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# ACETYLCHOLINE RECEPTOR CLUSTERING IN RAT MYOTUBES: REQUIREMENT FOR CA$^{2+}$ AND EFFECTS OF DRUGS WHICH DEPOLYMERIZE MICROTUBULES[1]

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## Abstract

The acetylcholine receptor (AChR) clusters which form in the plasma membranes of cultured rat myotubes disappear when the myotubes are exposed to medium depleted of $Ca^{2+}$. This loss of receptor clusters is reversible and depends both on extracellular $Ca^{2+}$ concentrations and on temperature. Other divalent cations ($Mg^{2+}$, $Ba^{2+}$, $Sr^{2+}$, $Co^{2+}$, $Mn^{2+}$) do not maintain receptor clusters when $Ca^{2+}$ is absent. Extracellular $Na^+$, which promotes $Ca^{2+}$ efflux from myotube cultures, is necessary for cluster loss to occur in $Ca^{2+}$-depleted medium.

Experiments using colchicine and other drugs which depolymerize microtubules suggest that the cytoskeleton is involved in AChR clustering. At submicromolar concentrations, these drugs afford partial protection against cluster loss caused by $Ca^{2+}$ depletion. Colchicine also alters the organization of vinculin in the vicinity of AChR clusters. It is concluded that receptor clustering probably depends on intracellular $Ca^{2+}$ and on a structure or mechanism indirectly affected by colchicine.

Acetylcholine receptors (AChRs) are an essential postsynaptic element of the vertebrate neuromuscular junction, and, because they can be specifically labeled with $\alpha$-bungarotoxin ($\alpha$-BT), they have proved to be a useful marker in studies of neuromuscular junction formation. Shortly after a motor neuron makes contact with a developing skeletal muscle, AChRs in the muscle membrane begin to accumulate in the regions of neuromuscular contact (Bevan and Steinbach, 1977; Burden, 1977; Brathwaite and Harris, 1979; Jacob and Lentz, 1979), probably by the aggregation there of extrajunctional AChRs (Anderson and Cohen, 1977; Anderson et al., 1977; Frank and Fischbach, 1979). Experiments utilizing *in vitro* culture techniques have established that neuromuscular contact can cause redistribution of AChRs in the muscle membrane (Anderson et al., 1977; Frank and Fischbach, 1979). This rearrangement does not, however, require functionally active AChRs or cholinergic neurons (Steinbach et al., 1973; Anderson et al., 1977; but see Cohen and Weldon, 1980, and Kidokoro et al., 1980). Indeed, the nerve may not be required at all for receptor aggregation to occur: AChR aggregates or clusters resembling those seen at the motor endplate form in extrajunctional regions of denervated muscle (Ko et al., 1977; Steinbach, 1981b) and in muscle cells grown in tissue culture in the absence of nerve (Vogel et al., 1972; Fischbach and Cohen, 1973; Sytkowski et al., 1973; Axelrod et al., 1976; Anderson and Cohen, 1977).

Several findings suggest that the AChR clusters which form in aneural cultures of rat myotubes are a useful model for studying the initial events of synapse formation in the rat. First, clusters formed *in vitro* morphologically resemble those induced by nerve *in vivo*: both are composed of aggregates of smaller entities which often appear, after staining with a fluorescent derivative of $\alpha$-BT, as tightly packed "speckles" over a darker background (Bloch and Geiger, 1980; Steinbach, 1981a). Second, receptor densities in these two structures are comparable (Axelrod et al., 1976; Land et al., 1977; Reiness and Weinberg, 1981). Third, they form by similar mech-

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anisms. Rat myotube clusters form by a process of receptor aggregation about several foci ("focal aggregation;" Bloch, 1979); nerve-induced clustering during neuromuscular junction formation occurs by a similar process (Kidokoro et al., 1980). Thus, studies of receptor clustering in cultured rat myotubes may reveal some of the mechanisms involved in the formation of nerve-induced aggregates at the developing motor endplate.

In the course of earlier work on the mechanism of AChR clustering (Bloch, 1979), I observed that drugs likely to affect Ca$^{2+}$ metabolism also altered AChR clusters. In particular, addition of the Ca$^{2+}$ chelator, EGTA, in amounts sufficient to decrease the free extracellular Ca$^{2+}$ concentration to about 0.3 mM, resulted in cluster loss in a small fraction of the myotubes in the culture. More strikingly, addition of Mn$^{2+}$ or Co$^{2+}$ to cultures completely prevented the re-formation of clusters which had previously been dispersed by energy metabolism inhibitors. Subsequent studies showed that the maintenance of AChR clusters induced by nerve at the developing motor endplate requires the presence of Ca$^{2+}$ (Bloch and Steinbach, 1981). Here I present more extensive studies on the role of Ca$^{2+}$ in AChR clustering in cultured rat myotubes.

## Materials and Methods

Cultures of rat skeletal muscle myotubes were prepared from the hindlimbs of neonatal Sprague-Dawley rats as described (Bloch, 1979; Bloch and Geiger, 1980). Cultures were used 6 to 7 days after plating, 2 to 4 days after the onset of myotube formation. All cultures were maintained at 37°C in an atmosphere of 94% air, 6% $CO_2$. Ca$^{2+}$-free medium consisted of Dulbecco-Vogt modified Eagle's medium (DMEM) prepared without $CaCl_2$, and supplemented with serum (5% v/v) which had been dialyzed extensively against phosphate-buffered saline (10 mM NaP, 145 mM NaCl, pH 7.4) and filter-sterilized before use.

The distribution of surface AChR was visualized after staining with $\alpha$-bungarotoxin coupled with tetramethylrhodamine (R-$\alpha$-BT), prepared as described (Ravdin and Axelrod, 1977). Staining was for 20 min at room temperature or at 37°C with a solution of 5 $\mu$g/ml of R-$\alpha$-BT in DMEM plus 5% fetal calf serum (reaction medium), buffered at pH 7.0, with 15 mM HEPES. After staining, cultures were usually fixed with cold (−20°C) 95% ethanol, then rehydrated and mounted in 90% glycerol for examination under fluorescence optics. In some cases R-$\alpha$-BT staining was performed at room temperature, after which cultures were mounted onto chambers (Anderson and Cohen, 1977) filled with reaction medium and observed periodically thereafter. Such cultures were kept at 37°C except when under observation. More details regarding materials and conditions for fluorescence microscopy and photomicrography can be found elsewhere (Bloch, 1979). Staining of myotube cultures for vinculin was performed as described (Bloch and Geiger, 1980). Antibodies were prepared and purified as reported elsewhere (Bloch and Hall, 1983).

Quantitation of fluorescence observations was done by determining the number of myotubes per culture displaying at least one AChR cluster greater than 40 $\mu m^2$ in area, then dividing this number by the total number of myotubes examined. A cluster was defined as a bright, nonlinear region of tetramethylrhodamine fluorescence having clear borders with less intensely stained surrounding membrane, which was not due to overlap of two cells and which was not on a process isolated from the myotube or the substratum (Bloch, 1979). Such structures are localized on the ventral myotube surface at sites of myotube substrate contact (Bloch and Geiger, 1980). Only these substrate-associated AChR clusters are considered here. For all fluorescence observations for which quantitation was done, including observations of vinculin immunofluorescence, a representative number of myotubes (usually 50) per culture were evaluated and at least two cultures were averaged for each value obtained. All experiments were performed at least twice.

