Regulation of Agrin-induced Acetylcholine Receptor Aggregation by Ca^{++} and Phorbol Ester

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Abstract. Agrin, a protein extracted from the electric organ of *Torpedo californica*, induces the formation of specializations on cultured chick myotubes that resemble the postsynaptic apparatus at the neuromuscular junction. The aim of the studies reported here was to characterize the effects of agrin on the distribution of acetylcholine receptors (AChRs) and cholinesterase as a step toward determining agrin's mechanism of action. When agrin was added to the medium bathing chick myotubes small (<4 μm^2) aggregates of AChRs began to appear within 2 h and increased rapidly in number until 4 h. Over the next 12–20 h the number of aggregates per myotube decreased as the mean size of each aggregate increased to ~15 μm^2. The accumulation of AChRs into agrin-induced aggregates occurred primarily by lateral migration of AChRs already in the myotube plasma membrane at the time agrin was added to the cultures. Aggregates of AChRs and cholinesterase remained as long as agrin was present in the medium; if agrin was removed the number of aggregates declined slowly. The formation and maintenance of agrin-induced AChR aggregates required Ca^{++}. Co^{++} and Mn^{++} inhibited agrin-induced AChR aggregation and increased the rate of aggregate dispersal. Mg^{++} and Sr^{++} could not substitute for Ca^{++}. Agrin-induced receptor aggregation also was inhibited by phorbol 12-myristate 13-acetate, an activator of protein kinase C, and by inhibitors of energy metabolism. The similarities between agrin's effects on cultured myotubes and events that occur during formation of neuromuscular junctions support the hypothesis that axon terminals release molecules similar to agrin that induce the differentiation of the postsynaptic apparatus.

THE postsynaptic apparatus at the vertebrate neuromuscular junction is characterized by high concentrations of specialized components of the extracellular matrix, the myofiber's plasma membrane, and the underlying cytoplasm (Chiu and Sanes, 1984; Birks et al., 1960; Miledi, 1960; Couteaux and Dechavassine, 1968; Hirokawa and Heuser, 1982). At developing neuromuscular junctions these specializations form at the site of nerve-muscle contact (Dennis, 1981). Similar postsynaptic specializations are induced to form on regenerating myofibers in adult muscles by molecules bound to the synaptic portion of the muscle fiber's sheath of basal lamina (McMahan and Slater, 1984; Anglister and McMahan, 1985). Thus it seems reasonable to hypothesize that molecules released by nerve terminals induce the formation of the postsynaptic apparatus at developing neuromuscular junctions and that these molecules become stably incorporated into the synaptic basal lamina where they function to maintain the postsynaptic apparatus in the adult and direct its differentiation during regeneration.

To identify molecules that induce differentiation of the postsynaptic apparatus and determine their mechanism of action, we isolated a basal lamina-containing fraction from the electric organ of *Torpedo californica* and examined its effects on chick myotubes in culture (Rubin and McMahan, 1982; Godfrey et al., 1984; Nitkin et al., 1983; Wallace, 1986; Wallace et al., 1985). We found that this fraction induced the formation of specializations on cultured myotubes at which three components of the postsynaptic apparatus, acetylcholine receptors (AChRs),[1] acetylcholinesterase (AChE), and butyrylcholinesterase (BuChE), were concentrated. We have shown that the accumulation of all three components is caused by agrin, a protein present in our extracts in two forms, having apparent molecular weights of 150,000 and 95,000 (Nitkin et al., 1987).

Several lines of evidence suggest that agrin is similar to molecules that direct formation of the postsynaptic apparatus at the neuromuscular junction. For example, antibodies to agrin bind in high concentrations to the synaptic basal lamina at the neuromuscular junction (Fallon et al., 1985; Reist et al., 1987) and to molecules in the cytoplasm of spinal motoneurons (Smith et al., 1987). Extracts of muscle and spinal cord contain molecules that cause the formation of AChR/AChE aggregates on cultured myotubes; these molecules are antigenically similar to agrin (Godfrey et al., 1984; Fallon et al., 1985; Smith et al., 1987). Agrin induces AChRs present in the myotube membrane to accumulate into aggregates

1. *Abbreviations used in this paper*: AChE, acetylcholinesterase; AChR, acetylcholine receptor; BuChE, butyrylcholinesterase; ChE, cholinesterase; TPA, phorbol 12-myristate 13-acetate.

by lateral migration (Godfrey et al., 1984), as is also the case, at least in part, for the accumulation of AChRs at developing synapses (Anderson and Cohen, 1977; Ziskind-Conhaim et al., 1984; Role et al., 1985). Thus, agrin induces changes in the distribution of AChRs, AChE, and BuChE on myotubes in vitro which resemble those occurring during formation of the postsynaptic apparatus at developing neuromuscular junctions and agrin is antigenically and functionally similar to molecules situated at positions in vivo where signals that direct synaptic differentiation are located.

The present studies were undertaken to examine the mechanism by which agrin induces formation of aggregates of AChRs and cholinesterase (ChE) on myotubes in culture and to compare it to AChR and ChE accumulation at developing neuromuscular junctions, to test the hypothesis that agrin is similar to molecules that direct the differentiation of the postsynaptic apparatus in vivo and to gain insight into how components of the postsynaptic apparatus are assembled and stabilized at neuromuscular junctions. Here we show that agrin-induced AChR aggregation requires Ca^{++} and metabolic energy, and is inhibited by phorbol 12-myristate 13-acetate (TPA), an activator of protein kinase C.

Materials and Methods

Chick Myotube Cultures

Myotube cultures were prepared from hindlimb muscles of 11- to 12-d White Leghorn chick embryos by the method of Fischbach (1972) with minor modifications (Godfrey et al., 1984; Wallace, 1986). Normal culture medium consisted of Eagle's minimum essential medium supplemented with 10% (vol/vol) horse serum and 2% chick embryo extract (Wallace, 1986). In some experiments cultures were transferred to a balanced salt solution consisting of 5.6 mM glucose, 0.44 mM KH_2PO_4, 1.34 mM Na_2HPO_4, 0.001% phenol red, 5.36 mM KCl, 137 mM NaCl, 1 mM $MgCl_2$, 0.8 mM $MgSO_4$, 25 mM Hepes, pH 7.4, containing either 0.1 mM EGTA or 0.03–10 mM $CaCl_2$, during a 6-h incubation with agrin. There were no obvious differences in the response of myotubes to agrin under these conditions, provided Ca^{++} was present, except that the absence of horse serum and embryo extract reduced by twofold the amount of electric organ extract required to elicit a half maximal response (see Wallace, 1986).

Agrin

Partially-purified preparations of agrin, a Cibacron extract (84 U/μg) or an agarose pool fraction (114 U/μg), were used in these studies. They were prepared from the electric organ of *Torpedo californica* as previously described (Nitkin et al., 1987) and the equivalent of 3–5 U were added to each culture.

Quantification of AChR Aggregation

AChR and ChE aggregates were visualized and counted as previously described (Wallace, 1986). Briefly, AChRs were labeled with rhodamine-α-bungarotoxin, rinsed, fixed, mounted in glycerol, and observed by fluorescence microscopy. ChE aggregates were stained histochemically (Karnovsky, 1964); alternatively, AChE aggregates were identified by immunohistochemistry using a monoclonal antibody specific for chick AChE (Rotundo, 1984). Cultures were examined at a magnification of 320× with a Zeiss microscope equipped with phase and fluorescence optics. The mean number of aggregates per 400 μm myotube segment was determined by counting 10 segments in each of three cultures; except as noted only patches whose shortest axis was >2 μm were counted. In some experiments only newly synthesized AChRs were fluorescently labeled by incubating cultures for 1 h at 37°C with 1.25×10^{-6} M α-bungarotoxin, rinsing three times with 1 ml culture medium, then incubating for varying lengths of times before labeling with rhodamine-α-bungarotoxin.

