

Regulation of Acetylcholine Receptor Synthesis at the Level of Translation in Rat Primary Muscle Cells

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Abstract. Previous studies have shown that rat primary muscle cells do not respond to crude rat brain extract or one of its active components, ascorbic acid, with a significant increase in surface acetylcholine receptor (AChR) number. We report here that, although little or no response is seen on the cell surface, rat primary muscle cells do respond to both crude brain extract and to ascorbic acid with an approximately threefold increase in AChR α -subunit mRNA. The response of the mRNA is similar to that

seen in the cloned L₅ cells. However, while in L₅ cells the increase in α -subunit mRNA is further translated into increased levels of α -subunit protein, there is no such increase in α -subunit synthesis in the primary cells. This study thus shows a regulation of surface AChR synthesis in rat primary cells at the level of α -subunit translation. This level of regulation is different from that involving subunit transcription or subunit assembly reported by others.

STUDIES on the regulation of surface acetylcholine receptor (AChR) in cultured muscle cells have shown that expression on the cell surface can be controlled by different events in the biosynthesis of this receptor. Regulation has been documented at the level of mRNA transcription (Klarsfeld and Changeux, 1985; Buonanno and Merlie, 1986; Evans et al., 1987; Moss et al., 1987; Baldwin et al., 1988; Harris et al., 1988; Horovitz et al., 1989), subunit assembly (Merlie and Lindstrom, 1983; Olson et al., 1983), and efficiency of insertion into the membrane (Carlin et al., 1986). In this study, we explore the regulation of surface AChR in rat primary cells and describe a different level of regulation.

Previous studies have shown that the rat-derived cloned muscle cells, L₆ and L₅, and chick primary cells respond to brain extracts with an increase in surface receptor (Podleski et al., 1978; Jessel et al., 1979; Neugebauer et al., 1985; Usdin and Fischbach, 1986). In the L₅ cells, this response was reported to be due to ascorbic acid found in the active brain extract fraction (Knaack and Podleski, 1985; Knaack et al., 1986). However, no increase in surface AChR occurs in the rat primary cells in response to crude brain extract or to ascorbic acid (Kalcheim et al., 1982; Salpeter et al., 1982; Knaack and Podleski, 1985; Knaack et al., 1986; Salpeter and Greenberg, manuscript in preparation). To gain greater understanding of the control of AChR expression in muscle, we examined the effect of these factors on the levels of mRNA, on AChR α -subunit synthesis, and on surface receptor number in rat primary muscle cells in culture. Our results show that brain extract or ascorbic acid selectively increase

the α -mRNA levels in the rat primary muscle cells as previously shown for the L₅ cells (Horovitz et al., 1989). However, in contrast to L₅ cells, the increase is not followed by a corresponding increase in α -subunit protein. These data suggest that expression of surface AChR in rat primary muscle cells is regulated at the level of cytoplasmic α -subunit translation.

Materials and Methods

Cell Cultures

Cultures of primary rat skeletal and L₅ muscle cells were prepared as previously described (Salpeter et al., 1982; Neugebauer et al., 1985). Briefly, rat primary cells were plated on rat tail-collagen-coated 100-mm tissue culture dishes or multiwell plates (containing 24 wells), at a density of 5×10^5 cells/ml growth medium (DME supplemented with 10% FCS). On the third day of plating, 10^{-5} M cytosine arabinoside (Ara-C) was added to minimize fibroblast proliferation. L₅ cells that adhere well to culture dishes were plated on noncollagen-coated plates at a density of 5×10^4 cells/ml using the same culture medium as for the primary cells. Since L₅ cultures contain no fibroblasts, no Ara-C was added to these cells. It has been found (Knaack et al., 1986), and further confirmed by us, that the response of AChR in L₅ cells to ascorbic acid is not significantly affected by Ara-C or by plating these cells on collagen-coated dishes.

