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 L5 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 L5 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 (t₁/₂ = 19 h) was not significantly different from that in untreated controls of 0.050 ± 0.001 h⁻¹ (t₁/₂ = 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) 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 L5 and L6 cloned cell lines (21, 23, 31, 34), and are produced by substance(s) of 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 L5.

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

L5 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 x 10⁴ cells/ml, into individual wells (0.5 ml/well)
of Falcon multiwell tissue culture plates (2.0 cm² = 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 H₂O 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**

(125I-)-Bungarotoxin (125I-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 receptor (AChR) myotubes (25). 125I-BGT was prepared in our laboratory (26) and used to assay whole culture AChR levels as reported previously (23). The total counts of 125I-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 125I-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**

L₅ cells in 35-mm dishes were labeled for 30 min using 20 nM (125I)-BGT in Tyrode buffer with 2 mg/ml bovine serum albumin. The labeled BGT was aspirated off and the cells were washed in Tyrode-BSA for a minimum of five 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 Podarowicz et al. (13). Each determination was made in triplicate. 10 µl of DME containing 1 µCi of [125I]Thy and 5 ng of unlabeled thymidine were added to each well of L5 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 230 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 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 Nielsen and Luddvigsen (32). Creatine kinase determination reagents (454 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 (125I)-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 ± 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. L5 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 μm² 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 ± SEM) 125I-BGT binding sites/square micrometer. The mean site density on cultures treated with 30 μg/ml of ascorbic acid was 270 ± 32 125I-BGT binding sites/μm². 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 L5 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**

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

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.
| Insertion into the Surface Membrane |

The addition of ascorbic acid induces myoblast proliferation in Lc 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 controls 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) (t₀ = 13.9 h) in untreated controls, to one of 0.037 ± 0.006 h⁻¹ (t₀ = 18.7 h) for ascorbate–treated cultures. Although these means are not significantly different at P = 0.1 (Students’ t test with Cochran’s 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 Lc 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):

\[
\frac{dA}{dt} = k_+ (t) - k_- A(t),
\]

where, at a given time (t), \( \frac{dA}{dt} \) 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 \( \frac{dA}{dt} \), 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...
Ascorbic Acid (μg/day)

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 (○). Media concentration of Ara-C in treated cultures was 10 μM. Percent increase = [(EXP - CTL)/CTL] × 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 mononucleated cell 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 (○). 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 L5 cells.

Ascorbic Acid Induces AChR Clustering

Previous studies have shown that brain extracts also induce the clustering of AChR on L5 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 μm² with average site densities >2,000 sites/μm²) 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) L5 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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Fetal rat brain extract</th>
<th>Ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of clusters (per 10⁶ μm² of myotube)*</td>
<td>2.87 ± 1.7</td>
<td>71 ± 17</td>
<td>15.8 ± 6</td>
</tr>
<tr>
<td>% of total clusters that are high density clusters*</td>
<td>0</td>
<td>22.2 ± 8</td>
<td>27.7 ± 6.5</td>
</tr>
<tr>
<td>Average size (μm²) of high density clusters*</td>
<td>0</td>
<td>148.6 ± 16 (31)</td>
<td>88.5 ± 21 (11)</td>
</tr>
<tr>
<td>% (× 10²) of myotube surface area that is &gt;2,000 sites/μm²</td>
<td>0</td>
<td>18.0 ± 0.2</td>
<td>2.1 ± 0.2</td>
</tr>
</tbody>
</table>

* 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 μm² containing an AChR site density ≥2,000 sites/μm². The number of high density clusters per μm² of myotube determined from low exposure autoradiograms (0.035–0.5 grains/μm²) was divided by the number of clusters per μm² 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 μm² 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 L5 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 L5 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 L5 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 L5 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 L5 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 L5 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 L5 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 L5 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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