To analyze in detail the number and size of AChR aggregates, myotubes were chosen at random under phase optics, the focal plane adjusted to view the lower surface of the myotube (where >90% of the agrin-induced aggregates were located), and the myotube segment photographed at 100× magnification under fluorescence optics. The negatives were projected at a final magnification of 1,000× and the outline of each distinct fluorescent patch along a 360-μm myotube segment was traced. The area of each patch was measured by tracing its outline with a HIPad Digitizer (Houston Instrument, Austin, TX) coupled with an Apple II computer (Apple Computer, Inc., Cupertino, CA). At each time interval a total of 40 myotube segments (eight from each of five cultures from two platings) were analyzed.

Results

Time Course of Formation of Agrin-induced AChR Aggregates

At developing neuromuscular junctions the accumulation of AChRs begins within a few hours of nerve-muscle contact (Dennis, 1981). Formation of agrin-induced AChR aggregates has been shown to begin within 2–4 h of adding agrin to chick myotubes in culture and to reach a plateau by 24 h (Godfrey et al., 1984). To determine the time course of aggregate formation in more detail, cultures were labeled with rhodamine-α-bungarotoxin at short time intervals after adding agrin, fixed, and examined by fluorescence microscopy. As illustrated in Fig. 1, little change in AChR distribution occurred during the first hour. Myotubes treated for 2 h with agrin were speckled with small (<4 μm^2) patches of AChRs. As judged by the focal plane of the fluorescent patches, ~90% were on the surface of the myotube that was in contact with the culture dish and often were concentrated along the edges of the myotubes. At later times, larger (≥4 μm^2) aggregates of AChRs were seen. The large aggregates were also found primarily (>95%) on the lower surface of the myotubes. At each time interval myotubes were selected at random, photographed, and the number and size of AChR aggregates measured. As shown in Figs. 2 and 3, aggregates began to appear between 1 and 2 h after adding agrin and increased rapidly in number until 4 h. Over the next 12–20 h the number of aggregates decreased, while the total area occupied by aggregates and the mean size of each aggregate continued to increase. Thus, agrin rapidly induced the formation of a large number of small aggregates of AChRs scattered over the lower surface of the myotubes. These were gradually replaced by a smaller number of large aggregates. It is not possible to infer from the available data whether the reduction in the number of aggregates that occurred between 8 and 16 h was due to the coalescence of smaller aggregates or the growth of some aggregates coupled with the disappearance of others. The total area occupied by aggregates continued to increase during this period (Fig. 3 *b*), while the density of AChRs within aggregates, as judged by the intensity of their fluorescence, appeared to remain constant or increase (Fig. 1). This suggests that agrin continues to cause the accumulation of diffusely distributed receptors into aggregates for at least the first 16 h of treatment.

Previous experiments have shown that the large agrin-induced AChR aggregates arise at least in part by lateral migration of receptors already in the plasma membrane at the time agrin is added (Godfrey et al., 1984). To determine if this is also true for the small aggregates that are induced by agrin during the first few hours, cultures were labeled with rhodamine-conjugated α-bungarotoxin, rinsed, and then agrin was added. For incubations of less than 12 h the number and apparent intensity of patches was similar whether AChRs were labeled before adding agrin or at the end of the

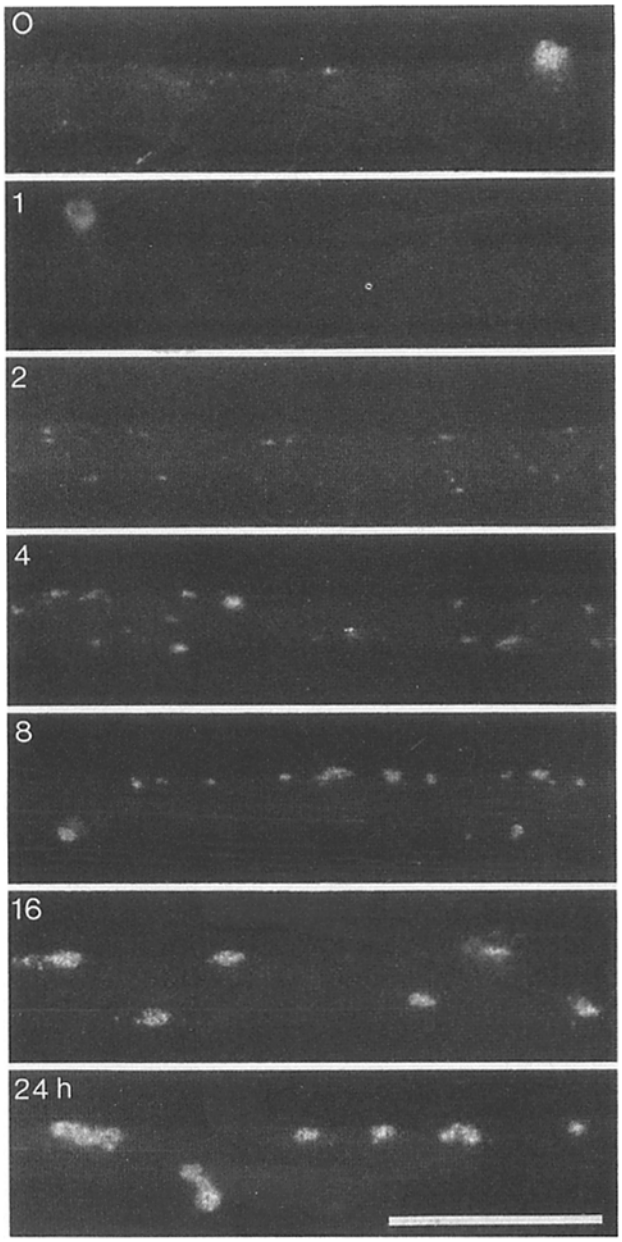

Figure 1. Changes in the distribution of AChRs after addition of agrin. Fluorescence micrographs of segments of cultured chick myotubes treated with agrin for 0–24 h and labeled with rhodamine-α-bungarotoxin to reveal the distribution of AChRs. Agrin induced the rapid (0–4 h) formation of a large number of small aggregates. Between 4 and 24 h aggregates decreased in number and increased in size. Bar, 50 μm.

