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A factor from neurons increases the number of acetylcholine receptor aggregates on cultured muscle cells

(synaptogenesis/neurotrophic factor/membrane receptor mobility)

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ABSTRACT **There is an increase in the number of acetylcholine (AcCho) receptor aggregates on striated embryonic mouse myotubes when they are cocultured with clonal neuroblastoma–glioma hybrid cells. Medium conditioned by hybrid cells contains a factor which increases the number of AcCho receptor aggregates on myotubes cultured from mouse, rat or chick muscle. AcCho receptor-aggregating activity was present in medium conditioned by the neuroblastoma parent clone but was not detected in medium conditioned by cells of the parent glioma clone, fibroblasts, or HeLa cells. The factor increased the aggregation of AcCho receptors within 24 hr without a significant increase in the total number of AcCho receptors, and its action did not depend on myotube protein synthesis. The factor appears to rearrange the distribution of myotube AcCho receptors either by aggregating mobile AcCho receptors or by stabilizing labile receptor aggregates.**

Acetylcholine (AcCho) receptors of the innervated skeletal muscle are localized at the neuromuscular junction (1) seemingly by two processes: (*i*) accumulation of AcCho receptors at the neuromuscular contact region, and (*ii*) elimination of AcCho receptors from noncontact regions (2). Activation of the muscle fiber by electrical stimulation or synaptic activity can decrease the number of extrajunctional AcCho receptors (3); however, there is only speculation concerning the mechanism of AcCho receptor accumulation at muscle regions aligned with presynaptic acetylcholine release sites (4, 5). In various cells, randomly dispersed surface receptors form patches or caps when the cells are treated with specific immunoglobulins or lectins (6, 7). We here report a similar phenomenon: a factor produced by the cholinergic neuroblastoma–glioma hybrid cell line NG108-15 increases the number of AcCho receptor aggregates on cultured myotubes. We propose that such a factor may be involved in the aggregation of AcCho receptors at the site of nerve contact during synapse formation.

MATERIALS AND METHODS

Cell Culture. Mouse muscle cultures were prepared from the hindlimbs of 18–20-day-old C57B/6N mouse embryos, as described (8), except that cell proliferation was suppressed by the addition of 10^{-5} M fluorodeoxyuridine on the fifth and sixth days of culture. Cultures were established by adding a suspension of 5×10^5 single cells to 35-mm plastic plates, or 1.5×10^5 cells to the 16-mm wells of plastic multiwell plates (Costar). Conditioned medium produced as described below was added to muscle cultures 10–21 days after plating.

Rat muscle cultures were prepared from Fischer strain rat embryos by using the same methods.

Chick muscle cultures were prepared from the pectoral muscle of 11-day-old chick embryos (9). A suspension of mechanically dissociated single cells was prepared in a medium of 85% Eagle's minimal essential medium, 10% horse serum, and 5% chick embryo extract, and 2.0×10^4 cells in 40 μl of medium were added to the 5-mm round wells in Teflon-covered glass slides (Roboz Surgical Instrument Co.). On the third day after plating, the cultures were fed with medium in which the concentration of chick embryo extract was reduced to 2%. Conditioned medium prepared as described below was added on the fifth day after plating.

Production of Conditioned Medium. Before they were used to condition medium, NG108-15 cells were induced to differentiate (see ref. 24) in collagen coated flasks for at least 1 week in the Dulbecco-Vogt modification of Eagle's minimal essential medium (DMEM) containing 1 mM N^6,$O^{2'}$-dibutyryl-adenosine 3′:5′-cyclic monophosphate (Bt_2cAMP), 0.1 mM hypoxanthine, and 16 μM thymidine (control medium) plus either 5% fetal calf serum or 5% horse serum. Conditioned medium was prepared by washing a tissue culture flask containing a nearly confluent culture of differentiated NG108-15 cells twice with DMEM and feeding with control medium (serum free). The amount of control medium was the minimal volume necessary to maintain the cells for 24 hr without a significant change in the pH of the medium; typically, this volume was 15 ml per 1×10^6 cells. One day after adding the control medium to NG108-15 cells, it was removed, centrifuged at $2,000 \times g$ for 5 min to remove cellular debris, and added to muscle cultures. To prepare medium conditioned by undifferentiated NG108-15 cells, log phase cultures of NG108-15 cells grown in plastic flasks and fed with control medium plus 10% fetal calf serum but without Bt_2cAMP were rinsed and fed with control medium lacking Bt_2cAMP.

