Acetylcholine Receptor α-Subunit mRNA Is Increased by Ascorbic Acid in Cloned L_5 Muscle Cells: Northern Blot Analysis and In Situ Hybridization

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Abstract. Ascorbic acid is the major factor in brain extract responsible for increasing the average acetylcholine receptor (AChR) site density on the cloned muscle cell line L_5. In the present study, we show that this effect of ascorbic acid requires mRNA synthesis, and that the mRNA level for the AChR α-subunit is increased to about the same level as are the surface receptors. We have found no increase in the mRNA levels of the β-, γ-, and δ-subunits, or in the mRNAs of other muscle-specific proteins, such as that of light chain myosin 2, α-actin, and creatine kinase. By in situ hybridization, we further show that the increase in α-mRNA in response to ascorbic acid is exclusively in myotubes and is located near clusters of nuclei. mRNA levels for the α-subunit in mononucleated cells are very low and do not significantly increase in response to ascorbic acid. The mononucleated cells are thus excluded as a possible source for the increase in α-subunit mRNA detected by Northern blot analysis. Our results indicate that there is a very specific action of ascorbic acid on the regulation of AChR α-mRNA in the L_5 muscle cells, and that the expression of surface receptors in these cells is limited by the amount of AChR α-subunit mRNA.

NUMEROUS studies have shown that the levels of acetylcholine receptor (AChR)[1] on the cell surface can be regulated in muscle cells by neural tissue and brain extracts (Podleski et al., 1978; Jessel et al., 1979; Connolly et al., 1982; Buc-Caron et al., 1983; Neugebauer et al., 1985). Several molecules, purified from neural tissue, seem to mediate this effect. One such factor, ascorbic acid (176 mol wt), has been reported to increase surface AChR levels in the rat-derived cloned L_5 muscle cell (Knaack and Podleski, 1985; Knaack et al., 1986). Other factors such as ARIA (43,000 mol wt), purified from chick brain (Usdin and Fischbach, 1986), and CGRP (3,806 mol wt), a neuropeptide present in many parts of the nervous system (Rosenfeld et al., 1983; Mason et al., 1984; Takaim et al., 1985*a*,*b*), have both been reported to increase surface AChR levels in chick primary cells (Usdin and Fischbach, 1986; New and Mudge, 1986; Fontaine et al., 1986). Although it has not yet been demonstrated that any of the above factors affect the synthesis of the AChR in vivo, they provide useful tools for studying the regulation of AChR synthesis.

Since ascorbic acid was shown to increase the rate of insertion of the AChR into the cell surface membrane, without affecting the degradation rate of AChR or increasing overall total protein synthesis (Knaack and Podleski, 1985; Knaack et al., 1986), the work in our laboratory has concentrated on understanding the molecular events involved in the effect of ascorbic acid on the AChR. In the present study, we examined the effect of ascorbic acid on the AChR mRNA in L_5 cells to establish the extent to which the increase in the surface AChR is transcriptionally regulated. Our results show that ascorbic acid induces an increase in α-subunit-specific mRNA which correlates with the increased expression of AChR on the cell surface. Since none of the other subunit mRNAs are elevated, we suggest that the increased expression of surface AChR in response to ascorbic acid is controlled by the availability of only the α-subunit mRNA. By in situ hybridization, we further show that the ascorbic acid-induced increase in α-subunit mRNA is not due to an increase in the number of AChRs producing myotubes nor to expression in mononucleated cells, but is a result of a net increase in α-mRNA per myotube. The α-mRNA molecules in the L_5 cells are predominantly located over, or in the vicinity of, clusters of nuclei along the myotubes. This localization is especially pronounced in the ascorbic acid-treated cells where a 4.6-fold increase in α-mRNA is expressed at such active nuclear clusters.

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; BGT, bungarotoxin.

Materials and Methods

Cell Cultures

L_5 muscle cultures (Yaffe, 1968; Catterall, 1976) were prepared as previously described (Knaack and Podleski, 1985; Neugebauer et al., 1985). Briefly, L_5 cells were plated at a density of 5×10^4 cells/ml on either 100-mm tissue culture dishes containing 10 ml of medium (for RNA preparation), or on multi-well plates containing 0.5 ml medium (for surface AChR studies). Cells were also plated at a density of $1–2 \times 10^4$ cells/ml on 35-mm culture dishes containing 2 ml of medium (for in situ hybridization). The growth medium was DME and 10% FCS. Under these conditions, L_5 cells fuse spontaneously starting on day 3 in culture.

Ascorbic Acid and Rat Brain Extract Treatment

Cells were treated, on either day 4 or on days 4 and 5 of plating, with ascorbic acid (30 μg/ml of growth medium) using a freshly prepared (1 mg/ml) ascorbic acid solution (Knaack and Podleski, 1985) or with rat brain extract (540 μg/ml of growth medium; Podleski et al., 1978; Salpeter et al., 1982). Control cells were treated with an equal volume of PBS. Cultures were harvested at different times after treatment indicated in Results.

Actinomycin D Treatment

Actinomycin D (Calbiochem-Behring Corp., San Diego, CA) at a final concentration of 2.5 μg/ml was added to L_5 cells at the same time as the ascorbic acid, or at different times after ascorbic acid addition. Incubation times with actinomycin D were between 16 and 24 h. At the end of the incubation period, cells were assayed for surface AChR and α-mRNA, as described below. We, as well as others (Yaffe and Feldman, 1964; Hartzell and Fambrough, 1973), observed that the actinomycin D treatment often caused a significant detachment of mononucleated cells with little effect on myotubes. Nevertheless, in each experiment after incubation with actinomycin D, the cultures were examined microscopically to determine that there was no obvious decrease in myotube numbers relative to untreated cultures.