Treatment with Rat Brain Extract (RBE) and Ascorbic Acid

On day 4 or on days 4 and 5 after plating, cells were treated with 30 μ g of freshly prepared ascorbic acid solution/1 ml of growth medium (Knaack and Podleski, 1985), or with 540 μ g of newborn RBE protein/1 ml of growth medium (prepared as previously described) (Podleski et al., 1978; Salpeter et al., 1982; Podleski and Salpeter, 1988). Control cells were treated with PBS. Cultures were harvested at different times after treatment and were analyzed for surface AChR, mRNA levels, and α -subunit synthesis, as described below.

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

Quantitation of Cell Surface AChR

Cells were assayed for cell surface AChR by ^{125}I - α -bungarotoxin (BGT) binding. ^{125}I - α -BGT (sp act, 50–100 Ci/mmol) was added to cells plated on multiwell plates (24 wells/plate) 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. The cells were solubilized in 0.9 ml of 2 M NaOH, and bound ^{125}I -BGT was determined by gamma counting (Knaack and Podleski, 1985). Nonspecific binding was determined by preincubating cells for 15 min with 10 μM unlabeled α -BGT, rinsing with HEPES-buffered DME, and incubating with ^{125}I - α -BGT, as described above.

RNA Preparation and Northern Blot Analysis

Total RNA from cells plated on 100-mm culture dishes was extracted by the LiCl-urea procedure (Auffray et al., 1980). 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 supplier's instructions.

Prehybridization and hybridization were performed at 55°C in 50% formamide, 10% dextran sulfate, 1% SDS, 1 M sodium chloride, and 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA. Hybridized blots were washed under stringent conditions of $0.1 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M NaCl} + 15 \text{ mM}$ sodium citrate), and 0.1% SDS at 65°C. The amount of mRNA in each band was quantified by densitometric scanning of autoradiograms using an ultrascan laser densitometer (LKB Instruments, Inc., Gaithersburg, MD) or on a gel scanner (model 1312; Isco, Inc., Lincoln, NE). Only exposure times giving optical density values proportional to the amounts of total RNA applied were used for quantitation.

AChR α -subunit was detected by using a ^{32}P -labeled RNA probe. This probe was obtained by the transcription of the 5' end of the 450-bp PstI fragment isolated from the pMAR α 5 plasmid (Boulter et al., 1985), provided by J. Patrick, subcloned in the pGEM3 vector. The β -, γ -, and δ -specific cDNA probes were used after nick-translation and labeling with [^{32}P]deoxyATP. Specific cDNA probes for the β - (BMB49; Patrick et al., 1987), and γ - (BMG419; Boulter et al., 1986) subunits were provided by J. Patrick (California Institute of Technology) and J. Boulter (Salk Institute), and the δ -subunit (p6H) was supplied by N. Davidson (LaPolla et al., 1984).