The other cell types were grown on plastic tissue culture flasks in DMEM containing 10% fetal calf serum. HeLa cell cultures and cultures of human diploid fibroblasts were switched directly to control medium. Cultures of N18TG-2 or C6BU-1 cells were treated for 1 week with the medium used to differentiate NG108-15 cells, and were then fed with control medium.

Dialyzed conditioned medium was prepared sterilely by introducing NG108-15 cell-conditioned medium into autoclaved dialysis tubing and dialyzing it for 16 hr against a 100-fold volume of fresh control medium. Lyophilized conditioned medium was prepared from conditioned medium exhaustively dialyzed against a solution of 1 mM sodium phosphate buffer (pH 7.4) and then against deionized water.

Staining of AcCho Receptor Sites. The distribution of AcCho receptors on mouse and rat myotube surfaces was visualized by indirect immunoperoxidase staining of bound α-bungarotoxin (α-btx), as described (10, 11), except that: (*i*)

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Abbreviations: AcCho, acetylcholine; α-btx, α-bungarotoxin; A/M, acetylcholine receptor aggregates per myotube; Bt_2cAMP, N^6,$O^{2'}$-dibutyryl-adenosine 3′:5′-cyclic monophosphate; DMEM, the Dulbecco-Vogt modification of Eagle's minimal essential medium.

cultures were washed twice with fresh medium prior to incubation with α-btx and (ii) 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.2 was used for the fixation step after α-btx incubation. For examination, the stained, osmium tetroxide-treated cultures were mounted in a glycerol mounting medium under a glass coverslip.

AcCho receptors on chick myotubes were stained with rhodamine-conjugated α-btx prepared from tetramethyl rhodamine isothiocyanate (Baltimore Biological Laboratories) as described (12).

Chick myotube cultures were incubated for 1 hr in medium containing 95% DMEM, 5% fetal calf serum, and tetramethyl rhodamine–α-btx at 1 μg ml^{-1}. The cultures were then washed for 5 min each in two changes of a solution of 95% medium and 5% fetal calf serum and in one change of medium alone; the cells were fixed in 80% ethanol at −20°. The cultures were mounted in buffered glycerol mounting medium (BBL), pH 8.0 or above, under a glass cover slip. The stained material was inspected with a Zeiss Photomicroscope II equipped with epi-illumination and a 40X Planapo oil objective (N.A. 1.0). Light from a 200 watt mercury arc bulb was passed through BP 546/9 and KP 600 excitation filters and a Kodak No. 23A barrier filter.

Microscopic Evaluation of AcCho Receptor Aggregates. To quantify the incidence of AcCho receptor aggregation in mouse or rat cultures, the number of AcCho receptor aggregates, defined as regions of intense immunoperoxidase staining on myotubes, and the number of myotube segments was counted in 50 fields in each muscle culture at a magnification of 320. For each muscle culture plate, the average number of AcCho receptor aggregates per myotube (A/M) was determined by dividing the total number of aggregates by the total number of myotubes observed. Because segments of some myotubes extended beyond the microscope field, the A/M is an underestimate of the total number of aggregates on each myotube.

The A/M of muscle cultures grown without NG108-15 cells or without the addition of conditioned medium was consistent within groups of cultures prepared at one time and fed with the same medium, but varied between groups from 0.11 to 0.76. Therefore, in each experiment, control and experimental cultures were taken from the same group and received the same medium. The results produced by various treatments are expressed as the ratio of the A/M in cocultures or conditioned-medium-treated cultures to the A/M of control muscle cultures (see Table 1). These numbers thus represent the fold change in the number of AcCho receptor aggregates per myotube produced by a particular treatment.