Quantitation of Cell Surface Acetylcholine Receptor

Surface AChR was determined by a ^{125}I-α-bungarotoxin (^{125}I-α-BGT) binding assay (Knaack and Podleski, 1985). ^{125}I-α-BGT (sp act, 50–100 Ci/mmol) was added to cells plated on multi-well dishes (24 wells/dish), at a final concentration of 20 nM. After a 30-min incubation at room temperature, the cell layer was rinsed several times with Hepes-buffered DME (pH 7.4) containing 1 mg/ml BSA, and the cells were solubilized in 0.9 ml of 2 M NaOH. Bound ^{125}I-BGT was determined by gamma counting. Nonspecific binding was determined by a 15-min preincubation with 10 μM unlabeled α-BGT followed by the addition of ^{125}I-α-BGT, as described above.

RNA Preparation, Northern Blot, and Slot Blot Analysis

Total RNA was extracted by the LiCl-urea procedure (Auffray et al., 1980). For Northern analysis, 10 or 20 μg total RNA was electrophoresed in 1.2% agarose-formaldehyde denaturing gel and transferred to GeneScreen Plus (New England Nuclear, Boston, MA) nylon membrane according to suppliers' instructions. Prehybridization and hybridization were performed at 55°C in 50% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl, and 100 μg/ml denatured salmon sperm DNA.

For the detection of AChR α-subunit mRNA, a ^{32}P-labeled RNA probe was used. This probe was made by transcribing and ^{32}P-labeling the 450-bp PstI fragment of a mouse α-subunit-specific cDNA, isolated from the pMARα15 plasmid (Boulter et al., 1985), and subcloning it into a pGEM3 vector.

The β-, γ-, and δ-subunits were detected by hybridization with ^{32}P-labeled nick-translated cDNA probes. Plasmids containing cDNA were provided by J. Patrick, and J. Boulter (Salk Institute) supplied the β- (BMB49; Patrick et al., 1987) and γ-subunits (BMG419; Boulter et al., 1986); and N. Davidson (California Institute of Technology) provided the δ-subunit (p6H; LaPolla et al., 1984).

Hybridized blots were washed with 0.1 × SSC (1 × SSC = 150 mM NaCl, 150 mM sodium citrate), 0.1% SDS at 65°C. The specific mRNA bands were quantified by densitometric scanning of autoradiograms using a densitometer (LKB Instruments Inc., Gaithersburg, MD). Only exposure times, giving optical density values proportional to the amounts of total RNA applied, were used for quantification.

For slot blot analysis, ~5 μg of total RNA was resuspended in 10 μl of formaldehyde-formamide solution denatured at 65°C for 15 min and applied to GeneScreen Plus (New England Nuclear) using a BRL Hybri-slot Manifold (1052MM). After RNA application, gentle vacuum was applied for 15 min until samples were completely adsorbed. Hybridization using a ^{32}P-labeled nick-translated α-specific cDNA (pA59) (Merlie et al., 1983) was performed, as for Northern blots, in 50% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl, and 100 μg/ml denatured salmon sperm DNA, overnight at 65°C. After hybridization, blots were washed for 2 h in 0.1 × SSC, 0.1% SDS at 70°C.

In Situ Hybridization

Preparation of ^{35}S-labeled RNA Probe. A 1.3-kb α-subunit-specific PstI cDNA fragment obtained from the pMARα15 plasmid (Boulter et al., 1986) was subcloned into the pGEM3 transcription vector. ^{35}S-labeled antisense RNA transcripts (sp act, $0.8–1 \times 10^9$ cpm/μg) were prepared by the Riboprobe procedure (Promega Biotec, Madison, WI) using 40 mCi/ml [^{35}S]uridine 5′-triphosphate (SJ40383; Amersham Corp., Arlington Heights, IL), and T7 polymerase. Since it was previously suggested that smaller probes are more efficient for in situ hybridization (Cox et al., 1984), the labeled probe was hydrolyzed with 40 mM $NaHCO_3$/60 mM Na_2CO_2 (pH 10.2) at 60°C for 38 min, which was the calculated time for obtaining 150-bp-long fragments (Cox et al., 1984). The hydrolyzed probe was ethanol precipitated, resuspended in hybridization buffer to a concentration of 1 ng/μl ($\sim 1 \times 10^6$ cpm/μl), and stored at −20°C for at most 2–3 d.

Hybridization. Cells grown on 35-mm dishes, as described above, were fixed with a freshly prepared 4% paraformaldehyde (pH 7.4) solution. To lower nonspecific background hybridization, cultures were acetylated for 10 min at room temperature using a mixture of 150 μl acetic anhydride and 50 ml of 0.1 M triethanolamine (pH 8.0). After acetylation, cells were rinsed 4–5 times with 2 × SSC, and were prehybridized at 55°C for 4 h in a buffer containing 50% formamide, 10% dextran sulfate, 1% SDS, 100 mM DTT, 1 M NaCl, and 100 μg/ml of each salmon sperm DNA and *Escherichia coli* tRNA. After prehybridization, the buffer was removed, and 10 μl of fresh hybridization buffer, containing 2 ng of ^{35}S-labeled α-subunit-specific antisense RNA probe, preheated to 65°C for 10 min, were applied to the center of each culture dish. The hybridized area was covered with an 18 × 18-mm coverslip. Culture dishes were placed in large, covered petri dishes and were hybridized at 55°C for 16–24 h in a water bath. After hybridization, coverslips were removed by 2 × SSC, 50% formamide. Plates were washed twice with shaking for 30 min each, first in 2 × SSC, 50% formamide, and then

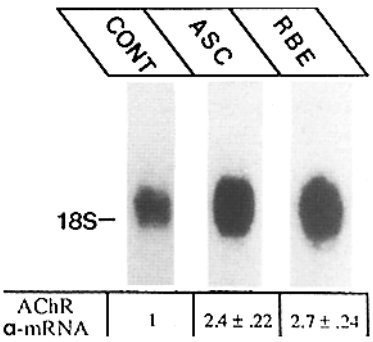

Figure 1. AChR α-subunit mRNA increases in response to crude brain extract and ascorbic acid. L_5 cells treated with crude brain extract (*RBE*), ascorbic acid (*ASC*), or PBS (*CONT*) on days 4 and 5 after plating were harvested on day 7. 10 μg of total RNA, extracted from treated and control cells, were analyzed by Northern blot hybridization using a ^{32}P-labeled α-subunit-specific RNA probe (see Materials and Methods). Position of 18S rRNA is indicated. Tabulated data (below blots) report average results for four experiments. Surface AChR, assessed on companion cultures by ^{125}I-α-BGT binding and gamma counting (see Materials and Methods) gave an increase over control values for ascorbic acid and brain extract of 2.9 ± 0.11 and 3.1 ± 0.23 respectively, within the range of two- to sixfold previously reported (Knaack and Podleski, 1985; Knaack et al., 1986; Neugebauer et al., 1985).

