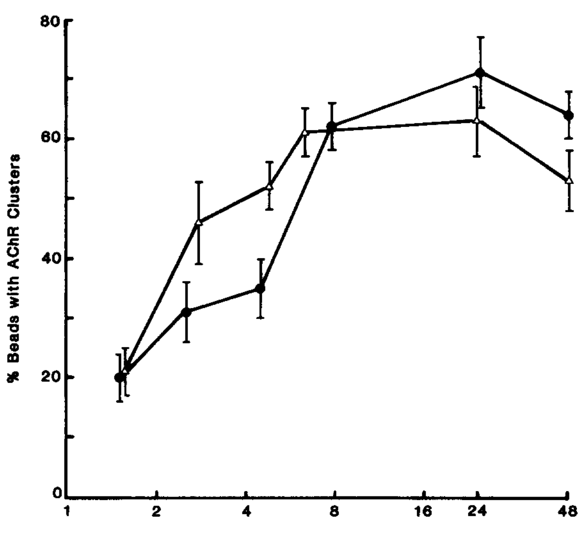

FIGURE 1 Time course of the formation of AChR clusters induced by polyornithine-coated latex beads. Data from two experiments (filled circles and open triangles) are represented here. Each point represents an average of 20 cells. The vertical bars denote the standard error of the mean.

Fig. 3 shows an example of a 2-d bead-muscle contact to illustrate the specializations associated with well-formed clusters. Both intracellular and extracellular specializations can be clearly seen in this area, including a basement membrane on the outside of the cell, invaginations of the cell membrane, coated vesicles, a membrane-associated cytoplasmic density, smooth-surfaced cisternae, and a meshwork of thin (5–6-nm) filaments. Previously we have shown that these specializations are associated with bead-induced AChR clusters (27). Uncoated beads which are ineffective in inducing AChR clusters do not cause the formation of these specializations (26, 27).

Because the beads mark the position of presumptive AChR clusters with over 60% of certainty (Fig. 1), we can then ask the question: Which one of these specializations develop first at the presumptive clusters? From studies such as that shown in Fig. 1, it was clear that only 20% of the clusters at the bead-muscle contacts were detectable at 1.5 h of co-culture; thus our ultrastructural studies were concentrated on 1-h bead-muscle contacts. During the course of this study, 20 cells from two different culture preparations at co-culture periods ranging from 1 h to 5 d were studied by serial thin sectioning. The time course of the AChR clustering in these two series was determined by parallel fluorescence microscopy as shown in Fig. 1. At the time when R-BTX was added to each fluorescence specimen, its sister culture was immediately fixed for EM. Among the cells studied, seven were from 1-h co-cultures with a total of more than 50 bead-muscle contacts.

The most prominent cytoplasmic specialization at the 1 h bead-muscle contact was a meshwork of filaments as shown in Fig. 4. The diameter of these filaments was compared with that of the thin filaments in the myofibrils within the same section: the meshwork filaments had a mean diameter of 5.4 nm (SD = 1.1 nm, $n = 30$) and the thin filaments had a mean diameter of 5.9 nm (SD = 0.9 nm, $n = 30$) when measured at a magnification of 80,000. Thus, this meshwork seems to be composed of actin filaments. The width of the meshwork ranged from 0.1 to 3.5 μm and they varied from small patches underneath the membrane (Fig. 4, inset) to a continuous meshwork underlying the entire bead-muscle contact (Fig. 4). The meshwork thickness ranged from 0.1 to 3 μm and its close juxtaposition to the membrane was apparent (Fig. 4, inset). This inset also illustrates areas of the membrane devoid of such meshwork, both within and outside the bead contact.

The thin filaments within the meshwork were randomly disposed in most cases. Occasionally, however, the filaments away from the membrane were arranged in loose parallel bundles (Fig. 5). This suggests that the cortical thin filament bundles might participate in the formation of this meshwork. Thick (myosin) filaments and microtubules were noticeably absent from the meshwork. Other organelles, such as polysomes, rough endoplasmic reticulum, mitochondria, and Golgi apparatus, were also excluded from this meshwork, although they were present in areas immediately adjacent to it. Coated vesicles, 0.1 μm in diameter, however, were occasionally detected within the meshwork (Fig. 5).