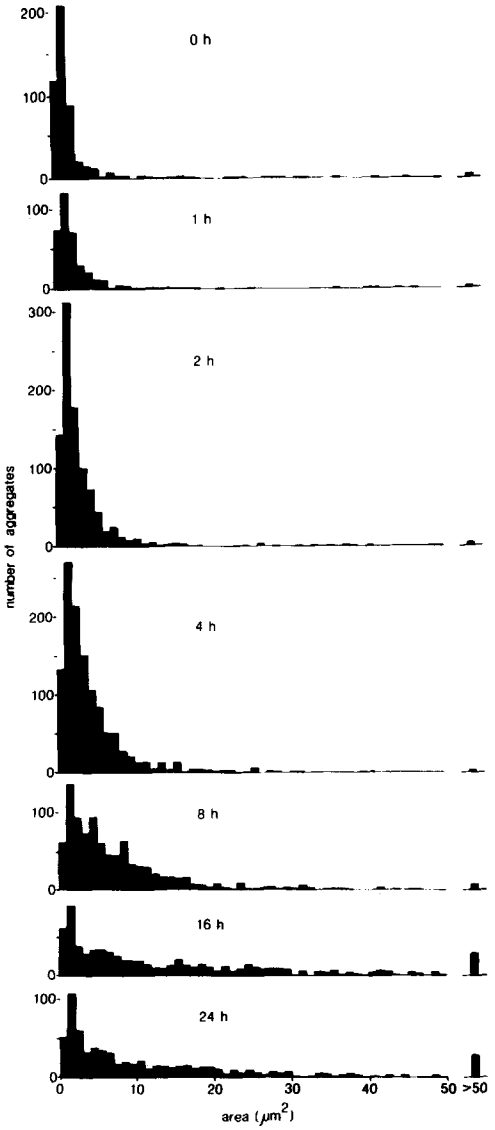

Figure 2. Size distribution histograms of AChR aggregates. Each histogram represents AChR aggregates on myotubes treated with agrin for the time indicated. The height of each column indicates the total number of aggregates of a given area observed on 40 myotube segments, each 360-μm long, taken from five cultures.

incubation period (Fig. 4). Thus, many of the receptors that accumulated in aggregates during this time were recruited from AChRs already in the plasma membrane. This was confirmed by blocking receptors on the myotube surface with unlabeled α-bungarotoxin, incubating myotubes with agrin for various lengths of time, and then labeling newly inserted AChRs with rhodamine-α-bungarotoxin. As expected, few AChR aggregates were detected during the first 6 h of agrin treatment; those that were seen fluoresced weakly (Fig. 4). With time there was a gradual increase in the fluorescent intensity of AChR aggregates (data not shown). Thus, newly inserted receptors contributed little to aggregates formed during the first few hours of exposure to agrin, apparently no more than would be expected from ongoing receptor turnover.

If the mechanism by which agrin induces ChE aggregates is the same as for AChR aggregation, then the time course of aggregation of these two components would be similar. Unfortunately, because of the high level of staining on myotubes in control cultures, it was not possible to distinguish agrin-induced formation of small aggregates of AChE or BuChE, labeled either histochemically or immunohistochemically. Large aggregates of ChE could first be detected reliably by ~6 h; thereafter the number of ChE aggregates increased with the same time course as AChR aggregates (see Fig. 2, Wallace, 1986).

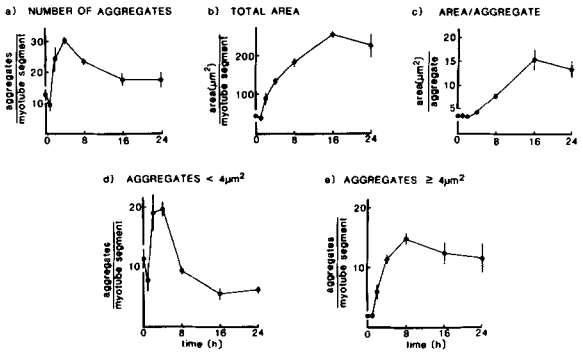

Figure 3. Time course of agrin-induced AChR aggregation. Analysis of data illustrated in Fig. 2, expressed as mean ± SEM (n = 5). (*a*) Number of AChR aggregates. (*b*) Total area occupied by aggregated AChRs. (*c*) Mean area per aggregate. (*d*) Number of aggregates <4 μm². (*e*) Number of aggregates ≥4 μm². During the first 4 h of treatment with agrin a large number of small aggregates of AChRs were induced to form. Thereafter the number of aggregates decreased as the mean size of each aggregate increased. In our previous studies and in most of the experiments reported here only aggregates ≥4 μm² were counted.

Agrin is Required for Both the Formation and Maintenance of AChR and ChE Aggregates

As described above, agrin-induced aggregation of diffusely distributed AChRs continued during at least the first 16 h of agrin treatment. If agrin was removed during this period, then the increase in the number and size of aggregates stopped within 1–2 h (data not shown). In the continued presence of agrin the number of aggregates remained constant from 24–72 h (Godfrey et al., 1984). If agrin was removed after 18–24 h, the number of large AChR aggregates fell (Fig. 5). The rate at which aggregates disappeared depended on the age of the myotubes; the number of AChR aggregates decreased more rapidly in younger cultures (Fig. 6). Thus 6 h after removing agrin from a 5 d muscle culture, only 54 ± 7% (mean ± SEM, n = 5) of the AChR-aggregates induced by a prior 18-h incubation remained, while 6 h after removing agrin from a 6 d muscle culture 83 ± 4% (n = 5) of the

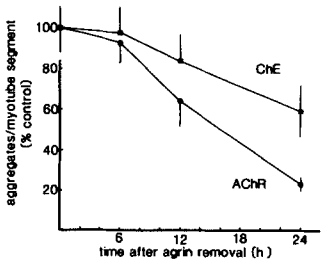

Figure 5. Agrin-induced AChR- and ChE-aggregates disappear if agrin is removed from the culture medium. Cultures were incubated for 18 h with agrin, then rinsed and returned to the incubator in fresh medium. After 0 to 24 h cultures were labeled with rhodamine-α-bungarotoxin or stained for ChE and the number of aggregates counted. Data, expressed as mean ± SEM (n = 3), is plotted as a percentage of the number of aggregates present at the end of the 18-h incubation with agrin.