Measurement of Total AcCho Receptor. Companion cultures of those described in Table 1, experiment C, were harvested and homogenized in phosphate buffered saline at 0.5 ml/plate. Aliquots of increasing volume of each homogenate were incubated with 8 nM di[^{125}I]iodo-α-btx (prepared according to ref. 13) for 60 min at 37°. Bound toxin was separated from unbound toxin by filtration through Millipore EGWP filters. Nonspecific binding, estimated by adding 5×10^{-3} M d-tubocurarine to incubation mixtures, was subtracted from each value. For each homogenate the number of binding sites was determined from the slope of a plot of radioactivity versus the volume of aliquot.

RESULTS

Distribution of AcCho Receptors. In mouse or rat muscle cultures stained for bound α-btx by the immunoperoxidase method, most myotube surfaces were lightly stained. In addition, small patches of heavy stain were found on many myotubes whether cocultured with NG108-15 cells or grown alone (Fig. 1). In cocultures patches were found in regions of nerve contact as well as in regions where no contact was observed. When the binding of α-btx to mouse myotubes was inhibited by 1 mM decamethonium, no patches were observed. The patches found by light microscopy on myotubes cocultured with NG108-15 cells were shown by electron microscopy to correspond to regions of high α-btx binding site density, and the patches did not result from local membrane infolding (see also ref. 11). Hence, the patches of intense staining on the myotubes in untreated muscle cultures or in muscle cultures treated with conditioned media were taken to represent AcCho receptor aggregates. Regions of intense fluorescence on chick myotubes stained with tetramethyl rhodamine–α-btx were likewise defined as AcCho receptor aggregates.

Cocultures. Mouse myotubes cocultured for 7 days with NG108-15 cells as described (8) had a 3.8-fold larger A/M than myotubes cultured alone. This increase in the number of AcCho receptor aggregates occurred without any noticeable change in the number or morphology of myotubes, and no regions of intense staining were detected on NG108-15 cells. Although the presence of Bt_2cAMP enhances synaptogenesis in NG108-15 cell–mouse myotube cocultures (14), approximately the same increase in AcCho receptor aggregates per myotube in cocultures occurred in the absence of Bt_2cAMP (Fig. 2).

The Effect of Conditioned Medium. Direct contact of NG108-15 cells with muscle cells was not required to produce an increase in the A/M. Some mouse muscle cultures were fed every day for one week with medium from NG108-15 cell myotube cocultures diluted 1:2 with fresh medium. These muscle cultures had an A/M 2.2-fold greater than that found in muscle cultures in which half of the medium was replaced daily with fresh medium.

The AcCho receptor-aggregation factor was produced by NG108-15 cells grown alone, and its production and action did not require serum. As shown in Table 1A, mouse muscle cultures that were fed daily for 6 days with serum-free medium that had been conditioned by contact for 1 day with cultures of differentiated NG108-15 cells had an A/M 4.7-fold larger than that of muscle cultures fed with serum-free fresh medium (control medium). In the three experiments described in Table 1, A–C, mouse myotube cultures treated with control medium had an average A/M of 0.335 (SEM = 0.032, $n = 6$), whereas cultures treated with NG108-15 cell-conditioned medium had an average A/M of 1.17 (SEM = 0.037, $n = 6$). The addition of

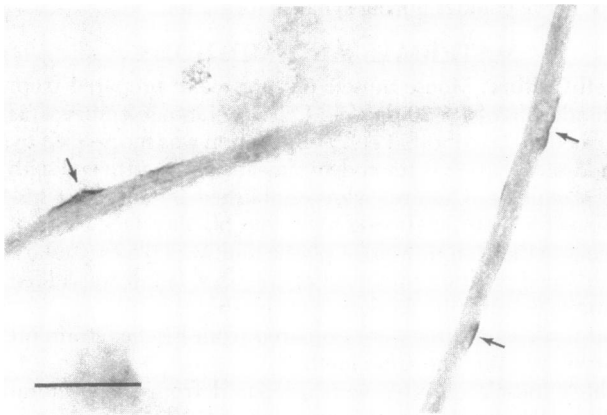

FIG. 1. Bright field photomicrograph of cultured mouse myotubes treated with NG108-15 cell conditioned medium and then stained by the α-btx–immunoperoxidase method to reveal AcCho receptor distribution. Three AcCho receptor aggregates can be seen (arrows). Aggregates are more easily detected at the edge of the muscle fibers (see ref. 11). Reference bar = 25 μm.