A second specialization observed at these 1-h contacts was a set of smooth endoplasmic reticulum cisternae within the meshwork (Figs. 4 and 6). These cisternae, sectioned either longitudinally or transversely, decorated the otherwise homogeneous meshwork of thin filaments. The cross-sectioned profile indicates that they often existed in tubular form (Fig. 6). They bear striking resemblance to the longitudinal sarcotubules of the sarcoplasmic reticulum (Fig. 7). These cisternae

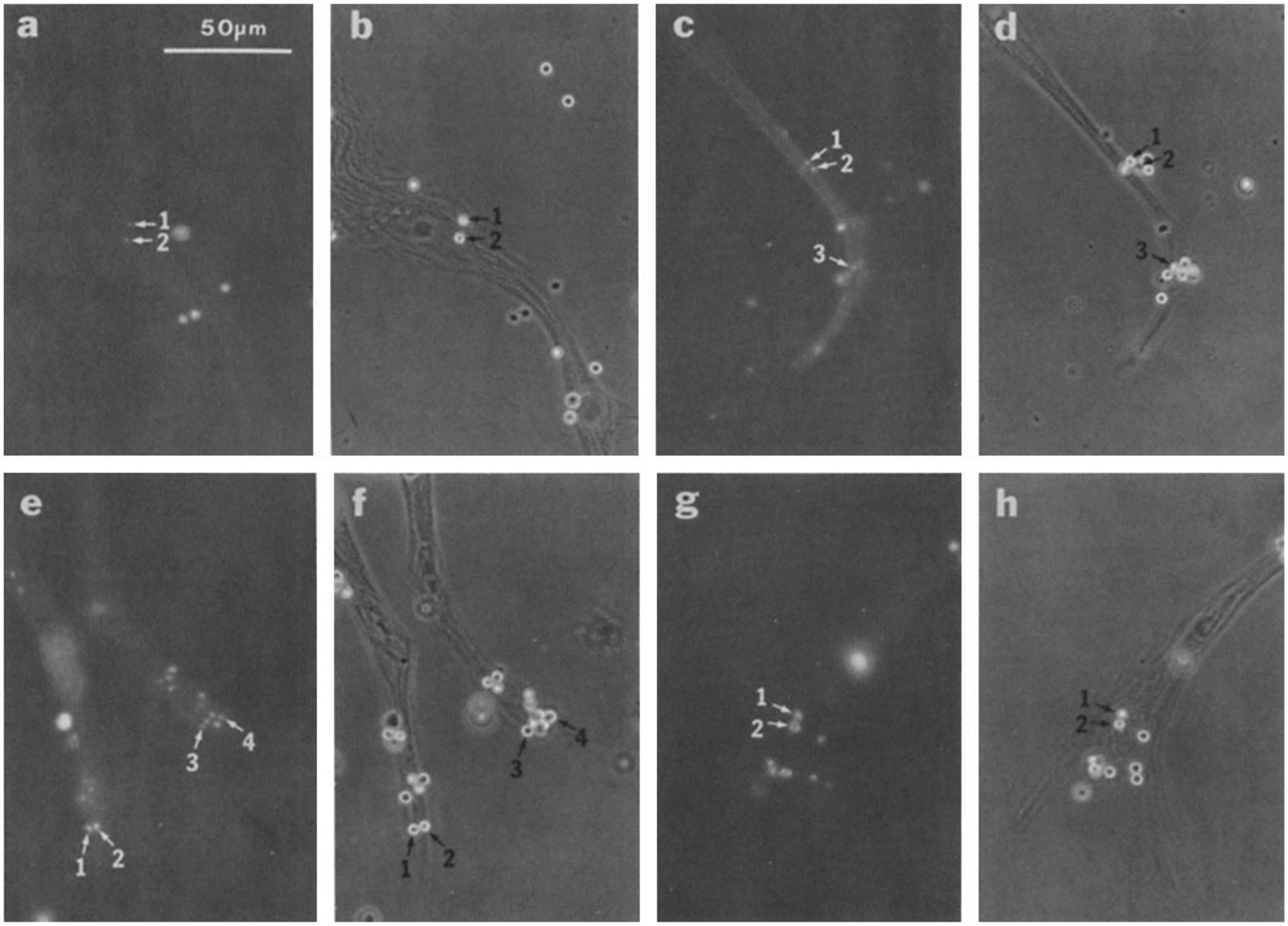

FIGURE 2 Examples of AChR clusters at the bead-muscle contacts from one series of experiments. (*a*, *c*, *e*, and *g*) R-BTX fluorescence micrographs. (*b*, *d*, *f*, and *h*) The corresponding phase-contrast images. Duration of bead-muscle co-culture: (*a* and *b*) 1.5 h; (*c* and *d*) 2.5 h; (*e* and *f*) 7.5 h; (*g* and *h*) 24 h. All fluorescence pictures were exposed and processed under identical conditions. × 348.

