Selective Effects of Ascorbic Acid on Acetylcholine Receptor Number and Distribution

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Abstract. Ascorbic acid in soluble extracts of neural tissue can account for the increase in surface acetylcholine receptors (AChR's) seen on L_5 myogenic cells treated with crude brain extract (Knaack, D., and T. R. Podleski, 1985, *Proc. Natl. Acad. Sci. USA.*, 82:575–579). The present study further elucidates the nature of the response of L_5 cells to ascorbic acid. Light autoradiography showed that ascorbic acid treatment affects both the number and distribution of surface AChR's. Ascorbic acid, like crude brain extracts, caused a three- to fourfold increase in average AChR site density. However, the number of AChR clusters induced by ascorbic acid was only one-fifth that observed with crude brain extract.

The rate constant for degradation of AChR in ascorbic acid–treated cells of 0.037 ± 0.006 h^{-1} ($t_{1/2} = 19$ h) was not significantly different from that in untreated controls of 0.050 ± 0.001 h^{-1} ($t_{1/2} = 14$ h). The increase in AChR site density is primarily due to a 2.8-fold increase in the average rate of AChR incorporation. Ascorbic acid also stimulates thymidine incorporation and increases the total number of nuclei per culture. However, cellular proliferation is not responsible for the increase in AChR's since 10 μM cytosine arabinofuranoside blocks the mitogenic effect without affecting the AChR increase. The specificity of ascorbic acid on AChR expression was established by showing that (*a*) ascorbic acid produced only a slight increase in total protein, which can be accounted for by the mitogenic effect, and (*b*) the normal increase seen in creatine kinase activity during muscle differentiation was not altered by the addition of ascorbic acid.

We conclude that the action of ascorbic acid on AChR number cannot be explained by changes in cell growth, survival, differentiation, or protein synthesis. Therefore, in addition to a minor stimulation of AChR clustering, ascorbic acid specifically affects some aspect of the AChR biosynthetic pathway.

WHEN spinal cord explants are co-cultured with myogenic cells, the expression of acetylcholine receptors (AChR's)[1] on myotubes is affected primarily in two ways. The overall receptor site density is increased and discrete receptor clusters are formed (9, 34). Several studies have suggested that the spinal cord explants could affect AChR number and distribution via the release of soluble factors (2, 7, 17, 34, 41).

Studies using partially purified brain and nerve-derived substances indicate that more than one factor may affect AChR expression. Formation of receptor clusters has been observed in a number of systems and has been associated with components having a range of molecular weights (2, 17, 28, 34). But according to Neugebauer et al. (31), clustering activity is greatest in the high molecular weight range. On the other hand, significant increases in the overall AChR site density have only been demonstrated on primary chick skeletal muscle (17) and the L_5 and L_6 cloned cell lines (21, 23, 31, 34), and are produced by substance(s) with apparent molecular weight(s) of <1,500 (5, 17, 21, 23, 31). Knaack and Podleski

(23) have recently identified ascorbic acid as the low molecular weight component from fetal calf brain responsible for increasing the number of AChR's on the myogenic cell line L_5.

The present studies were undertaken to further elaborate the effect of ascorbic acid treatment on AChR site density and clustering, and to determine whether ascorbic acid treatment can fully account for the effects of brain extract on these parameters. We find that the increase in AChR site density observed with brain extract can be accounted for by ascorbic acid. This increase is due primarily to an increase in the rate of AChR insertion into surface membrane rather than to a decrease in the rate of degradation. Furthermore, general effects of ascorbic acid on protein levels, cell survival, or the fusion of myoblasts cannot account for the AChR increase produced by ascorbic acid. The main difference between ascorbic acid and brain extract is the ability of brain extract to induce a greater number and density of AChR clusters.

Materials and Methods

Cell Culture

L_5 cells were cultured as reported previously (23). Briefly, cells were plated in Dulbecco's modified Eagle's medium (DME) plus 10% heat-inactivated fetal calf serum, at a density of 5×10^4 cells/ml, into individual wells (0.5 ml/well)

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[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor; Ara-C, cytosine arabinofuranoside; α-BGT, α-bungarotoxin; DME, Dulbecco's modified Eagle's medium; [^{3}H]Thy, tritiated thymidine.

of Falcon multiwell tissue culture plates (2.0 cm^2 = growth area). Unless indicated otherwise, 15 μl of a freshly prepared 1 mg/ml ascorbic acid (ICN K&K Laboratories Inc., Plainview, NY) stock, prepared in H$_2$O and sterilized through a 0.45-μm filter (Gelman Sciences, Inc., Ann Arbor, MI), was added to triplicate wells, one dose per day, for two consecutive days beginning either the fourth or fifth day after plating. This dose yields a maximal increase in AChR's (see Fig. 2).

Total Culture–AChR Assay

^{125}I-α-Bungarotoxin (^{125}I-BGT) binding was used to measure AChR levels. In this study, AChR site density refers to the acetylcholine or BGT binding site density, since specific BGT binding has been shown to correlate with acetylcholine sensitivity in rat myotubes (25). ^{125}I-BGT was prepared in our laboratory (26) and used to assay whole culture AChR levels as reported previously (23). The total counts of ^{125}I-BGT bound for a given treatment were calculated as the mean for triplicate determinations per culture. Nonspecific binding was determined for each multiwell plate by preincubating triplicate wells with 100 nM unlabeled BGT for 15 min prior to labeling with ^{125}I-BGT. The mean counts bound per well were taken as the level of nonspecific binding. Specific binding was determined by subtracting the mean nonspecific binding for the respective dish from the mean total binding for a given determination. Standard errors of the mean (SEMs) for specific BGT binding were calculated from the square root of the sums of squares of the standard errors for the nonspecific and total binding.