To examine the dependence of cluster loss on extracellular Na$^+$ concentrations, two kinds of media were prepared that were depleted of Na$^+$. One medium consisted of DMEM prepared without NaCl. The Na$^+$ concentration in this medium, before addition of serum, was calculated to be 44 mM, due to the use of $NaHCO_3$ and NaP salts. After addition of dialyzed serum (Na$^+$ = 155 mM), the basal Na$^+$ concentration was 52 mM. Isotonicity was established by addition of sucrose (to 260 mM) or NaCl (to 130 mM). The second medium consisted of 25 mM HEPES, 10 mM glucose, 2 mM $NaCO_3$, 4 mM KCl, 2 mM $MgCl_2$, adjusted to 7.2 with NaOH, to give basal Na$^+$ concentrations of approximately 17 mM. Isotonicity was established by addition of sucrose (to 260 mM) or NaCl (to 130 mM).

To determine the Na$^+$ dependence of Ca$^{2+}$ efflux from rat myotube cultures, cultures, grown in 35-mm tissue culture dishes (Bloch, 1979), were exposed at 37°C to $^{45}CaCl_2$ (Amersham Corp., Arlington Heights, IL) for 5 hr, at a specific activity of 13 Ci/mol. Cultures were then washed four times with 2 ml of 10 mM HEPES, 290 mM sucrose, adjusted to pH 7.3 with KOH. They were next covered with 2 ml of the same solution, at room temperature, supplemented with 0.1 mM $CaCl_2$ and $3 \times 10^{-7}$ M tetrodotoxin, in 10 mM HEPES, 145 mM NaCl, adjusted to pH 7.3 with KOH and supplemented similarly, or in a mixture of these two solutions to give an intermediate concentration of NaCl. Aliquots (100 ml) were collected and counted in Aquasol in a Beckman liquid scintillation counter. Similar results were obtained if $CaCl_2$ and tetrodotoxin were omitted from the efflux buffers.

The sources for most materials referred to in this communication have already been reported (Bloch, 1979). Lumicolchicine was prepared by ultraviolet irradiation of an ethanolic colchicine solution (Wilson and Friedkin, 1966). *N*$^6$,*O*$^2$-Dibutyryl-guanosine 3′:5′-cyclic monophosphate and demecolcine were obtained from Sigma Chemical Co. (St. Louis, MO). Podophyllotoxin was from Aldrich Chemical Co. (Milwaukee, WI).

## Results

*Ca$^{2+}$ requirement.* Most primary rat myotubes observed 5 to 7 days after plating contain large clusters of AChR in their surface membranes which may be visualized using fluorescent derivatives of $\alpha$-BT (Anderson and Cohen, 1974; Axelrod et al., 1976). When cells are main-

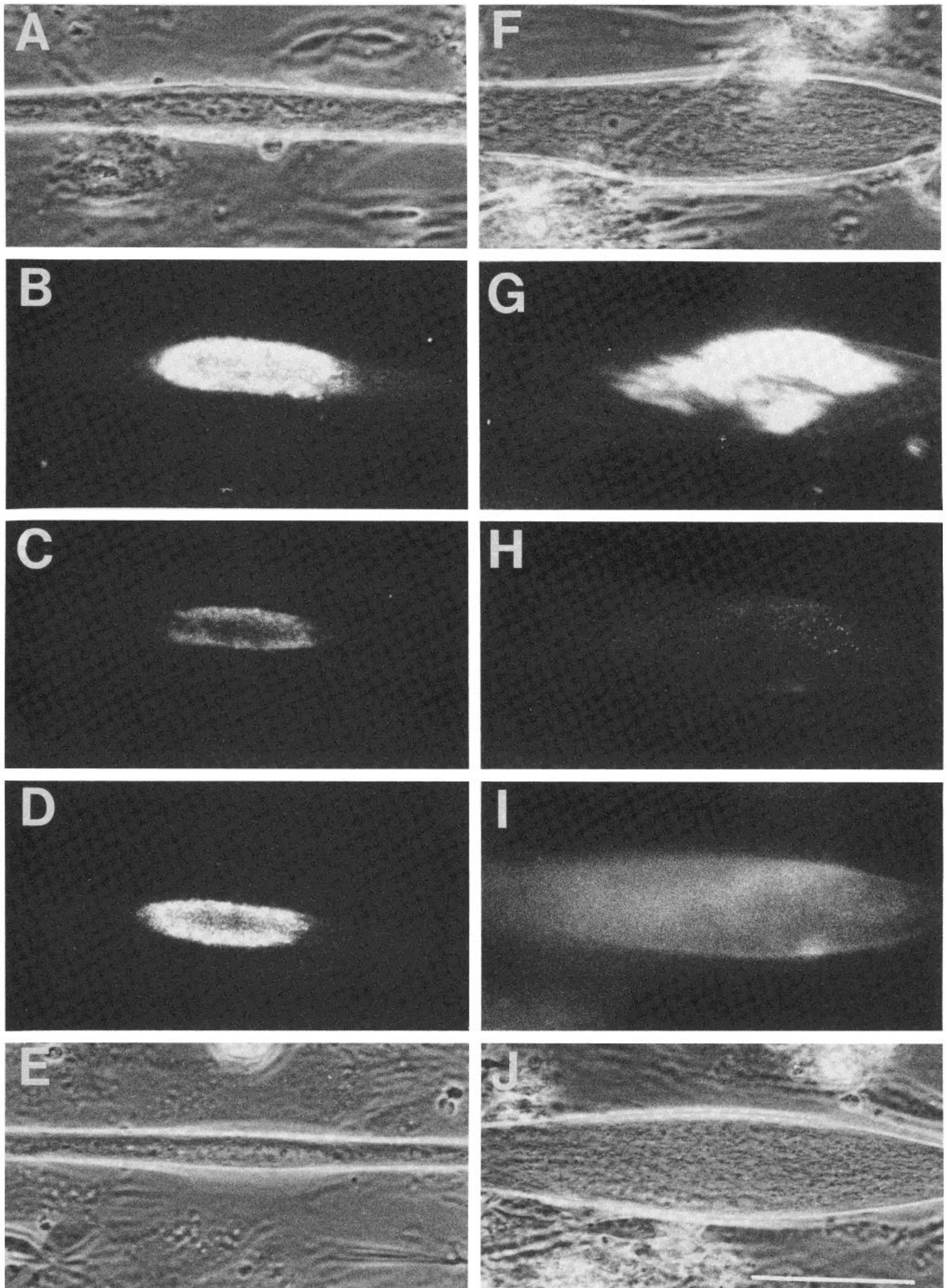


*Figure 1*

tained for a short time in the absence of $Ca^{2+}$, however, AChR clusters are lost. Figure 1 shows an example in which an AChR cluster, present at the beginning of the experiment (Fig. 1*G*) has been lost during 6 hr of incubation in medium from which $Ca^{2+}$ was omitted (Fig. 1*H*). Loss was not the result of dissociation of R-$\alpha$-BT from AChR, as restaining in the presence of $Ca^{2+}$ with a fresh solution of R-$\alpha$-BT did not cause the original cluster to reappear (Fig. 1*I*). Although it lost its cluster of AChR, the myotube did not undergo any other significant morphological changes observable by phase microscopy (cf. Fig. 1, *F* and *J*). Results similar to those illustrated in Figure 1 were obtained with a large number of identified cells. In all, AChR clusters on 110 cells treated in $Ca^{2+}$-free medium were observed in this manner, and 63% of them were lost over a 6-hr period. In contrast, studies of clusters on 77 control cells incubated in the presence of 1.8 mM $Ca^{2+}$ showed that only 14% were lost (see Fig. 1, *A* to *E*).