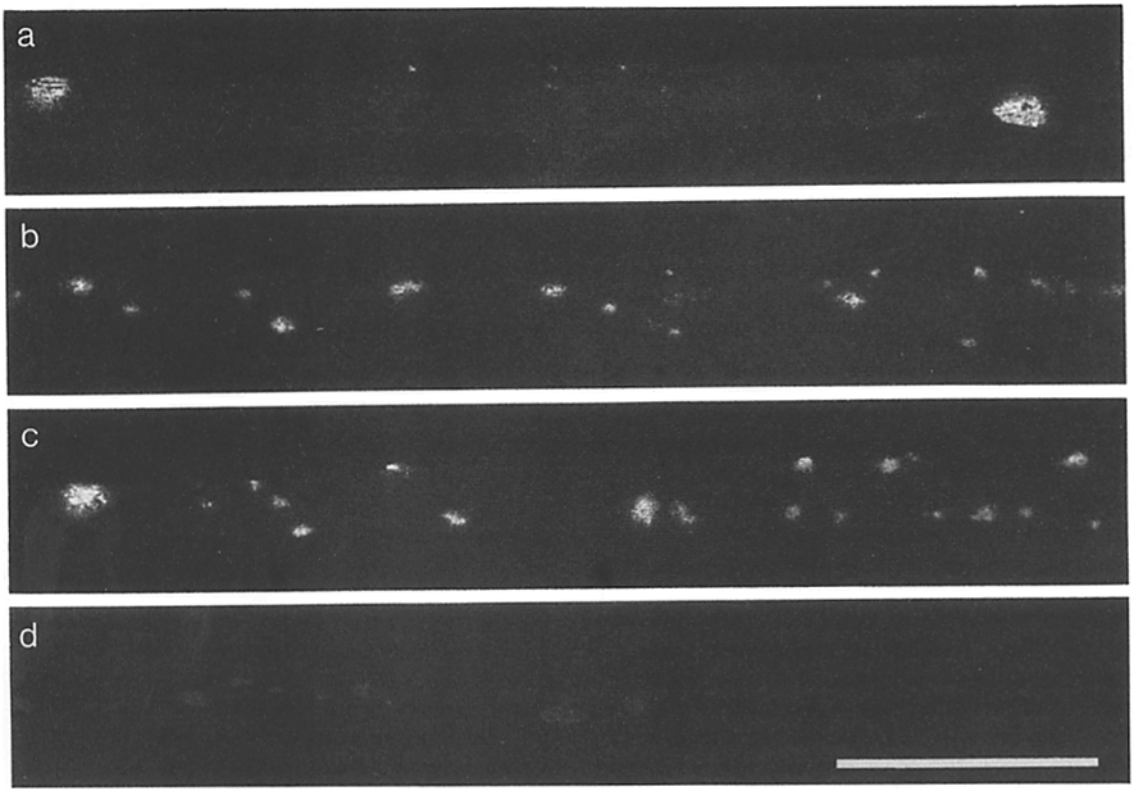

Figure 4. Agrin induces AChR aggregation by lateral migration. Fluorescence micrographs of segments of cultured chick myotubes labeled with rhodamine-α-bungarotoxin. (*a*) Myotube not treated with agrin. (*b*) Myotube labeled at the end of a 6-h incubation with agrin. (*c*) Myotube labeled with rhodamine-α-bungarotoxin, incubated with agrin for 6 h, and then fixed. (*d*) Myotube treated with unlabeled α-bungarotoxin to block exposed AChRs, incubated for 6 h with agrin, and then labeled with rhodamine-α-bungarotoxin to reveal distribution of newly inserted AChRs. Very few newly inserted AChRs were observed in agrin-induced aggregates. Thus most of the AChRs detected in aggregates after a 6-h incubation with agrin must have been present in the myotube membrane at the time agrin was added and therefore must have accumulated into aggregates by lateral migration. Bar, 50 μm.

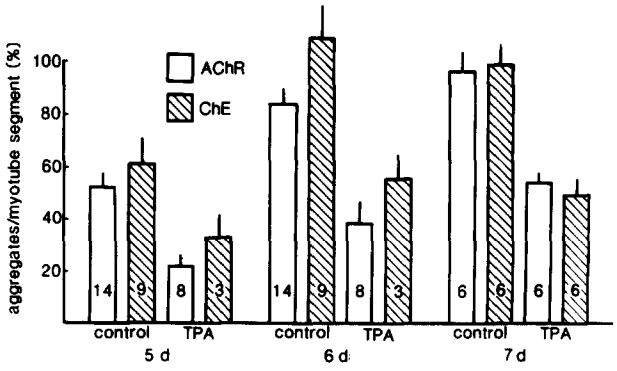

Figure 6. Effects of age of culture and TPA on the rate of AChR- and ChE-aggregate disappearance. Cultures were treated with agrin for 18-h beginning on day 4, 5, or 6 of culture, then rinsed, incubated with fresh medium (control) or medium supplemented with TPA (*TPA*) for an additional 6 h, and the number of AChR- and ChE-aggregates remaining was determined. Data is expressed as a percentage of the number of aggregates present at the end of the 18-h incubation. The bars indicate the SEM, the number of observations is indicated within the columns.

aggregates induced by treating the cultures with agrin for the previous 18 h remained. The number of ChE patches also decreased after removal of agrin; the rate of disappearance of ChE aggregates was slower than that of AChR clusters (Fig. 5). As with AChR aggregates, the decrease in ChE aggregates was more rapid in younger cultures; 61 ± 17% (n = 3) of ChE aggregates remained 6 h after removing agrin from 5 d cultures, 100 ± 14% (n = 4) in 6 d cultures (Fig. 6). Thus, in response to agrin, more mature myotubes formed more stable aggregates of AChRs and ChE.

Agrin-induced AChR Aggregation Requires Ca^{++}

It has been shown in nerve-muscle cocultures that neurites fail to induce accumulation of AChRs in the absence of Ca^{++} (Henderson et al., 1984). To determine if agrin-induced

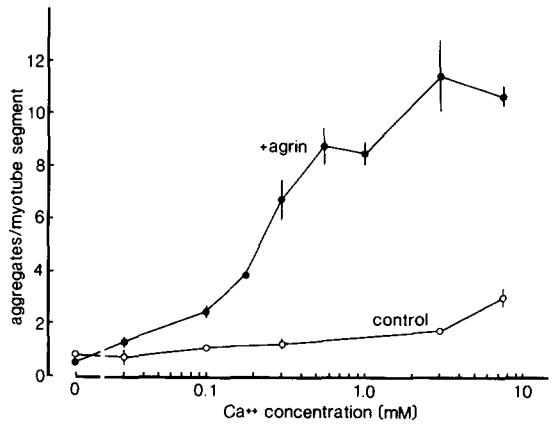

Figure 7. Ca^{++}-dependence of agrin-induced AChR aggregation. Cultures were incubated with or without agrin for 6 h in a balanced salt solution (5.6 mM glucose, 0.44 mM KH_2PO_4, 1.34 mM Na_2HPO_4, 0.001% phenol red, 5.36 mM KCl, 137 mM NaCl, 1 mM $MgCl_2$, 0.8 mM $MgSO_4$, 25 mM Hepes, pH 7.4) containing 0.1 mM EGTA (0 Ca^{++}) or 0.03-10 mM $CaCl_2$. AChRs were then labeled and the number of aggregates determined. Data is expressed as mean ± SEM (n = 3).

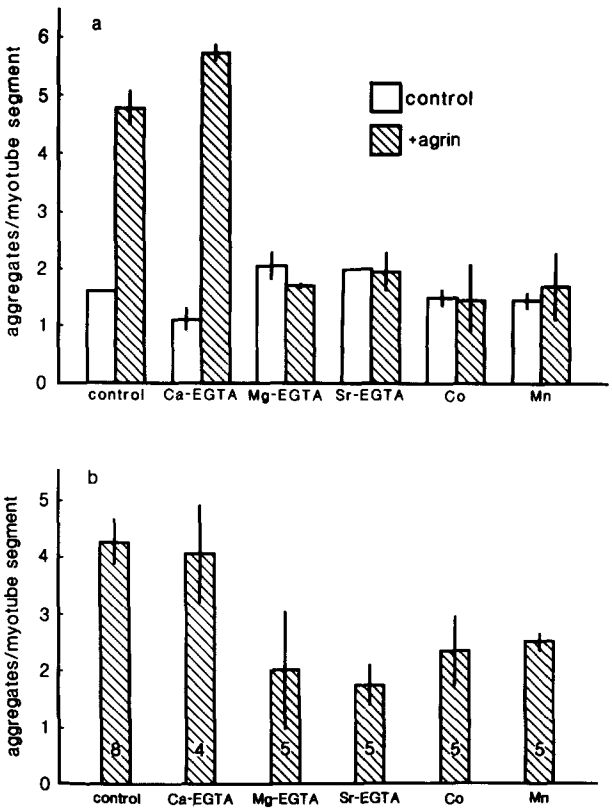

Figure 8. The effects of divalent cations on the formation and maintenance of agrin-induced AChR-aggregates. (*a*) Cultures were incubated with or without agrin for 6 h in normal medium (control) or medium containing 3 mM of the supplements indicated, then AChRs were labeled and the number of aggregates determined. Normal medium contained 1.8 mM Ca^{++}. Addition of 3 mM Ca^{++}-EGTA did not inhibit agrin-induced AChR aggregation, indicating that EGTA itself was not inhibitory. Data is expressed as mean ± SEM (n = 3). (*b*) Cultures were incubated for 16 h with agrin, rinsed, and incubated for an additional 6 h in normal medium (*control*) or medium containing 3 mM of the supplements indicated, then AChRs were labeled and the number of aggregates determined. Data is expressed as the increase in number of aggregates per myotube segment compared with cultures not treated with agrin. Error bars indicate SEM; the number of observations is given within the columns.