Autoradiography

L$_5$ cells in 35-mm dishes were labeled for 30 min using 20 nM ^{125}I-BGT in Tyrodes buffer with 2 mg/ml bovine serum albumin. The labeled BGT was aspirated off and the cells were washed in Tyrodes–BSA for a minimum of five rinses of 5 ml each over the course of 30 min. The cultures were then fixed for 30 min in 2% glutaraldehyde in 0.1 M sodium phosphate buffer (dibasic). Glutaraldehyde was removed with three rinses in 0.1 M sodium phosphate plus 0.2 M sucrose. Distilled water was used for the final rinse. After air drying, the cells were prepared for autoradiography by the stripping film method described by Land et al. (25) (for greater detail see reference 35). In this method, a preformed monolayer of Ilford L4 emulsion, dried on a collodion-coated slide, is stripped and applied to the cultures. (The sensitivity of the emulsion had been previously assessed using a known source of iodine 125 [12].) Different exposure times were used for various autoradiograms. In one, the emulsions were overexposed to facilitate identification and counting of receptor clusters. In the other, exposure times were chosen to allow the counting of individual developed grains in regions of the muscle not containing very high density receptor clusters. These exposure times were calculated to give grain densities in the linear part of the dose response curves for Ilford L4 emulsion developed with Kodak D19 (i.e., 0.03 to 0.5 grains/μm^2) (37).

Scanning Electron Microscopy and Autoradiography

Scanning electron microscopy and autoradiography were performed according to the methods outlined by Neugebauer et al. (31).

AChR Site Density Assessed by Grain Counting

Grain counting was performed as described by Neugebauer et al. (31). A grid in the microscope ocular (total area = 7,600 μm^2; subdivision of 39 μm^2 each) was used for grain counting. Myotubes were selected by placing the microscope objective (1,000×, oil immersion) over a randomly chosen area of the dish. The stage was then moved until the first myotube appropriate for counting was intersected with the upper left-hand corner of the gradicule. To be considered appropriate for counting, a myotube area of at least 2,500 μm^2 in size had to be free of overlying debris and mononucleated cells. Any myotube appearing phase bright or damaged was not counted. Average AChR site densities for a 2,500 μm^2 area in a minimum of eight myotubes from each of duplicate dishes were calculated using the equation given in Matthews-Bellinger and Salpeter (29).

Assessment of Receptor Clustering

(a) The number of clusters per square micrometer of myotube was determined from overexposed autoradiograms. Clusters were subjectively defined as discrete regions of intense grain density identified with bright field optics at low magnification. A grid in the ocular (total area = 119 × 10^3 μm^2; subdivision of 600 μm^2) was used to define the dimensions of a field. Clusters substantially less in area than one-fourth of a grid subdivision (i.e., $\ll$ 150 μm^2) were not scored. The myotube surface area present in a particular field was determined

under phase-contrast optics by determining the fraction of grid intersections that fell over myotubes (see reference 31). The number of clusters and myotube surface area were determined for each of 15 fields on duplicate dishes for each treatment.

(b) High density receptor clusters were studied using dishes exposed for grain counting (see above). Clusters were located by systematically scanning sequential fields on a given dish with bright field optics (1000×, oil immersion). A field was defined by the grid in the ocular (7,600 μm^2). Clusters were identified subjectively (as outlined above), and only those greater than one grid subdivision (39 μm^2) in area were scored. Once a cluster was identified, the grain density for the entire cluster was determined as outlined for grain counting. The boundaries of a cluster were defined subsequently as only those grid subdivisions containing a grain density of greater than three times the average intercluster site density previously determined for the entire dish (see ref. 31 for calculation of intercluster site density).

Site densities of 2,000 sites per square micrometer in these experiments are comparable to the high density clusters of Neugebauer et al. (31) with ratios of cluster to intercluster site densities of greater than 9.

^{3}H-Thymidine ([^{3}H]Thy) Incorporation

[^{3}H]Thy incorporation was studied by a modification of the method of Gospodarowicz et al. (13). Each determination was made in triplicate. 10 μl of DME containing 1 μCi of [^{3}H]Thy and 5 ng of unlabeled thymidine were added to each well of L$_5$ cells in multiwell tissue culture dishes. After 4–6 h of incubation at 37°C the medium was aspirated, and the wells were rinsed twice with 1 ml of DME. Phosphate-buffered saline (PBS) (100 μl) was then added to each well and each well was swabbed with a 5-cm-long cotton tipped applicator. Microscopic examination of the wells demonstrated that no cells remained. The wooden ends of the applicators were then inserted into a styrofoam holder, which was inverted so that the tips of the applicators were immersed into a glass bath containing 250 ml of cold 10% trichloroacetic acid. A magnetic stir bar provided gentle stirring. After 20 min, the applicators were removed and washed in the same way for 10 min in cold 5% trichloroacetic acid. A third wash was performed in cold 95% ethanol for 15 min. The applicators were then dried in a 37°C oven. The cotton tips were cut and placed into 10 ml of Aquasol (New England Nuclear, Boston, MA) filled scintillation vials, and counted in a Beckman LS-230 scintillation counter.