Synthesis of AChR α -Subunit

Cells, with or without ascorbic acid added on days 4 and 5 of plating, were labeled on day 7 for 10, 15, or 20 min or 1, 2, or 4 h at 37°C with 50 μCi [^{35}S]methionine ($\sim 1,000 \text{ Ci}/\text{mmol}$; Amersham Corp, Arlington Heights, IL) in 2 ml labeling medium containing DME, 5% FCS, and 10 μM L-methionine. After labeling, cells were rinsed with PBS, scraped, pelleted at 2,000 g , and treated as described by Merlie and Lindstrom (1983). Ascorbic acid-treated or control cells, obtained from one or two 100-mm dishes, were extracted while being vortexed for 10 min with 1.0 ml of PBS, containing 1% Triton X-100, 1 mM EDTA, and 1 mM of each of the following protease inhibitors: PMSF, *N*-ethylmaleimide, and *p*-aminobenzamide. Insoluble material was removed by centrifugation for 10 min at 15,000 g . Total extracted protein was determined by the Bradford method (Bradford, 1976) using a commercial Bradford reagent (Bio-Rad Laboratories, Cambridge, MA). [^{35}S]Methionine incorporation was determined by scintillation counting after precipitation with 10% TCA on nitrocellulose filters (HVLP; Millipore Continental Water Systems, Bedford, MA). The amount of α -subunit in each extract was determined by immunoprecipitation with a specific monoclonal antibody mAb 61 (kindly provided by Dr. J. Lindstrom, Salk Institute) that was shown to recognize and precipitate all forms of the α -subunit (Merlie and Lindstrom, 1983). Before the immunoprecipitation, the extracts were preincubated for 15 min at 4°C with 150 $\mu\text{l}/\text{ml}$ extract of a 10% *Staphylococcus aureus* (Sigma Chemical Co., St. Louis, MO) suspension in PBS, containing 1% Triton, to partially remove proteins that bind nonspecifically to the *S. aureus*. After the removal of *S. aureus* by centrifugation, 0.3 ml of the prepurified extracts were immunoprecipitated for 60 min at 4°C with 20 μl of 5% dilution of mAb 61 (initial concentration, 0.43 μM). Subsequently, 20 μl of 10% *S. aureus* suspension, preadsorbed with rabbit anti-rat IgG (ICN Biomedicals Inc., Irvine, CA), was added, for 1 h at 4°C to precipitate the mAb anti- α -complex. The optimal amounts of mAb 61 and immunosorbent rabbit anti-rat IgG *S. aureus* for these studies were established in separate experiments on immunoprecipitation of Triton-solubilized rat primary muscle cell AChRs labeled with ^{125}I - α -BGT. Immunoprecipitates were collected by centrifugation at 15,000 g for 10 min, washed four times with 0.5 M NaCl, 0.01 M sodium phosphate

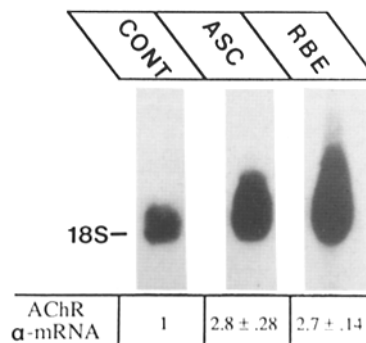


Figure 1. AChR α -subunit mRNA in rat primary muscle cells increases in response to RBE and ascorbic acid (ASC). Cultures were treated with ASC, RBE, or PBS (CONT) on days 4 and 5 of plating, and harvested 72 h later (on day 7). 10 μg of total RNA was analyzed by Northern blots. Position of the 18-S rRNA is indicated. Tabulated data (below dots) is based on densitometric quantitation from three different experiments. In contrast, analysis of surface AChR based on ^{125}I - α -BGT binding in parallel cultures gave no increase for surface AChR: values of 1.1 ± 0.04 and $1.3 \pm 0.12 \times$ control were obtained for ascorbic acid-treated and RBE-treated cells, respectively (see also Christian et al., 1978; Salpeter et al., 1982, and Neugebauer et al., 1985).

buffer (pH 7.4, 0.1% Triton X-100 at 4°C), and once with distilled H₂O at 4°C. The α -subunit mAb 61 immunocomplex was eluted by incubation with 40 μl of SDS sample buffer at 100°C for 3 min and analyzed by SDS-PAGE (Ryrie and Gallagher, 1979; Spitsberg, 1987), according to a modification of the Laemmli technique (Laemmli, 1970). Immunoprecipitation of non-specific protein bands was determined using irrelevant mAbs. After electrophoresis, gels were processed for fluorography using Autofluor (National Diagnostics, Inc., Somerville, NJ). The amount of α -subunit was determined by densitometric quantitation of the obtained autoradiogram using a gel scanner (model 1312; Isco, Inc.).

Results

Effect of Rat Brain Extract and Ascorbic Acid on Levels of Surface AChR and AChR mRNA

Fig. 1² shows that rat primary muscle cells respond to both crude RBE and ascorbic acid by a substantial increase in α -mRNA levels. Densitometric quantification of Northern blots from three different experiments indicate a 2.7 ± 0.14 -fold increase in α -subunit mRNA with RBE and 2.8 ± 0.25 -fold increase with ascorbic acid. In contrast, surface AChR levels did not increase significantly. Since the responses were identical to ascorbic acid and to crude RBE, all subsequent experiments evaluated only the effect of ascorbic acid.

