Genetic Variants of C2 Muscle Cells That Are Defective in Synthesis of the α-Subunit of the Acetylcholine Receptor

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Abstract. We have analyzed two genetic variants of C2 muscle cells that have reduced levels of binding activity for α-bungarotoxin and have found that both synthesize only low levels of the α-subunit of the acetylcholine receptor. In both variants the uptake of ^{22}Na in response to carbachol is diminished in proportion to the reduction in toxin-binding activity. In addition, the kinetic and sedimentation properties of the residual toxin-binding activity in both is indistinguishable from that seen in wild-type cells. Immunoblotting experiments on extracts of the variants using subunit-specific antibodies to α- and β-subunits of the acetylcholine receptor demonstrated that the β-subunit was present, but failed to detect α-subunit. In both variants, the amount of α-subunit accumulated after a 5-min period of labeling with [^{35}S]methionine was reduced by over 90%, leading to the conclusion that the α-subunit is synthesized at greatly reduced rates. Northern blot and S1 nuclease analysis showed no differences between the α-subunit mRNA in wild-type and variant cells.

The acetylcholine receptor (AChR)[1] is the major constituent of the postsynaptic membrane in muscle cells and mediates the activation of muscle cells by nerves. Both the concentration and location of this protein on the cell surface are regulated during muscle cell differentiation and during the formation of the neuromuscular junction (Fambrough, 1979). The mechanisms controlling the synthesis and insertion in the membrane of the AChR are thus of major physiological significance.

The AChR consists of four related polypeptide chains, in the ratio $\alpha_2\beta\gamma\delta$. These subunits extend across the membrane to form a channel whose opening is regulated by the binding of ACh to the two α-subunits (Conti-Tronconi and Raftery, 1982; Changeux et al., 1984; McCarthy et al., 1986). Each of the individual polypeptides crosses the membrane several times and is thought to contribute one transmembrane segment to form the wall of the ion channel (Numa et al., 1983; Claudio et al., 1983, Finer-Moore and Stroud, 1984).

Relatively little is understood about how this protein is assembled and transported to the cell surface. Experiments in an in vitro protein synthesis system have shown that each of the chains is derived from a larger precursor that is inserted into the membrane and cleaved without association with the other chains (Anderson and Blobel, 1981). The steps after synthesis and cleavage have been investigated for the α-subunit in BC3H-1, a nonfusing mouse muscle cell line. Merlie and his collaborators have used mAbs to show that, after synthesis and before assembly with the other subunits, this subunit undergoes a change in structure that is associated with the acquisition of binding activity for α-bungarotoxin (α-BuTx) (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983; Carlin et al., 1986*a*; Smith et al., 1987). Their experiments also suggest that posttranslational regulation plays a role in determining the amount of active receptor in BC3H-1 muscle cells (Olson et al., 1983*a*; Carlin et al., 1986*b*).

One experimental approach that has been used to investigate the assembly and insertion of complex membrane proteins is the study of genetic variants (Goldstein et al., 1985; Krieger et al., 1985). We have recently described a method by which genetic variants of the C2 mouse muscle cell line (Yaffe and Saxel, 1977) may be recognized and isolated, and have described three clones that are deficient in the surface expression of the AChR (Black and Hall, 1985). Here we report that two of these cell lines fail to synthesize the α-subunit of the receptor at normal rates.

Materials and Methods

Antibodies

Four mAbs and an antiserum against the AChR were used either to immunoprecipitate AChR subunits or as probes to recognize them on immunoblots. mAbs 35 and 61, which recognize the α-subunit, were produced from rats immunized with AChR from *Electrophorus electricus* (Tzartos et al., 1981); mAb 210, which also recognizes the α-subunit (Ratnam et al., 1986*a*), was

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; α-BuTx, α-bungarotoxin.

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prepared using rats immunized with a mixture of AChRs from fetal bovine muscle and the mouse muscle cell line BC3H-1 (Hochschwender, S., and J. Lindstrom, unpublished results); and mAb 124, which recognizes the β-subunit, was derived from rats immunized with *Torpedo* AChR (Gullick and Lindstrom, 1983). mAbs 35 and 210 bind to the main immunogenic region located on the extracellular surface at α46-127 of the α-subunit, and mAb 61 binds to a region located on the intracellular surface at α371-386 (Ratnam et al., 1986*a*, *b*). mAb 124 binds to an intracellular domain of the β-subunit (Gullick and Lindstrom, 1983; Sargent et al., 1984).