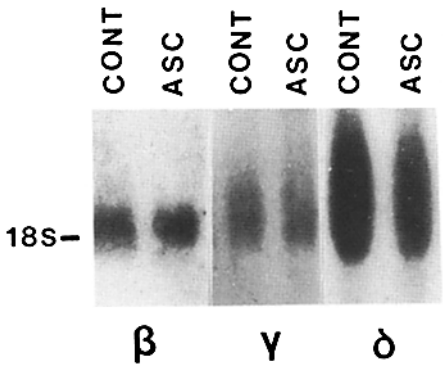

Figure 2. Ascorbic acid does not increase the β-, γ-, and δ-subunit mRNA levels. Northern blot analysis of 10 μg total RNA from control (*CONT*) or ascorbic acid–treated (*ASC*) cells as in Fig. 1, hybridized to ^{32}P-labeled nick-translated β-, γ-, and δ-subunit–specific cDNA probes (see Materials and Methods). Position of the 18S rRNA is indicated.

in 1 × SSC, 50% formamide at 55°C, and, finally, in 1 × SSC at room temperature. Cells were dehydrated through 70, 95, and 100% ethanol, and air dried. Duplicate plates for each condition were treated with 100 μg/ml RNase for 30 min at 37°C before the acetylation step, and were used as controls for nonspecific binding of the probe.

Detection of Hybridization. Autoradiograms were prepared by the flat substrate monolayer stripping film method to avoid artefacts caused by uneven drying of liquid emulsion over and around individual cell contours. The stripping film using Ilford L4 emulsion was made and applied as previously described (Land et al., 1977; Salpeter, 1981). Developing was for 4 min at 20°C using D19 developer.

Autoradiograms were examined by both oil immersion phase microscopy (microscope with anoptral-contrast objectives; Reichert Scientific Instruments, Buffalo, NY) and by scanning EM, according to the procedures previously described (Neugebauer et al., 1985; Salpeter et al., 1988). For phase microscopy, nuclei of cells in developed autoradiograms were stained with Harris' modified hematoxylin (Fisher Scientific, Rochester, NY), according to manufacturers recommendation, for 6 min, and counterstained with Eosin Y (1 g in 70% ethyl alcohol plus 0.5% glacial acetic acid). Since it was not determined whether under the conditions used (0.2 μg probe/ml hybridization mixture for 16–24 h) saturation of hybridization was achieved, no absolute quantitation of α-mRNA was attempted.

Fine Structure

Cells were fixed and prepared for transmission EM, as previously described (Land et al., 1977; Salpeter et al., 1982).

Results

Ascorbic Acid Treatment and Cellular Transcript Levels

The effect of crude brain extract and of ascorbic acid on the levels of the α-subunit mRNA is illustrated in Fig. 1.[2] Densitometric quantitation of α-mRNA levels in four different RNA preparations from ascorbic acid and brain extract–treated cultures gave a mean increase of 2.4 ± 0.22 for ascorbic acid and 2.7 ± 0.24 for rat brain extract compared to PBS-treated controls. These results show that α-mRNA levels are increased by both brain extract and ascorbic acid, and that the extent of this increase is similar to the increase observed in surface AChR. Fig. 1 further shows that, as previously reported for surface receptors, ascorbic acid induces the same response in α-subunit mRNA as the crude brain extract does.

In contrast to the increase in AChR α-subunit mRNA in response to ascorbic acid, there was no change in the levels of the β- or γ-subunit mRNAs, and there was a slight decrease in the δ-subunit mRNA (Fig. 2). Other muscle-specific mRNAs such as α-actin, myosin light chain 2, and creatine phosphokinase were also not increased (not shown).

The response to ascorbic acid was further examined by determining the time course required for the ascorbic acid–induced changes in α-mRNA and in surface AChR. Using slot-blot analysis, an increase in α-mRNA levels was detected by 20–25 h after ascorbic acid treatment (Fig. 3 *A*). Similarly, the earliest time that a significant increase (1.3–1.5-fold) in surface AChR could be detected by ^{125}I-α-BGT binding and gamma counting was 20–25 h after the addition of ascorbic acid (Fig. 3 *B*). The above results show that there is a relatively long delay after the addition of ascorbic acid before any significant increase is seen in either α-subunit mRNA or surface receptor.

Ascorbic Acid Effect on AChR Requires mRNA Synthesis

To determine whether the ascorbic acid–induced increase in surface AChR and α-subunit mRNA levels require de novo RNA synthesis, actinomycin D, an inhibitor of mRNA synthesis, was added at different times after ascorbic acid. If added to treated cultures up to 24 h after the addition of ascorbic acid, actinomycin D completely blocked the increase in surface AChR (Fig. 4 *A*). Furthermore, no increase in α-subunit mRNA was detected (data not shown). If added 24 h or later after ascorbic acid treatment (after some increase in surface receptor has already occurred as shown in Fig. 3 *B*), actinomycin D arrested any further increase in surface AChR levels (Fig. 4 *B*).

