Participation of Calcium and Calmodulin in the Formation of Acetylcholine Receptor Clusters

H. BENJAMIN PENG
Department of Anatomy, University of Illinois at Chicago, Chicago, Illinois 60680

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ABSTRACT The formation of acetylcholine receptor (AChR) clusters can be experimentally induced in cultured *Xenopus* myotomal muscle cells by positive polypeptide-coated latex beads (Peng, H. B., P.-C. Cheng, and P. W. Luther, 1981, *Nature* [*Lond.*], 292:831–834). This provides a simple procedure for studying the cellular process of AChR clustering. In this study, the involvement of calcium and calmodulin in this process was examined. A deprivation in extracellular calcium by calcium-free medium or by the addition of calcium antagonists such as divalent cations Co^{2+} and Ni^{2+} (1–5 mM) or organic compounds verapamil and D-600 (0.1–0.5 mM) suppressed the formation of AChR clusters induced by the latex beads in a largely reversible manner. Antagonists against calmodulin, including trifluoperazine (1–5 μM) and the naphthalene sulfonamide W-7 (20 μM), also suppressed AChR clustering. However, the effect of W-7 was much weaker than that of trifluoperazine (TFP). Although the formation of AChR clusters is inhibited by these drugs, the stability of the existent clusters is relatively insensitive to them. These data suggest that the clustering of AChR involves a Ca^{2+} and calmodulin-activated process. Immunofluorescence studies using an antibody against calmodulin indicate that calmodulin is diffusely distributed in the cytoplasm in addition to its localization at the I-bands. Thus I propose that a local rise in intracellular calcium caused by a locally applied stimulus, exemplified here by the polypeptide-coated latex beads, may trigger the formation of AChR clusters. Furthermore, the cellular machinery for this process may involve calmodulin and is diffusely distributed in the muscle cell.

The formation of the acetylcholine receptor (AChR)[1] clusters is one of the earliest events during the innervation of the skeletal muscle (3, 17, 38, 49). Despite the fact that several cholinergic neurons can form functional neuromuscular contacts with the muscle as demonstrated by the appearance of end-plate potentials (26, 34, 45), not all can induce this postsynaptic differentiation. In *Xenopus* nerve-muscle co-cultures, it has been found that only neurons isolated from the spinal cord can induce the formation of AChR clusters; neurons isolated from dorsal root ganglia or sympathetic ganglia are ineffective (11, 27).

The process that triggers the AChR clustering and its neuronal specificity are intriguing questions on synaptogenesis. Recently it has been shown that AChR clustering can also be induced by positive polypeptide-coated latex beads in cultured *Xenopus* muscle cells (39, 40). In addition, a set of structural specializations characteristic of the postsynaptic membrane, such as the in-foldings, the cytoplasmic density, and the basement membrane, also develops at the bead-muscle contacts (40). Thus this latex bead-muscle co-culture offers a simple model system for elucidating the cellular mechanism of the formation of AChR clusters.

In this paper, we are concerned with what triggers the AChR clustering at the bead-muscle contacts. Previously Gingell et al. (18, 19) showed that local application of polycations to the surface of *Xenopus* eggs causes a muscle-like contraction of the cortical cytoplasm and this process appears to be mediated by calcium. Thus I hypothesize that polypeptide-coated latex beads trigger AChR clustering by a Ca^{2+}-mediated event. The bead may cause an increase in the membrane permeability, and this allows a local influx of Ca^{2+} into the cell.

I tested this hypothesis through the use of calcium antagonists, agents that interfere with the Ca^{2+} influx into the cell. My results show that these agents can reversibly suppress the AChR clustering induced by latex beads. Since many of the Ca^{2+}-regulated cellular processes are mediated by the ubiqui-

[1] *Abbreviations used in this paper:* AChR, acetylcholine receptors; PBS, phosphate-buffered saline; R-BTX, tetramethyl rhodamine-conjugated α-bungarotoxin; TFP, trifluoperazine.

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tous calcium-binding protein, calmodulin (9, 30), I have also tested its involvement through the use of inhibitors against calmodulin. Here, again, I found that the calmodulin inhibitors suppress the latex bead-induced AChR clustering. These results support the hypothesis that a Ca^{2+}-calmodulin system is involved in the formation of AChR clusters.

These studies were previously reported in two abstracts (35, 41).

MATERIALS AND METHODS

Cell Cultures: Myotome cells were isolated from stage 20–22 *Xenopus laevis* embryos (33) according to previous methods (2, 37). They were cultured on coverglass squares (18 × 18 mm) in Steinberg's solution, consisting of 60 mM NaCl, 0.7 mM KCl, 0.4 mM $Ca(NO_3)_2$, 0.8 mM $MgSO_4$, and 10 mM HEPES, pH 7.4, supplemented with 10% L-15 (Leibovitz) medium and 1% fetal bovine serum (Gibco Laboratories, Grand Island, NY). The cultures were maintained at 22°C.