The antiserum was raised in rabbits immunized with AChR purified from denervated rat muscle by concanavalin A-Sepharose and cobra toxin affinity chromatography as described by Froehner et al. (1977).

Culture Conditions

C2 cells were cultured according to the general procedures previously described (Inestrosa et al., 1983). In most experiments, cells were plated at a density of 4×10^3 cells/cm^2, and were maintained in a medium containing 20% FCS and 0.5% chick embryo extract until they approached confluence (2-3 d). The medium was then changed to one containing 10% horse serum (fusion medium), and the cultures were analyzed 2-3 d later. The muscle cells at this time were extensively fused into myotubes and AChR expression was at its peak (Inestrosa et al., 1983).

Preparation of C2 Extracts

Myotube cultures were scraped into ice-cold buffer (extraction buffer) containing 10 mM Tris-HCl (pH 7.4)/1 mM EDTA/1 mM EGTA/1% Triton X-100 /1 mM sodium tetrathionate/1 mM *N*-ethylmaleimide/0.5 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/20 U per ml of aprotinin/ 20 μg/ml of leupeptin. 10 ml of extraction buffer per cm^2 of culture surface were used. The cells were homogenized in a ground glass homogenizer, the homogenate was centrifuged for 20 min at 4°C at 25,000 *g* and the supernatant fraction was used as a crude AChR preparation. In some cases the myotubes were taken up and homogenized in extraction buffer without Triton X-100, and the homogenate centrifuged for 1 h at 4°C at 138,000 *g* in an SW 50.1 rotor. The pellet was then homogenized in extraction buffer, and insoluble material was removed by low-speed centrifugation as described above. Both extraction procedures gave essentially the same results in the experiments described below.

Radioactive Labeling of Myotube Proteins

Myotube cultures in 75-cm^2 dishes were incubated either overnight or for 5 min with 0.5 mCi [^{35}S]methionine (5-15 Ci/mmol) in 5-ml fusion medium containing 10 μM unlabeled methionine. After the incubation, the medium was removed, the cultures were rinsed once with PBS, and the cells were extracted as described above.

Assays

AChR and acetylcholinesterase assays were carried out as previously described (Inestrosa et al., 1983; Brockes and Hall, 1975). Creatine phosphokinase assays were performed according to Olson et al. (1983*b*). Protein assays were performed by the method of Lowry et al. (1951).

Kinetics of α-BuTx-AChR Binding

To determine the rate of AChR-α-BuTx association, crude extracts containing AChR (~500 fmol) were incubated at room temperature with 1 nM ^{125}I-α-BuTx in a total volume of 600 μl. Aliquots (80 μl) of the reaction mixture were then diluted at various times into 320 μl ice-cold buffer containing 0.1 μM unlabeled toxin. Toxin-receptor complex was detected by DEAE filtration, and the data were analyzed as previously described (Brockes and Hall, 1975). To determine the rate of dissociation of the toxin-receptor complex, ~500 fmol AChR was incubated in a total volume of 200 μl with 5 nM ^{125}I-α-BuTx for 60 min at 37°C. To block further formation of labeled toxin-receptor complex, unlabeled toxin was added to give a final concentration of 0.5 μM. Aliquots were then taken at various times, and the amount of labeled toxin-receptor complex determined by DEAE filtration.

Sucrose Gradients

Extracts were prepared from cultures in 25-cm^2 plates, as described above, and were layered on a 5-ml linear gradient of 5-20% sucrose in extraction buffer. The gradients were centrifuged in an L-5 centrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 10°C for 14 h at 116,000 *g* in an SW 50.1 rotor. Fractions of ~0.35 ml were then collected, and each fraction assayed for ^{125}I-α-BuTx binding activity by DEAE filtration.

Affinity Purification

An extract made from two 75-cm^2 myotube cultures was incubated for 3 h at 4°C with 0.3 ml of cobra toxin-Sepharose (0.3 mg/ml) prepared as described in Brockes and Hall (1975). The resin was then transferred to a column, washed twice with a 0.5-ml extraction buffer, once with 1 ml 1 M NaCl in extraction buffer, and once with an additional 1 ml extraction buffer. Three successive elutions were then carried out at 30-min intervals with 0.15 ml 1 M carbamylcholine in extraction buffer. The eluants were combined and 40% TCA added to a final concentration of 10%. The precipitated protein was collected by centrifugation and dissolved in 150 μl of SDS-PAGE sample buffer (Laemmli, 1970). After addition of a few microliters of 1 M NaOH to neutralize the sample, it was dialyzed twice for 4-8 h against 600 ml of sample buffer to reduce the ionic strength. A 40-μl aliquot was then subjected to SDS-PAGE according to the procedures of Laemmli (1970).