We first determined the time course of the increase in α -subunit mRNA (Fig. 2) and found that a 24-h incubation with a single dose of ascorbic acid was sufficient to induce a threefold increase in α -mRNA and that this increase was maintained up to 72 h (no longer time was examined). However, as previously shown (Kalchheim et al., 1982) and confirmed by us in the present study (see legend, Fig. 1), surface AChR levels in response to ascorbic acid are not increased in the primary cells even after 72 h of treatment. Unlike the mRNA for the α -subunit, mRNA levels for the β -, γ -, and

2. The data of Figs. 1 and 3 were presented in preliminary form (1987. *Proc. UCLA Symp. Muscle Development, Steamboat Springs, CO*; Horovitz and Salpeter, 1988).

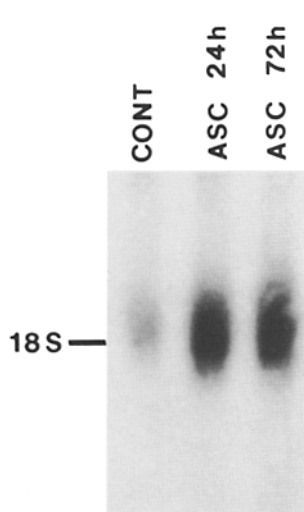


Figure 2. The increase in α -mRNA levels in rat primary muscle occurs by 24 h after incubation in ascorbic acid (ASC). One dose of ASC was added to the cultures on day 4, and cells were assayed either 24 or 72 h later by Northern blot analysis. Control cells (CONT) were in culture for the same length of time. The position of the 18S rRNA is shown. Densitometric scans of autoradiograms from three such experiments show an increase, after the dose of ascorbic acid, over control cells of 3.0 ± 0.42 and 2.8 ± 0.25 at 24 h and 72 h, respectively.

δ -subunits showed no significant increase in response to ascorbic acid (Fig. 3).

Synthesis of α -Subunit Protein Does Not Increase in Response to Ascorbic Acid

To determine at which posttranscriptional level the uncoupling between the response of the mRNA and the surface receptor occurs, we compared the synthesis of the α -subunit in control and ascorbic acid-treated cells. Control cells and cells treated with ascorbic acid for 72 h were labeled with [35 S]methionine for periods of 10 min to 4 h, and the amount of newly synthesized α -subunit was determined by immunoprecipitation with mAb 61, a specific anti- α -subunit mAb (Tzartos et al., 1981) (see Materials and Methods).

As shown in Figs. 4 and 5 A, no change in the amount of newly synthesized α -subunit could be detected after ascorbic acid treatment of rat primary muscle cells at any of the times tested. From densitometric measurements of α -AChR protein in 10 experiments, we obtained an average value for the ratio of ascorbate-treated to control cells of 1.1 ± 0.13 . For

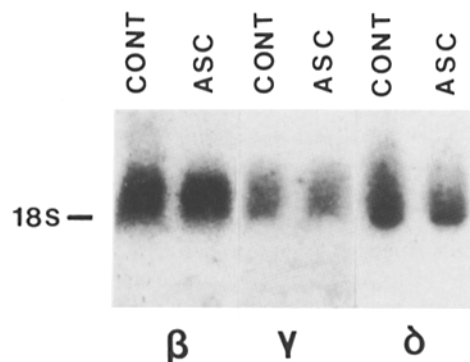


Figure 3. Ascorbic acid does not increase the β -, γ -, and δ -subunit mRNA levels in rat primary cells. Northern blot analysis of 10 μ g total RNA from control (CONT) or ascorbic acid-treated cells (ASC) as in Fig. 1, hybridized to 32 P-labeled nick-translated β -, γ -, and δ -subunit-specific cDNA probes (see Materials and Methods). Position of the 18S rRNA is indicated.