Comparable results were found using a different experimental procedure in which cultures were first treated in $Ca^{2+}$-depleted medium and then stained with R-$\alpha$-BT at the termination of the experiment. After staining, samples were fixed and stored at −20°C. In this way, larger numbers of cultures could be processed simultaneously for subsequent observation.

Using this alternative protocol, I established some of the basic characteristics of cluster loss caused by withdrawal of $Ca^{2+}$. (*i*) Approximately 6 hr are required for nearly complete loss of receptor clusters to occur (Fig. 2). (*ii*) The extent of cluster loss depends upon how much $Ca^{2+}$ was left in the medium. $Ca^{2+}$ concentrations were altered in two ways: $Ca^{2+}$-free medium was supplemented with known amounts of $CaCl_2$, or EGTA was added to medium containing 1.8 mM $Ca^{2+}$. Comparable results were obtained in both cases: the apparent $Ca^{2+}$ concentration at which half-maximal cluster loss occurs after a 6-hr incubation is 250 ± 60 $\mu$M ($n = 3$) using the former method, and 210 ± 70 $\mu$M ($n = 3$) using the latter (Fig. 3). Although not investigated further, these values are probably a function of the incubation conditions and of time. (*iii*) Cluster loss caused by withdrawal of $Ca^{2+}$ was reversible upon restoration of $Ca^{2+}$ to the medium (Fig. 2, *arrow*). Recovery of clusters was at a rate of about 6% myotubes with clusters/hr and approached control levels by 15 hr after reintroduction of $Ca^{2+}$. Recovery of clusters was not the result of the appearance of a new population of myotubes in the culture, because 60 to 76% of the myotubes in cultures in which receptors were stained with R-$\alpha$-BT before treatment in $Ca^{2+}$-free medium subsequently re-formed receptor clusters when allowed to recover for 16 hr. As these cultures were not restained with R-$\alpha$-BT, all of the receptors visualized in re-formed clusters were present at the beginning of the experiment.

*Assessment of damage.* The reversibility of the effect of $Ca^{2+}$ deprivation suggests that removal of $Ca^{2+}$ from the culture medium does not severely damage myotubes. Further experimental evidence against damage is: (*i*) Cultures treated for 6 hr in $Ca^{2+}$-free medium retain their permeability barrier to $^{22}Na^+$ and respond normally by fluxing $^{22}Na^+$ in the presence of an agonist of the AChR, carbachol (Table I). (*ii*) Quantitation of AChR number, measured using radiolabeled $\alpha$-BT (Patrick et al., 1977), showed no significant change in cultures exposed to $Ca^{2+}$-depleted medium (Table I). (*iii*) Synthesis and insertion of intracellular pools of AChR into the surface membrane (Devreotes and Fambrough, 1975; Hartzell and Fambrough, 1973; Patrick et al., 1977) are not affected by $Ca^{2+}$-free medium during a 6-hr treatment (Table I). (*iv*) Cell shape and number are not significantly altered by $Ca^{2+}$-free treatment (e.g., Fig. 1). (*v*) Myotube membrane potential, kindly measured by Dr. Y. Kidokoro, is not significantly changed under conditions which promote cluster loss. Impalements were made at room temperature; all results obtained, in single cultures, were averaged for each of the conditions used. Control membrane potentials were −58 ± 2 mV ($n = 5$ cells). Incubation in $Ca^{2+}$-free medium for 5 hr, followed by impalement in buffered saline lacking $Ca^{2+}$, gave membrane potentials of −41 ± 5 mV ($n = 5$ cells). However, incubation and impalement in the absence of $Ca^{2+}$ but in the presence of 2 mM $SrCl_2$, which does not protect against the loss of AChR clusters (see below), gave membrane potentials of −53 ± 3 mV ($n = 5$ cells). The results show that under conditions where cluster loss is observed, membrane potential is only slightly decreased.

EGTA treatment generally caused more cell loss and distortion of cell shape than did treatment with $Ca^{2+}$-depleted medium (see also Axelrod et al., 1978). (These effects of EGTA were observed by simple microscopic

*Figure 1.* AChR cluster loss observed on identified cells treated in $Ca^{2+}$-free medium. Cultures on collagen-coated glass coverslips were washed once with HEPES-buffered medium and stained for 30 min at room temperature with a fresh solution of R-$\alpha$-BT. They were then washed again and mounted on sealed chambers filled with reaction medium (*A* to *E*) or with reaction medium prepared free of $Ca^{2+}$ (*F* to *J*). Cultures were observed at room temperature (22 to 24°C). Cells showing AChR clusters were photographed under phase and fluorescence illumination. Initial fluorescence exposures were limited to 5 sec. After photography, the cell was sketched and its microscope coordinates were noted. Once a sufficient number of examples were taken (usually 12 to 18/culture, requiring about 1 hr), the culture was incubated at 37°C. After 6 hr, the same cells were relocated and rephotographed. Cultures were then restained with R-$\alpha$-BT and observed once more. *A* and *F*, Phase images of cells at the start of the experiment; *B* and *G*, fluorescence images at the start of the experiment; *C* and *H*, fluorescence images after 6 hr at 37°C; *D* and *I*, fluorescence images upon restaining cultures with R-$\alpha$-BT after the 6-hr incubation; *E* and *J*, phase images after the 6-hr incubation and restaining. All fluorescence images were exposed, developed, and printed identically. The *bar* in *J* represents 50 $\mu$m. The results show the loss of the cluster in the cell deprived of $Ca^{2+}$, but no comparable loss in the control. The apparent decrease in intensity of staining seen in the control cluster (compare *B* and *C*) is probably due to normal AChR turnover together with a change in the particular region of the myotube observed (compare *A* and *E*). This is exaggerated here by the use of high contrast processing and printing methods.

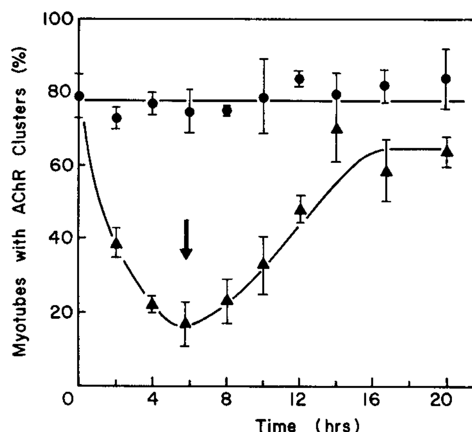


*Figure 2.* Time course of AChR cluster loss in $Ca^{2+}$-free medium and of recovery upon reintroduction of $Ca^{2+}$. Cultures on collagen-coated coverslips were washed, placed into 1 ml of control reaction medium (●), or reaction medium prepared free of $Ca^{2+}$ (▲), then incubated at 37°C. After 6 hr, 2 mM $Ca^{2+}$ was reintroduced to the $Ca^{2+}$-free medium (*arrow*). As a function of time after onset of incubation, samples were withdrawn, washed, stained, with R-α-BT, then fixed in ethanol for subsequent quantitation. The results show the reversible nature of AChR cluster loss.