Clustering of AChRs by Latex Beads: The method to produce AChR clusters by polypeptide-coated latex beads has been reported (39, 40). Briefly, 4–6 μm polystyrene latex beads (Polysciences, Inc., Warrington, PA) were incubated with 1 mg/ml poly-L-α-ornithine (30,000 mol wt [Sigma Chemical Co., St. Louis, MO]) in phosphate-buffered saline (PBS) overnight at 4°C. After extensive washing with PBS, the beads were applied to muscle cultures in medium with or without the test drug. After a period of bead-muscle co-culture, ranging from 2 h to overnight, the cultures were labeled with tetramethyl rhodamine-conjugated α-bungarotoxin (R-BTX) (2, 46), fixed with 95% ethanol at −20°C, and mounted with a mixture of polyvinyl alcohol and glycerol. The association of AChR clusters with the latex beads was assayed by phase-contrast and fluorescence microscopy.

R-BTX fluorescence associated with bead-muscle contacts usually became detectable after 2 h of co-culture. Both the size of the clusters and the percentage of contacts exhibiting clusters increased with time (Table II, experiment *2* and unpublished results). To facilitate the collection of data, I examined most preparations after 18–20 h of coculture, unless otherwise noted in Results.

Immunofluorescence with Antibodies against Calmodulin: To examine the distribution of calmodulin in the cells, I fixed cultures with 3% paraformaldehyde in PBS for 10 min, permeabilized with 95% ethanol at −20°C for 10 min, reacted with an antibody against calmodulin (kindly provided by Dr. Jan de Mey, Janssen Pharmaceutica, Belgium), and then labeled with FITC-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). For the co-localization of AChR clusters, the culture was first treated with R-BTX before the aldehyde fixation. The specimens were examined with a microscope equipped with rhodamine and FITC fluorescence optics.

Chemicals: Verapamil (Isoptin) and D-600 were kindly provided by Mr. H. E. Grahm and Dr. A. Oberdorf of Knoll Pharmaceutical (Whippany, NJ). Nifedipine (Procardia) was kindly provided by Dr. D. Poorvin of Pfizer Pharmaceuticals (New York, NY). Dr. J. Atkinson, Jr., of SmithKline & French Laboratories (Philadelphia, PA) kindly provided the trifluoperazine (Stelazine). W5 and W7 were obained from Caabco, Inc. (Houston, TX).

RESULTS

Effects of Divalent Cations

As a first test of the effect of calcium deprivation, I studied the effect of Ca^{2+}-free condition on the formation of AChR clusters. The control culture was incubated in Steinberg's solution only, without serum or L-15 supplement. In this and subsequent experiments, all cells having low background fluorescence within randomly chosen fields were scored. As shown in Table I (experiment *1*), AChR clusters were seen at 58% of the bead-muscle contacts. A sample micrograph is shown in Fig. 1, *a* and *b*. In test cultures that were incubated in Ca^{2+}-free Steinberg's solution plus 0.4 mM EGTA or Ca^{2+}, Mg^{2+}-free Steinberg's solution plus 0.4 mM EDTA, the for-

TABLE I

*Effect of Calcium Antagonists on the Clustering of AChR**

Experiment	Treatment	% Beads associated with AChR clusters[‡]	Ratio: test/control
1	Control	57.9 ± 3.9 (20/455)	1
	Ca, Mg-free + EDTA, 0.4 mM	15.6 ± 5.8 (14/171)	0.27
	Ca-free + EGTA, 0.4 mM	2.9 ± 1.2 (20/271)	0.05
2	Control	68.1 ± 4.4 (20/249)	1
	$CoCl_2$, 2.5 mM	23.5 ± 5.5 (20/145)	0.34
	$CoCl_2$, 5.0 mM	7.2 ± 2.4 (20/218)	0.11
	$CoCl_2$, 10.0 mM	7.7 ± 2.5 (20/135)	0.11
3	Control	79.0 ± 4.1 (20/239)	1
	$NiCl_2$, 1.0 mM	64.9 ± 6.3 (20/166)	0.82
	$NiCl_2$, 2.5 mM	44.2 ± 7.7 (20/160)	0.56
	$NiCl_2$, 5.0 mM	2.6 ± 1.0 (20/352)	0.03
4	Control	87.8 ± 5.2 (20/239)	1
	$MnCl_2$, 1.0 mM	89.8 ± 6.7 (20/368)	1.02
	$MnCl_2$, 5.0 mM	75.0 ± 3.7 (20/225)	0.85
5	Control	64.8 ± 3.2 (20/281)	1
	Verapamil, 0.10 mM	54.9 ± 6.5 (20/182)	0.85
	Verapamil, 0.25 mM	7.7 ± 2.1 (20/202)	0.12
	Verapamil, 0.50 mM	4.9 ± 2.1 (10/115)	0.07
6	Control	60.6 ± 4.2 (20/496)	1
	D-600, 0.25 mM[§]	5.9 ± 1.3 (20/389)	0.10
	D-600, 0.50 mM	3.5 ± 1.3 (18/243)	0.06
7	Control	70.3 ± 5.3 (20/187)	1
	Nifedipine, 0.25 mM[§]	68.3 ± 6.6 (20/150)	0.97
	Nifedipine, 0.50 mM	53.6 ± 6.3 (20/249)	0.76

* AChR clusters were induced by 6-μm polyornithine-coated latex beads. The control cultures, except for experiment *1*, were incubated in Steinberg's solution supplemented with 1% fetal bovine serum and 10% L-15 (Leibovitz) medium. Test cultures were incubated in this medium plus the antagonist specified. In experiment *1*, all cultures were incubated in Steinberg's solution with the specified modification only, without serum or L-15. After an overnight coculture with the beads, the cultures were labeled with R-BTX, fixed with 95% ethanol at −20°C, and mounted on slides. The location of AChR clusters and the beads was assayed with a Leitz Orthoplan microscope equipped with epifluorescence and phase-contrast optics.