In Situ Hybridization

Fig. 5 and Table I illustrate the ascorbic acid–induced increase in α-subunit mRNA as detected on the single cell level by in situ hybridization. These results establish that the α-mRNA is exclusively detected in myotubes and that in both control and ascorbic acid–treated mononucleated cells only grain densities close to background can be detected. Furthermore, in myotubes, the α-mRNA is not uniformly distributed but is predominantly located over and in the vicinity of nuclear clusters. This nonuniform distribution of grains cannot be because of uneven drying of the emulsion since a predried monolayer of emulsion was used. A gradient of grains is usually observed away from the labeled nuclear clusters (Figs. 5 *B* and 6). This gradient is variable and is steeper in ascorbic acid–treated cells than in controls. As Table I shows, treatment (for 72 h) with ascorbic acid can increase the α-mRNA levels near and over labeled nuclei by 4.6-fold, whereas away from nuclear clusters a smaller (1.6-fold) increase in α-mRNA is detected. Although the increase in α-mRNA is observed in most of the ascorbic acid–treated myotubes, there is a considerable cell-to-cell variation, and, even in the same myotube, not all areas containing nuclear clusters are highly labeled.

2. The data of Fig. 1 were presented in preliminary form in proceedings of meetings. Knaack, D., S. Admon, T. Podleski, R. Oswald, and M. M. Salpeter. 1985. *Soc. Neurosci. Abstr.* 11:771; see also Knaack et al., 1987; Horovitz and Salpeter, 1988.

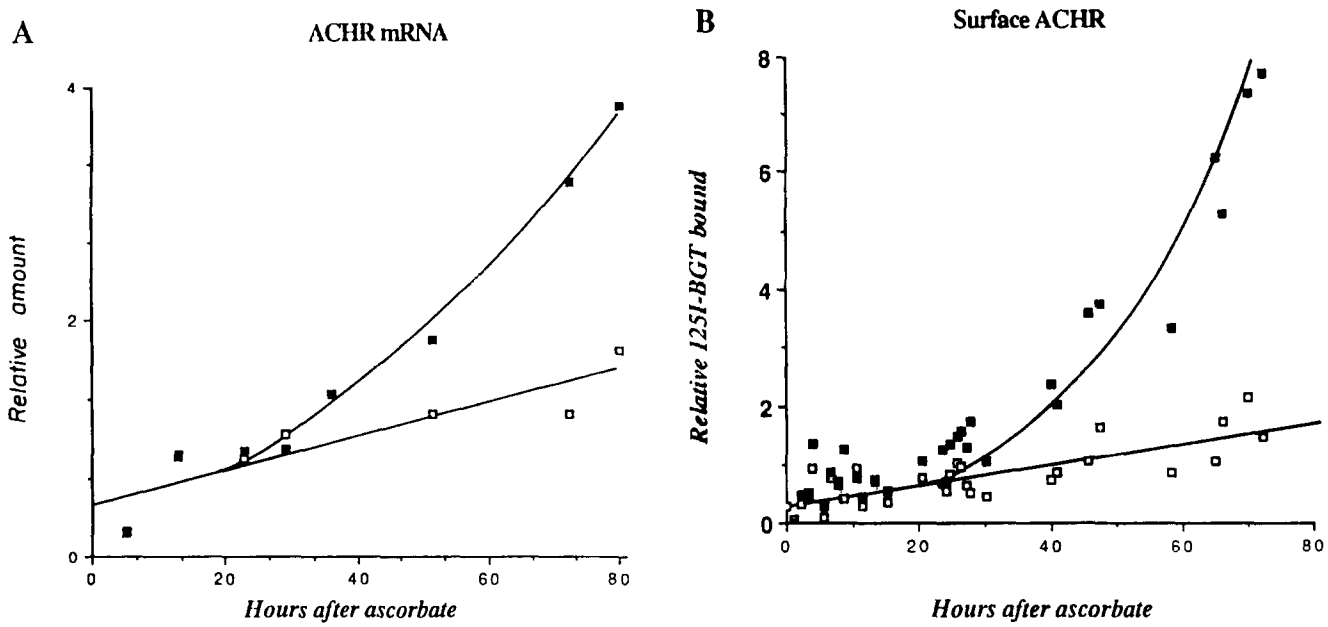

Figure 3. Ascorbic acid effect on α-subunit mRNA and surface AChR. (*A*) The increase in mRNA for the AChR α-subunit in L_5 cells is plotted against time in ascorbic acid. Total mRNA for the α-subunit of AChR was determined by slot-blot hybridization using a ^{32}P-labeled nick-translated α-subunit-specific probe (see Materials and Methods). Curves are normalized to midpoint of PBS control curves at 40 h. PBS-treated control cells (□); ascorbic acid–treated cells (■). The control and ascorbic acid data overlap at the first two data points. Data is average of two experiments. (*B*) ^{125}I-α-BGT binding to L_5 myotube cell surface evaluated by gamma counting is plotted against time in ascorbic acid. Time 0 is day 4. All data were normalized to the midpoint of the control curve (at 40 h). PBS-treated control cells (□); ascorbic acid–treated cells (■). Data are from six experiments. Each time point represents one or an average of three experiments. Note that in this figure, cultures used for mRNA and AChR were not plated at the same time in contrast to those used for Fig. 1.

In contrast to the localization of the α-mRNA around and over the myotube nuclei, the surface AChR (labeled with ^{125}I-BGT) does not show preferential localization around nuclear rich areas. Grain counts from autoradiograms of ascorbic acid and control cells showed a complete independence in the localization of AChR clusters relative to nuclei. As was previously shown, the AChR distribution in control L_5 cells has very few clusters (Neugebauer et al., 1985), and ascorbic acid–treated cells have only slightly more (Knaack et al., 1986). We found, however, that, even after crude brain extract, which contains considerably more clustering activity than ascorbic acid does (Knaack et al., 1986), the AChR clusters did not coincide with regions of nuclear clusters. Thus, there seems to be a redistribution of receptors, either in the cytoplasm before insertion into the membrane or in the membrane after insertion, resulting in a distribution of receptor on the myotube surface unrelated to the nuclear clusters.