Nuclei in Myotubes

Ascorbic acid–treated cultures received 15 μg/d of ascorbic acid according to the protocol used for AChR determination. The distribution of nuclei was determined on day 7 as reported previously (21). In summary, cells were fixed in 95% ethanol for 5 min. The ethanol was then removed and the dishes were allowed to air dry prior to staining for 8 h with hematoxylin. After the hematoxylin stain had been removed, the dish was rinsed several times with ethanol and tap water.

The number of nuclei per myotube was determined under phase-contrast at 240×. For a given treatment, all the nuclei in myotubes for five randomly chosen fields of 53 × 10^3 μm^2 each, were counted in each of two duplicate cultures. The nuclei in nonfused cells were also counted. The means and standard errors for 10 fields in each of triplicate cultures were determined.

Creatine Kinase Determination

Cultures were grown in multiwell plates as outlined above. Duplicate sets of triplicate (day 6 and 7) or quadruplicate (days 3, 4, and 5) wells were dosed with ascorbic acid according to the AChR assay protocol. Cultures were harvested on the specified days by washing each well twice with cold PBS (pH 7.4). The cells from replicate cultures were then harvested with a rubber policeman and pooled. The empty wells were rinsed with 100 μl lysis buffer (50 mM glycylglycine-HCl, pH 6.7, 0.1% 2-mercaptoethanol, and 1% Nonidet P-40, and 0.9 ml PBS). The PBS washes for each culture were added to the harvested cells, sonicated for 15 s at 4°C, and stored in liquid nitrogen. Creatine kinase activity was determined by the spectrophotometric coupled assay of Nielson and Ludvigsen (32). Creatine kinase determination reagents (#45-UV) and purified rabbit creatine kinase standard were purchased from Sigma Chemical Co., St. Louis, MO.

AChR Degradation

Degradation of surface membrane AChR's was determined according to the method of Devreotes and Fambrough (10). Growth media was aseptically removed from all the cultures at the start of an experiment and saved at 38°C. The medium was replaced with Tyrodes-BSA solution, and cultures were labeled with ^{125}I-BGT as described above. The Tyrodes solution from the final

wash of the labeling procedure was aspirated, the reserved media were returned to the cultures, and the labeled cultures were returned to the incubator. Nonspecific binding was determined by pre-treating cultures with unlabeled BGT as outlined above for AChR assay. The amount of BGT bound to the cells was determined at various times over the next 40 h. At each time point, the media was aspirated and each well was rinsed twice with fresh DME without serum supplement. Triplicate determinations of total and nonspecific binding remaining on the tissue culture cells were made for each time point. Cultures were digested overnight in 2.0 N NaOH and harvested as described above.

Protein Assay

The protein content in the cell sonicates prepared for creatine kinase assay was determined by the method of Lowry et al. (27), using bovine serum albumin as a standard. The cell sonicates were diluted ten times with water before adding to the reaction mixture.

Fetal Brain Extracts

0.2 M ammonium acetate fetal calf brain extract was prepared as described previously (23). Newborn rat brain extract was prepared as described by Podleski et al. (34) except using PBS, pH 7.4. Data for crude extract were obtained using the same dosage regime as for ascorbic acid. Extracts were added at doses yielding maximum AChR increases (21, 34).

Results

Ascorbic Acid Increases Total Culture AChR and Surface Membrane AChR Site Density