‡ Expressed in mean ± SEM. (No. of cells scored/total No. of bead-muscle contacts scored). In each cell every bead-muscle contact was scored.

§ The stock solutions for D-600 and nifedipine were prepared in ethanol. In D-600 experiment both the control and the test cultures also contained 2% ethanol, and in nifedipine experiment they contained 1% ethanol.

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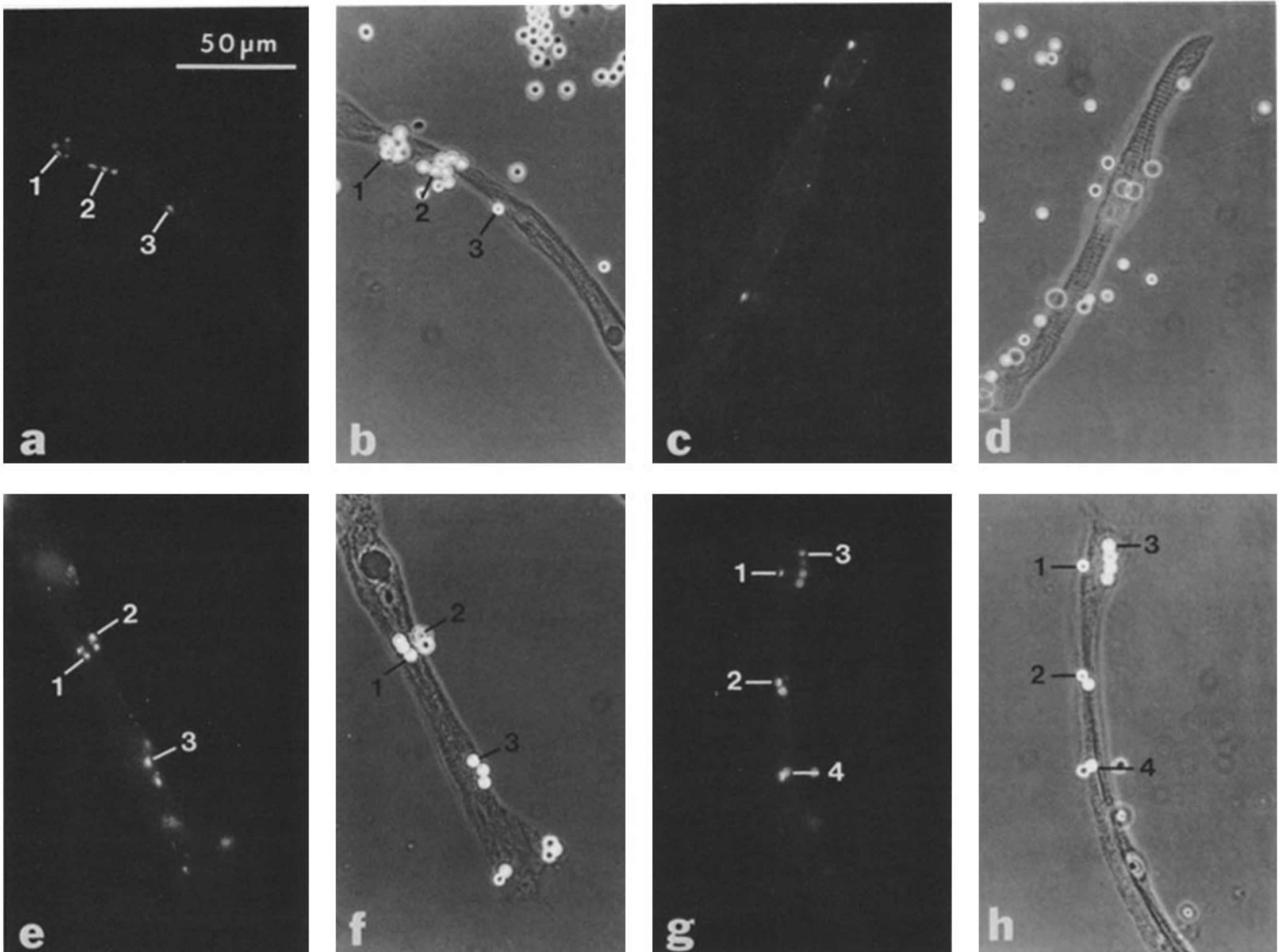

FIGURE 1 Sample micrographs showing the effects of calcium and calmodulin antagonists on the formation and the stabilization of AChR clusters. *a*, *c*, *e*, and *g*, are fluorescence micrographs; *b*, *d*, *f*, and *h*, the corresponding phase-contrast images. In the control (*a* and *b*), polyornithine-coated latex beads induced the formation of AChR clusters as indicated by the correspondence in the numbered positions in *a* and *b*. The beads failed to induce AChR clustering in the presence of 0.25 mM verapamil (*c* and *d*). In *c*, the fluorescence spots not in association with the beads presumably are existent clusters which are unaffected by verapamil. In *e–h*, the clusters were first generated by an overnight bead-muscle co-culture in normal medium and then 0.2 mM verapamil (*e* and *f*) or 5 μM TFP (*g* and *h*) was added and the micrographs were taken 24 h after the drug treatment. The correspondence between the R-BTX fluorescence spots and the positions of the beads indicates that the existent clusters are stable under these condtions. × 330.

mation of AChR clusters was greatly suppressed (Table I, experiment *1*). Although long-term incubation in the test solution was harmful to the cells, as shown by an increase in cytoplasmic fluorescence, thus indicating the permeation of the plasma membrane, the cells survived the short-term treatment as indicated by the intactness of cross-striations and by the fact that AChR clusters not induced by the beads persisted through the treatment. In this respect, it is known that a dispersal of the AChR cluster occurs if the cell is deprived of its metabolic energy (6, 8).