AChR aggregation required Ca^{++}, myotubes were incubated in a HEPES-buffered balanced salt solution containing either 0.1 mM EGTA (0 Ca^{++}) or 0.03-7.5 mM $CaCl_2$. As illustrated in Fig. 7, agrin did not induce AChR aggregation in the absence of Ca^{++}. The number of AChR aggregates formed during a 6-h incubation with agrin increased with increasing Ca^{++} concentration, reaching a plateau at 3 mM. The concentration of Ca^{++} required to give a 1/2 maximal response was 0.3 mM. To characterize further the Ca^{++}-dependence of agrin-induced AChR aggregation, the effects of other divalent cations were tested. Fig. 8 *a* shows that if Ca^{++} was replaced by Mg^{++} or Sr^{++} ions, no receptor aggregation occurred. Thus, neither Mg^{++} nor Sr^{++} could substitute for Ca^{++}, although neither inhibited AChR aggregation in the presence of Ca^{++} (data not shown). Two inorganic Ca^{++} antagonists, Co^{++} and Mn^{++}, did inhibit agrin-induced AChR aggregation, even in the presence of Ca^{++}. Thus, ex-

Table I. Effects of Drugs on Agrin-induced AChR Aggregation

Target and Treatment*	Concentration	Activity‡	
Ca^{++} channel			
Verapamil	10^{-4} M	110 ± 11	(5)
Nifedipine	10^{-5} M	70 ± 9	(6)
Diltiazem	10^{-4} M	78 ± 19	(5)
Cromolyn	2×10^{-4} M	133 ± 12	(5)
Flunarizine	10^{-4} M	108 ± 8	(5)
Intracellular Ca^{++} concentration			
A23187	$2-5 \times 10^{-7}$ M	94 ± 11	(6)
Ruthenium red	5×10^{-6} M	76 ± 15	(6)
Ca^{++}-calmodulin			
Trifluoperazine	10^{-5} M	91 ± 9	(6)
Calmidazolium	10^{-6} M	92 ± 6	(8)
G proteins			
Cholera toxin	20 μg/ml	116 ± 10	(9)
β-γ-methyleneguanosine 5′-triphosphate	10^{-3} M	105 ± 8	(3)
Cyclic nucleotide concentration			
Forskolin	2×10^{-5} M	112 ± 16	(6)
Theophylline	10^{-3} M	109 ± 5	(6)
N^6,O^2,-dibutyryladenosine 3′:5′-cyclic monophosphate§	10^{-3} M	118 ± 9	(15)
8-bromoadenosine 3′:5′-cyclic monophosphate§	10^{-3} M	136 ± 26	(4)
$N^2,O^{2'}$-dibutyrylguanosine 3′:5′-cyclic monophosphate§	10^{-3} M	109 ± 12	(9)
Cytoskeleton			
Cytochalasin D	0.4 μg/ml	84 ± 9	(9)
	4 μg/ml	91 ± 16	(6)
	8 μg/ml	64 ± 17	(6)
Colchicine	10^{-6} M	134 ± 9	(6)
Demecolcine	10^{-5} M	106 ± 9	(6)
Podophyllotoxin	10^{-6} M	116 ± 16	(6)
Local anesthetics			
Procaine	10^{-3} M	82 ± 7	(5)
Lidocaine	10^{-3} M	111 ± 8	(5)
Protein/collagen synthesis			
Puromycin	20–100 μg/ml	77 ± 9	(11)
Cycloheximide	100 μg/ml	77 ± 4	(25)
Cis-4-hydroxy proline	200 μg/ml	96 ± 5	(6)
α,α′-Dipyridyl	3×10^{-4} M	112 ± 6	(9)
Other			
4-methylumbelliferyl-β-xyloside Tunicamycin	10^{-3} M	97 ± 10	(3)
	1 μg/ml	81 ± 6	(4)
5′-deoxy-5′-methylthioadenosine	2×10^{-4} M	111 ± 11	(4)
GRGDS peptide	0.5 mg/ml	69 ± 7	(3)
Energy metabolism			
Dicumarol	10^{-4} M	105 ± 21	(6)
Sodium azide	5×10^{-3} M	111 ± 11	(9)
Potassium cyanide	10^{-3} M	115 ± 20	(6)
Dinitrophenol	10^{-3} M	45 ± 17	(12)
Oligomycin	1 μg/ml	36 ± 13	(14)
22°C		4 ± 7	(6)
pH 6.9		29 ± 6	(9)
pH 6.4		11 ± 3	(9)
Protein kinase C			
TPA	5×10^{-8} M	4 ± 3	(7)

* Myotube cultures were routinely incubated for 6 h with agrin and the drug listed, then labeled with rhodamine-α-bungarotoxin, fixed, and the number of aggregates per myotube segment determined. With puromycin and cycloheximide, similar results were obtained if myotubes were pretreated for as long as 2 h before adding agrin. None of the drugs caused a significant increase in the number of AChR aggregates in cultures not treated with agrin.

‡ Data, expressed as percent of control, is mean ± SEM. The number of observations is given in parentheses.

§ Medium also contained 1 mM theophylline.

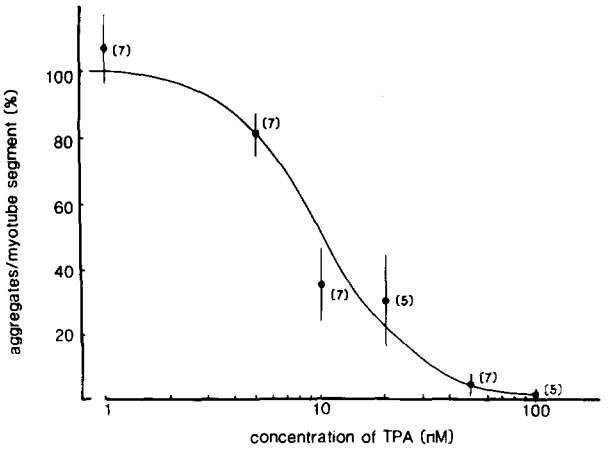

Figure 9. The number of agrin-induced AChR aggregates is reduced in cultures treated with TPA. Cultures were incubated for 6 h with agrin in the presence of the indicated concentrations of TPA, then AChRs were labeled and the increase in the number of aggregates determined. Data, expressed as a percentage of the increase observed in cultures not treated with TPA, is plotted as the mean ± SEM; the number of observations is indicated in parentheses. The curve was fit to the points by eye.

ternal Ca^{++} ions are required for agrin-induced AChR-aggregate formation.