Morphology of the Labeled Areas

We examined the fine structure of the L_5 cells to determine whether there are any unique specializations in the areas over and adjacent to nuclear clusters that might explain the accumulation of the α-mRNA at these sites. The fine structure of the L_5 cells is similar to that of the L_6 cells (Land et al., 1977; Klier et al., 1977; Podleski et al., 1979). The mononucleated cells have large nuclei and a cytoplasm with extensive membranous endoplasmic reticulum as well as mitochondria. The myotubes are rich in mytochondria with only occasional and small strands of rough endoplasmic reticulum and relatively low levels of myofilaments with no Z bands. The only organelle more frequently encountered near nuclei was the Golgi complex. Although no quantitation was attempted, we saw no obvious preferential localization of other organelles, such as rough endoplasmic reticulum near the nuclei (Fig. 6). As previously described for rat primary cells (Salpeter et al., 1982), treatment with brain extract causes no demonstrable change in the fine structure or apparent morphological differentiation in either myotubes or mononucleated cells.

Discussion

Ascorbic acid was shown earlier to be a major active component in brain extract involved in increasing surface AChR levels in the L_5 cloned muscle cell (Knaack and Podleski, 1985; Knaack et al., 1986). In the present study, we show that ascorbic acid specifically increases the mRNA for the α-subunit of the AChR by about twofold, with higher increases (~4.6-fold) in localized regions (Table I). However, it does not increase any of the other AChR subunit mRNAs. Since actinomycin D blocks the increase in both α-mRNA and surface AChR, the ascorbic acid–induced increase in the L_5 cells is dependent upon de novo synthesis and is not merely a posttranscriptional stabilization. However, it is not clear whether the synthesis required is for α-AChR or that of another mRNA species. The time course required for the ascorbic acid action (20–25 h) raises the possibility that one or more intermediate steps may be involved.

Our finding that only the α-subunit mRNA is increased is similar to, but an extension of, reports on chick primary muscle cells. Harris et al. (1988) showed that ARIA increases mRNA for the α-subunit in chick cells but not that for the γ- and δ-subunits. In that study, the β-subunit was not

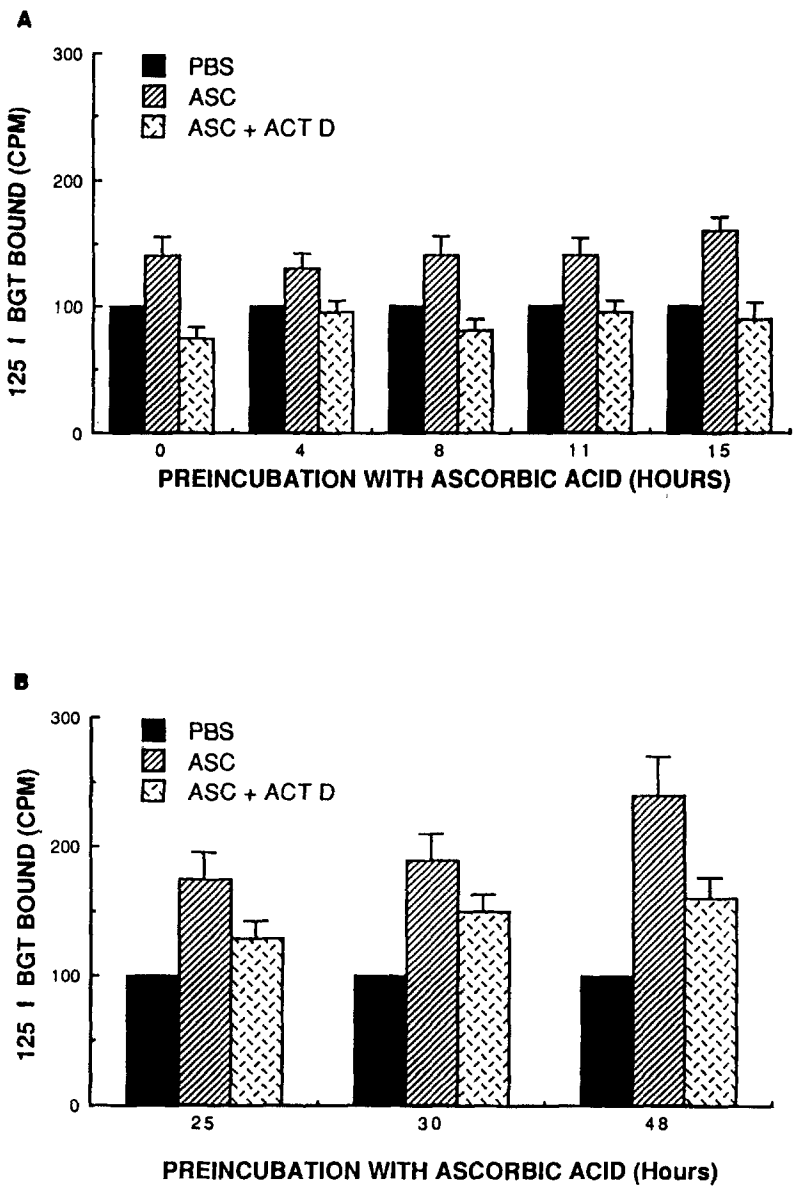

Figure 4. Actinomycin D prevents the ascorbic acid-induced increase in surface AChR measured by ^{125}I-BGT binding and gamma counting. Cells were pretreated with ascorbic acid for different times (preincubation with ascorbic acid) before addition of actinomycin D (*ASC + ACT D*). Surface AChR was assessed 16–24 h later. For all time points, a total treatment of at least 24 h was used before surface AChR was assayed. In parallel ascorbic acid-treated cells (*ASC*) and PBS-treated control cells (*PBS*) were incubated for an equal total length of time (preincubation time plus 16–24 h). Actinomycin D treatment of control cells (*PBS + ACT D*) decreased surface receptor levels at most by 30% (data not shown). (*A*) Cells preincubated with ascorbic acid for up to 15 h. (*B*) Cells preincubated with ascorbic acid for 24, 30, and 48 h. Since by 24 h some increase in surface AChR is already produced by ascorbic acid (see Fig. 3 *B* and also ascorbic acid columns in *A*), the addition of actinomycin D at this stage does not prevent but only arrests further increase.

checked. Similarly, CGRP also increases the α-subunit mRNA although other subunits were not tested (Fontaine et al., 1987). Thus, several different factors, present in brain and spinal cord, stimulate specific increases in the α-subunit mRNA. This regulation is thus distinctly different from the regulation of extrajunctional receptors seen after denervation or during differentiation when mRNA for all the AChR subunits are synchronously increased (Buonanno and Merlie, 1986; Evans et al., 1987; Moss et al., 1987; Baldwin et al., 1988; Goldman et al., 1988).