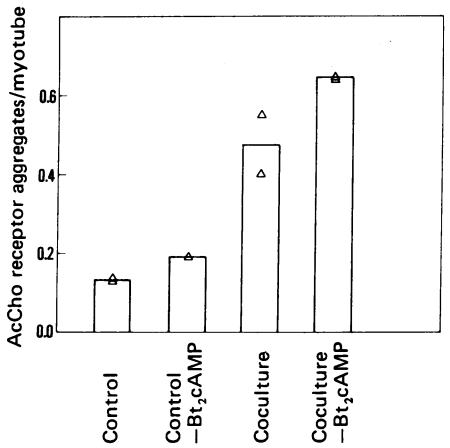

FIG. 2. The effect of coculture with NG108-15 cells on the number of AcCho receptor aggregates per myotube found on mouse muscle. Cocultures of mouse myotubes and predifferentiated NG108-15 cells were prepared as described (8). All cultures were x-irradiated (8000 Rad) 2 days after coculture and then Bt_2cAMP was removed from the medium of the cultures indicated. One week later all cultures were stained by the immunoperoxidase procedure. Cultures were not x-irradiated in subsequent experiments.

horse serum to control medium produced only a 1.4-fold increase in the A/M of muscle cultures.

Cellular and Species Specificity. Medium conditioned by cells of the hybrid parent mouse neuroblastoma clone N18TG-2(15) increased the A/M 2.4-fold in mouse myotube cultures; this effect is comparable to that of NG108-15 cell-conditioned medium. Medium conditioned by cells of the other hybrid parent clone, the rat glioma C6BU-1(16), had no effect on the A/M (Table 1B). Two non-neural cell types did not produce AcCho receptor aggregating activity. Conditioned medium obtained from cultures of HeLa cells did not alter the A/M, and medium from cultures of human diploid fibroblasts decreased the A/M (Table 1C). NG108-15 cells secrete AcCho into the culture medium (17). However, the addition of AcCho (1 mM) to muscle cultures in control medium did not increase the A/M, but decreased it to one-third of the control A/M.

Undifferentiated NG108-15 cells also produced receptor-aggregating activity. Medium conditioned by log phase NG108-15 cells that had not been treated with Bt_2cAMP caused an increase in the A/M of rat muscle cultures similar to that found in rat cultures treated with medium conditioned by differentiated NG108-15 cells (Table 1D).

NG108-15 cell-conditioned medium increased the A/M of cultures of rat or chick muscle. The mean increase in the A/M of rat muscle cultures from three separate experiments was 2.5 (SEM = 0.31, $n = 4$) after a 24 hr treatment with conditioned medium (see also Table 1D). The mean increase in the A/M of chick myotubes in three separate experiments was 4.37 (SEM = 0.54, $n = 4$) after treatment with conditioned medium for 24–36 hr.

Properties of the AcCho Receptor-Aggregation Factor. The AcCho receptor-aggregating activity of NG108-15 cell-conditioned medium was reduced by dilution with control medium. A 1:5 dilution reduced the activity by approximately one-half. After dialysis of NG108-15 cell-conditioned medium against 100 volumes of control medium for 16 hr, the receptor-aggregating activity was retained by the dialysis membrane. The activity was also retained after exhaustive dialysis against water prior to lyophilization. Treatment at 100° for 15 min abolished the receptor-aggregating activity of NG108-15 cell-conditioned medium. The conditioned medium retained its activity when passed through a 0.45 or 0.22 μm pore size filter (Millipore), provided that bovine serum albumin at 1 mg/ml was added to the medium.