Immunoprecipitations

Myotube extracts were immunoprecipitated with mAbs 35, 210, and 124, and with an antiserum to denervated rat muscle AChR. For samples in immunoblot experiments, 200-μl aliquots of extract were incubated for 2 h at 4°C with an amount of primary antibody that would precipitate over 50% of the α-BuTx binding activity in extracts of wild-type cells. An IgG fraction of rabbit anti-rat IgG (Cappel Laboratories, Cochranville, PA) conjugated to protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) by the method of Schneider et al. (1982) was then added and the incubation continued for 1 h at 4°C. The material bound to the Sepharose beads was washed three times with 0.5 M NaCl, 0.1% Triton X-100, 10 mM sodium phosphate, pH 7.4, once with 10 mM sodium phosphate, pH 7.4, and then eluted in SDS-PAGE sample buffer by boiling for 4-5 min.

For extracts of [^{35}S]methionine-labeled cells, 100-μl aliquots were incubated with 100 μl of a 10% suspension of fixed *Staphylococcus aureus* (Calbiochem-Behring Corp., La Jolla, CA) for 15 min at 4°C and then, after removal of the *S. aureus*, with primary antibody as described above. When mAbs were used as the first antibody, an IgG fraction of a rabbit anti-rat IgG serum was then added for 60 min at 4°C, followed by a 30-min incubation with fixed *S. aureus* that had been preincubated with unlabeled cell extract. When the rabbit serum was used as a first antibody, preabsorbed *S. aureus* was added without prior addition of second antibody. The immunoprecipitated material was then washed and processed for SDS-PAGE as described above.

Immunoblots

Each immunoprecipitated sample was divided between two gel lanes and subjected to SDS-PAGE. Proteins in the gel were then transferred to nitrocellulose and the nitrocellulose was blocked for 30 min with 0.15 M NaCl, 0.3% Tween 20, 20 mM sodium phosphate, pH 7.4. The nitrocellulose was then incubated overnight in 10 ml of the blocking solution containing either mAb 210 or mAb 124, washed four times with blocking solution, and incubated 2 h with 2×10^6 cpm of ^{125}I-goat anti-rat IgG antibody. Finally, the nitrocellulose was washed four times with the blocking solution.

Fluorography

Gels with [^{35}S]methionine-labeled samples were treated with Enhance (New England Nuclear, Boston, MA) according to the manufacturer's instructions, and exposed to film. Exposures were 3 d for the gels shown in Fig. 4, and 5 d for those shown in Fig. 5.

S1 Nuclease Analysis

RNA was isolated from C2 cells using the guanidine thiocyanate procedures of Chirgwin et al. (1979). Poly(A)$^+$ RNA was selected by chromatography over an oligo (dT) cellulose column (Aviv and Leder, 1972). Hybridization of Poly(A)$^+$ RNA to the cDNA encoding the mouse muscle α-subunit (Boulter et al., 1985) and subsequent S1 nuclease digestion and analysis of S1-resistant products were performed as described previously (Goldman et al., 1985).

Table I. Expression of Activities Characteristic of Differentiated Myotubes in Wild-Type and Variant C2 Cultures

Activity	Wt	1R⁻	2R⁻
Creatine phosphokinase (*μmol/min per mg protein*)	0.63	0.49	1.42
Acetylcholinesterase (*μmol/min per mg protein*)	283	515	594
AChR (*pmol/mg protein*)	0.15	<0.01	<0.01

Myoblasts (10^5 cells) were cultured in 25-cm^2 flasks for 2 d in growth medium and then changed to fusion medium. 5 d after the addition of fusion medium, the cells were scraped from the dish and assays performed as described in Materials and Methods. Each value represents the average of two determinations.