The mechanism by which these neurally derived factors effect the AChR α-mRNA is not yet clear. In chick primary cells, CGRP rapidly increases cAMP levels at the same concentrations that it induces an approximately fourfold increase in α-AChR mRNA. Thus, cAMP was suggested as a possible second messenger in the biosynthesis of the AChR mRNA (Fontaine et al., 1987). We found that CGRP similarly induces a fivefold increase in cAMP levels in L_5 cells and an increase in surface receptor of only 1.4–1.6-fold. However, ascorbic acid has no effect on cAMP levels in L_5 cells. Yet ascorbic acid elevates the α-mRNA levels in L_5 cells by as much as CGRP does in the chick cells and produces a much greater increase in surface AChR than CGRP does (our manuscript in preparation). Therefore, not only different neuronal factors but also different second messenger systems seem to be involved in the regulation of the α-subunit mRNA (and thus of the surface AChR).

In situ hybridization for the α-subunit mRNA conclusively shows that ascorbic acid increases α-mRNA levels per cell and that the increase is exclusively located in the myotube. Thus, the effect of ascorbic acid is specific for the differentiated muscle cell and is not because of an increase in the expression of α-mRNA in mononucleated cells. The α-mRNA is localized around nuclear clusters in the myotube, but the surface AChR is not colocalized with the α-mRNA around the nuclei.

Since Podleski et al. (1978) first showed that nerve extracts can stimulate the increase in surface AChR, these data, and

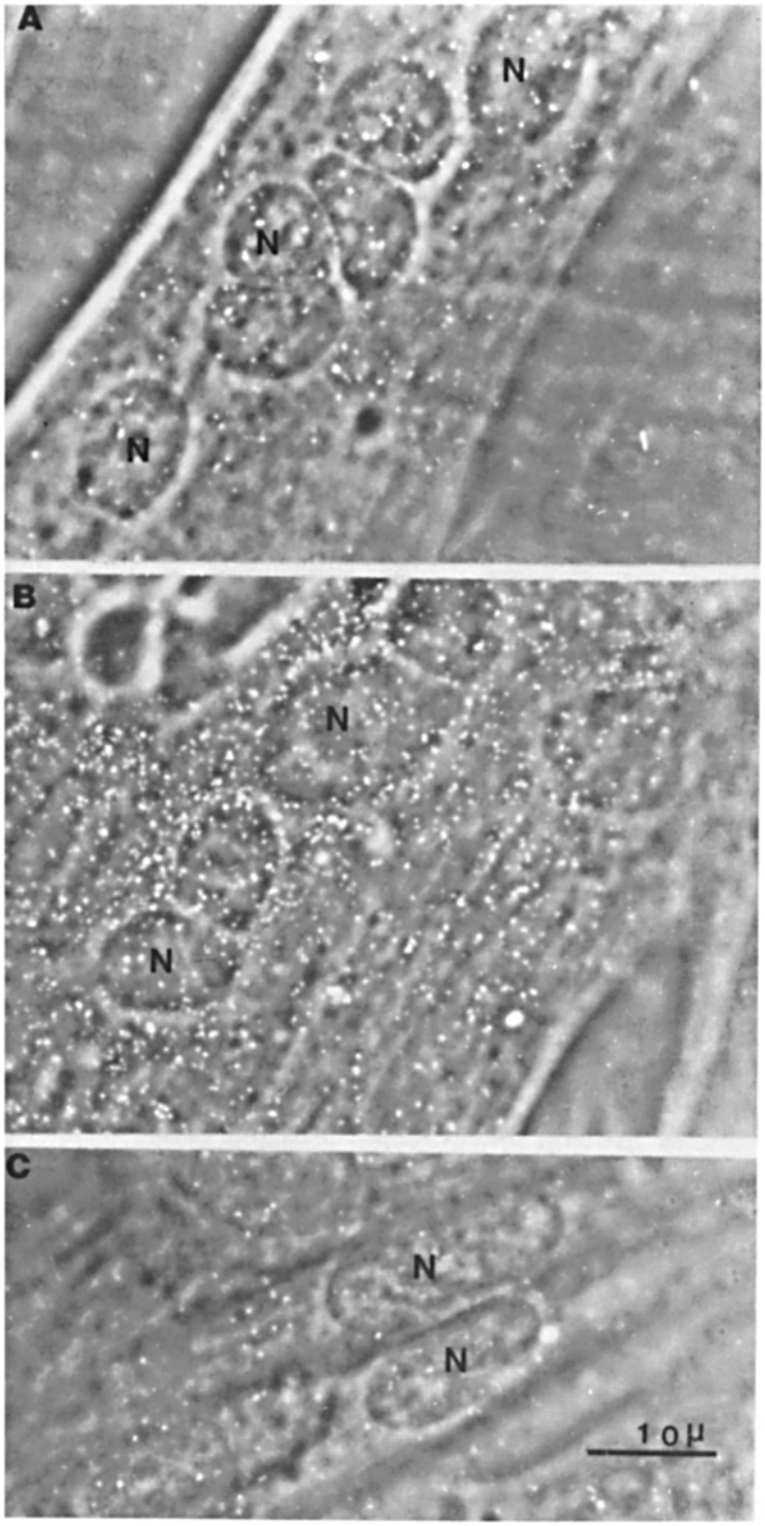

Figure 5. In situ hybridization shows AChR α-subunit mRNA localization in vicinity of nuclei in myotubes. Light microscope autoradiograms of L_5 cells hybridized in situ by an α-specific mRNA probe (see Materials and Methods). The autoradiograms were photographed with a microscope using an anoptral-contrast objective (Reichert Scientific Instruments), accounting for the white autoradiographic grains. (*A*) Cluster of nuclei in a myotube of control cells. (*B*) Cluster of nuclei in ascorbic acid–treated cells. (*C*) Mononucleated cells. In each panel a few nuclei are marked *N*. Bar, 10 μm.