One explanation for the effects of divalent cations is that agrin increases the permeability of myotubes to Ca^{++} and the influx of Ca^{++} triggers AChR aggregation. However, the calcium ionophore A23187 did not itself induce AChR aggregate formation or inhibit agrin-induced AChR aggregation at concentrations as high as 400 nM (Table I). Compounds that raise cytoplasmic Ca^{++} concentration by releasing Ca^{++} from mitochondrial stores, such as ruthenium red and metabolic inhibitors, likewise did not induce AChR aggregate formation. Moreover, none of the organic Ca^{++}-channel antagonists tested (verapamil, nifedipine, diltiazem, cromolyn, flunarizine) was effective in inhibiting agrin-induced AChR aggregation (Table I). Thus, although our results do not exclude the possibility that AChR aggregation is the result of a localized increase in intracellular Ca^{++} brought about, for example, by agrin opening a channel that allows Ca^{++} influx, agrin-induced AChR aggregation does not appear to be mediated by a uniform increase in cytoplasmic Ca^{++} concentration. Consistent with this conclusion is the observation that inhibitors of the interaction of Ca^{++} with calmodulin (trifluoperazine and calmidizolium), a common mechanism mediating the effects of increased cytoplasmic Ca^{++}, did not prevent agrin-induced AChR aggregation (Table I).

The effects of Ca^{++} removal on the stability of pre-formed aggregates was tested by treating myotubes overnight with agrin to induce AChR-aggregate formation, then incubating the myotubes in medium with or without various divalent cations. As illustrated in Fig. 8 *b*, adding Co^{++} or Mn^{++} or replacing Ca^{++} with Mg^{++} or Sr^{++} did reduce somewhat the number of AChR aggregates that remained after 6 h. Thus, Ca^{++} is required both for the formation and maintenance of agrin-induced AChR aggregates, but the effect of Ca^{++} removal on the rate at which aggregates disappeared was much smaller than on aggregate formation.

Effects of Metabolic Inhibitors

As listed in Table I, a wide range of pharmacological agents that either activate or inhibit a variety of cellular processes neither induced the formation of AChR aggregates nor inhibited or augmented agrin-induced AChR aggregation, including drugs that activate GTP-binding proteins, increase cyclic nucleotide concentration, destabilize microtubules or actin filaments, act as local anesthetics, or inhibit protein synthesis, methylation, or glycosylation. The effects of treatments that interfere with energy metabolism were variable, some metabolic inhibitors (dinitrophenol and oligomycin) prevented agrin-induced AChR aggregation while others (sodium azide and potassium cyanide) did not. Lowering the temperature to 22°C also inhibited agrin-induced AChR aggregation, as did reducing the pH below 7. It seems reasonable to conclude from these results that agrin-induced AChR aggregation requires metabolic energy and that the variability observed in the extent of inhibition by different metabolic inhibitors was owing to differences in the efficacy with which they interfered with the myofiber's energy metabolism.

Inhibition of Agrin-induced AChR Aggregation by TPA

Anthony et al. (1984) have demonstrated that transformation of myotubes by infection with the Rous sarcoma virus inhibits both spontaneous and extract-induced formation of AChR aggregates. Their evidence suggests that activity of the sarc gene product, a serine protein kinase, is responsible for this effect. To determine if AChR aggregation in normal myotubes might also be regulated by protein phosphorylation, myotubes were treated with agents known to either directly or indirectly stimulate or inhibit endogenous protein kinases. Forskolin, membrane permeable derivatives of cAMP and cGMP, calmidazolium, and trifluoperazine had little effect on the number of AChR aggregates on control or agrin-treated myotubes (Table I), suggesting that neither cyclic nucleotide- nor Ca^{++}-calmodulin-dependent protein kinases regulate AChR aggregation. On the other hand, treating cultures with the phorbol ester TPA, an activator of protein kinase C (Nishizuka, 1984), greatly reduced the number of AChR aggregates formed in response to agrin (Table I).

Dose Dependence of TPA Inhibition

To provide evidence that TPA inhibition of agrin-induced AChR aggregation was due to activation of protein kinase C, the effects of different concentrations of TPA were examined. Fig. 9 shows that as the concentration of TPA was increased, the mean number of aggregates of AChRs induced by agrin decreased, reaching control levels at 50 nM TPA. This concentration of TPA also caused a slight reduction in the number of AChR aggregates in myotubes not exposed to agrin. The concentration of TPA that produced half maximal inhibition was ~10 nM, which is in good agreement with the concentration of TPA required for half-maximal stimulation of protein kinase C (Nishizuka, 1984). Thus, inhibition of agrin-induced AChR-aggregation by TPA is likely to be due to changes in protein phosphorylation mediated by an endogenous protein kinase C.

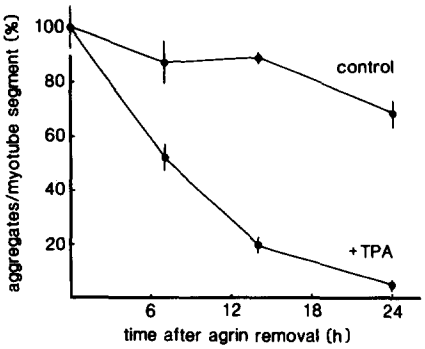

Figure 10. TPA increases the rate of disappearance of agrin-induced AChR aggregates. Cultures were incubated with agrin for 16 h, rinsed, and returned to the incubator in fresh medium with or without 20 nM TPA. After 0–24 h AChRs were labeled and the number of aggregates determined. Data, expressed as the mean ± SEM (n = 3), is plotted as a percentage of the number of aggregates present at the end of the 16-h incubation with agrin.

TPA Inhibits Formation and Accelerates Disappearance of AChR Aggregates

To determine whether TPA was inhibiting agrin-induced AChR aggregate formation or causing the dispersal of aggregates after they formed, cultures were treated overnight with agrin, rinsed, and incubated in culture medium with or without TPA. As shown in Fig. 10, TPA increased the rate of disappearance of AChR aggregates in 7-d cultures. The effect was also seen in younger cultures in which the normal disappearance of AChR aggregates is faster (Fig. 6). However, the effect of TPA on aggregate disappearance was smaller than its effect on aggregate formation. During a 6-h incubation with agrin, 20 nM TPA inhibited formation of AChR aggregates more than 70% (Fig. 9); exposing agrin-pretreated myotubes to 20 nM TPA for 6 h caused only a 50% decrease in the number of AChR aggregates compared with controls (Figs. 6 and 10). Thus TPA both inhibited AChR aggregate formation and increased the rate at which aggregates disappeared.

To confirm that TPA inhibited AChR aggregation in addition to increasing the rate of aggregate disappearance, the rate of appearance of agrin-induced AChR aggregates was assayed. For this experiment all distinct fluorescent patches, not only those with shortest axis >2 μm, were counted (see Materials and Methods). As illustrated in Fig. 11, the number of aggregates on cultured myotubes was analyzed after 2- or 4-h incubation with agrin. Adding TPA together with agrin during the first 2 h reduced the number of aggregates observed by ≥90%. Adding TPA during the second 2-h period caused the number of aggregates to remain relatively constant, indicating that the rate of disappearance of aggregates, even in the presence of TPA, was too slow to be detected during a 2-h incubation. Thus, the reduction in the number of aggregates seen after treatment with TPA could not have been due to rapid dispersal of aggregates after they appeared but rather to inhibition of aggregate formation.

The rapid time course with which TPA inhibited agrin-induced AChR aggregation suggests that inhibition by TPA is a direct consequence of protein phosphorylation by activation of protein kinase C, rather than an indirect effect due to TPA-induced changes in gene expression; in chick myotubes newly synthesized proteins, such as AChRs and AChE, require 2–3 h to reach the cell surface (Devreotes and Fambrough, 1975; Rotundo and Fambrough, 1980). To provide further evidence that inhibition of agrin-induced AChR aggregation by TPA was not due to changes in gene expression, myotubes were incubated with inhibitors of protein synthesis for 1 h before the addition of TPA and agrin. As illustrated in Fig. 12, TPA still prevented agrin-induced AChR aggregation when protein synthesis was inhibited >97%. Thus, the effects of TPA on AChR aggregation appear to be a direct consequence of protein kinase C–mediated protein phosphorylation.