Results

A screen of ~20,000 mutagenized C2 muscle cells, using the replica technique (Raetz et al., 1982; Black and Hall, 1985), yielded two stable variant lines with decreased levels of α-BuTx binding activity. The two variants, which were derived from separate screens, were designated $1R^-$ and $2R^-$ to denote the apparent deficiency in AChR. Toxin-binding activity in variant $1R^-$, measured either on the surface or in extracts of differentiated cells, ranged from 5 to 25% of that in wild-type cells, while activity in $2R^-$ cultures was consistently <10%. Both variants fused to form myotubes in fusion medium and myotubes of both expressed approximately normal amounts of acetylcholinesterase and creatine phosphokinase, two enzymes whose levels are dramatically increased after fusion (Table I). Thus, the variants are not defective in overall differentiation, but have a specific defect in expression of α-BuTx binding activity.

Properties of the AChR in R^- Variants

To determine whether the low level of α-BuTx binding activity in the variants represented a correspondingly low level of functional AChRs, we measured the uptake of ^{22}Na induced by carbamylcholine. In both variants, carbamylcholine-induced ^{22}Na uptake was reduced in proportion to the decrease in toxin-binding activity (Table II). We then examined whether the low levels of toxin binding that we observed could be due to the presence of AChR with altered properties rather than to a deficiency in the number of AChRs. When we examined the kinetics of binding of α-BuTx, neither the association rate of the toxin-binding reaction nor the rate of dissociation of toxin–receptor complex was significantly altered in variant cells. The half-time for dissociation was ~150 h for both wild-type and variant myotubes, and the association rate constant in all three cases was $\sim 5 \times 10^4\ M^{-1}\ S^{-1}$. These values are in good agreement with those obtained earlier for C-2 cells (Gu et al., 1985). The toxin-binding activity in extracts of wild-type and variant cells was also examined by sedimentation in sucrose gradients; in all cases the peak of activity occurred at 9 S (data not shown). The toxin-binding activity present in the variants appears to be associated with receptors whose properties resemble those of the wild-type AChR. We conclude that the reduced toxin-binding activity reflects a reduced amount of AChR.

Table II. Physiological Activity of AChR in Variant C2 Myotubes

	AChr	Percent	^{22}Na uptake	Percent
	fm		*cpm*	
Wt	75	100	2,560	100
$1R^-$	15	20	625	24
$2R^-$	2.2	3	76	3

5-d myotube cultures in 24-well culture plates were assayed for carbamylcholine-stimulated ^{22}Na uptake as described in Maricq et al. (1985). Uptake was measured after 20 s. AChR was assayed by binding of ^{125}I-α-BuTx as described in Materials and Methods. Each value represents the average of two cultures.

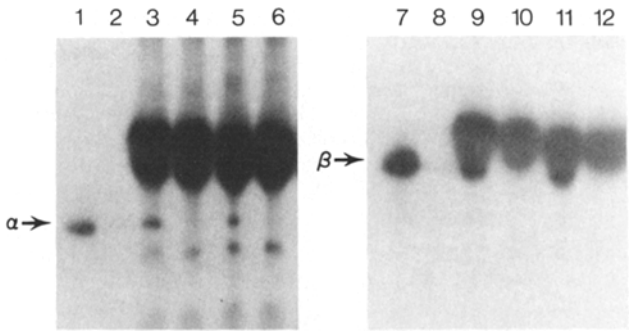

Figure 1. Identification of AChR subunits in C2 cells by immunoblotting. Immunoblotting of extracts of wild-type C2 cells or of partially purified AChR from the extracts was carried out as described in Materials and Methods. In all cases the blots were probed with either mAb 210 to detect the α-subunit or mAb 124 to detect β-subunit. In lanes *1* and *7*, AChR that had been purified from C2 extracts with a cobra toxin-affinity column (see Materials and Methods) was probed with anti-α-antibody or anti-β-antibody, respectively. Lanes *2* and *8* are the same except that α-BuTx was added to the C2 extract before affinity chromatography to prevent the adsorption of receptor to the column. Lanes *3* and *9* show extracts precipitated with anti-α-antibody (mAb 35) and probed with anti-α- or anti-β-antibodies, respectively. Lanes *4* and *10* show extraction buffer analyzed as described for lanes *3* and *9*. Lanes *5* and *11* show extracts precipitated with anti-α-antibody (mAb 124), and probed with anti-α or anti-β-antibodies, respectively. Lanes *6* and *12* show extraction buffer analyzed as described for lanes *5* and *11*.