Measurements of total culture AChR levels (using gamma counting) in untreated cultures showed that BGT binding increased from 0.98 ± 0.50 fM (mean $\pm$ SEM; $n = 3$) on day 4 in culture to 6.33 ± 0.70 fM on day 7. This increase is most likely due to an increase in the total number of myotubes since fusion is occurring rapidly during this period. If ascorbate was added to matched cultures on days 4 and 5, the total BGT bound on day 7 was increased to 20.7 ± 1.2 fM, representing a 3.2-fold increase over that in controls. To determine the effect on AChR site density per unit area of myotube, autoradiography and grain counting were used. L_5 myotubes are very branched (see Fig. 1), and it is impossible to count grains over an entire tube. Average site densities were obtained for at least 2,500 μm^2 per myotube as described in Materials and Methods. Autoradiography showed that ascorbic acid treatment causes a 3.6-fold increase in AChR site density per square micrometer of myotube surface (Table I). This increase was of the same magnitude as the increase in AChR per total culture observed with gamma counting techniques and is not significantly different from that occurring after treatment with crude fetal calf or rat brain extract. Control levels of AChR site density on day 7 for three experiments were 73.6 ± 11 (mean $\pm$ SEM) ^{125}I-BGT binding sites per square micrometer. The mean site density on cultures treated with 30 $\mu g/ml$ of ascorbic acid was 270 ± 32 ^{125}I-BGT binding sites/μm^2. These data indicate that the AChR increase observed in response to ascorbic acid is not due to a survival- or differentiation-promoting effect of ascorbic acid, leading to a greater number of differentiated myotubes, each with normal AChR levels. Rather, the increase in AChR's is due to an increased level of surface AChR expression in individual differentiated myotubes.
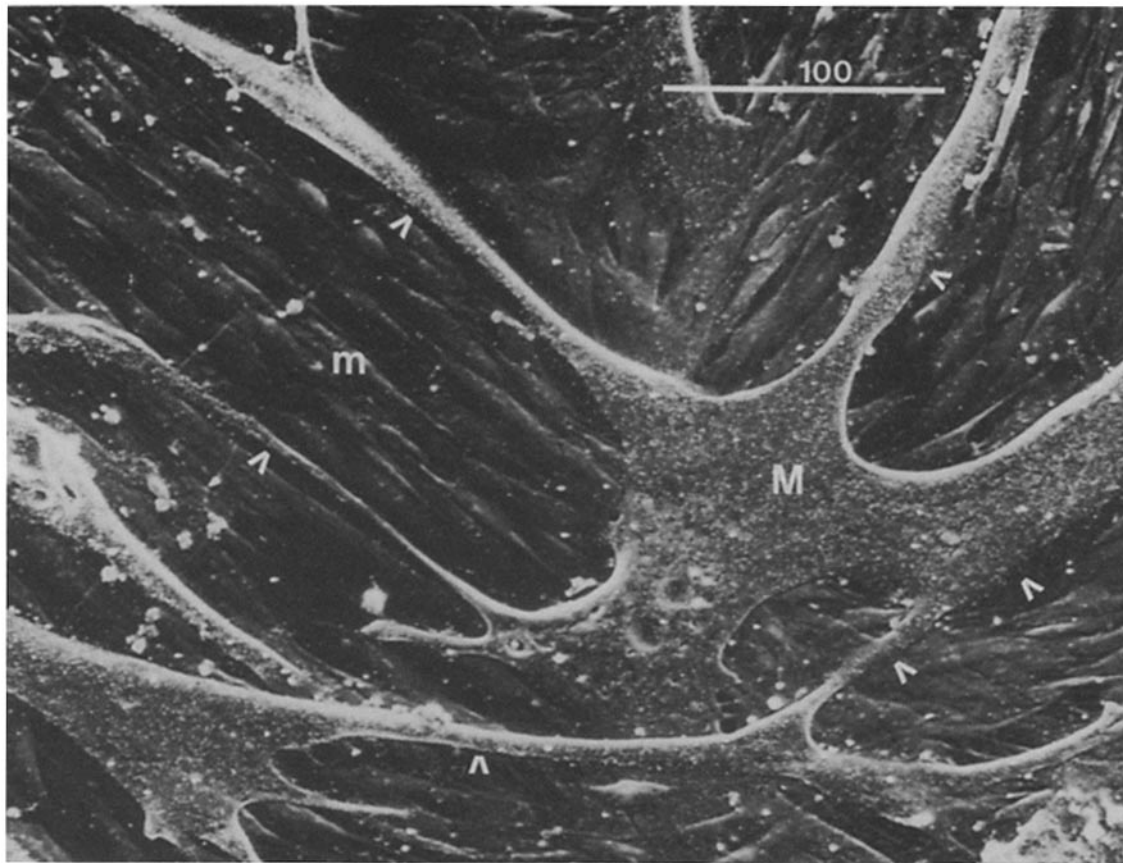

Figure 1. Scanning electron microscope autoradiogram of L_5 cell. This micrograph illustrates the highly branched nature of the myotubes (*M*) from these cells. Arrowheads help delineate the cell outline. Developed grains are seen as fine white specks generally uniformly distributed throughout myotube, but not present on mononucleated cells (*m*). Bar, 100 μm.

Table I. Crude Extract and Ascorbic Acid: A Comparison of Cellular Effects

Parameter measured	Crude brain* extract/control (± SEM)	Ascorbic acid/control[‡] (±SEM)
Total protein[§]	1.39 ± 0.04 (5)	1.25 ± 0.04 (4)
CK activity/well	1.10 ± 0.10 (5)	1.18 ± 0.10 (4)
[³H]Thy incorporation (day 6)	3.10 ± 0.50 (4)	2.66 ± 0.45 (4)
AChR total culture[∥]	4.10 ± 0.80 (5)	3.50 ± 0.45 (5)
AChR site density[¶]	2.83 ± 0.45 (2)	3.67 ± 0.48 (3)

The values given are the mean values for extract- or ascorbic acid–treated cultures, divided by the value for untreated controls. The values given were determined on day 7, using the standard dosage regime, except for thymidine incorporation which was determined on day 6. The number in parentheses refers to the number of experiments done.

* Fetal calf brain extract was used for all determinations except AChR site density, in which newborn rat brain extract was used.

[‡] See Fig. 3 and Results for actual experimental values.

[§] The level of total protein in ascorbic acid–treated cultures was significantly different ($P < 0.05$) from the level in extract-treated cultures. No other determinations were significantly different between extract- and ascorbic acid–treated cultures.

[∥] By gamma counting.

[¶] By light microscope autoradiography.

AChR Accumulation Is Independent of Myoblast Proliferation

The addition of ascorbic acid induces myoblast proliferation in L_5 cultures. This was established by determining the level of [³H]Thy incorporated into chromosomal DNA during 4-h pulse periods as outlined in Materials and Methods. 24 h after the first dose of ascorbic acid, the amount of [³H]Thy incorporated over the 4-h pulse period was 2.0 ± 0.28 (mean ± SEM, $n = 4$) times the amount of untreated controls. If [³H]Thy was added at 48 h after the first dose of ascorbic acid, incorporation over the 4-h pulse period was 2.66 ± 0.45 (mean ± SEM, $n = 4$) times control, and if added at 72 h (the time of the AChR assay), [³H]Thy incorporation was approximately equal to controls.