Discussion

Comparison of Agrin-induced AChR/ChE Aggregation and the Accumulation of AChR/AChE at Developing Neuromuscular Junctions

Time Course of AChR Aggregation. Results of experiments described in this report demonstrate that agrin-induced AChR aggregation begins with the formation of small clusters of AChRs which, over the course of 16–24 h, increase in size to form large patches with sharp boundaries. The results also confirm and extend our previous finding (Godfrey et al., 1984) that this accumulation of AChRs occurs by lateral migration of receptors already in the plasma membrane. Accumulation of AChRs at developing neuromuscular junctions also occurs, at least in part, by lateral migration (Anderson and Cohen, 1977; Ziskind-Conhaim et al., 1984; Role et al., 1985) and follows a similar time course; a loose cluster of small aggregates of AChRs appears beneath the developing axon terminal and during the ensuing ~24 h matures into a large, relatively uniform and circumscribed "plaque" (Anderson and Cohen, 1977; Steinbach, 1981). Thus, the formation of agrin-induced AChR aggregates on cultured myotubes resembles the accumulation of AChRs at developing neuromuscular junctions.

Stability of AChR/ChE Aggregates

If agrin is removed from cultured myotubes, AChR and ChE aggregates slowly disappear (Figs. 5 and 6). Similarly, if

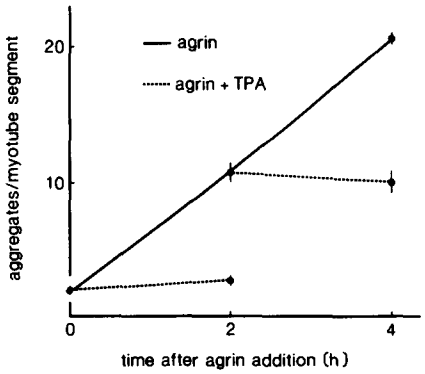

Figure 11. TPA inhibits agrin-induced AChR aggregation. Cultures were incubated with agrin with or without 20 nM TPA for 2–4 h as indicated. AChRs were then labeled and the number of aggregates, including those <4 μm², was determined. Data is expressed as mean ± SEM (n = 3).

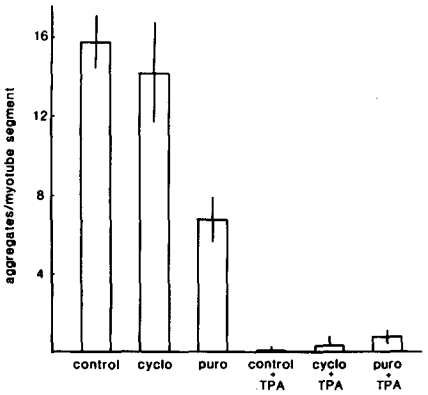

Figure 12. TPA inhibits agrin-induced AChR aggregation in the absence of protein synthesis. Myotubes were incubated for 1 h in normal medium (*control*) or medium containing 100 μg/ml of either cycloheximide (*cyclo*) or puromycin (*puro*). Agrin was then added to all the cultures; in addition, 20 nM TPA (*TPA*) was added to one set of cultures. After an additional 6 h of incubation AChRs were labeled and the increase in the number of aggregates compared to cultures not treated with agrin was determined. Data is expressed as mean ± SEM, $n = 3$. The extent to which protein synthesis was inhibited was determined by measuring the incorporation of [^{35}S]methionine into TCA-precipitable material; within 30 min cycloheximide and puromycin were each found to have inhibited incorporation by >97%.

newly formed neuromuscular junctions in vivo are denervated, there is a marked dispersal of junctional AChR and AChE clusters (Slater, 1982; Moss and Schuetze, 1987). Thus, newly formed AChR/ChE aggregates induced by agrin, like newly formed aggregates induced by axon terminals, are labile. The morphological stability of AChR and AChE aggregates on myofibers in vivo increases after birth; by 2 wk of age denervation leads to little change in the distribution of AChRs and AChE at the neuromuscular junction, although the levels of AChE decrease due to lack of electromechanical activity (Dennis, 1981; Slater, 1982). This developmental change is consistent with the hypothesis that by 2 wk of age sufficient agrin has become stably incorporated into the myofiber's synaptic basal lamina to maintain AChR distribution in the absence of the axon terminal. In culture, the amount of basal lamina elaborated by myotubes and/or the amount of agrin that becomes bound to the basal lamina apparently is not sufficient to maintain AChR/ChE aggregates when agrin is removed from the culture medium.

Ca^{++}-dependence of AChR Aggregation

Our results show that Ca^{++} is necessary both for the formation and maintenance of agrin-induced AChR aggregates (Figs. 7 and 8). AChRs also fail to accumulate at sites of synaptic contact between cultured nerve and muscle cells if the medium lacks Ca^{++} (Henderson et al., 1984). Moreover, AChRs aggregated at developing neuromuscular junctions in vivo are dispersed if Ca^{++} ions are removed (Bloch and Steinbach, 1981). Thus the formation and maintenance of both agrin- and nerve-induced AChR aggregates requires Ca^{++}. With age, aggregates of AChRs at developing neuromuscular junctions in vivo become increasingly insensitive to dispersal by Ca^{++} removal (Bloch and Steinbach, 1981); we do not yet know whether prolonged exposure to agrin would likewise render AChR clusters on cultured myotubes resistant to dispersal by removal of Ca^{++}.

Lack of Dependence on Electromechanical Activity

We found that agrin caused AChR aggregation on cultured myotubes in the absence of any induced electromechanical activity and even in the presence of lidocaine or procaine (Table I), which prevented any visible spontaneous contractions. Similarly, although electromechanical activity regulates many aspects of neuromuscular development (Dennis, 1981), AChRs aggregate at sites of nerve-muscle contact in the absence of activity (Cohen, 1972; Steinbach, 1974; Anderson and Cohen, 1977; Obata, 1977; Davey and Cohen, 1987). Moreover, after 2 wk of age, AChRs remain accumulated at neuromuscular junctions after denervation (Slater, 1982). Thus, electromechanical activity is not necessary for the formation or maintenance of either agrin- or nerve-induced AChR aggregates.

Agrin also induces the formation of aggregates of ChE on cultured chick myotubes in the absence of electromechanical activity, although the level to which ChE accumulates is much lower than at neuromuscular junctions (Wallace, 1986). We do not yet know if electromechanical activity would enhance ChE accumulation at agrin-induced aggregates on cultured myotubes, as it does at the neuromuscular junction (Weinberg and Hall, 1979).

In summary, agrin-induced aggregation of AChRs on myotubes in culture resembles the accumulation of AChRs at developing neuromuscular junctions; both occur with a similar time course, arise at least in part by lateral migration of existing receptors, are at first readily reversible, require Ca^{++}, and are not dependent on electromechanical activity. These observations provide additional support for our hypothesis that molecules similar or identical to agrin are released from axon terminals at developing neuromuscular junctions, induce the formation of the postsynaptic apparatus, and become associated with the synaptic basal lamina where they function to maintain the postsynaptic apparatus in the adult and direct its differentiation during regeneration.