subsequent confirmation in other muscle cells (Jessel et al., 1979; Connolly et al., 1982; Buc-Caron et al., 1983; Neugebauer et al., 1985), raised an apparent contradiction. It has been known for some time that denervation, with its associated loss of muscle activity, stimulates AChR synthesis and an increase in extrajunctional AChR (see Klarsfeld and Changeux, 1985; Carlin et al., 1986; for reviews see Fambrough, 1979; Salpeter, 1987). Furthermore, denervation causes an increase in AChR mRNA that precedes the increase in extrajunctional surface AChR (Moss et al., 1987; Goldman et al., 1988). Thus, nerve factors would not be expected to cause an increase in AChR. However, muscle activ-

Table I. The Effect of Ascorbic Acid on α-mRNA Levels in L_5 Muscle Cells as Determined by In Situ Hybridization

Location	Control	+Ascorbic acid	Ascorbic acid/control
In mononucleated myoblasts	1.0 ± 0.17	1.2 ± 0.09	1.2
In myotubes on and around nuclei	2.2 ± 0.33	10.0 ± 0.83	4.6
In myotubes away from nuclei	1.1 ± 0.13	1.8 ± 0.3	1.6

Data normalized to myoblast grain density, which was 1.3 grains/39 μm^2. All these values are after subtracting background, which was on average 1 grain/39 μm^2. Cultures treated with RNase before hybridization had no label above background. Cells were dosed with ascorbic acid on day 4 and fixed on day 7.

ity does not significantly affect junctional AChR densities, as evidenced by the high AChR levels maintained at the innervated neuromuscular junction in electrically active muscle (for review, see Salpeter and Loring, 1985). Specific mRNAs for the AChR are also higher near the junction (Merlie and Sanes, 1985; Witzemann et al., 1987; Fontaine et al., 1988). These observations suggest that different mechanisms can be involved in regulating local levels of surface AChR. It is possible that factors released at the neuromuscular junctions maintain the high junctional AChR levels in electrically active muscle. Although none of the neural factors shown to regulate AChR levels in vitro have yet been conclusively identified as being released at the junction, these factors do allow us to study the different steps in the regulation of AChR synthesis in different muscle cells (see also Horovitz et al., 1989).

In this study, we have demonstrated that brain extract and ascorbic acid–induced increase in surface AChR are correlated with an increase in α-subunit mRNA on single myotube level, suggesting that surface AChR in response to these factors is regulated by α-subunit transcript availability. Furthermore, since the increase in surface AChR is dependent on de novo RNA synthesis, a transcriptional regulation is indicated.

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Note Added in Proof: Since this manuscript was submitted, a report by D. A. Harris, D. L. Falls, and G. D. Fischbach was published (*Nature (Lond.)*. 1988. 337:173–176. This paper similarly shows that a selective localization of AChR α-subunit mRNA exists over nuclear clusters in chick muscle treated with ARIA and that not all nuclei are equally involved. That report differs from ours in that it found a colocalization between surface AChR clusters and mRNA concentrations, and the magnitude of the increase in local mRNA density is less than that reported in our Table I. These differences may be because of the different muscle species or neural factors used.

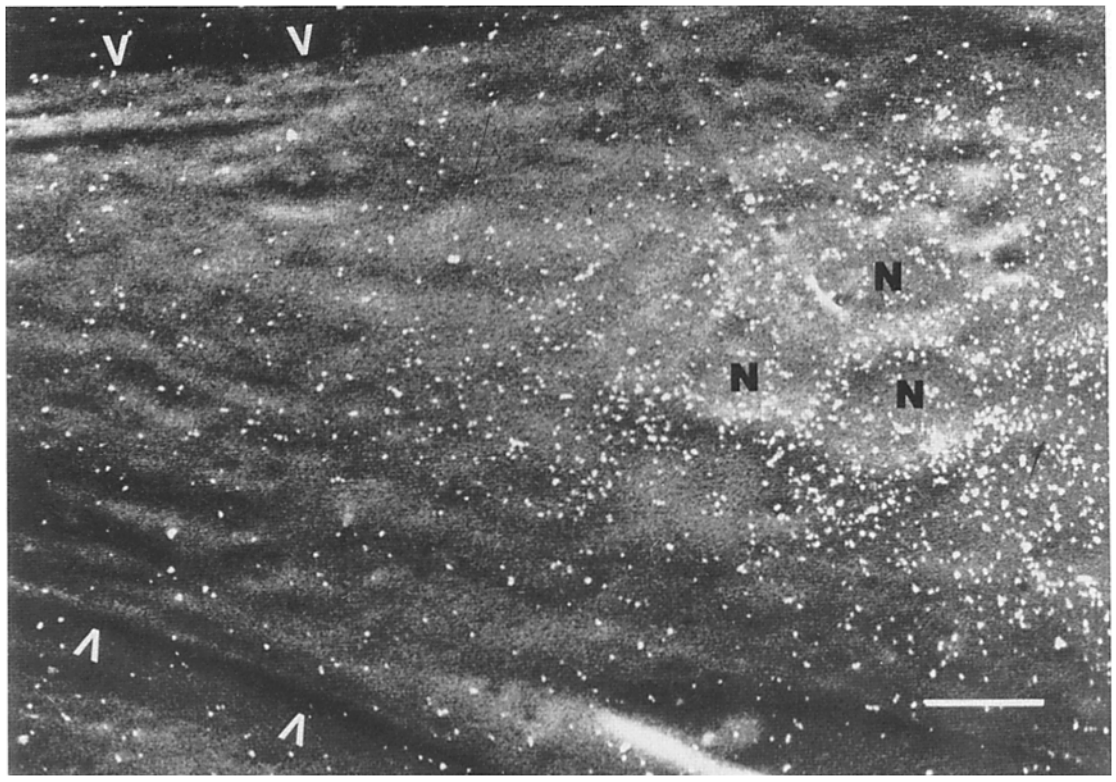

Figure 6. Gradient of α-mRNA seen in SEM autoradiogram. Scanning EM autoradiogram of an L_5 myotube hybridized in situ with an α-AChR-specific probe as in Fig. 5. Nuclei can be seen in this preparation as circular craters (a few marked *N*). Edge of cell marked with caret. Note heavy label over and in the vicinity of nuclei with marked gradient away from the nuclear cluster. Bar, 10 μm.