were located either within the meshwork or close to the plasma membrane at the bead-muscle contact.

The fact that this meshwork of thin filaments is specifically associated with the site of AChR clustering is further supported by the following observations: (*a*) In areas away from the bead-muscle contacts, the cortex was unspecialized. Fig. 8 shows an area on the upper cell surface lateral to the bead-muscle contact as shown in Fig. 4. In contrast to the bead contacts, organelles including mitochondria, polysomes, smooth and rough endoplasmic reticulum were not excluded from the cortex of the bead-free areas. Thin filaments were also present in this area, but they did not form extensive meshwork structures. Even when two beads were situated within a diameter (4.5 μm) to each other, the meshwork associated with each bead still existed as discrete entities (Fig. 5), reflecting the discreteness of the AChR clusters on the cell surface. (*b*) This meshwork was observed at an average of 70% bead-muscle contacts (out of 35 contacts scored) at 1 h, whereas <20% of the contacts exhibited AChR clusters at this time (Fig. 1). Thus, the appearance of this meshwork preceded the AChR clustering and the percentage of beads exhibiting this meshwork at the onset of AChR clustering (1 h) agrees well with the percentage of beads which eventually become cluster-positive (24 h, cf. Fig. 1).

At mature AChR clusters, such filamentous meshwork were also detected. It was usually contiguous with the membrane-associated cytoplasmic density (Fig. 3) and was most clearly seen when the density is sectioned tangentially as reported previously (see Fig. 9 in reference 27). However, meshworks as extensive as those shown in Figs. 4 and 5 were rarely observed at mature bead-induced clusters. Rather, it was often separated into patches by invaginations of the membrane. The first appearance of the invaginations was several hours after the onset of the meshwork (Peng, unpublished results) and they eventually became coextensive as the early meshwork (Fig. 3 and 4). On the basis of these observations we conclude that this meshwork of thin filaments is an integral component of the AChR cluster-associated specializations. Concomitant with the maturation of the cluster, the meshwork becomes more closed apposed to the receptor-rich membrane.

DISCUSSION

In this study we have shown that the formation of AChR clusters proceeds rapidly following its induction by the polyornithine-coated beads in cultured *Xenopus* muscle cells. Clusters, when visualized with R-BTX labeling, can be detected as early as 1.5 h after the initiation of this process and by 7 to 8 hours it has reached the saturation level (Fig. 1). The aggregation of receptors continues, however, as shown