Table 1. Changes in the number of AcCho receptor aggregates per myotube produced by conditioned media and various treatments

Exp.	Treatment	A/M	(A/M)/(A/M)con
A	Six-day exposure to:		
	1. Control medium	0.25	1.0
	2. NG108-15 cell-conditioned medium	1.17	4.7
	3. Control medium + 5% horse serum	0.36	1.4
B	24-hr exposure to:		
	1. Control medium	0.38	1.0
	2. NG108-15 conditioned medium	1.09	2.87
	3. N18TG-2 conditioned medium	0.92	2.42
	4. C6BU-1 conditioned medium	0.38	1.0
C	36-hr exposure to:		
	1. Control medium	0.37	1.0
	2. NG108-15 conditioned medium	1.27	3.4
	3. Human diploid fibroblast-conditioned medium	0.18	0.49
	4. HeLa cell-conditioned medium	0.41	1.11
	5. Control medium + 1 mM AcCho	0.11	0.29
D	36-hr exposure to:		
	1. Control	0.76	1.0
	2. Differentiated NG108-15 cell-conditioned medium	1.62	2.89
	3. Control medium (no Bt_2 cAMP)	0.56	1.0
	4. Undifferentiated NG108-15 cell-conditioned medium	1.90	3.39

Mouse muscle cultures were prepared in 35-mm tissue culture plates as described in *Materials and Methods*. In experiment A, mouse muscle cultures received daily feedings for 1 week with NG108-15 cell-conditioned medium, control medium plus 5% horse serum, or control medium alone. The muscle cultures in experiment B were prepared from C3H/HEN strain mice. All conditioned media were prepared by adding control medium (containing Bt_2cAMP) to cells grown in plastic tissue culture flasks untreated with collagen. In experiment C, mouse muscle cultures were grown for 4 days in DMEM containing no serum, and then they were given one feeding with the indicated medium 36 hr before staining. Conditioned media were prepared from a confluent log phase culture of human diploid fibroblasts and a subconfluent log phase HeLa cell culture grown in tissue culture flasks not treated with collagen. In experiment D, 8-day-old rat muscle cultures grown in 16-mm wells were washed for 1 hr in DMEM and then treated for 36 hr with control or conditioned media.

Mechanism of Action of the Aggregation Factor. The increase in the A/M produced by NG108-15 cell-conditioned medium was not due to an increase in the number of myotube AcCho receptors. The total number of AcCho receptors in muscle cultures was determined by an ^{125}I-labeled α-btx binding assay (Table 2). Muscle cultures treated with NG108-15 cell-conditioned medium showed a 3.4-fold increase in A/M when compared to muscle cultures receiving control medium, but these cultures showed only a minor increase (<20%), if any, in the total number of α-btx binding sites. NG108-15 cell-conditioned medium had little effect on the average size of the immunoperoxidase stained receptor aggregates. The average

Table 2. A comparison of AcCho receptor aggregates per myotube with the quantity of α-btx bound per culture

Source of medium	A/M	*n*	Ratio	α-btx bound, fmol/dish	*n*
Control medium	0.37	2	1.0	44 ± 15	3
NG108-15 conditioned medium	1.27	2	3.4	52 ± 7	3

Companion plates to the mouse muscle cultures described in Table 1, exp. C, 1 and 2, were labeled with ^{125}I-labeled α-btx, as described in *Materials and Methods*.

length of AcCho receptor aggregate profiles was 9.1 μm (SEM = 0.69; n = 74) in mouse muscle cultures treated with NG108-15 cell-conditioned medium versus 9.8 μm (SEM = 0.80, n = 53) in muscle cultures treated with control medium. Therefore, if a similar density of AcCho receptor in the aggregates of either condition is assumed, treatment of muscle cultures with NG108-15 cell-conditioned medium results in a greater proportion of AcCho receptors found in aggregates.

The NG108-15 cell factor was effective in the absence of myotube protein synthesis. NG108-15 cell-conditioned medium added to muscle cultures along with cycloheximide (100 μg ml^{-1}) produced a 4.6-fold increase in the A/M within 21 hr. The effect of cycloheximide in control medium over this time period was negligible (Table 3A).