I then tested the effects of three divalent cations, Co^{2+}, Ni^{2+}, and Mn^{2+}, on the clustering of AChR. It is well known that these ions interfere with the Ca^{2+} influx into the cell. As shown in Table I (experiments *2* and *3*), both Co^{2+} and Ni^{2+} suppressed the formation of new clusters at the bead-muscle contacts at a concentration of 2.5 mM and nearly abolished it at a concentration of 5 mM. Both of these ions (especially Co^{2+}) also caused a shift in pH toward a more acidic range. However, the effect can not be attributed to this pH change as the same was observed even when both the control and the test solutions were titrated to the same pH of 7.

In Co^{2+} or Ni^{2+}-treated cells, cross-striations, and existent AChR clusters not associated with the beads persisted, thus indicating the survival of the cells under such treatment. However, the reversibility of the Co^{2+} effect was tested. The results of one experiment are listed in Table II (experiment *1*). In the control, the bead-induced clustering of AChR was already apparent after a 2-h bead-muscle co-culture. $CoCl_2$ at 5 mM suppressed the clustering process to 35% of the 2-h control level and this suppression was maintained after an overnight co-culture in the continued presence of Co^{2+}. However, when Co^{2+} was removed after 2 h, the clustering process returned to 78% of the control value after an overnight incubation in normal medium. In two other reversal experiments with 5 mM $CoCl_2$, we obtained values of 51% and 68%. Thus this effect is largely reversible.

I also tested the effect of Mn^{2+} on the bead-induced AChR clustering. As shown in Table I (experiment *4*), $MnCl_2$ at a

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Table II
Reversal of the Effects of Calcium Antagonists and Trifluoperazine on AChR Clustering

Experiment	Treatment*	% Beads associated‡ with AChR clusters	Ratio: test/control
1	Control (24 h)	83.5 ± 3.9 (20/140)	1
	Control (2 h)	72.8 ± 4.8 (20/150)	0.87
	$CoCl_2$, 5 mM (24 h)	24.3 ± 5.1 (18/127)	0.29
	$CoCl_2$, 5 mM (2 h)	25.6 ± 6.1 (20/137)	0.31
	$CoCl_2$, 5 mM (2 h) → Control (22 h)	64.8 ± 4.5 (20/139)	0.78
2	Control (24 h)	66.0 ± 2.8 (20/281)	1
	Control (2 h)	36.9 ± 4.2 (20/231)	0.56
	Verapamil, 0.2 mM (24 h)	8.4 ± 2.2 (20/351)	0.13
	Verapamil, 0.2 mM (2 h)	14.3 ± 3.0 (20/325)	0.22
	Verapamil, 0.2 mM (2 h) → Control (22 h)	65.5 ± 4.1 (20/157)	0.99
3	Control (24 h)	62.0 ± 3.2 (20/310)	1
	Control (2 h)	63.5 ± 4.5 (20/256)	1.02
	Trifluoperazine, 5 μM (24 h)	24.8 ± 2.2 (20/539)	0.40
	Trifluoperazine, 5 μM (2 h)	25.3 ± 4.0 (18/412)	0.41
	Trifluoperazine, 5 μM (2 h) → Control (22 h)	47.4 ± 4.4 (20/384)	0.76

* The first samples were taken 2 h after the beads were applied to the muscle cells. At this time, one test culture was changed to control medium and allowed to develop to next day. The other antagonist-treated culture and a control sister culture were also maintained to the next day. The AChR clusters were assayed by R-BTX as described in Table I.

‡ Expression similar to that in Table I.

concentration of 5 mM also suppressed the cluster formation relative to the control. However, this effect was weak as compared to Co^{2+} and Ni^{2+}. Higher concentrations of Mn^{2+} were found to be toxic to the cells, as indicated by the loss of cross-striations and the appearance of clumps in the cytoplasm.

Effects of Organic Compounds

Several organic compounds have been found to be potent blockers of calcium current through the cell membrane in a variety of systems. Three of these compounds, verapamil, D-600, and nifedipine, were tested in this study. As shown in Table I (experiments *5–6*), both verapamil and its methoxy derivative D-600 strongly suppressed the formation of new AChR clusters induced by the latex beads at a concentration of 0.2 mM. Thus they are ten times more potent than the divalent cations described above. The details of one verapamil experiment are plotted in Fig. 2. In this experiment, the slope of the regression line is 0.78 for the control and 0.33 for culture treated with 0.2 mM verapamil. Representative micrographs are shown in Figure. 1, *c* and *d*.

Cultures appeared healthy after the verapamil or D-600 treatment at the concentrations and duration used in this study. The effect is almost completely reversible as shown in Table II (experiment *2*). Here the clustering process was suppressed to 39% of the control level in the presence of 0.2 mM verapamil after 2 h. But after the removal of the antagonist at this time, it returned to the control level after an overnight incubation, whereas the level remained suppressed in the continued presence of the drug. In a second reversal experiment with 0.2 mM verapamil, the clustering induced by latex beads returned to 90% of the control level after the drug removal.