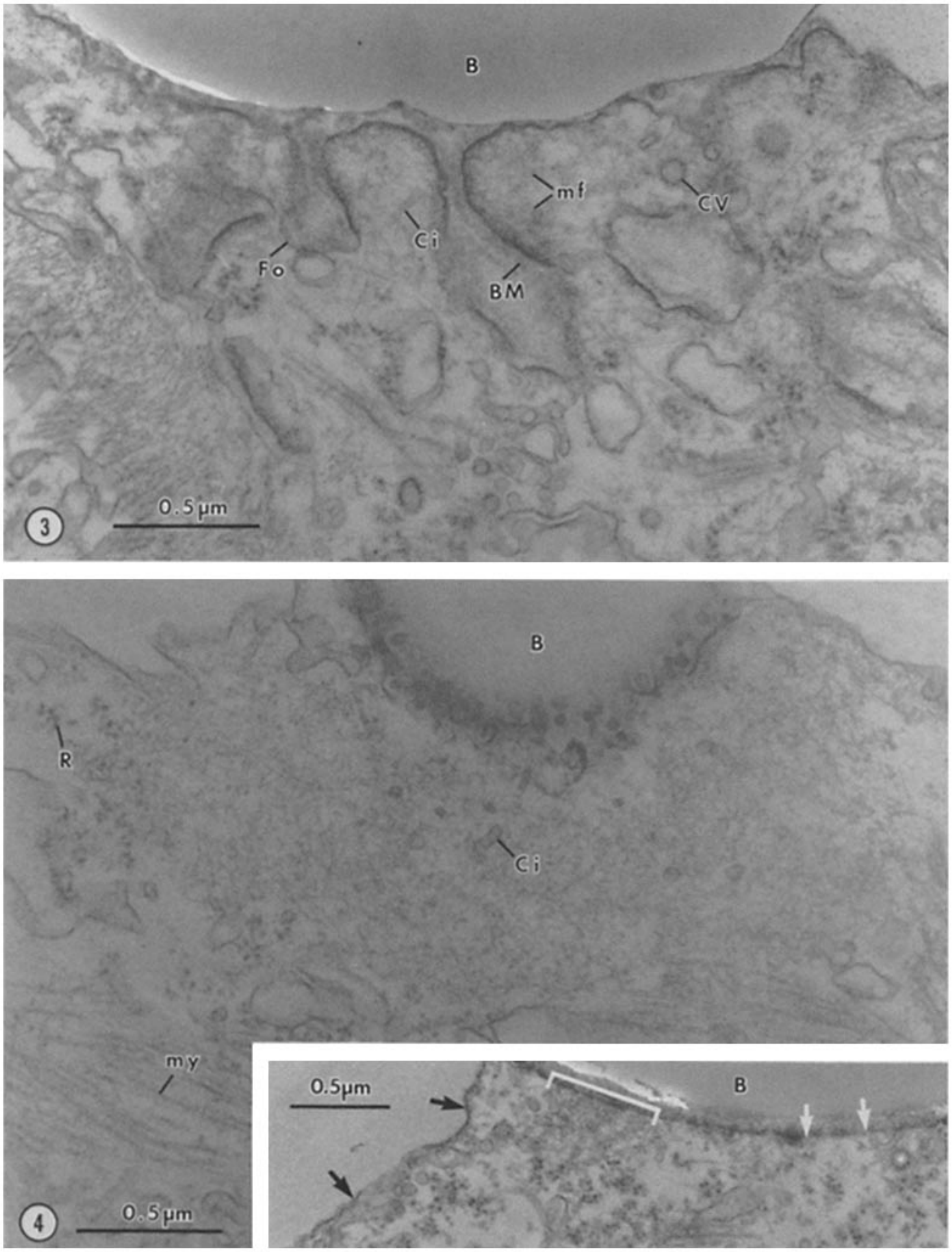

FIGURE 3 and 4 Fig 3: Ultrastructural specializations developed at the bead-muscle contact after 48 h of co-culture. *B*, bead; *BM*, basement membrane; *Fo*, infolding; *Ci*, smooth membrane cistern; *mf*, meshwork of filaments; *CV*, coated vesicles. × 51,500. Fig. 4: Filamentous meshwork at 1-h bead-muscle contact. Other organelles, except membrane cisternae (*Ci*), are excluded from this meshwork. *B*, bead; *R*, polysomes; *my*, thick (myosin) filaments. × 51,000. (*Inset*) Another example of the meshwork which was composed of a smaller patch (bracket). This specialized cortical area is in sharp contrast to the meshwork-free area both within the contact (white arrows) and outside the contact (black arrows). × 28,000.

by a gradual increase in the size of the clusters at least through the first 24 h (Fig. 2). These results clearly demonstrate that new clusters are induced by the beads and rule out the possibility that the beads may somehow become associated with the existent clusters.

Fluorescence microscopy with R-BTX is a highly sensitive way of detecting AChR clustering. Previous freeze-fracture studies have demonstrated that R-BTX fluorescence patches, even in submicrometer dimensions, can be matched precisely with arrays of putative AChR intramembranous particles and areas devoid of R-BTX fluorescence are always associated with a low-density, diffuse intramembranous particle distribution (3, 4, 17). Recently Olek et al. (19) also reported that the formation of AChR clusters in rat myotubes can occur with a rapid time course comparable to that reported here after the addition of brain extract.