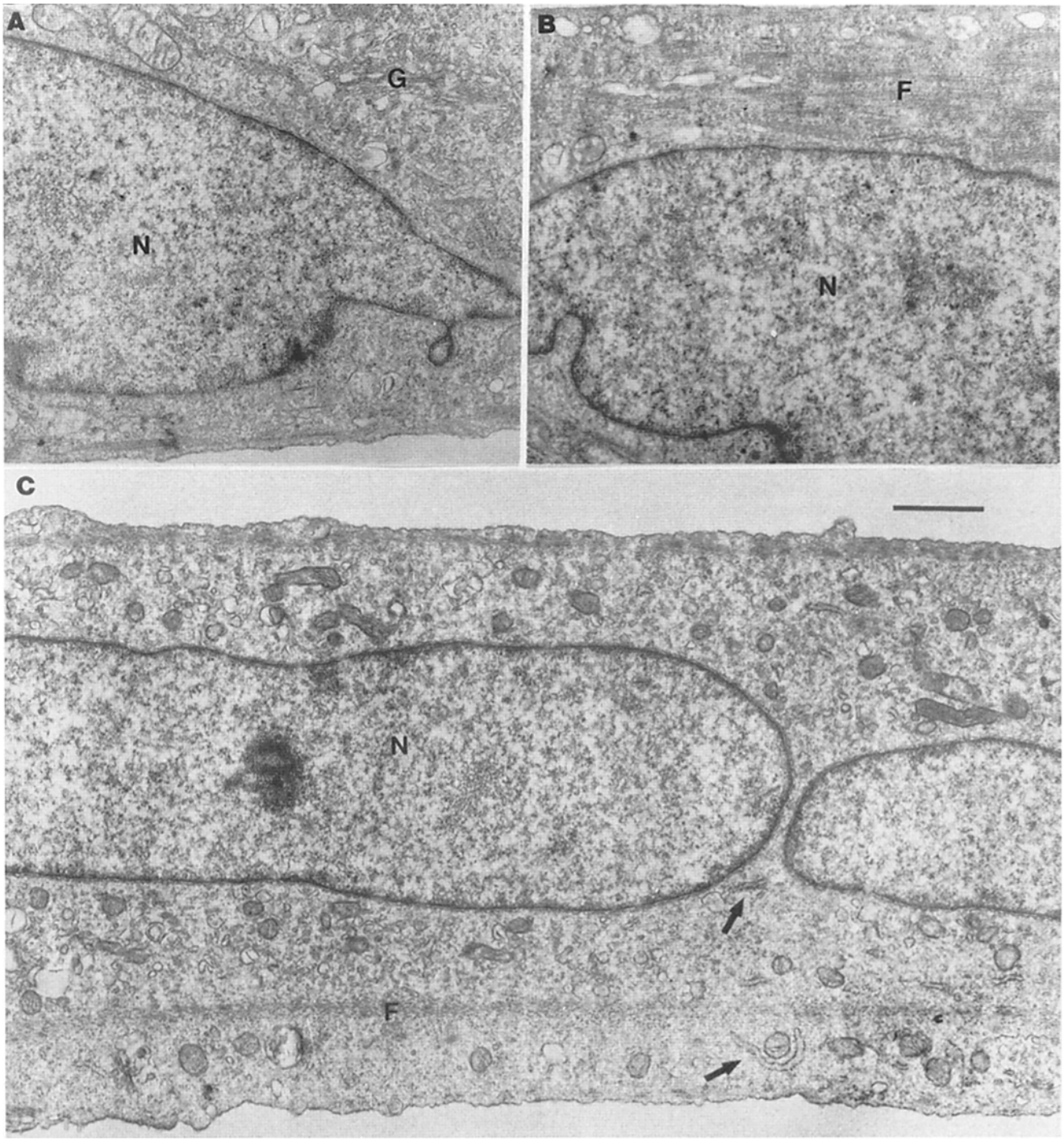

Figure 7. Fine structure of L_5 cells is not altered by brain factor. Electron micrograph of L_5 myotubes at region of nuclear clusters (to be compared with Fig. 8 without nuclei). (*A* and *B*) Control cells. (*C*) Cells treated with a partially purified low molecular weight active fraction of brain extract such as shown to contain ascorbic acid (Knaack and Podleski, 1985; Neugebauer et al., 1985). A greater than twofold increase in surface AChR had been obtained in parallel cultures after this treatment. Note lack of unique specialization in region of nuclei except for pronounced Golgi complex (*G*). Strands of rough endoplasmic reticulum (*arrows*) are sparsely distributed. Myofilaments (*F*) are not as developed in L_5 cells as in primary cells and lack Z bands (see also Podleski et al., 1979; Salpeter et al., 1982). Cells treated with crude brain extract have a similar appearance. Bar, 10 μm.

Figure 8. Fine structure of L_5 myotubes does not differ in regions with and without nuclear clusters. As in Fig. 7, *A* is from control cultures, and *B* is from cultures treated with the low molecular weight brain extract fraction. Figs. 7 and 8 show that there is no significant difference in fine structure between cells near or away from nuclear clusters, nor between control and brain extract-treated cells.

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