The effect of nifedipine is much less pronounced than the two other compounds described above. In solution, nifedipine is sensitive to light and relatively unstable. Thus these experiments were performed under subdued light and the stock solution was always prepared fresh just before experiment. As shown in Table I (experiment 7), AChR clustering induced by the beads was not affected by nifedipine at 0.25 mM and

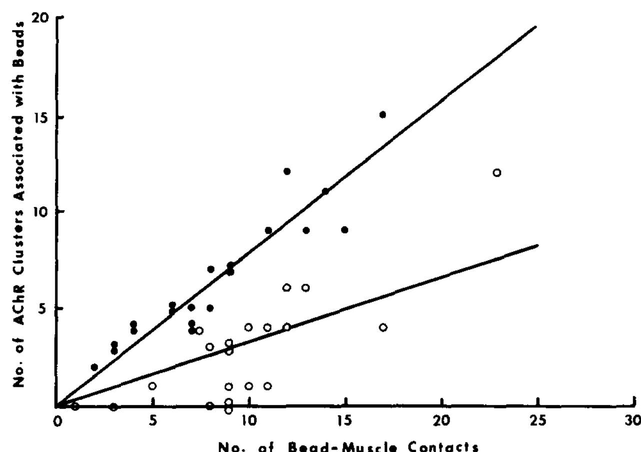

Figure 2 The effect of verapamil on the formation of AChR clusters. Each point represents a single cell. (●) Normal culture medium; (○) medium containing 0.2 mM verapamil. The slope of the regression lines is 0.78 for the control and 0.33 for the verapamil-treated culture.

reduced to 76% of the control level at 0.5 mM, which was found to be the saturation concentration. The cells appeared normal in the presence of the drug.

Effects of Calmodulin Inhibitors

In these experiments, I have tested the effects of three compounds, trifluoperazine (TFP), W-7, and its less potent derivative W-5. The results are summarized in Fig. 3. Here, the data of two TFP experiments, one W-7 and one W-5 experiment are presented. It can be seen that TFP exerted a strong inhibitory effect on the clustering of AChR induced by polyornithine-coated beads. A 50% inhibition was reached at TFP concentration of 3 μM. This is 100 times the effectiveness of verapamil or D-600. W-7 also suppressed the AChR clustering, but to a much less extent than TFP. At 20-μM concentration, W-7 reduced the clustering to ~60% of the control value. W-5 exerted a even weaker effect than W-7.

The reversibility of the inhibition by TFP was also tested as shown in Table II (experiment *3*). In this experiment, the

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formation of AChR clusters was suppressed to 40% of the control value in the presence of 5 μM TFP when assayed either at 2 of 24 h of incubation. However, after the removal of the drug at 2 h, the clustering returned to 76% of the control value when assayed on the following day, nearly doubling the drug-inhibited level. Thus, this inhibitory effect is largely reversible.

Effects on the Existent AChR Clusters

The persistence of AChR clusters not in association with the bead-muscle contacts in the presence of Ca^{2+} antagonists or calmodulin inhibitors (e.g., Fig. 1, *c* and *d*) suggested that these treatments have little effect on the existent clusters. However, whether these clusters were formed before the drug treatment could not be ascertained. Thus, I studied the effects of these drugs on the stability of the bead-induced AChR clusters. After an overnight bead-muscle co-culture in normal growth medium, the test cultures were treated 5 mM $CoCl_2$, 0.2 mM verapamil or 5 μM TFP and, following an overnight incubation, the AChR clusters still associated with the bead-muscle contacts were assayed. As shown in Table III, 70% of the clusters formed at the bead-muscle contacts remained intact after Co^{2+} treatment and 90% were still seen after verapamil or TFP treatment. Representative micrographs of the verapamil and TFP treatments were shown in Fig. 1, *e–h*. At corresponding concentrations, these compounds strongly inhibited the formation of AChR clusters (Table I and Fig. 3).

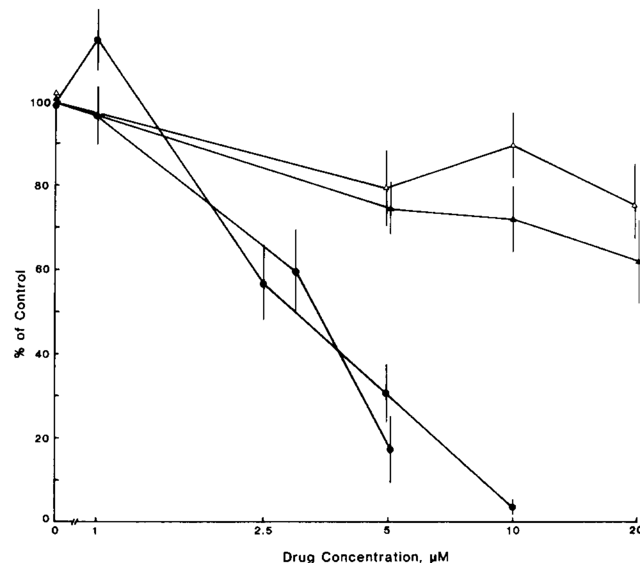

FIGURE 3 Effects of calmodulin antagonists on the formation of AChR clusters induced by polyornithine-coated latex beads. For each point, the AChR clusters associated with the beads in 20 cells were scored with fluorescence and phase-contrast microscopy, and the mean percentage of beads with AChR clusters was calculated. The results are expressed as the ratio (%) between the test culture and the control (ordinate). The bars represent standard error of the mean (*SEM*). The results of two TFP experiments (●), one W-7 (▲), and one W-5 (△) experiment are presented. The values of SEM for the controls are 4.2% and 7.2% for the TFP experiments, 6.3% for the W-7 experiment, and 6.3% for the W-5 experiment.