Mechanism of Agrin-induced AChR Aggregation and Comparison with other AChR-aggregating Factors

Comparison of AChR-aggregating Factors. Of the many factors that cause the formation of aggregates of AChRs on myotubes in culture (for review see Godfrey et al., 1984; Nitkin et al., 1987), only agrin (Nitkin et al., 1987), CGRP (New and Mudge, 1986), ARIA (Usdin and Fischbach, 1986), sciatin/transferrin (Markelonis et al., 1982; Oh and Markelonis, 1982), and ascorbate (Kalcheim et al., 1982) have been purified and characterized. These molecules share no known structural similarities (Nitkin et al., 1987). CGRP, ARIA, and sciatin/transferrin increase the rate of appearance of new AChRs and thus they may mediate the increased rate of AChR synthesis and insertion reported to occur during formation of neuromuscular synapses (Role et al., 1985) and at the mature neuromuscular junction (Merlie and Sanes, 1985). Agrin does not increase AChR synthesis (Godfrey et al., 1984), but compared with CGRP, ARIA, sciatin/transferrin, and ascorbate causes a more extensive redistribution of AChRs. Thus, agrin is structurally and functionally distinct from other identified AChR-aggregating molecules.

The effects of agrin, embryonic brain extract, and positively-charged latex beads on AChR distribution appear similar, suggesting that they might trigger a common mechanism. Indeed, we find agrin-like molecules in extracts of embryonic chick brain and spinal cord (Smith et al., 1987). Thus agrin might account for some or all of the AChR-aggregating activity in the brain extracts described by others, although such brain extracts also cause an increase in the number of AChRs (Bauer et al., 1981; Buc-Caron et al., 1983; Bursztajn and Fischbach, 1984; Connolly et al., 1982; Jessel et al., 1979; Podleski et al., 1978), and thus clearly contain active components in addition to agrin.

Comparison of Induced and Spontaneously Occurring AChR Aggregates

The properties of AChR aggregates that occur spontaneously on rat myotubes differ from those of aggregates induced by nerve, agrin, or brain extracts. Spontaneous AChR aggregates are much larger (Bruner and Bursztajn, 1986; Englander and Rubin, 1987), have a lower density of AChRs (Salpeter et al., 1982), differ in their response to colchicine and cytochalasin (Bloch, 1979, 1983; Connolly, 1984; Englander and Rubin, 1987), are on the bottom rather than on the top of the myotubes (Salpeter et al., 1982; Bloch and Geiger, 1980), and are associated with adhesion plaques (Bloch and Geiger, 1980). Thus, spontaneously occurring AChR aggregates may not be a good model for the postsynaptic apparatus.

Model for Agrin-induced AChR Aggregation

We propose the following working hypothesis for agrin-induced AChR aggregation. Most AChRs (and perhaps other components of the postsynaptic apparatus as well) on the surface of untreated myotubes have been shown to be in small aggregates, composed of 5–25 receptors (Cohen and Pumplin, 1979). Each of these aggregates, or "speckles", is a ~0.1-μm wide patch that is seen as a cluster of particles in freeze-fracture (Cohen and Pumplin, 1979; Peng and Nakajima, 1978; Yee et al., 1978) or as a "mound" in conventional electron microscopy (Burrage and Lentz, 1981). Speckles appear to be free to diffuse laterally in the myotube plasma membrane (Axelrod et al., 1976). We propose that myotubes also have on their surface receptors for agrin, in chick muscle cultures the agrin receptors would be scattered over the bottom surface of the myotubes. As a consequence of the binding of agrin to its receptor, AChR speckles in the immediate vicinity of the agrin-receptor complex are immobilized, thus forming "microaggregates" (clusters $<4\ \mu m^2$). This process requires Ca^{++} (Figs. 7 and 8) and metabolic energy (Table I), but not protein synthesis (Table I, Fig. 12), is inhibited by protein kinase C-mediated protein phosphorylation (Figs. 9–12), and does not depend on colchicine- or cytochalasin D-sensitive components of the cytoskeleton (Table I; but see Connolly, 1984; Englander and Rubin, 1987). The dependence on metabolic energy indicates that agrin-induced AChR aggregation is a more complex process than simple crosslinking of surface receptors by a multivalent ligand, such as occurs during patching on lymphocytes, which does not rely on energy metabolism (De Petris, 1977). As more speckles are trapped, microaggregates grow and merge to form larger patches (Figs. 1–3), until a steady state is reached at which the rate of removal of receptors from the patch, either by internalization or diffusion away, equals the rate of addition of new receptors. Such a large patch would remain so long as agrin is bound to its receptor and Ca^{++} and energy supplies are available to allow addition of new receptors. Removal of agrin, Ca^{++}, or energy would lead to the disappearance of the patch by blocking addition of new speckles. The finding that aggregates disappear more rapidly if Ca^{++} and agrin are both removed (Fig. 8) indicates that Ca^{++} plays a role in stabilizing aggregates as well.

Brain extract-induced AChR aggregates on rat myotubes form in a similar way, microaggregates merging to give rise to large patches over the course of several hours by a process requiring metabolic energy (Salpeter et al., 1982; Olek et al., 1983, 1986*a*, *b*). However, in rat muscle cultures extract-induced AChR aggregates are on the top surface of the myotubes (Salpeter et al., 1982; Olek et al., 1983, 1986*a*, *b*). Accordingly, we propose that rat muscle cultures differ from chick in that agrin receptors are localized to the tops of the myotubes. In both rat and chick, components of the cytoskeleton probably play a role in stabilizing aggregates once they have formed; however, the timing and nature of this stabilization appears to differ in myotubes from different species (Table I; Connolly, 1984; Englander and Rubin, 1987; Olek et al., 1986*a*, *b*).

Role of Calmodulin

Two inhibitors of calmodulin, trifluoperazine and calmidazolium, did not significantly reduce agrin-induced AChR aggregation (Table I). Although we did not assay directly the effectiveness of these drugs, they caused obvious changes in myotube morphology and, at the concentrations used, they have been shown by others to interfere in established calmodulin-mediated processes (Prozialeck and Weiss, 1982, 1985). Trifluoperazine has been reported by Peng (1984) to block the aggregation of AChRs on cultured *Xenopus* myocytes induced by positively charged latex beads (Peng and Cheng, 1982), although he also reported that two other calmodulin antagonists, the naphthalene sulfonamides W-7 and W-5, caused little inhibition. The difference in sensitivity to trifluoperazine could be due to underlying differences in the mechanism of charged bead vs. agrin-induced aggregate formation, to species differences (*Xenopus* vs. chick myotubes), or to nonspecific effects of trifluoperazine.

Role of Protein Kinase C

Prolonged treatment of cultured myotubes with phorbol esters that activate protein kinase C, such as TPA, produces many changes in myotube morphology and metabolism (Croop et al., 1982), including a 50% decrease in the rate of synthesis and a 30% increase in the rate of degradation of AChRs (Mishkin et al., 1978). Our finding that TPA inhibited agrin-induced AChR aggregation as rapidly as it could be measured (2 h, Fig. 11) and even when new protein synthesis was prevented (Fig. 12) suggests that inhibition of agrin-induced AChR aggregation by TPA is a direct consequence of protein kinase C–mediated phosphorylation, rather than an indirect effect due to changes in myotube morphology, metabolism, or gene expression. Thus it is tempting to speculate that a protein phosphorylated by protein kinase C is involved in agrin-induced aggregation of AChRs.

In this regard, it would be interesting to identify proteins phosphorylated both by TPA and by the sarc gene product, a serine protein kinase, which is responsible for the inhibition of AChR aggregation in myotubes transformed with the Rous sarcoma virus (Englander and Rubin, 1987).

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