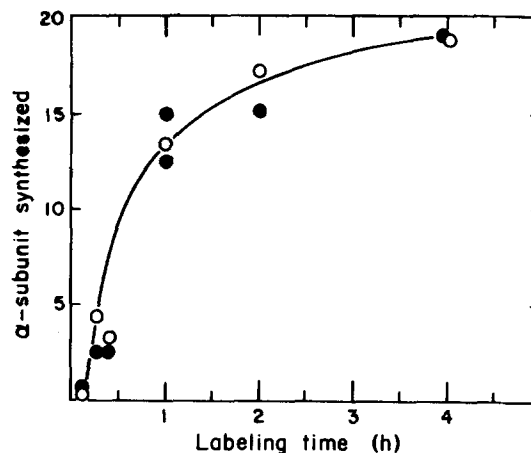


Figure 4. No difference is seen in rate of α -subunit synthesis between ascorbic acid-treated and control rat primary muscle cells. 72-h ascorbic acid-treated (\bullet) and control (\circ) cells were labeled with [35 S]methionine for 10, 15, and 20 min, and 1, 2, and 4 h. After a Triton extraction, the α -subunit was immunoprecipitated with mAb 61, and analyzed by 10% SDS-PAGE (as described in Materials and Methods). The relative amounts of protein synthesized were determined by densitometric scanning. The data are a composite of two experiments. Since the 1-h time point was the only one in which the two experiments overlapped, the data was normalized to the 1-h control value of the first experiment.

comparison, the synthesis of the AChR α -subunit was also studied in ascorbic acid-treated L_5 cells (Fig. 5 B) in which, as previously shown (Knaack et al., 1986; Horovitz et al., 1989), both surface AChR and α -subunit mRNA levels are increased after treatment. In these cells, a 2.2 ± 0.2 -fold increase in α -subunit protein was determined after treatment with ascorbic acid. This increase in the L_5 cells is somewhat lower than is seen in the surface AChR and may indicate that further regulation occurs in the assembly and insertion of this protein (a phenomenon also reported for rat primary cells in response to muscle inactivity by Carlin et al., 1986). It is nevertheless clear that, unlike the rat primary cells, the ascorbic acid-induced increase in AChR α -subunit mRNA in L_5 cells is further translated to give increased levels of α -subunit protein.

We also considered the possibility that in the rat primary cells the lack of increase of α -subunit protein after ascorbic acid is a net result of an increase in both synthesis and degradation. Although no increase in synthesis was seen in Figs. 4 and 5 A, pulse chase experiments were performed to determine whether any major difference in degradation of newly synthesized α -subunit could be detected. Control cells and cells treated with ascorbic acid for 72 h were pulse labeled with [35 S]methionine for 20 min and chased with nonradioactive methionine (Merlie and Lindstrom, 1983) for up to 3 h (Fig. 6). The degradation curves of the α -subunit are similar for control and ascorbic acid-treated cells.

Discussion

Synthesis and expression of surface AChR involves transcription of the four subunit mRNAs (α , β , γ , δ), their further translation, posttranslational processing and assembly of the subunit proteins, and, finally, receptor insertion into