Thus these results suggest that the stability of the existent AChR clusters is much less sensitive to the Ca^{2+} antagonists or the calmodulin inhibitor than the formation of new clusters. I therefore conclude that the inhibition of AChR clustering by these agents is primarily due to their effects on the process of formation rather than on the process of stabilization.

Antibody Staining

To understand the distribution of calmodulin in *Xenopus* muscle cells, I labeled the cells with an antibody against calmodulin and examined its localization with the indirect immunofluorescence technique.

As a control, I studied the staining pattern of certain nonmuscle cells. As shown in Fig. 4, *a* and *b*, the antibody labeled the stress fibers, the nucleus, and the perinuclear area, in accordance with other published results (e.g., in reference 20). It often decorated the stress fibers in a linear punctate pattern (Fig. 4*a*). Muscle cells were generally more intensely stained by the antibody than the nonmuscle cells. Besides a diffuse cytoplasmic staining, the I-bands were also intensely stained (Fig. 4, *c* and *d*). This I-band staining is also consistent with previous reports (10).

When muscle cells were double-labeled with both R-BTX and the antibodies, no specific correlation in the staining patterns were seen. Fig. 5*a* shows two AChR clusters induced by latex beads (indicated by arrows in Fig. 5*c*) and Fig. 5*b* shows the corresponding antibody staining at the same focal plane. Fig. 5*d* shows a spontaneously occurring AChR cluster located on the bottom of the cell and Fig. 5*e* shows the corresponding antibody staining. In both cases, the antibodies labeled the cytoplasm and the cross-striations. At the AChR clusters, the staining was not intensified.

DISCUSSION

In this study, I used calcium and calmodulin antagonists to probe the nature of the interaction between polypeptide-coated latex beads and cultured muscle cells that leads to the formation of AChR clusters and other postsynaptic specializations. Calcium antagonists, including the divalent cations

TABLE III

Effects of Calcium Antagonists and Trifluoperazine on Existent AChR Clusters

Experiment	Treatment*	% Beads associated‡ with AChR clusters	Ratio: test/control
1	Control	69.0 ± 3.7 (20/264)	1
	Control (1 d) → $CoCl_2$, 5 mM (1 d)	48.4 ± 5.8 (10/158)	0.70
	Control (1 d) → Verapamil, 0.2 mM (1 d)	62.0 ± 3.0 (20/283)	0.90
2	Control	93.6 ± 2.4 (20/119)	1
	Control (1 d) → Trifluoperazine, 5 μM (1 d)	83.3 ± 3.1 (20/192)	0.89

* AChR clusters were induced by polyornithine-coated latex beads. After 1 d of bead-muscle coculture, the test cultures were treated with Ca^{2+} antagonists or trifluoperazine. The AChR clusters were assayed the following day.

‡ Expression similar to that in Table I.

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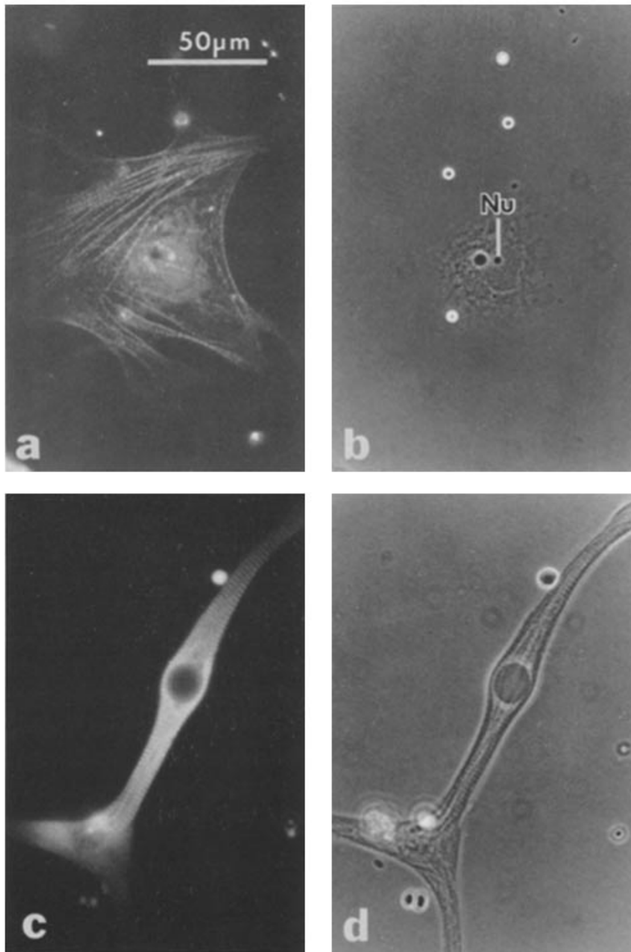