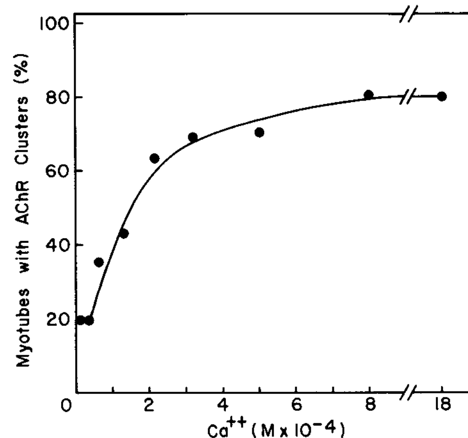


*Figure 3.* AChR cluster loss as a function of extracellular $Ca^{2+}$ concentration. Cultures were treated for 6 hr at 37°C, as described in the legend to Figure 2, in reaction medium containing various concentrations of $Ca^{2+}$ (prepared by adding $Ca^{2+}$ to $Ca^{2+}$-free medium). After staining and fixation, the fraction of total myotubes displaying AChR clusters was determined. The results show that half-maximal loss of AChR clusters occurs at extracellular $Ca^{2+}$ concentrations of about 0.2 mM.

inspection of the cultures and were not quantitated.) For most experiments reported here, therefore, the latter method was used. However, when EGTA was used (e.g., Table III, column B), the results obtained were confirmed using $Ca^{2+}$-free medium.

*Effects of other ionic changes.* To determine whether other divalent cations can replace $Ca^{2+}$ in maintaining AChR clusters, I treated cells for 6 hr in $Ca^{2+}$-depleted medium, or in this medium supplemented with other divalent cations. No divalent cations were capable of substituting for $Ca^{2+}$ to a significant extent (Table II). In $Ca^{2+}$-free conditions, both $Ba^{2+}$ and $Co^{2+}$ at 2 mM had detrimental effects on the myotubes.

In an additional set of experiments, I investigated the importance of extracellular $Na^{+}$ in cluster loss caused by $Ca^{2+}$ deprivation. Figure 4 shows that, when cells are exposed to $Ca^{2+}$-free medium containing low concentrations of $Na^{+}$ (sucrose was used as a substitute for NaCl), most AChR clusters were not lost. The apparent half-maximal $Na^{+}$ concentration to maintain AChR clusters in the absence of $Ca^{2+}$ was 65 mM. It is clear that removal of $Na^{+}$ and not of $Cl^{-}$ is the important factor, as the use of LiCl instead of sucrose as a NaCl substitute also prevents AChR cluster loss in $Ca^{2+}$-free medium (not shown). To learn whether, as with other cells (Blaustein and Nelson, 1981), $Ca^{2+}$ efflux from myotube cultures was dependent on extracellular $Na^{+}$, I determined the rate at which myotube cultures lost $^{45}Ca^{2+}$. The results, shown in Figure 5, suggest that the initial rate of $Ca^{2+}$ efflux from myotube cultures is highly dependent on $Na^{+}$. Unlike cluster loss, initial rates of $Ca^{2+}$ efflux did not saturate at extracellular $Na^{+}$ concentrations up to 145 mM. It should be noted, however, that the times used for the two experiments are very different; therefore, comparisons between $^{45}Ca^{2+}$ efflux and cluster loss are at best only qualitative.

It could be argued that removal of $Ca^{2+}$ from culture medium is not a specific method for dispersing AChR clusters and that removal of any medium component would have a similar effect. Although this possibility seems unlikely, I controlled for it by testing the effects of medium lacking $Mg^{2+}$, $K^{+}$, glucose, choline, amino acids, vitamins, or fetal calf serum. Depletion of none of these substances had a significant effect on AChR clusters.

*Effects of drugs.* To learn whether particular cellular functions were involved in AChR clustering, I tested a large number of drugs to determine their effects on cluster stability, on cluster dispersal by EGTA, and on cluster re-formation after dispersal in $Ca^{2+}$-free medium. Results are presented in Table III.

Column A of Table III summarizes the effects of drugs on the stability of AChR clusters. As reported elsewhere (Bloch, 1979), $5 \times 10^{-3}$ M sodium azide and $10^{-4}$ M carbachol cause extensive (3- to 6-fold) loss of clusters. The magnitude of their effect is similar to that caused by $Ca^{2+}$ withdrawal. Other than these, none of the drugs or conditions tested had a large effect on AChR clusters.

Column B of Table III summarizes the effects of the same drugs on cluster loss caused by chelation of $Ca^{2+}$ with EGTA. Several treatments successfully prevent dispersal under these conditions: (*i*) Restoration of free $Ca^{2+}$ to the medium completely prevents cluster loss (see also Fig. 3). (*ii*) Prefixation, lowering the temperature, or, with more variability, addition of concanavalin A, also block cluster loss, perhaps by decreasing the mobility of AChRs within the sarcolemma. (*iii*) Slight but significant ($p < 0.02$) protection against cluster loss was also afforded by D600 and tetrodotoxin. (*iv*) Colchicine, podophyllotoxin, and demecolcine afforded significant protection of AChR clusters. Lumicolcine, a stereoisomer of colchicine unable to bind to tubulin (Wilson and Friedkin, 1966), had no effect on cluster loss.

Column C of Table III presents the effects of the same

TABLE I

*[$^{125}$I]α-Bungarotoxin binding and $^{22}Na^+$ influx in $Ca^{2+}$-deprived cells*

Cultures were treated for 6 h in either control reaction medium or reaction medium prepared free of $Ca^{2+}$. For quantitation of total AChR, two or more cultures were incubated with $2 \times 10^{-8}$ M [$^{125}$I]α-BT for 30 min at 22°C in the presence or absence of $10^{-4}$ M *d*-tubocurarine (Patrick et al., 1977). In the absence of *d*-tubocurarine, 90% of AChRs are saturated under these conditions (Patrick et al., 1977). For measurements of incorporation of AChR into the sarcolemma, cells were presaturated with α-BT to block all AChRs already in the sarcolemma. Cultures were then exposed to control medium or to $Ca^{2+}$-free medium for 6 hr and assayed for [$^{125}$I]α-BT binding as just described (Patrick et al., 1977). As α-BT does not readily dissociate from AChRs (Hartzell and Fambrough, 1973), [$^{125}$I]α-BT binds only to those receptors inserted into the surface membrane during the 6-hr incubation . $^{22}Na^+$ flux determinations were performed at 22°C using $10^{-4}$ M carbachol as described (Stallcup and Cohn, 1976), except that $MgSO_4$ and $NaH_2PO_4$ were omitted from the assay buffer. Total flux is the average of values obtained at equilibrium (1 to 4 min after flux onset) and gives a measure of total intracellular space made accessible by addition of carbachol. Cultures used for the toxin binding and flux experiments were prepared independently, and thus should not be compared. Values for $^{22}Na^+$ influx in the absence of carbachol are subject to 20 to 50% SD; other values are reliable to within ± 15%.

| | [$^{125}$I]α-BT Bound (pmol/dish × $10^2$) | | $^{22}Na^+$ Influx (μmol/dish) | | |
|---|---|---|---|---|---|
| | | | Initial rate ($min^{-1}$) | | |
| | Total | Newly incorporated | −Carbachol | +Carbachol | Total +Carbachol |
| Control (+$Ca^{2+}$) | 8.6 | 3.7 | 0.032 | 0.85 | 0.47 |
| $Ca^{2+}$-depleted | 9.4 | 3.2 | 0.036 | 0.75 | 0.43 |

TABLE II

*Inability of other divalent cations to substitute for $Ca^{2+}$*

Rat myotubes were treated as described in the legend to Figure 2 for 6 hr in $Ca^{2+}$-free medium or in medium supplemented with the concentration of divalent cation noted. Addition of the same concentrations of divalent cation to control medium had little or no effect on AChR cluster stability (see Table III and the text).