Since increased [³H]Thy incorporation over the first 48 h indicated that there was an induction of myoblast division following the addition of ascorbic acid, there was a possibility that ascorbic acid caused some change in the newly divided mononucleated cells, which lead to increased AChR expression. Two experimental results indicate that the mitogenic effect is not responsible for mediating the effects of ascorbic acid on AChR levels. (a) Although the total number of mononucleated cells formed in the presence of ascorbic acid is 2.30 ± 1.34 (mean ± SEM, $n = 3$) times control, most of these myoblasts had not fused by the time the AChR determination was made. On day 7 the number of nuclei present in myotubes of ascorbic acid–treated cultures was 1.38 ± 0.29 times control. (b) The mitogenic effect of ascorbic acid can be blocked by the addition of 10 μM cytosine arabinofuranoside (Ara-C) without altering the effect of ascorbic acid on the level of AChR's. In four paired experiments, the maximum response to ascorbic acid in Ara-C–treated cultures was 3.6 ± 0.63 (mean ± SEM) times control which was not significantly different from the maximum response of 3.8 ± 0.18 (mean ± SEM) times control obtained from cultures in the absence of Ara-C. Representative dose response curves with and without Ara-C are shown in Fig. 2. Under these

conditions, Ara-C reduced thymidine incorporation over the pulse period by greater than 90%, from 16,933 ± 972 to 616 ± 265 cpm (mean ± range; $n = 2$) in ascorbate-treated cultures and from 6,396 ± 1,022 to 593 ± 243 cpm in untreated controls.

AChR Induction Is Due to an Increased Rate of AChR Insertion into the Surface Membrane

Autoradiography and the total culture AChR assay by gamma counting are static measures of the number of AChR on the myotube membrane surface. The ascorbic acid–induced AChR increase could be due to either an increased rate of insertion of receptors into the muscle membrane surface, or to a slowing of the degradation rate of surface AChR's. The rates of receptor degradation and accumulation were therefore measured in ascorbate-treated and control cultures. A slight decrease in degradation rate was found after ascorbate treatment, from a mean rate constant of 0.050 ± 0.001 h⁻¹ (mean ± SEM; $n = 5$) ($t_{\frac{1}{2}} = 13.9$ h) in untreated controls, to one of 0.037 ± 0.006 h⁻¹ ($t_{\frac{1}{2}} = 18.7$ h) for ascorbate-treated cultures. Although these means are not significantly different at $P = 0.1$ (Students' t test with Cochrans' distribution for nonhomogeneous variance [8]), the variances are ($P < 0.05$) (8). These data suggest a small, but not consistent, effect of ascorbate on receptor degradation rate under our experimental conditions. Both the means and the change in variance are in close agreement with the values that were reported by Neugebauer et al. (31) for L_5 cells with and without brain extract treatment.

Given the observed rates of receptor degradation, the extent to which the increased receptor site density must be due to an increase in the rate of receptor insertion was calculated using the equation of Schimke and Doyle (38):

$$dA/dt = k_+(t) - k_-A(t),$$

where, at a given time (t), dA/dt is the average net AChR accumulation rate, A equals the concentration of AChR, k_+ is the AChR incorporation rate and k_- is the degradation rate for AChR. Fig. 3A illustrates that AChR levels in untreated control dishes increased continuously over the experimental period. This increase may be due to the formation of new myotubes during the experiment or to an ongoing increase in the synthesis of receptors. Such an ongoing increase in AChR levels suggests an accelerating k_+ when assessed as insertion rate per dish. The average net AChR accumulation in controls dishes for three such experiments was found to be 0.087 ± 0.034 fM/h per dish (SEM). Using this value for dA/dt, and the degradation rate determined above, the average AChR insertion rate (k_+) in control cultures over the 72-h experimental period was calculated to be 0.31 ± 0.09 (mean ± SEM; $n = 3$) fM/h per dish. With this calculated insertion rate, it is clear that the AChR levels observed experimentally at 72 h after the first addition of ascorbate (i.e., a 3.5-fold increase) cannot be achieved merely by decreasing the degradation rate from the 0.050 h⁻¹ to the 0.037 h⁻¹ value obtained after ascorbate treatment. Such a change in degradation rate would increase receptor site density only 1.25-fold in 72 h. In fact, if the insertion rate calculated for control cultures remained the same after ascorbate, there is no rate of degradation which could produce a 3.5-fold increase in receptors in 72 h or less. Using the above equation and the degradation rate measured in ascorbate-treated cells, the average insertion
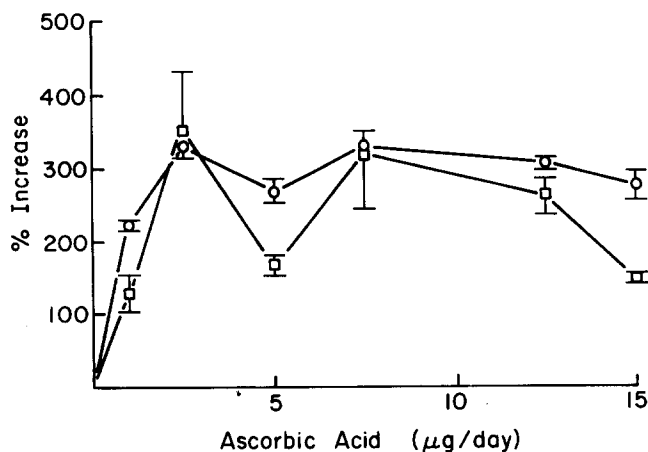
Figure 2. Effects of Ara-C on the ascorbic acid–induced AChR increase. AChR level is plotted as a function of dose of ascorbic acid. AChR levels are given for cultures receiving 24-h pre-treatment with Ara-C (□) or no Ara-C (O). Media concentration of Ara-C in treated cultures was 10 μM. Percent increase = $[(EXP - CTL)/CTL] \times 100$, where EXP is the mean specific counts bound to cultures receiving a given dose of ascorbic acid and CTL is the mean specific counts bound to control cultures not receiving ascorbic acid. Individual control values were determined for cultures receiving Ara-C and for those that did not. Associated relative errors were calculated as the square root of the sums of squares of the relative error for controls and experimentals.