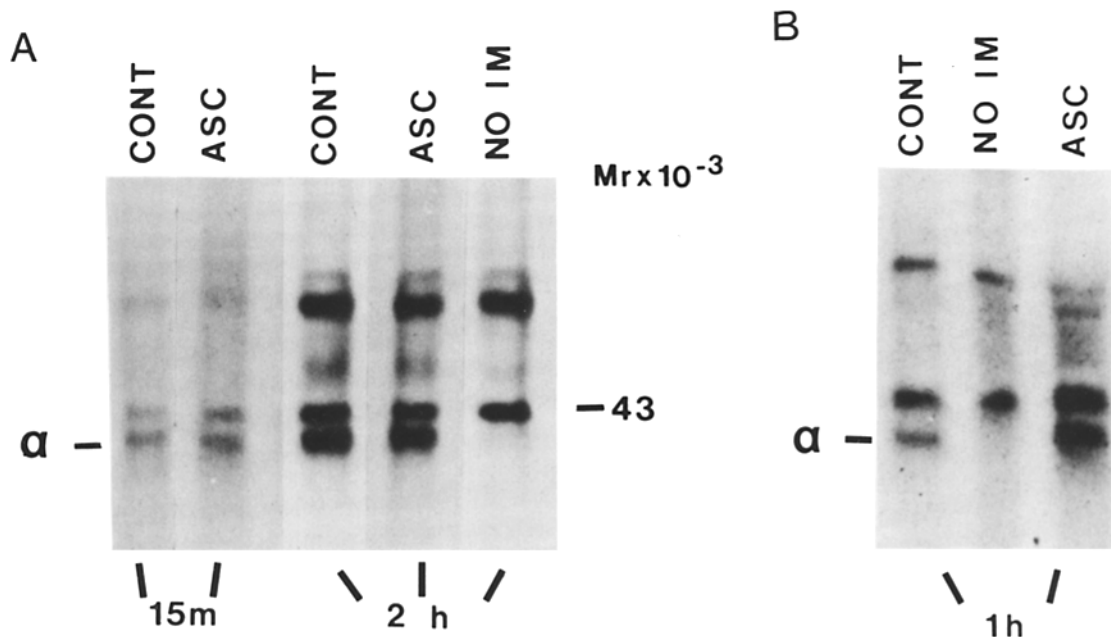


Figure 5. The amount of α -subunit synthesized is not increased in ascorbic acid-treated (ASC) rat primary muscle cells (A) but is increased in the rat-derived cloned L₅ cells (B). Control (CONT) and ASC-treated cells, labeled with [³⁵S]methionine for between 15 min and 2 h, were immunoprecipitated with mAb 61 or with a nonrelevant antibody (NO IM), as described in Materials and Methods. Triton extracts were immunoprecipitated and were analyzed by 10% SDS-PAGE. Positions of 43-kD protein standard and the α -subunit are indicated. Densitometric analysis of 10 experiments with primary cells and six experiments with L₅ cells give values over control of 1.1 ± 0.23 and 2.2 ± 0.2 , respectively.

the cell membrane (see Merlie and Sebanne, 1981; Merlie et al., 1982; Merlie and Lindstrom, 1983). Studies on the regulation of AChR in the cloned L₅ muscle cells have shown that the RBE or ascorbic acid-induced increase in surface AChR is accompanied by an increase in the mRNA levels for the α -subunit and that actinomycin D prevents the ascorbic acid-induced increase in both α -mRNA and surface AChR (Horovitz et al., 1989). These studies had suggested that in the L₅ cells, surface AChR regulation by neural factors is controlled by the availability of α -mRNA transcripts since no increase was seen in the mRNA levels of the β -, γ -, or δ -subunits. A similar conclusion that the

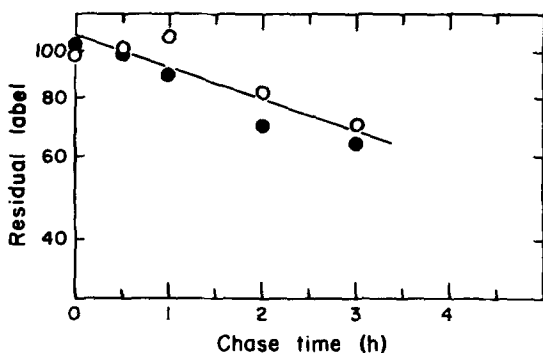


Figure 6. Degradation of α -subunit in ascorbic acid-treated (●) rat primary muscle cells is similar to that in control (○) cells. Cells were labeled with [³⁵S]methionine for 20 min and then chased with nonradioactive methionine for up to 3 h. Residual label on y-axis is based on densitometric evaluation of autoradiograms from SDS gels normalized to 100% at 0 time. Data combines two experiments, normalized to the average value at 0.5 h after labeling.

α -subunit regulates surface AChR was suggested for the increase in surface AChR in response to a brain-derived factor, ARIA, in chick cells (Harris et al., 1988).

Rat primary muscle cells, however, do not respond to either RBE or ascorbic acid with more than a 30% increase in surface receptor (see also Salpeter et al., 1982; Kalchheim et al., 1982; Neugebauer et al., 1985). The present study was aimed at determining whether this lack of response of the surface AChRs was at the level of α -subunit transcript accumulation or at some later stage in the biosynthesis of the AChR.