| Sample | Concentration of Divalent Cation Added (mM) | Myotubes with AChR Clusters (%) |
|---|---|---|
| $Ca^{2+}$-free medium | 0[a] | 21 ± 6 (5)[b] |
| Control (+$Ca^{2+}$) | 1.8 | 78 ± 9 (5) |
| + extra $MgCl_2$ | 4 | 20 ± 3 (3) |
| + $CoCl_2$ | 1 | 17 ± 9 (3) |
| | 2 | 5 ± 4 (3)[c] |
| + $MnCl_2$ | 1 | 16 ± 15 (3) |
| | 2 | 12 ± 7 (3) |
| + $BaCl_2$ | 1 | 18 ± 1 (3) |
| | 2 | 26[c] |
| + $SrCl_2$ | 1 | 31 ± 15 (3) |
| | 2 | 32, 28 |

[a] Total $Ca^{2+}$ in this medium was found by atomic absorption spectroscopy to be 17 μM (kindly performed by the Analytical Laboratory, Veteran's Administration Hospital, La Jolla, CA). This medium and all others listed here also contained 2 mM $MgCl_2$.

[b] Values are means ± SD followed by the number of determinations in parentheses. The combined results of five experiments are reported here.

[c] $BaCl_2$ at 2 mM causes extensive cell loss when $Ca^{2+}$ is omitted from the medium. $CoCl_2$ also causes some cell damage.

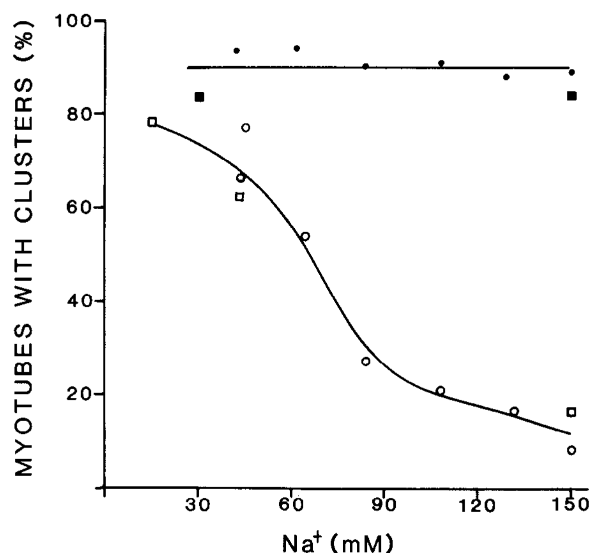


*Figure 4.* Cluster loss in $Ca^{2+}$-depleted medium as a function of extracellular $Na^+$ concentration. Cells were cultured in one of two media (see below, and "Materials and Methods") for 6 hr at 37°C, then stained and assayed for AChR clusters ("Materials and Methods"). The *open symbols* (○, □) represent results obtained in the absence of added $Ca^{2+}$ in the medium. The *solid symbols* (●, ■) represent results obtained in medium containing 1.8 mM $CaCl_2$. ○, ●, medium consisting of DMEM prepared with varying concentrations of NaCl and sucrose; □, ■, medium consisting of a HEPES-buffered solution prepared with varying concentrations of NaCl and sucrose. It is clear that AChR clusters are not significantly affected by reducing the extracellular $Na^+$ concentration at normal extracellular $Ca^{2+}$ concentrations. In medium lacking $Ca^{2+}$, reducing extracellular $Na^+$ helps to maintain AChR clusters.

drugs on cluster re-formation after dispersal in $Ca^{2+}$-free medium. Cluster re-formation is blocked by: (*i*) treatments which presumably alter AChR mobility (concanavalin A, room temperature, prefixation), and (*ii*) drugs which depolymerize microtubules (podophyllotoxin and colchicine, but not lumicolchicine). In addition, drugs which affect (*iii*) protein synthesis (cycloheximide), (*iv*) intracellular cation composition (ouabain, carbachol, and, perhaps, $BaCl_2$, $MnCl_2$, $CoCl_2$ and EGTA), and (*v*) energy metabolism ($5 \times 10^{-3}$ M sodium azide) also prevent cluster re-formation. Smaller inhibitory effects of D600 and tetrodotoxin were also observed. It is noteworthy that the effects of these drugs on re-formation were much more marked than on cluster stability (Table III, column A). This suggests that the drugs did not act by dispersing clusters after they had re-formed, but instead inhibited re-formation itself. It is unlikely that they prevented cluster re-formation solely by blocking myotube $Ca^{2+}$ metabolism, as most of these same drugs also inhibited cluster re-formation after dispersal by sodium azide (Bloch, 1979). (See "Discussion" for further comparisons of the effects of $Ca^{2+}$-deprivation and sodium azide.)

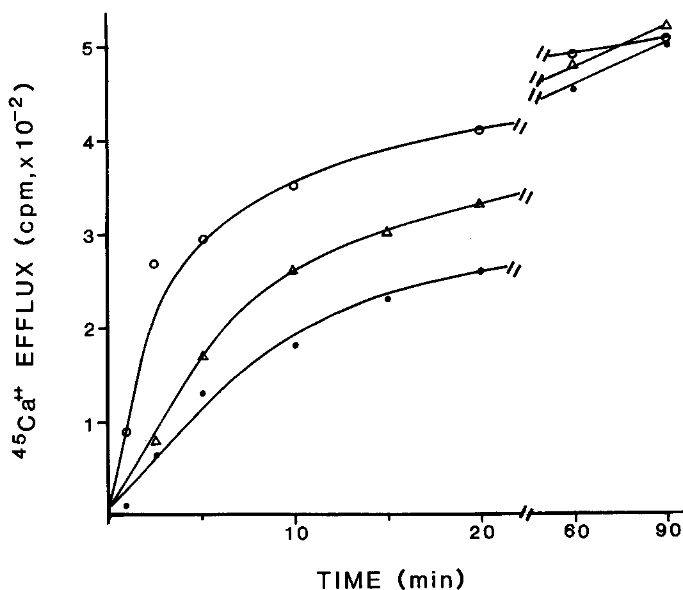


*Figure 5.* $^{45}Ca^{2+}$ efflux from rat myotube cultures at different extracellular $Na^+$ concentrations. Myotube cultures were used in which 63% of the nuclei (visualized with methylene blue) were present in myotubes, the remainder in mononucleate cells. Cultures were loaded with $^{45}Ca^{2+}$ and assayed for efflux of the radioisotope as described under "Materials and Methods." ●, efflux in medium prepared free of NaCl, and containing 290 mM sucrose; △, efflux in medium containing 58 mM NaCl and 174 mM sucrose; ○, efflux in medium containing 145 mM NaCl. Initial rates of efflux were (in nanomoles/minute/dish): 0.47, 0.74, and 1.9, respectively. The results show that $^{45}Ca^{2+}$ efflux is accelerated by increasing concentrations of extracellular $Na^+$.

Other drugs tested which had no effect on either cluster dispersal or re-formation are: cAMP and cGMP, and their dibutyryl derivatives, all assayed at 1 mM in the presence or absence of theophylline; theophylline (1 mM); caffeine (2 mM); *d*-tubocurarine (0.1 mM); $SrCl_2$ (2 mM); $MgCl_2$ (4 mM added to that already in medium); phalloidin (10 μg/ml); cytochalasins A (1 μg/ml), B (2 μg/ml), C (1 μg/ml), or D (0.1 μg/ml); insulin (25 μg/ml); the anesthetic, QX314 (0.25 mM); and the ionophore, A23187 (0.5 μg/ml).