We have identified that a meshwork of thin (5–6 nm) filaments and a system of smooth endoplasmic reticulum cisternae which are suspended within this meshwork are the earliest (1 h) specializations detectable at the bead-muscle contacts. Two sets of data indicate that these EM specializations are located at presumptive AChR clusters: (*a*) Previous whole-mount stereo EM studies have shown that both the filament meshwork and the cisternae are associated with the AChR cluster identifiable with R-BTX labeling (22). (*b*) The percentage of bead-muscle contacts exhibiting these EM specializations at 1 h compares closely with the percentage of the contacts that eventually develop into AChR clusters. Thus, a strong spatial and temporal correlation between the meshwork and the process of AChR clustering exists. Although this meshwork is less prominent at mature AChR clusters in thin sections (Fig. 3), results obtained through whole-mount (22) and freeze-etching (12, 13) techniques have clearly shown its existence.

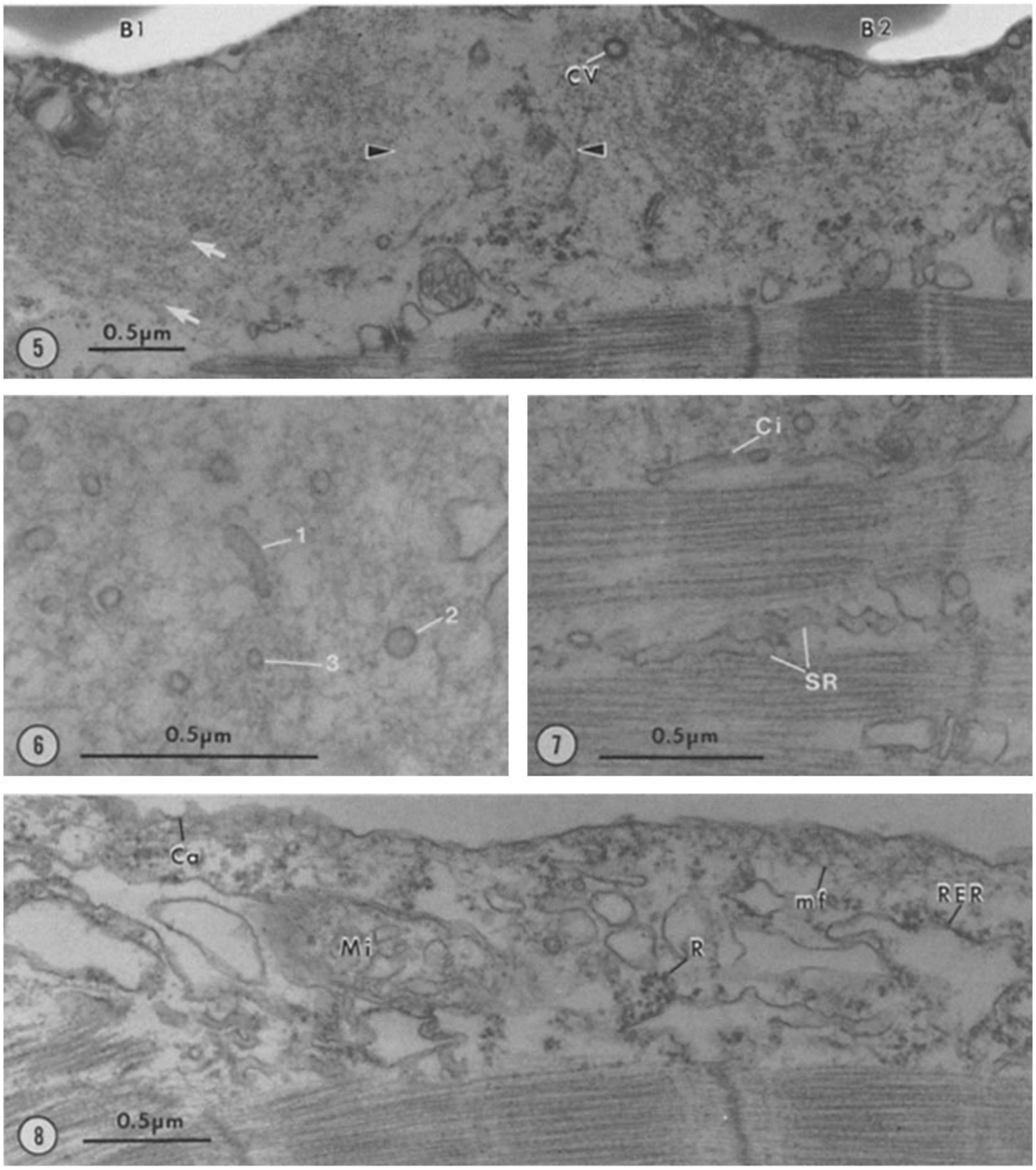

FIGURE 5–8 Fig. 5: Meshwork in a muscle cell contacted by two beads (*B1* and *B2*) at 1 h. In the area between the beads (arrowheads), the cytoplasm was free of the meshwork. Under *B1*, some of the filaments appeared in a parallel array as indicated by the white arrows. *CV*, coated vesicles. × 33,000. Fig. 6: The bead-induced meshwork at 1 h. The smooth endoplasmic reticulum-like membrane cisternae were suspended in this meshwork. They were sectioned either longitudinally (*1*) or transversely (*2* and *3*). Some of the cisternae (e.g., *3*) appeared to be ensheathed by the filaments. × 80,000. Fig. 7: Comparison between the meshwork-associated cisternae (*Ci*) and the SR. *Ci* was located at the lower edge of the meshwork. × 56,000. Fig. 8: An area of the cell not occupied by the beads. *Ca*, caveolae; *Mi*, mitochondria; *R*, polysomes; *RER*, rough endoplasmic reticulum; *mf*, thin filaments. × 35,200.

This meshwork of thin filaments reported here resembles the network of actin filaments observed at the leading edge (lamellipodium) of motile nonmuscle cells (32) and also at the site of phagocytosis in macrophages (21). The dynamic nature of this meshwork in nonmuscle cells has been exemplified by the fact that it can be assembled or disassembled in minutes at the lamellipodium in response to a change in the direction of cell movement (18) and by the observation that actin molecules within the meshwork exchange rapidly with those in the cytoplasmic pool (16). The rapidity of the assembly of this meshwork at the bead-muscle contacts observed in this study suggests that it may also play a role in the motility process involved in the formation of AChR clusters.