FIGURE 4 Immmunofluorescent labeling with anti-calmodulin antibody. In nonmuscle cells (*a* and *b*), the antibody stained the stress fibers in a linear punctate pattern, besides its staining of the nucleus and its peripheral area. The rest of the cytoplasm showed little antibody staining. *Nu*, the nucleolus. In muscle, the antibody labeled the myofibrils (I-bands), giving them the characteristic cross-striated pattern, as well as the sarcoplasm (*c*). *a* and *c* are the fluorescence micrographs with FITC optics, and *b* and *d* are their respective phase-contrast images. × 320.

and the organic compounds involved in this study, have been extensively used to investigate the physiology of Ca^{2+} channels in neurons and muscles (reviewed in references 15, 16, 21). In vertebrate skeletal muscles these agents (including Co^{2+}, Ni^{2+}, D-600, and nifedipine) can suppress the Ca^{2+} currents (1). Although Mn^{2+} also blocks Ca^{2+} currents, the Ca^{2+} channels in frog skeletal muscle are also permeable to Mn^{2+} (1). Thus Mn^{2+} seems to be a weaker antagonist than Co^{2+} or Ni^{2+}. This fact may account for its weak effect on the AChR clustering (Table I). My results also show that the clustering is relatively insensitive to nifedipine (Table I). Recent studies have shown that D-600 can cause frog skeletal muscle paralysis, probably by interfering with the Ca^{2+}-mediated excitation-contraction coupling (13). However, this paralysis can not be produced by nifedipine (E. W. McCleskey and W. Almers, personal communication). This suggests that there are also nifedipine-insensitive Ca^{2+} channels in amphibian skeletal muscles. The effective concentration of antagonists (verapamil and D-600) for blocking the AChR clustering is about ten times higher than that required for the suppression of Ca^{2+} currents (1) or the excitation-contraction coupling (13). However, since the latex beads used in this study adheres strongly to the cell membrane, it may exert a constant stimulation on a patch of membrane as large as 5 μm in diameter. Thus one may expect that such an interaction can only be overcome at a higher antagonist concentration. My results are consistent with the previous data of Bloch (6) who showed that the reformation of AChR clusters following their dispersal by sodium azide can be blocked by EGTA, $MnCl_2$, and $CoCl_2$ in rat myotubes. But he found that D-600 (2×10^{-5} g/ml) had no effect on this process, presumably because of the low concentration used (an order of magnitude smaller than used in the present study).

These Ca^{2+} deprivation studies thus suggest that the bead-induced AChR clustering is mediated by Ca^{2+}. In thin-section electron microscopy, we observed that the polypeptide-coated beads can come into close contacts with the cell both in the control (40) and in the presence of Co^{2+} or verapamil (unpublished results). Thus these effects are not due to a lack of association between the beads and the cell. Since the stability of the existent clusters is largely unaffected by the Ca^{2+} antagonists (Table III), their effects on the formation of the clusters seem to be specific. Deprivation of extracellular calcium does suppress AChR synthesis in rat myotubes (29). However, this does not seem to affect significantly the surface pool of AChR clusters within a 20-h period (14, 29). This surface pool alone can contribute to the formation of new AChR clusters in both *Xenopus* (3, 40) and rat muscle cultures (6). The half life of *Xenopus* surface AChR is 52 h (7), much longer than the duration of our experiments. Thus these effects can not be attributed to a depletion of the AChR pool.

On the basis of these results, I propose that the polypeptide bead-induced formation of AChR clusters involves a local influx of Ca^{2+} into the cell at the sites of bead-muscle contacts. This is probably mediated by an increase in the local membrane permeability to Ca^{2+} caused by these highly charged polybasic molecules as first suggested by Gingell et al. (18, 19).

Involvement of Calmodulin

Calmodulin inhibitors, including phenothiazines (50), such as TFP, and naphthalene sulfonamide (24), such as W5 and W7, recently have been widely used to elucidate the role of calmodulin in a variety of cellular processes, including cell growth and phagocytosis (25), receptor-mediated endocytosis (47), immunoglobulin capping (32), exocytosis (5, 12), maturation of oocytes (31), aggregation of pigment granules (28) and the change in gap junctions between lens fibers (42). Out of the three compounds tested, I found that TFP has the highest potency and W7 is more potent than its weaker analog W5. Consistent with the Ca^{2+} antagonist data, I also found that the stability of the existent clusters is rather insensitive to calmodulin inhibitors. This again indicates that Ca^{2+} is involved in the formation of new AChR clusters.

Together, these two lines of pharmacological evidence suggest that a local increase in Ca^{2+} concentration at the bead-muscle contacts may activate the calmodulin molecules to trigger the formation of AChR clusters. It is interesting to note that the effective concentration of TFP for the suppression of AChR clustering is 100- to 1,000-fold lower than that of the calcium antagonists. This indicates that the binding of Ca^{2+} to calmodulin is a pivotal step in the formation of AChR clusters. My immunofluorescence studies have shown that calmodulin seems to be diffusely distributed in the cell, besides