A number of the drugs used here are likely to alter $Ca^{2+}$ metabolism in cultured rat myotubes, but they apparently do not do so in the same manner as $Ca^{2+}$ deprivation (see "Discussion;" R. Bloch, manuscript in preparation).

*Effects of colchicine and podophyllotoxin.* The data in Table III indicate that drugs which promote microtubule depolymerization enhance the stability of AChR clusters in medium depleted of free $Ca^{2+}$. To characterize their effects further, I examined the concentration dependence of their effect on cluster stability. As shown in Figure 6, colchicine and podophyllotoxin exert their half-maximal effects at concentrations in the range of $10^{-6}$ M to $10^{-7}$ M and $10^{-7}$ M to $10^{-8}$ M, respectively. These values are close to those reported for the $K_d$'s of these drugs interacting with tubulin (Olmsted and Borisy, 1973; Margolis and Wilson, 1977). To determine whether the cytoskeletal organization in regions of AChR clustering is altered by microtubule-depolymerizing drugs, I treated cultures with $10^{-5}$ M colchicine in the presence or absence of $Ca^{2+}$ and then stained muscle cells with R-α-BT and with antibodies to vinculin (Geiger, 1979), a cytoskeletal protein intimately associated with AChR clusters (Bloch and Geiger, 1980). I observed a significant change in the organization of vinculin in myotubes treated with colchicine. First, vinculin at focal contacts of the myotube with the substrate appeared to be more common in treated cells than in controls. This observation proved difficult to quantitate, however. Second, many more myotubes displayed AChR clusters which were surrounded by a ring of vinculin staining (Fig. 7). This observation was quantitated by determining the fraction of AChR clusters at least half of whose perimeters were bordered by bright vinculin staining. This value was found to be 33% in controls and in $Ca^{2+}$-deprived cells, and 67% in cells treated with colchicine either in the presence or the absence of $Ca^{2+}$. Thus, a change in the organization of vinculin in the vicinity of AChR clusters occurs when cultures are treated with colchicine. When the same experiment was repeated using different concentrations of colchicine, the concentration which gave a half-maximal effect on the fraction of AChR clusters ringed by vinculin was between $10^{-6}$ and $10^{-7}$ M colchicine. This concentration dependence resembles that presented in Figure 6 for the dependence of AChR cluster integrity on colchicine concentration.

## Discussion

*$Ca^{2+}$ requirement.* The results reported above indicate that, to maintain their large, substrate-apposed AChR clusters, cultured rat myotubes require extracellular $Ca^{2+}$. Withdrawal of $Ca^{2+}$ from the culture medium causes a rapid and reversible loss of AChR clusters. Under conditions which cause cluster loss, however, $Ca^{2+}$ withdrawal does not cause large changes in other myotube properties, such as membrane potential, ion permeability, or AChR function and metabolism, nor does it cause significant cell death. This suggests that $Ca^{2+}$ deprivation does not cause cluster loss by grossly altering the integrity or viability of myotubes.[3]

The results presented here are similar to those reported elsewhere. Recently, Bursztajn et al. (1982) showed that lowering extracellular $Ca^{2+}$ to 25 μM causes the AChR clusters of rat myotubes to be lost, whereas raising extracellular $Ca^{2+}$ to 15 mM appears to enhance cluster size. Axelrod et al. (1978) reported no effect on AChR clusters of $Ca^{2+}$ chelation at room temperature, but they did not perform a similar experiment at 37°C. Peng (1982), using cultured *Xenopus* myocytes, showed that AChR clustering in response to contact with latex beads also requires the presence of extracellular $Ca^{2+}$. McManaman et al. (1981) reported that withdrawal of $Ca^{2+}$ from cultured rat myotubes begins to depress AChR synthesis after 4 hr of incubation but has no significant effect on receptor degradation, creatine phosphokinase levels, or protein synthesis. Bloch and Steinbach (1981)

[3] Membrane potentials and ion flux measurements were performed at room temperature. Quantitative comparison of these results with those obtained for AChR cluster dispersal, which was done at 37°C, is therefore not possible. Qualitatively, however, they do suggest that myotubes are not severely affected by withdrawal of $Ca^{2+}$.

TABLE III

*Effects of drugs on AChR clusters*

To study the effects of drugs on clusters or on cluster loss, clusters were treated for 6 h at 37°C in 1 ml of reaction medium containing the appropriate drug at the designated concentration (A: drug alone) or containing the drug together with 2.2 mM EGTA (B: drug + EGTA). To study the effects on cluster re-formation, clusters were first treated for 6 h in $Ca^{2+}$-free medium, after which $Ca^{2+}$ was reintroduced into the culture medium to 1.8 mM. Two hours later, the appropriate drug was added, and incubation was continued for 6 more h (C: recovery). The 2-h lag was used to allow maximum possible recovery of clusters during exposure to drugs without changing the length of exposure used to obtain the data in A and B. Cultures were stained, fixed, and quantitated as described in the legend to Figure 2.

| Drug | Concentration | Myotubes with Cluster (%) | | |
|---|---|---|---|---|
| | | A: Drug alone | B: Drug + ETGA | C: Drug during recovery |
| 0 | | 84 ± 3 (8)[a] | 12 ± 7 (8) | 50 ± 8 (3) |
| $CaCl_2$ | $2 \times 10^{-3}$ M[b] | 81, 81 | 74 ± 9 (6) | 58, 58 |
| $BaCl_2$ | $2 \times 10^{-3}$ M | 79, 68 | ND[c] | 32, 34 |
| $MnCl_2$ | $2 \times 10^{-3}$ M | 78, 68 | ND | 23, 28 |
| $CoCl_2$ | $2 \times 10^{-3}$ M | 73, 62 | ND | 20, 22 |
| D600 | $2 \times 10^{-5}$ gm/ml | 76, 74 | 27 ± 5 (3) | 42, 28 |
| EGTA | $1.5 \times 10^{-3}$ M | 67, 73[d] | ND | 16, 24 |
| Ouabain | $5 \times 10^{-3}$ M | 69, 62[d] | 2, 8 | 17, 11 |
| Carbachol | $10^{-4}$ M | 29 ± 13 (17) | 16, 2 | 4, 4 |
| Tetrodotoxin | $10^{-5}$ gm/ml | 80, 88 | 29 ± 3 (3) | 40 ± 10 (3) |
| Colchicine | $10^{-5}$ M | 69 ± 6 (3) | 41 ± 5 (6) | 12, 11 |
| Podophyllotoxin | $10^{-5}$ M | 69 ± 6 (3) | 41 ± 5 (5) | 14, 24 |
| Demecolcine | $10^{-5}$ M | 62, 73 | 38, 35 | ND |
| Lumicolchicine | $10^{-5}$ M | 84, 84 | 21, 20 | 41, 36 |
| Cycloheximide | $5 \times 10^{-5}$ gm/ml | 66 ± 11 (3)[d] | 4, 7 | 18, 21 |
| Sodium azide | $5 \times 10^{-3}$ M | 13 ± 8 (16)[d] | ND | 2, 8 |
| | $5 \times 10^{-4}$ M | 83, 76 | ND | 43, 46 |
| Concanavalin A | $10^{-5}$ gm/ml | 91, 83[d] | 35 | 16, 32 |
| | $2.5 \times 10^{-5}$ gm/ml | 84, 67 | 82, 30[e] | 20 |
| Prefixation with paraformaldehyde | 2%, 5 min, 22°C | 70, 73 | 73, 81 | 10, 4 |
| Temperature at 22°C | | 76, 76 | 84, 87 | 14, 23 |

[a] Values presented are means ± SD, followed by the number of determinations in parentheses.