AcCho receptors that were in the muscle surface membrane before exposure to the NG108-15 cell-conditioned medium were subsequently incorporated into receptor aggregates under the action of the factor. This was shown by an experiment in which AcCho receptors on the surface of myotubes were labeled with α-btx before the addition of NG108-15 cell-conditioned medium (Table 3B). Because the toxin binds tightly to the AcCho receptors, those receptors later inserted into the myotube plasma membrane should not have contributed to the subsequent immunoperoxidase staining. The NG108-15 cell-conditioned medium produced approximately the same increase of the A/M in cultures prelabeled with toxin and treated with conditioned medium as it did in cultures treated with conditioned medium and subsequently labeled with the toxin.

Table 3. Effect of protein synthesis inhibition and prelabeling with α-btx on AcCho receptor aggregation

Exp.	Treatment	A/M	(A/M)/(A/M)con
A	21-hr exposure to:		
	1. Control medium	0.52	1.0
	2. NG108-15 conditioned medium + cycloheximide	2.45	4.6
	3. Control medium + cycloheximide	0.48	0.92
B	Pre- versus post-labeling with α-btx		
	1. Control medium, post-labeled	0.38	1.0
	2. NG108-15 conditioned medium, post-labeled	1.27	3.3
	3. Control medium, pre-labeled	0.23	0.61
	4. NG108-15 conditioned medium, pre-labeled	1.13	3.00

Mouse muscle cultures in experiment A were grown for 1 day in DMEM and then given control or conditioned medium containing cycloheximide at 100 μg ml^{-1}. The (A/M)con used to compute the fold change in A/M was taken from muscle cultures given control medium minus cycloheximide. In experiment B, all muscle cultures were grown for one day in DMEM containing no serum. Cultures to be prelabeled were incubated for 1 hr with 0.2 nM α-btx, washed four times with DMEM containing bovine serum albumin at 1 mg ml^{-1}, and then treated for 24 hr with either NG108-15 cell-conditioned medium or control medium. All cultures were then fixed and stained for bound α-btx. The fold change in A/M is related to muscle cultures treated with control medium and post-labeled with α-btx.

DISCUSSION

NG108-15 cells synthesize and release AcCho (17) and form functional cholinergic synapses with striated myotubes derived from embryonic skeletal muscle of various species (8, 18) and with myotubes derived from a myogenic cell line (19). In a previous study, iontophoretic application of AcCho showed that cultured mouse myotubes have a generally low sensitivity to AcCho but possess a few small areas of high AcCho sensitivity. It appeared that at functional synapses NG108-15 cell AcCho release sites are juxtaposed to areas on mouse myotubes with a high sensitivity to AcCho (19). The present study shows that coculturing mouse myotubes with NG108-15 cells produces a 3-fold increase in the number of AcCho receptor aggregates per myotube. Furthermore, NG108-15 cells release a factor which increases the number of receptor aggregates found on myotubes.

The distribution of myotube AcCho receptors, visualized by staining with the α-btx–immunoperoxidase method or with tetramethyl rhodamine–α-btx, was non-uniform, and many myotubes had one or more small (~10μm diam.) patches of high receptor concentration (see also refs. 9, 13, 20, 21), which we call AcCho receptor aggregates. NG108-15 cell-conditioned medium increased the number of myotube AcCho receptor aggregates but did not alter their average size or appearance.

The receptor-aggregation factor elaborated by NG108-15 cells is a heat-labile macromolecule. The AcCho receptor-aggregating activity does not result from the removal of a compound from the control medium by NG108-15 cells, because the activity remained even after the conditioned medium was dialyzed, lyophilized, and reconstituted with 100% control medium. The aggregating activity is not the result of nonspecific proteins released into the medium by NG108-15 cells, because serum added to chemically defined medium does not produce a substantial increase in the number AcCho receptor aggregates. The detection of aggregating activity in NG108-15 cell-conditioned medium that was dialyzed and lyophilized indicates that the factor is not a small metabolite released from NG108-15 cells and that it probably has a molecular weight greater than 10,000. Passage of the factor through sterilizing filters indicates that it is not a cell fragment larger than 0.22 microns in diameter.