Analysis of AChR Subunits

The α-subunit of the AChR binds α-BuTx in the absence of other subunits (Haggerty and Froehner, 1981; Gershoni et al., 1983; Oblas et al., 1983). We therefore examined the level of this subunit in variant cells, along with the β-subunit for comparison. Because γ- and δ-subunits are readily degraded in muscle cell extracts, their levels were not determined. To detect α- and β-subunits, we used mAb 210, which is specific for the α-subunit (Ratnam et al., 1986*a*), and mAb 124, which is specific for the β-subunit (Gullick and Lindstrom, 1983). When tested on immunoblots with AChR that was partially purified from wild-type C2 cells by cobra toxin-affinity chromatography, each of these antibodies recognized a single band, of ~40 and 50 kD, respectively (Fig. 1, lanes *1* and *7*). Binding to both bands was eliminated if the AChR was prevented from binding to the affinity column by incubation of the C2 extract with excess α-BuTx (Fig. 1, lanes *2* and *8*).

Because extracts of the variant cells contain little toxin-binding activity, purification of receptor subunits from these

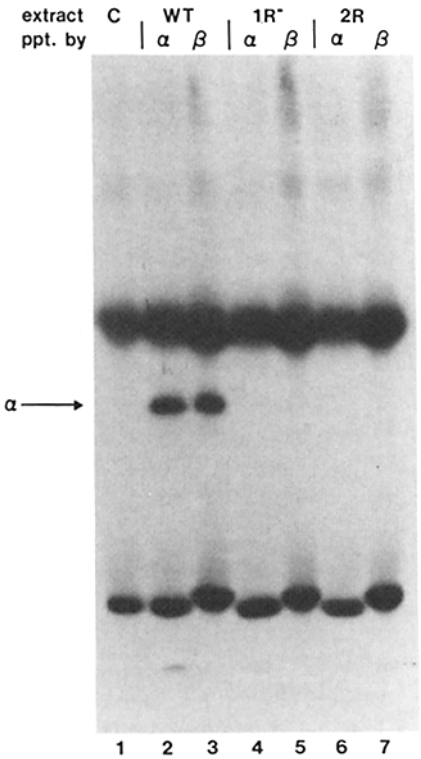

Figure 2. α-Subunit in wild-type and R⁻ variants. Immunoblotting of extracts of wild-type and variant cells was performed as described in Materials and Methods. The anti-α-antibody used for immunoprecipitation was mAb 35 and the anti-β-antibody was mAb 124. In all cases the antibody used as probe to detect the α-subunit was mAb 210. Lane *1*, extraction buffer precipitated with anti-α-antibody; lanes *2* and *3*, wild-type extract precipitated with anti-α and anti-β-antibodies, respectively; lanes *4* and *5*, 1R⁻ extract precipitated with anti-α and anti-β-antibodies, respectively; lanes *6* and *7*, 2R⁻ extract precipitated with anti-α and anti-β-antibodies, respectively.

cells using cobra toxin-affinity chromatography was not possible. It was also not possible to detect α- and β-subunits reliably in immunoblots of crude extracts of either wild-type or variant cells. We therefore devised a procedure in which the subunits were immunoprecipitated from crude extracts using mAbs to α- or β-subunits. These partially purified preparations were subjected to SDS gel electrophoresis and transferred to nitrocellulose. The immunoblots were then incubated with the mAb probe, followed by ^{125}I-second antibody. This method has the disadvantage that immunoglobulin from the immunoprecipitation is recognized by the ^{125}I-second antibody and forms a strong background band on the film. Both α- and β-subunits were separated well enough from the immunoglobulin bands, however, to be clearly recognized (Figs. 1 and 2).

In extracts of wild-type cells treated by this procedure, the α-subunit was precipitated both by anti-α and by anti-β-antibodies, indicating that the two subunits were part of a larger complex that is presumably the intact AChR (Fig. 2, lanes *1–3*). In the case of both variants, we detected little or no α-subunit after precipitation with either antibody (Fig. 2, lanes *4–7*).

When the anti-β-mAb was used as the probe for immunoblotting, β-subunit could also be detected in extracts of wild-type cells after immunoprecipitation with either anti-α or anti-β-mAbs (Fig. 3, lanes *1* and *2*). In the case of the variants, β-subunit was detected after immunoprecipitation with anti-β-antibody, but was present in reduced amounts (Fig. 3, lanes *4* and *6*). Significantly, β could not be detected in the variants after immunoprecipitation with anti-α-antibody (Fig. 3, lanes *3* and *5*). The results with antibodies to both α- and β-subunits thus indicate that a drastically reduced amount of α-subunit that is recognized by mAb 210 is accumulated in the variant cell lines.