rate (k_+) was calculated for the ascorbate-treated cultures. A 2.8-fold increase was obtained for the average k_+, from 0.31 ± 0.09 fM/h per dish in control cells to 0.85 ± 0.2 fM/h per dish in ascorbate-treated cells. Since the measurements of creatine kinase (Table I) and nuclei in myotubes (see previous section) indicate ascorbic acid does not stimulate myoblast fusion, the 2.8-fold increase in k_+ applies to the insertion rate per unit area of myotube even though the actual k_+ values are a composite rate for both the appearance of new myotubes and the insertion rate per myotube.

Acetylcholine Receptor Induction Is a Specific Response to Ascorbic Acid

To determine whether the stimulation of new receptors by ascorbic acid, described above, was due to an overall effect on protein synthesis, two experimental approaches were used. First, the effect of ascorbic acid on cellular total protein levels was determined. Total protein levels in ascorbic acid and control cultures were followed for a number of days. 3 d after the first addition of ascorbic acid and 2 d after the final addition, the total protein level was increased to 170 ± 4 μg/dish (mean ± SEM; n = 3), ~25% greater than the 138 ± 8 μg measured in untreated controls (Fig. 3B). This increase in total protein is an order of magnitude less than the increase in AChR (Fig. 3A). Furthermore, the increase in total protein must be due to the mitogenic effect of ascorbic acid described above. We determined that there is ~0.5 ng of protein per myoblast in exponentially growing cells. With the total increase in mononucleated cells on day 7 in ascorbate-treated cultures (~7 × 10⁴ cells), an increase in total protein of 36 μg was expected. This compares well to the observed total protein increase of 32 μg. Thus ascorbic acid does not stimulate a general increase in total protein synthesis in differentiated myotubes.
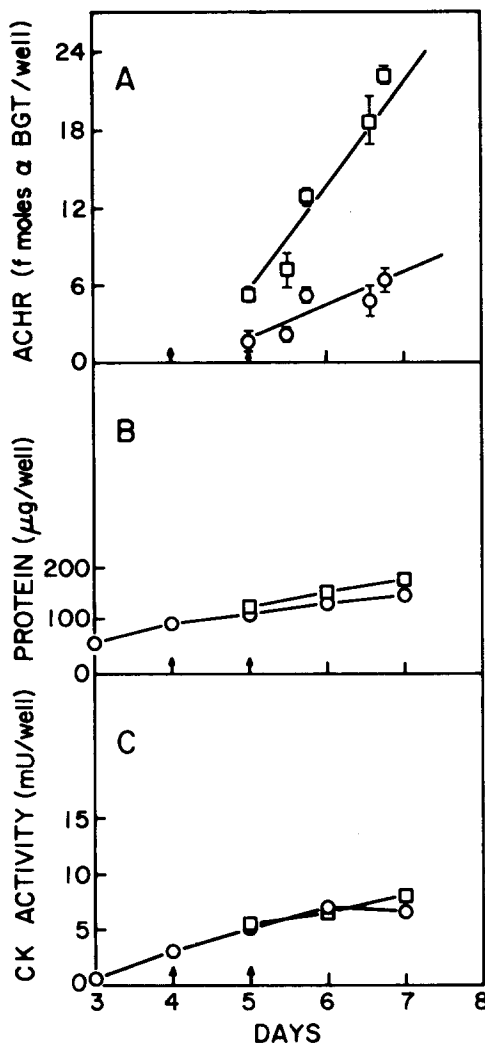
Figure 3. Time course of ascorbic acid effects on AChR's (A), total protein (B), and creatine kinase (C). The level of each parameter measured is presented here as a function of days in culture. In each panel the responses to ascorbic acid are indicated by (□) and untreated controls by (O). 15 μg of ascorbic acid were added on days 4 and 5 (arrows). The data in A is from a single experiment for which most data points were available. Average data from three such experiments is given in Results. B and C represent the pooled data from three experiments. Error bars in A are SEMs for triplicate determinations. Error bars in B and C (determined as for A) are within the size of the symbols and thus not shown.

The second test for the specificity of the ascorbate effect was the determination of creatine kinase levels, another developmentally regulated protein of differentiating muscle (39). The development of creatine kinase activity in L₅ cells during differentiation is shown in Fig. 3C. Between days 3 and 7, when the cells are fusing and differentiating into myotubes, there is a 14-fold increase in creatine kinase activity from 0.50 ± 0.01 U/well (mean ± SEM; n = 3) to 6.80 ± 0.40. No significant difference, however, was observed between cells treated with ascorbic acid and the untreated control cells. The levels of creatine kinase activity are known to correlate well with the extent of muscle differentiation (39). These results not only confirm that ascorbic acid treatment induces no general increase in total protein synthesis but also that it does not stimulate the differentiation of new myotubes.