[b] Added to medium already containing 1.8 mM $CaCl_2$.

[c] ND, not determined.

[d] Data taken from Bloch (1979), Table III.

[e] The reason for the variability of the effects of concanavalin A in this experiment is not clear. It was not remarked in early experiments using sodium azide (Bloch, 1979) and is therefore probably a result of the use of EGTA. Concanavalin A is a metalloprotein which is inactivated by chelation of its bound metals (Agrawal and Goldstein, 1968; Kalb and Levitski, 1968). The same experiment performed using medium prepared free of $Ca^{2+}$, rather than using EGTA, gave values of 55, 59, and 53% myotubes with clusters, a more consistent set of results. This suggests that concanavalin A partially prevents the loss of AChR clusters in medium depleted of $Ca^{2+}$.

showed that nerve-induced AChR aggregates at developing rat neuromuscular junctions disperse in the absence of $Ca^{2+}$ and re-form if $Ca^{2+}$ is reintroduced in the culture medium. In that system, too, muscle continued to synthesize protein at control levels despite the withdrawal of $Ca^{2+}$, suggesting that nonspecific effects were minimal.

Although $Ca^{2+}$ withdrawal seems not to alter myotubes greatly, it is premature to argue that its effect on the stability of AChR clusters is specific: $Ca^{2+}$ is too ubiquitous a modulator of muscle metabolism. An interesting feature related to the specificity of the effect, however, is the inability of other divalent cations, including $Sr^{2+}$, to substitute for $Ca^{2+}$. Many biological functions of $Ca^{2+}$ can be performed by $Ba^{2+}$ or $Sr^{+}$, but these cations cannot maintain AChR clusters in $Ca^{2+}$-free medium. Thus, the clustering mechanism shows a strong preference for $Ca^{2+}$.

The requirement for $Ca^{2+}$ in AChR clustering is distinctive, too, in that it differs from the requirement for energy metabolism (Bloch, 1979). As in the case of $Ca^{2+}$ deprivation, energy metabolism inhibitors such as sodium azide cause loss of AChR clusters, and this loss is reversible. Using either of the two treatments, a similar time course for cluster loss and re-formation may be observed. Also, incubation at room temperature inhibits both effects. Furthermore, cluster re-formation after dispersal by low $Ca^{2+}$ or azide poisoning is prevented by the same drugs, such as colchicine and cycloheximide. Despite these similarities, $Ca^{2+}$ deprivation and inhibition of energy metabolism clearly have different effects on some parameters in this system (cf. Tables I and III here with Tables I and III of Bloch, 1979). (*i*) $Ca^{2+}$ depletion has little effect on AChR incorporation into sarcolemma, which, however, is depressed by energy metabolism inhibitors (Hartzell and Fambrough, 1973; Bloch, 1979). (*ii*) Recovery of AChR clusters after azide poisoning but not after $Ca^{2+}$ deprivation is inhibited by low concentrations (0.5 mM) of sodium azide. (*iii*) Cluster loss in $Ca^{2+}$-depleted medium is antagonized by colchicine, which has no effect on cluster loss caused by energy metabolism inhibitors. These differences suggest that the two methods for disrupting clusters act on the mechanism involved in AChR clustering in different ways.

*The role of $Na^{+}$.* The fact that extracellular $Na^{+}$ is

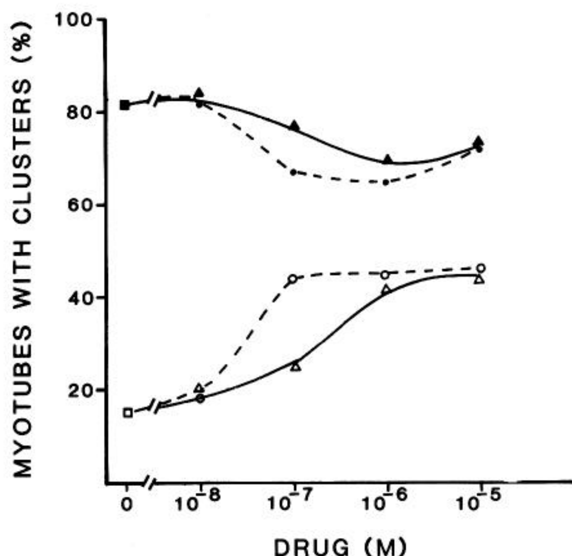


*Figure 6.* Effect of colchicine and podophyllotoxin on AChR clusters in the presence and absence of $Ca^{2+}$. Cultures were incubated for 6 hr at 37°C in reaction medium (*solid symbols*) or in reaction medium containing 2 mM EGTA (*open symbols*), supplemented with either colchicine (△, ▲) or podophyllotoxin (○, ●) at the concentration noted. After staining and fixation, AChR clusters were quantitated as described under "Materials and Methods." The results show that both drugs lower the control number of AChR clusters slightly, but, more significantly, both drugs protect against cluster loss in medium depleted of $Ca^{2+}$.

required for cluster loss to occur in $Ca^{2+}$-free medium places some constraints on the kinds of hypotheses that can be advanced to explain the effects of $Ca^{2+}$ withdrawal. Three likely possibilities are that $Ca^{2+}$ withdrawal causes a weakening of cell-substrate attachment, an increase in intracellular $Na^+$, or a decrease in intracellular $Ca^{2+}$. The evidence now available argues against the former two possibilities and in favor of the latter.

Divalent cations are believed to promote cellular adhesion in a wide variety of cells (Okada et al., 1974). In myotubes, AChR clusters form in regions of the plasma membrane which are closely apposed to the tissue culture substrate (Axelrod, 1980; Bloch and Geiger, 1980). If the mechanism of receptor clustering is linked to the mechanism promoting cell-substrate adhesion, then a perturbation in one might affect the other. In this case, withdrawal of extracellular $Ca^{2+}$ would be postulated to destabilize cell-substrate contact in a manner requiring the presence of high concentrations of $Na^+$. Arguing against this hypothesis is the observation that myotubes do not readily detach from the substrate in medium prepared without $Ca^{2+}$. In addition, when viewed with the light microscope, cell-substrate contact sites, and their associated regions inside the cell which are rich in vinculin, are not significantly altered by $Ca^{2+}$ deprivation (Bloch and Geiger, 1980). The light microscope would probably not detect slight changes or changes in ultrastructural organization, however. In additional experiments (R. Bloch, unpublished observations) I have found that the