Metabolic Labeling

We attempted to detect the α-subunit in a different way by metabolically labeling the proteins in C2 myotubes with [^{35}S]methionine. After labeling the cells for 16 h, aliquots of cell extracts were incubated in parallel experiments with two mAbs that recognize α-subunit (mAb 210 and mAb 35), one that recognizes β-subunit (mAb 124), and a rabbit antiserum that was raised against purified AChR from denervated rat muscle and that recognizes principally the α-subunit. The immunoprecipitates were then analyzed by SDS-PAGE and fluorography. In extracts of wild-type cells, each of these antibody preparations precipitated a protein of ~40 kD (Fig. 4, lanes *1*, *4*, *7*, and *13*). This protein, which

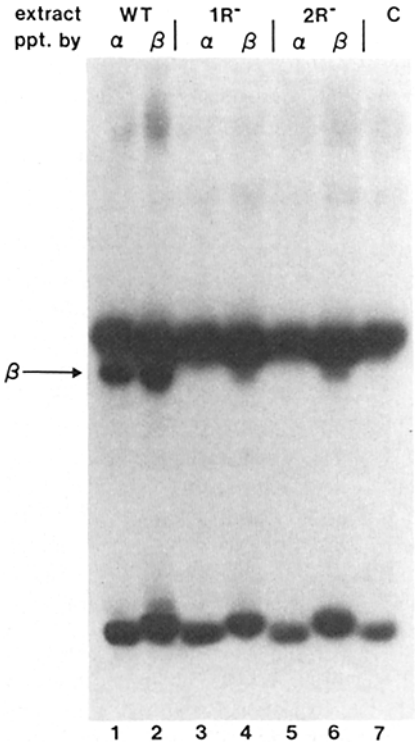

Figure 3. β-Subunit in wild-type and R⁻ variants. Immunoblotting of extracts of wild-type and variant cells was performed as described in Materials and Methods. The anti-α-antibody used for immunoprecipitation was mAb 35 and the anti-β-antibody was mAb 124. In all cases the antibody used as probe to detect the β-subunit was mAb 124. Lanes *1* and *2*, wild-type extract precipitated with anti-α and anti-β-antibodies, respectively; lanes *3* and *4*, 1R⁻ extract precipitated with anti-α and anti-β-antibodies, respectively; lanes *5* and *6*, 2R⁻ extract precipitated with anti-α and anti-β-antibodies, respectively; lane *7*, extraction buffer precipitated with anti-α-antibody.

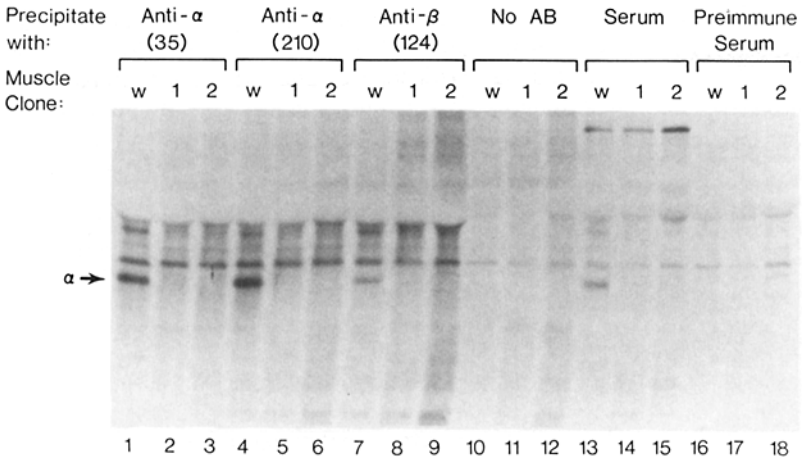

Figure 4. Immunoprecipitation of α-subunit from wild-type and variant cells after long-term labeling with [^{35}S]methionine. 3 d after addition of fusion medium, the cells were incubated with [^{35}S]-methionine for 16 h as described in Materials and Methods. Extracts of the cells were then immunoprecipitated with different antibodies and the immunoprecipitates analyzed by SDS gel electrophoresis as described in Materials and Methods. Lanes *1–3*, radioactive proteins precipitated by the anti-α-mAb 35 from wild-type, 1R$^-$ and 2R$^-$ extracts, respectively; lanes *4–6*, proteins precipitated by the anti-α-mAb 210 from wild-type, 1R$^-$ and 2R$^-$ extracts; lanes *7–9*, proteins precipitated by the anti-β-mAb 124 from wild type, 1R$^-$ and 2R$^-$ extracts; lanes *10–12*, proteins precipitated in the absence of first antibody from wild-type, 1R$^-$ and 2R$^-$ extracts; lanes *13–15*, proteins precipitated by an anti-AChR antiserum from wild-type, 1R$^-$ and 2R$^-$ extracts; lanes *16–18*, proteins precipitated by the preimmune serum from wild-type, 1R$^-$ and 2R$^-$ extracts.