Ascorbic Acid and Fetal Brain Extract Induce AChR Expression with Similar Specificity

Since ascorbic acid was found to be the major component in the fraction of brain extract causing receptor induction (23), brain extract was compared with ascorbic acid to see if it has the same specificity with respect to the other parameters measured. The data are given in Table I. Among those parameters studied, only the total protein levels were significantly different between cultures treated with ascorbic acid and those treated with brain extract. The difference, however, was only 10%. It is concluded from this comparison that ascorbic acid and brain extract have similar specificity in their induction of AChR expression in L_5 cells.

Ascorbic Acid Induces AChR Clustering

Previous studies have shown that brain extracts also induce the clustering of AChR on L_5 cells. Those clusters produced by crude brain extract are induced mainly in response to high molecular weight components. Clusters in extract-treated cultures occur more frequently and with higher site densities than those produced by a partially purified low molecular weight active component (31). Therefore, ascorbic acid was compared to brain extracts for its effect on receptor clustering.

Overexposed autoradiograms were prepared for both ascorbic acid and newborn rat brain extract-treated cultures (see Materials and Methods). Dishes were scanned and clusters identified subjectively (see Fig. 4). The frequency of occurrence of clusters was determined for cells treated with either ascorbic acid or brain extract, as well as for untreated control cells. Extract-treated cultures had ~25 times more clusters per unit area of myotube than control cultures, and 4.5 times more clusters than cultures receiving ascorbic acid (Table II). The effect of ascorbate was thus similar to that of the low molecular weight component of brain extract (31).

Duplicate autoradiograms were also prepared with shorter exposure times intended to be appropriate for grain counting. Clusters were often found to have high density cores (defined as at least 39 μm^2 with average site densities >2,000 sites/μm^2) which tended to locally overexpose the emulsion. Clusters with such high density cores, which we will call "high density clusters," were easily identified subjectively. High density clusters were not seen in untreated controls and represented 20–30% of the total number of clusters in dishes receiving ascorbic acid or brain extract (Table II). Again, there were ~5 times more high density clusters per unit area on brain extract than on ascorbic acid–treated cells. To estimate
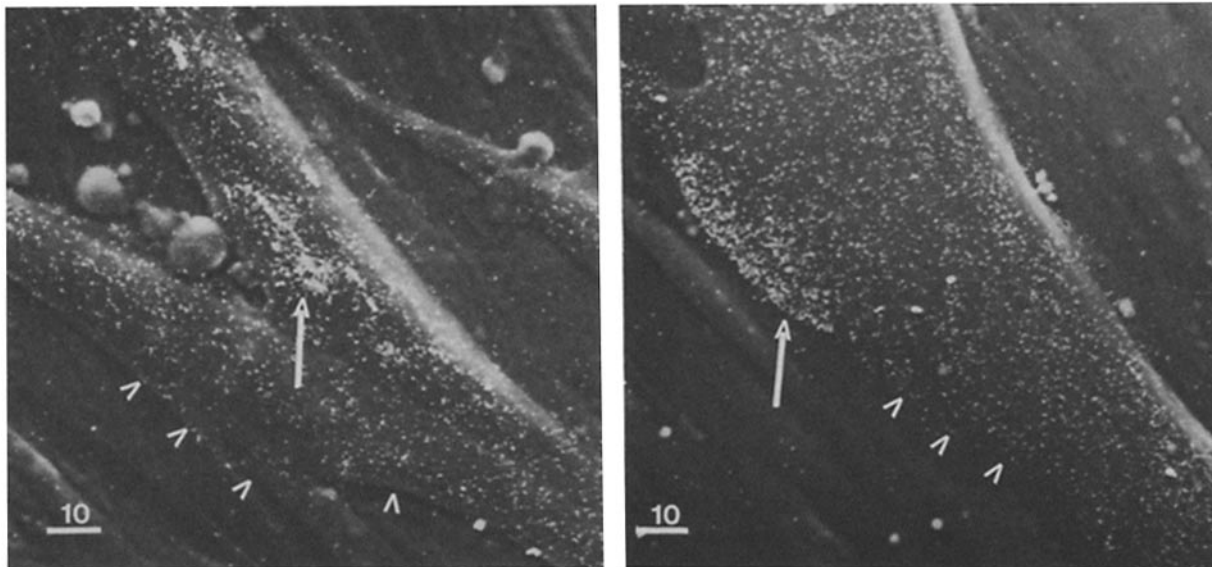

Figure 4. Overexposed scanning electron microscope autoradiogram of AChR clusters on extract-treated (*left*) and ascorbic acid–treated (*right*) L_5 cell. Arrowheads help delineate cell margins and arrows point to receptor clusters illustrating variation in cluster shape. Both types of clusters were seen on either treatment. Bar, 10 μm.

Table II. Induction of AChR Clusters

	Control	Fetal rat brain extract		Ascorbic acid	
Number of clusters (per 10^6 μm^2 of myotube)*	2.87 ± 1.7	71 ± 17		15.8 ± 6	
% of total clusters that are high density clusters‡	0	22.2 ± 8		27.7 ± 6.5	
Average size (μm^2) of high density clusters§‖	0	148.6 ± 16	(31)	88.5 ± 21	(11)
% (× 10^2) of myotube surface area that is >2,000 sites/μm^2	0	18.0 ± 0.2		2.1 ± 0.2	

* Determined from overexposed autoradiograms (see Materials and Methods for details).