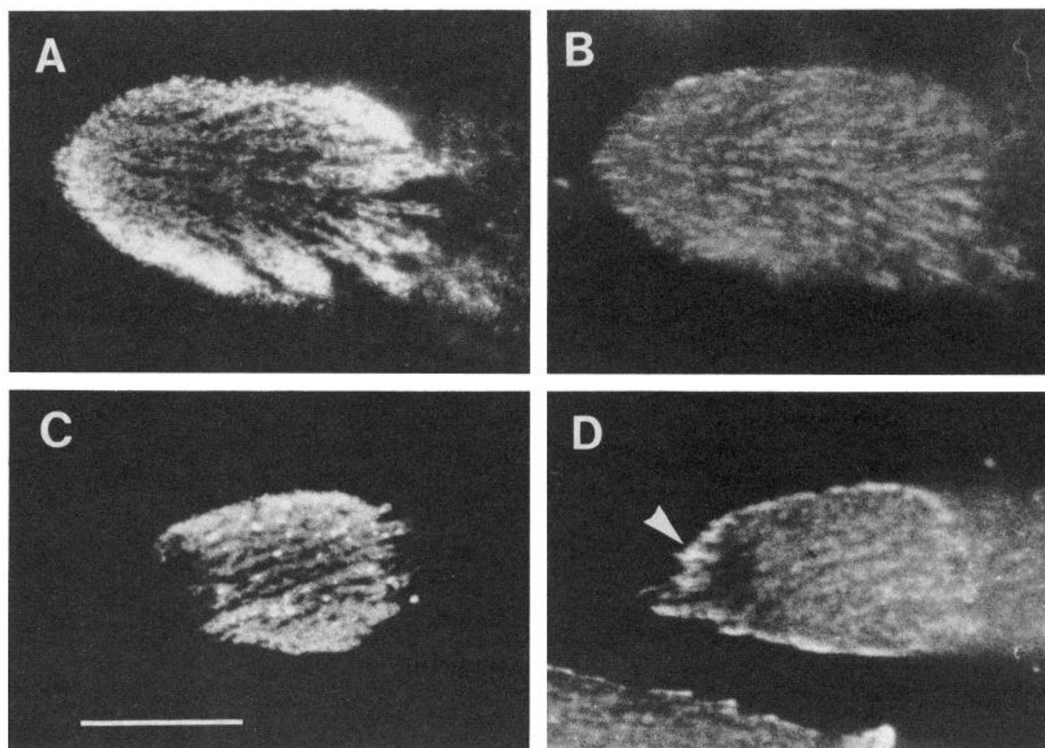


*Figure 7.* Effect of colchicine on vinculin distribution at AChR clusters. Cells were incubated in reaction medium for 6 hr at 37°C in the absence (*A* and *B*) or presence (*C* and *D*) of $10^{-5}$ M colchicine. They were then stained with R-$\alpha$-BT, permeabilized with a solution containing 0.5% Triton X-100, fixed in paraformaldehyde, and stained with 12 $\mu$g/ml of affinity-purified antibody to vinculin (Bloch and Geiger, 1980). Counterstaining was with fluorescein-conjugated goat anti-rabbit IgG (N. L. Cappel Laboratories), used at a dilution of 1:100. *A* and *C*, R-$\alpha$-BT staining of AChRs; *B* and *D*, antibody staining of vinculin. The *scale bar* represents 20 $\mu$m. The results show that colchicine increases vinculin staining around the periphery of AChR clusters (*D*, *arrowhead*).

initial adhesion of myotubes to tissue culture plastic does not require the presence of extracellular $Ca^{2+}$. It is not clear whether the same mechanisms are involved in maintaining long-term attachments. Nevertheless, the evidence presently available suggests that myotube-substrate interactions are not greatly altered in $Ca^{2+}$-deprived cultures.

$Ca^{2+}$ also regulates membrane permeability. In particular, when $Ca^{2+}$ is withdrawn from some cells, an increase in $Na^+$ influx occurs, resulting in depolarization and elevated intracellular $Na^+$ concentrations (Deitmer and Ellis, 1978). Removing extracellular $Na^+$ as $Ca^{2+}$ is withdrawn would reduce $Na^+$ influx. Although $Na^+$ influx is linked to cluster loss under some experimental conditions, e.g., in the presence of carbachol (R. Bloch, manuscript in preparation), several lines of evidence suggest that $Na^+$ influx is not responsible for cluster loss in $Ca^{2+}$-deprived cells. $Na^+$ influx into rat myotubes is stimulated by carbachol, which disrupts AChR clusters, but cluster loss under these conditions is partially blocked by $Ba^{2+}$ and is not affected by colchicine (R. Bloch, manuscript in preparation). On the other hand, cluster loss caused by withdrawal of $Ca^{2+}$ is partially blocked by colchicine but not by $Ba^{2+}$ (Tables II and III). Furthermore, cluster loss induced by carbachol is accompanied by depolarization of myotubes to 0 to −10 mV; smaller depolarizations induced by carbachol, to −35 mV, do not cause extensive cluster loss (R. Bloch, unpublished observation). In contrast, cluster loss in $Ca^{2+}$-free medium is accompanied by only slight depolarization of myotubes (see "Results"). Finally, a significant increase of $Na^+$ influx was not observed in $Ca^{2+}$-deprived cultures (Table I; see also Footnote 3).

Removal of $Ca^{2+}$ from cells is also likely to cause the gradual depletion of intracellular $Ca^{2+}$. In this case, $Na^+$ would be required in part for $Ca^{2+}$ extrusion, for example via $Na^+$-$Ca^{2+}$ exchange. $Na^+$-dependent $Ca^{2+}$ efflux has been documented in many excitable tissues (Blaustein and Nelson, 1981). In addition, the data in Figure 5 show that the initial rate of $Ca^{2+}$ efflux from myotube cultures is greatly enhanced by extracellular $Na^+$. When extracellular $Ca^{2+}$ is depleted, the $Na^+$-$Ca^{2+}$ exchange pump is likely to continue to function, driving $Ca^{2+}$ out of the cell until a new equilibrium is reached, at lower intracellular $Ca^{2+}$ concentrations. In agreement with this hypothesis, McManaman et al. (1981) have presented electrophysiological evidence that removal of extracellular $Ca^{2+}$ reduces intracellular $Ca^{2+}$ levels in rat myotubes. If this hypothesis correctly accounts for the effect of $Ca^{2+}$ deprivation and its requirement for $Na^+$, then some minimal level of intracellular $Ca^{2+}$ is probably required to maintain the AChR clusters of rat myotubes.

*Microtubule-depolymerizing drugs.* Colchicine and related drugs stabilize AChR clusters in $Ca^{2+}$-deprived cells but prevent cluster re-formation after reintroduction of $Ca^{2+}$. Microtubules are not generally enriched in the immediate vicinity of myotube AChR clusters (R. Bloch, unpublished observation; M. Daniels, personal communication); therefore, it is likely that the effect of colchicine on AChRs is indirect. Depolymerization of microtubules in mononucleate cells can alter the organization of intermediate filaments (Gaskin and Shelanski, 1976) and of microfilaments (Schliwa and van Blerkom, 1981), including the microfilaments which insert into the plasmolemma at sites of cell-substrate contact (Lloyd et al., 1977). A change of microfilament organization in myotubes at regions of cell-substrate attachment may account for the increased appearance of AChR clusters ringed with structures rich in vinculin. Changes in the contact-associated cytoskeleton might stabilize AChR clusters by helping to create an additional diffusion barrier around receptors or by enhancing direct interactions of clustered receptors with nearby cytoskeletal elements. Re-formation of AChR clusters after dispersal by sodium azide or $Ca^{2+}$ withdrawal might be blocked by similar mechanisms, for example by reduced receptor diffusion into the site of clustering, or by enhanced receptor binding to cytoskeletal structures outside the potential clustering sites.

Colchichine may also have other effects on the mechanism governing AChR clustering. For example, colchicine inhibits the synthesis and insertion of intracellular pools of AChRs into the sarcolemma (Devreotes and Fambrough, 1975). If a small number of newly synthesized AChRs or receptor-associated proteins are needed for receptor clustering to occur, then colchicine could block cluster reformation by preventing their incorporation into the sarcolemma. Although such a requirement is hypothetical, it would account for the observation (Table III, and Bloch, 1979) that cycloheximide also blocks cluster re-formation.

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