Either coculture with NG108-15 cells or treatment with NG108-15 cell-conditioned medium increases the number of myotube AcCho receptor aggregates in muscle cultures. It remains to be determined whether the increased number of myotube AcCho receptor aggregates produced by innervation (22) or the formation of AcCho receptor aggregates under a presynaptic neurite (23) are caused by the contact of pre- and postsynaptic membranes or by the local action of a soluble factor released from the presynaptic neurite.

Of the cells tested in the present study, only neuronal cell types produced detectable amounts of AcCho receptor-aggregating activity, although the aggregation factor was expressed independently of the cells' ability to synthesize and release AcCho. The parent mouse neuroblastoma clone N18TG-2, when differentiated, expresses the neuronal characteristics of process extension and electrical excitability (15) but has little choline acetyltransferase activity (EC 2.3.1.6). Cells of this clone produced the AcCho receptor-aggregation factor. Treatment of log phase NG108-15 cells with Bt_2cAMP promotes

the formation of long neurites and electrical excitability (24) and increases the synthesis and secretion of AcCho (17). Log phase NG108-15 cells also produced detectable amounts of aggregation factor, although they synthesize and release much less AcCho than NG108-15 cells induced to differentiate with Bt_2cAMP. Thus the production of the aggregation factor is not correlated with the synthesis of AcCho. C6BU-1 and HeLa cells as well as diploid fibroblast cells lack neuronal characteristics and did not release quantities of aggregation factor detectable by the AcCho receptor-aggregation assay. Cells of other clones and cells from normal tissues must be assayed to determine the general cellular specificity of the production of AcCho receptor-aggregating activity.

The effect of the AcCho receptor-aggregation factor produced by the NG108-15 cells is not species specific. Medium conditioned by NG108-15 cells produced an increase in A/M on the myotubes of mouse, rat, or chick. This finding is consistent with the proposed role of the factor in synaptogenesis, because NG108-15 cells form cholinergic synapses when cultured with mouse, rat, or chick myotubes (18). Interspecies synaptogenesis has been shown to take place between cultured myotubes of various species and explants of normal spinal cord (25, 26).

The mechanism of action of the receptor-aggregation factor seems to involve the redistribution of receptors in the myotube membrane and does not require the synthesis of new protein. Receptors can move laterally in the plasma membranes of cultured myotubes (21), and within hours AcCho receptor aggregates can form or disappear (23, 27). Apparently, the NG108-15 cell factor induces the aggregation of diffusely distributed mobile muscle AcCho receptors, or it stabilizes AcCho receptor aggregates once they have formed. Aggregation can occur while the AcCho binding sites are occupied by α-btx, as was found for the localization of AcCho receptors at the regions of contact of presynaptic neurons on myotubes (27–29).

Various mechanisms have been proposed to explain the aggregation of cell surface receptors by diffusely applied substances (6, 7). There are AcCho receptor aggregates in muscle cultures even without aggregation factor, thus the aggregation factor may be activating a mechanism which is partially activated even in untreated myotubes. On the other hand, the factor may provide another mechanism for AcCho receptor aggregation, such as by making a covalent modification of AcCho receptors (30) that increases their affinity for one another, or by binding to and cross linking AcCho receptors in a lectin-like fashion (31). It remains to be seen whether the aggregation factor is specific to AcCho receptors or whether it affects other cell surface components.

What role might the AcCho receptor-aggregation factor play in synaptogenesis and synaptic specificity? It is an attractive hypothesis that the factor is attached to or released from the presynaptic cell in the region of developing AcCho release sites, thereby producing a local aggregation of myotube AcCho receptors and a juxtaposition of the NG108-15 cell AcCho release sites with regions of high AcCho receptor density on normal mouse myotubes. A similar factor may be involved in the interaction between motor neurons and muscle during the development of the neuromuscular junction.

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