‡ A high density cluster is defined as one with a minimum core area of 39 μm^2 containing an AChR site density ≥2,000 sites/μm^2. The number of high density clusters per μm^2 of myotube determined from low exposure autoradiograms (0.035–0.5 grains/μm^2) was divided by the number of clusters per μm^2 of myotube determined from overexposed autoradiograms.

§ Determined from autoradiograms exposed for grain counting (see Materials and Methods). These numbers represent the average area of the high density clusters. See Materials and Methods for determination of total cluster boundaries by the "3 times" criteria. We assayed 2.38–2.57 × 0.06 μm^2 of myotube per dish per experiment (520–552 counting fields). Numbers in parentheses indicate the total number of high density clusters examined.

‖ In addition to a larger overall size of the high density clusters in extract-treated cells, the size of the high density core increased as well, making the % area of the high density clusters occupied by the high density core roughly the same (60–70%) in both extract- and ascorbate-treated cultures.

the size of these high density clusters their boundaries were defined quantitatively relative to average site density (see Materials and Methods). Table II shows that the high density clusters on brain extract–treated cells were ~60% larger than those formed on ascorbate acid–treated ones. The high site density cores of these clusters were also ~60% larger on brain extract–treated cells than on ascorbic acid–treated cells. This increase in core size, in addition to the larger number of high density clusters, resulted in an overall 8.5-fold larger surface area of myotubes occupied by the high density core regions in the extract-treated cultures than in those treated with ascorbic acid.

Discussion

Ascorbic acid was demonstrated previously to be the primary active component in fetal calf brain extract causing increased AChR levels on L_5 cells in vitro (23). This effect was shown to be specific for ascorbic acid and not to be a general property of reducing agents. In the present work the action of ascorbic acid has been studied in greater detail. It was shown that while ascorbic acid can act as a mitogen, the mitogenic effect can be blocked without altering the increase in AChR's. In addition, total protein and creatine kinase levels, whose half-lives are roughly equal to that of AChR's (16, 42, 43), are not increased by ascorbic acid to the extent that the AChR is. Taken together, these results indicate that general effects of ascorbic acid on protein synthesis, cell growth, or fusion cannot account for the more than threefold increase seen in the receptor number. It was also shown that the ascorbic acid–induced elevation of surface AChR site density on L_5 cells must be due primarily to an accelerated rate of receptor insertion (2.8-fold) into the surface membrane since only a slight decrease in degradation rate of receptors is seen. This conclusion is similar to that previously reached for brain extracts on L_5 cells (31) and chick primary muscle cells (17). Preliminary results from our laboratory (22) show an increase in mRNA levels for the alpha subunit of AChR in L_5 cells treated with ascorbate; thus it is likely that elevated levels of receptor synthesis account for the increased receptor insertion.

A number of different studies have reported that, like AChR's, the ascorbic acid levels of skeletal muscle undergo changes both as a consequence of normal cellular differentiation, and after denervation. For example, in both chick and rat muscle, ascorbic acid levels are high before birth and undergo perinatal declines (11, 20, 33), and in several animals ascorbic acid levels have been shown to increase after denervation (14, 24). Levels of AChR in skeletal muscle are also higher in embryonic and denervated muscle than in postnatal and innervated muscle (1, 3, 6, 30). Furthermore, we have found (data not shown) that the ascorbic acid–induced AChR increase in L_5 cells is accompanied by an elevation in intracellular ascorbic acid levels from a value of <0.03 µg/ml total protein to greater than 1.5 µg/mg, a value similar to embryonic skeletal muscle levels (11, 20, 33). This information suggests a direct relationship between intracellular levels of ascorbic acid and AChR synthesis.

Ascorbic acid also causes the formation of AChR clusters on L_5 cells. We found (data not shown) that, as in the case of clusters formed by brain extract (36), ascorbic acid–induced clusters are formed by a redistribution of pre-existing receptors. However, unlike the effect on receptor site density,

ascorbic acid is much less effective than crude brain extract in causing receptor cluster formation. The observations made in the present work are compatible with previous observations (31) that a low molecular weight fraction of crude brain extract, which causes the major increase in surface receptors on L_5 cells, is not the major cluster-inducing substance. Cluster-inducing activity in crude extract has a range of apparent molecular weights but predominates in high molecular weight fractions.

Neugebauer et al. (31) found that unlike L_5 cells, rat primary muscle cells are insensitive to the low molecular weight fraction. Consistent with this, we found that rat primary cells also show no increase in receptor site density after ascorbate (Knaack, D., I. Shen, M. M. Salpeter, and T. R. Podleski, unpublished observations). However, Kalcheim et al. (18, 19) found that ascorbate causes receptor clusters in rat primary cells, which is not compatible with the results predicted from the work by Neugebauer et al. (31). We have not yet examined the cluster response of primary rat cells to ascorbic acid under our culturing conditions.

There are several possible sources of ascorbic acid in vivo which could influence muscle. Blood plasma is one, and the levels of ascorbic acid that we have used are in the range of those found in serum (15). The nervous system may also be a primary source of ascorbate. Brain levels of ascorbic acid are among the highest in the body (15). Furthermore, neuronal release of ascorbic acid has been demonstrated in brain preparations (4), and it has been reported that ciliary ganglia release an ascorbic acid–like substance (40). Since ciliary ganglia contain predominantly cholinergic motor neurons, it is possible that motor neurons could serve as a source of ascorbic acid for skeletal muscle. How ascorbic acid in serum and neural tissue could interact to produce the changes associated with innervation and denervation is not known.

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