is the α-subunit, was not seen in the absence of antibody or with preimmune serum (Fig. 4, lanes *10* and *16*). No radioactive band at 40 kD, or at any other position, was specifically precipitated from extracts of the variant cells by any of the antibodies that we tested (Fig. 4, lanes *2*, *3*, *5*, *6*, *8*, *9*, *14*, and *15*). Both the immunoblot experiments and the long-term metabolic labeling experiments thus show that the variant cells fail to accumulate significant steady-state levels of α-subunit.

In the metabolic labeling experiments, a radioactive band in the expected position for the β-subunit was seen in wild-type, but not variant cells after immunoprecipitation with either anti-α-antibodies (Fig. 4, lanes *1–6*) or with the serum (Fig. 4, lanes *13–15*). Radioactivity was also seen in the β region in both wild-type and variant cells after immunoprecipitation with the anti-β antibody. Although these results are consistent with the results of the immunoblots, their interpretation is complicated by the presence of nonspecifically precipitated proteins in the 50-kD region that prevent the unequivocal identification of the β-subunit in each case.

The virtual absence of the α-subunit in long-term metabolic labeling experiments could arise from a defect in synthesis or from the rapid degradation of an altered or abnormal subunit. To distinguish these possibilities, we labeled cells with [^{35}S]methionine for only 5 min and immunoprecipitated the α-subunit. Under these conditions, the amount of labeled α-subunit should reflect the rate of its synthesis. The immunoprecipitation was carried out using either the anti-AChR serum described above or mAb 61, which has been shown by Merlie and Lindstrom (1983) to recognize the primary transcription product of the α-subunit. Similar results were obtained in both cases. In experiments with the serum, labeled α-subunit was immunoprecipitated from wild-type cells after a 5-min pulse of [^{35}S]methionine but little or no labeled α-subunit was detected in either variant (Fig. 5). Also with mAb 61, only a small amount of labeled subunit was immunoprecipitated from the variants (data not shown). In these experiments the radioactivity in the α-subunit relative to that of the contaminating actin band was compared in the three cases. The ratio of α to the reference band was 0.98 in the case of wild-type and 0.05 and 0.08 in the case of 1R$^-$ and 2R$^-$. We conclude from the results of experiments with both the antiserum and with mAb 61 that the α-subunit is synthesized at an abnormally low rate in the variant cells.

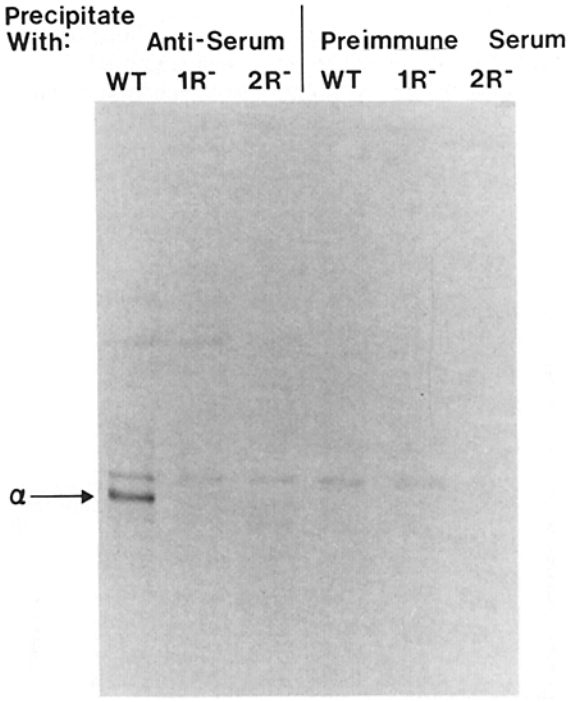

Figure 5. Immunoprecipitation of α-subunit after pulse-labeling of wild-type and variant cells with [^{35}S]methionine. The experiment was performed as described in Fig. 4, except that the cells were labeled for 5 min with [^{35}S]methionine. Lanes *1–3*, proteins precipitated by an anti-AChR antiserum from wild-type, 1R$^-$ and 2R$^-$ extracts, respectively; lanes *4–6*, proteins precipitated by the preimmune serum from wild-type, 1R$^-$ and 2R$^-$ extracts.