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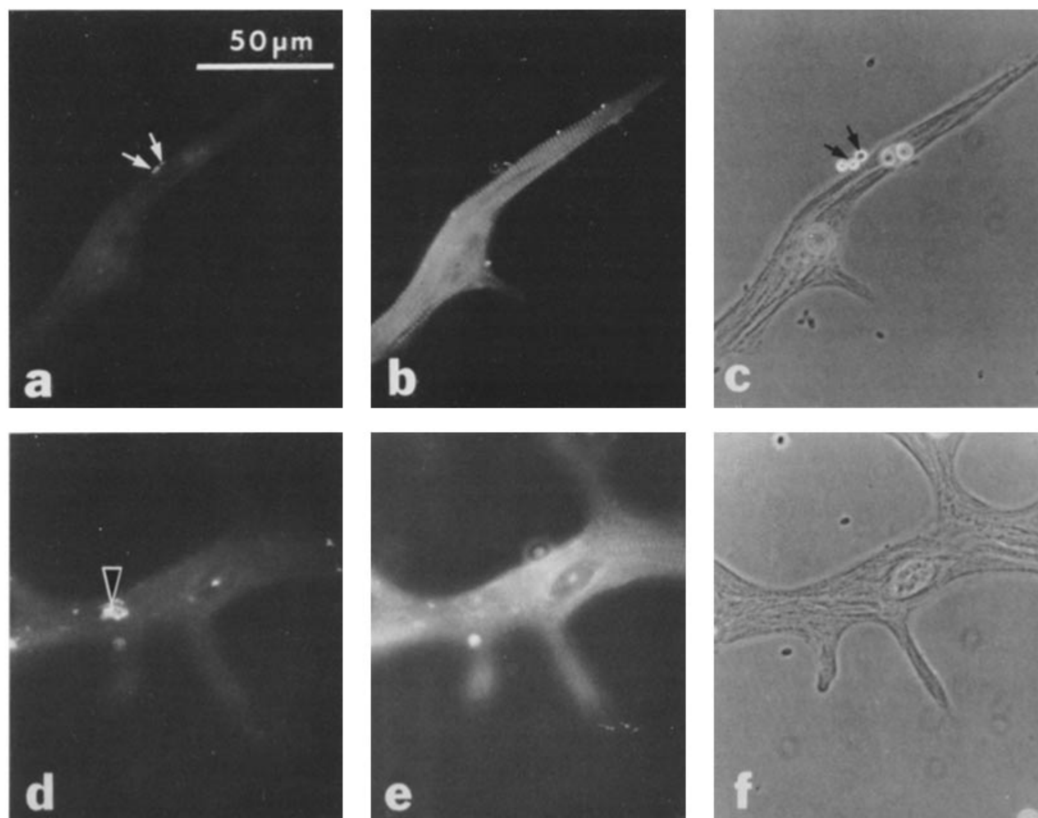

FIGURE 5 AChR clusters and anticalmodulin antibody localization. In *a–c*, two AChR clusters were formed at the bead-muscle contacts (arrows in *a* and *c*). Despite a diffuse cytoplasmic staining of the antibody against calmodulin (*b*), there is little preferential concentration of antibody labeling at the bead-induced AChR clusters. In *d–f*, a large AChR cluster located on the bottom of the cell facing the substrate (arrowhead in *d*) was not preferentially stained by the antibody (*e*); the myofibrillar staining by the antibody is obvious (*e*). *a* and *d* are rhodamine fluorescence images; *b* and *e* are FITC fluorescence images; *c* and *f* are phase-contrast images. All images of each cell were taken at the same focal plane. × 360.

its staining of cross-striated myofibrils. This suggests that the cellular machinery for the formation of AChR clusters, of which calmodulin is presumably a component, may be spread out in the cell and that it is locally activated by the latex beads in our experiments or by the neurites during innervation. In the latter case, it is also known that in tissue culture the neurite can induce AChR clustering along its length at any site on the muscle (3, 17).

Formation of AChR Clusters

The cellular process of the clustering of AChRs is not known. Current evidence indicates that receptors residing on the cell surface can undergo a lateral movement to become concentrated at the site of cluster formation (3, 6, 40). Several lines of data point to the involvement of cytoskeleton in this process: (*a*) The AChR within a cluster are essentially immobile, whereas, in the diffuse state, they can move freely in the plane of the membrane (4, 43). This suggests that the clustered receptors are anchored. Evidence indicates that they seem to be anchored to the cytoskeleton not extractable by Trition X-100 treatment (44). (*b*) Electron microscopy has shown that the AChR cluster is associated with a meshwork of filaments (23, 36), and immunofluorescence microscopy has shown that actin antibody stains the postsynaptic area at the rat neuromuscular junction (22).

There is also increasing evidence that suggests that Ca^{2+} may be involved in the regulation of cytoskeletal proteins. Examples are several of the actin-binding proteins such as α-actinin, myosin light-chain kinase, and gelsolin (reviewed in reference 48). In our model the Ca^{2+}-calmodulin interaction at the bead-muscle contact may locally activate a cytoskeletal system to cause the aggregation of AChR. Future works with this bead-muscle system should further our understanding of this process.

To extrapolate these results to the development of the neuromuscular junction, one can imagine that the early interaction between the in-growing neurite and the muscle cell may also involve a Ca^{2+}-mediated process similar to the one proposed here. A local increase in Ca^{2+} concentration at the presumptive postsynaptic membrane can result from the release of neurotransmitter or a trophic factor at the growth cone, or a recognition of certain molecule(s) on the neuronal cell surface. Furthermore, the failure of certain types of neurons to induce AChR clustering may result from a lack of a mechanism to elicit this Ca^{2+} response in the target cell.

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