Previously we showed that the formation of AChR clusters at the bead-muscle contacts can be blocked by Ca^{2+} channel blockers and the calmodulin inhibitor trifluoperazine (23). These results suggest that a local increase in Ca^{2+} level at the contact area may activate the clustering process. In view of our current findings, it is reasonable to speculate that this activation may first involve an assembly of the thin filament meshwork. The involvement of Ca^{2+} in this process is further implicated by the co-localization of sarcoplasmic reticulum–like cisternae within the meshwork at the early stage of cluster formation. These cisternae, similar to sarcoplasmic reticulum (7), may participate in regulating the Ca^{2+} level by acting as a mechanism for Ca^{2+} sequestration. This system of cisternae is maintained at mature AChR clusters (see Fig. 3 and reference 22) and perhaps also at the subsynaptic area at neuromuscular junction (9, 20) where it may be involved in the Ca^{2+} regulation during synaptic transmission (8).

Measurements on the lateral diffusion coefficients of AChRs within the plane of the membrane have shown that isolated receptors move freely in the membrane, whereas clustered receptors are essentially immobile (2, 28). As Edwards and Frisch (6) first suggested, the formation of AChR clusters can be accounted for by a simple diffusion-trap hypothesis. The membrane-associated thin filament meshwork at the bead-muscle contact could act as a trap so that the AChRs randomly moving into this area will be immobilized by the meshwork. Such a control of the mobility of integral membrane proteins by cyto-matrix has been demonstrated in the erythrocyte membrane (15). According to this model, the structural organization of a cluster would be determined by the organization of the filament meshwork underneath. This is supported by the often patchy appearance of both the clusters (Fig. 2*g* and see Fig. 5 in reference 27) and the meshwork (Fig. 4, inset). After this initial event of receptor concentration via the diffusion-trap mechanism, a slower process which stabilizes the clusters may set in. This may be manifested by the development of the postsynaptic density that underlies the receptor-rich membrane at the mature clusters (Fig. 3).

Our results do not rule out a local insertion of new AChRs from intracellular pools during the cluster formation (31). Our previous work (27) has shown that new receptors inserted into the membrane after the addition of the beads are also used in the formation of new clusters. However, these new receptors may be diffusely incorporated into the membrane and move to the site of new cluster formation by lateral diffusion (1).

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