Our results show that rat primary cells do respond to crude RBE and ascorbic acid with an approximately threefold increase in the α -subunit mRNA. However, no subsequent increase in newly synthesized α -subunit levels was detected. Since no difference between ascorbic acid-treated and control cells was observed in either the synthesis or the degradation rate of the α -subunit, our results indicate that in the primary rat cells, unlike L₅ cells, the increase in α -mRNA is not further translated into increased levels of α -subunit protein. In general, other studies to date have not evaluated the regulation of surface AChR at the level of translation, although a defect in AChR α -subunit translation has been described for a mutant muscle cell by Black et al. (1987).

The reason for the lack of increase in α -subunit synthesis in ascorbic acid-treated rat primary muscle cells is not clear. One possibility is that the high AChR site density already existing on the surface of these cells (850 sites/ μm^2 [Salpeter et al., 1982] as compared to ~ 150 sites/ μm^2 in L₅ cells [Neugebauer et al., 1985]) suppresses, by some feedback mechanism, any further increase in α -subunit synthesis.

A lack of correlation between the amounts of mRNA and surface receptor levels has also been demonstrated in several

studies using chick primary cells. For instance, in these cells inactivation of activity by tetrodotoxin increases α -subunit mRNA levels 13-fold with only a twofold increase in surface AChR (Klarsfeld and Changeux, 1985; however, see also Harris et al., 1988). In another example, treatment with the chick brain-derived factor ARIA causes, on average, higher increases in α -subunit mRNA than in surface receptors (Harris et al., 1988); and, finally, calcitonin gene-related peptide increases α -mRNA levels fourfold and surface AChR levels only 30–40% (Fontaine et al., 1987). The level of regulation at which this uncoupling between mRNA and surface receptor levels occurs in the chick cells has not yet been determined, although Harris et al. (1988) suggest the lack of stoichiometry between the α and other subunits as being a possible limiting factor. In light of our current results, it would be interesting to evaluate whether some regulation at the level of translation may not also contribute to the control of surface receptor expression in response to neural factors in chick cells. Similarly, it is interesting that after inactivation of muscle activity by tetrodotoxin, the increases in α -mRNA exceed or equal those in surface receptors (Klarsfeld and Changeux, 1985; Harris et al., 1988) while the increase in α -subunit synthesis is not as great as that in surface receptor (Carlin et al., 1986). Although the differences in these studies could be due to species differences (the mRNA studies having been performed in chick cells and the protein studies in rat cells), the possibility is raised that some of the regulation of surface AChR by muscle activity may also be at the level of translation.

In conclusion, previous studies on regulation of surface AChR levels in muscle cells have described regulation at the level of mRNA transcription in response to nerve factors (e.g., Knaack et al., 1987; Harris et al., 1988; Horovitz et al., 1989; Fontaine et al., 1987), differentiation and innervation (Buonanno and Merlie, 1986; Moss et al., 1987; Evans et al., 1987; Baldwin et al., 1988), and muscle activity (Klarsfeld and Changeux, 1985). Other studies have found evidence for posttranslational regulation, such as the level of subunit assembly and receptor insertion (Merlie et al., 1982; Olson et al., 1983; Carlin et al., 1986). The results of the present study document yet another level of regulation of surface AChR. We show that in rat primary muscle cells treated with ascorbic acid the surface AChR is regulated at the level of translation of the α -subunit. We conclude that in both the rat primary cells (as shown here) and in the L₅ cells (Horovitz et al., 1989) ascorbic acid specifically regulates the level of α -subunit mRNA. Posttranslational regulation then determines the final fate of the AChR on the surface of the cell. Since in L₅ cells the increase in α -mRNA is further translated and expressed on the cell surface whereas in the rat primary cells there is no increase beyond the level of mRNA, further studies comparing the responses of these two cell types should provide a good system for studying factors that control the complex process of surface AChR expression.

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