α-Subunit mRNA

We examined the mRNA coding for the α-subunit of the AChR in wild-type and variant cells using a probe prepared from a cDNA clone described by Boulter et al. (1985). This clone was obtained from a cDNA library prepared from the mouse muscle cell line, BC3H-1 (Schubert et al., 1977). When poly(A)$^+$ RNA was extracted from myotube cultures of each of the three cell types and analyzed by Northern blots, a single band of 2 kb and of approximately equal intensity was found in each case (data not shown).

To determine whether the mRNA coding for the α-subunit in the variants was altered, we carried out an S1 nuclease digestion of heteroduplexes formed between the mRNA and regions of the cDNA clone coding for the α-subunit as described by Maniatis et al. (1982). This clone codes for the entire mature α-subunit and includes 26 bases coding for the leader peptide at its 5′ end. Three fragments of this clone were subcloned into the single-stranded phage M13 (Messing and Vierra, 1982) and used for the analysis. The first contains 450 bases at the 5′ end of the clone; the second contains the remaining 1,270 bases at the 3′ end; and the third contains 540 bases between two Rsa I sites near the extreme 3′ end (Fig. 6 *A*).

For the analysis, mRNA prepared from wild-type cells and from the two variants was hybridized to single-stranded DNA from each of the subclones. After digestion with S1 nuclease, the hybrids were subjected to electrophoresis through denaturing polyacrylamide gels and electroblotted to gene-screen plus, and the mRNA sequences surviving S1 nuclease digestion were visualized by hybridization with a nick-translated probe prepared from the entire cDNA clone (Fig. 6 *B*).

The results of these experiments showed that both variants made approximately normal levels of α-subunit mRNA, and that in each case the protected fragments seen with all three probes were indistinguishable from those seen with wild-type mRNA. The multiple fragments protected by the 3′-1,270 and 3′-540 nucleotide-long probes likely reflect the expression of α-subunit mRNAs that use different polyadenylation signal sequences (Goldman et al., 1985). Thus, the S1 analysis revealed no differences from the wild-type in α-subunit mRNA made by either of the two variants.

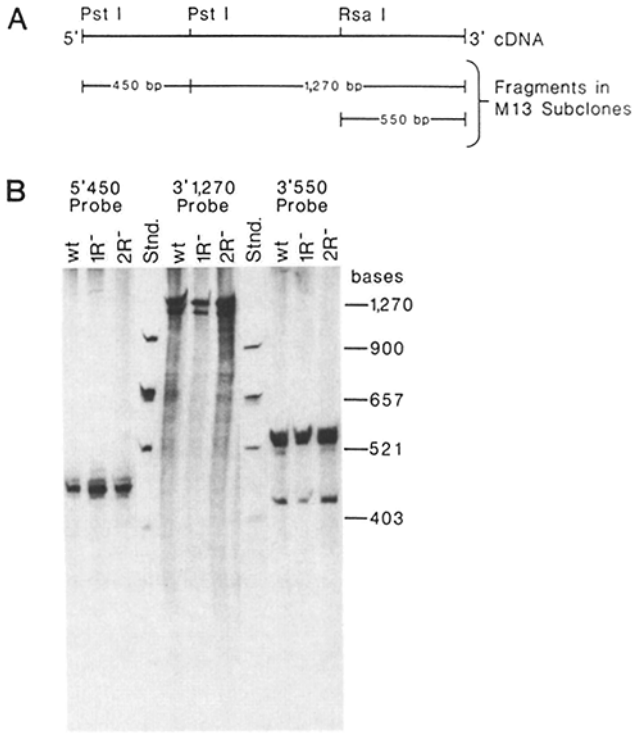

Figure 6. S1 nuclease analysis of C2 mRNAs. (*A*) Fragments of cDNA subcloned into M13 for hybridization with mRNA. (*B*) Gel profile of S1 nuclease–protected fragment generated by S1 nuclease digestion of heteroduplexes formed between the wild-type and the two mutant C2 cell line mRNAs (5 μg) with the 5′-450, 3′-1,270, and 3′-546 nucleotide-long subclones.

Discussion

We have attempted to define the defect in two variants of the C2 muscle cell line that were isolated by a replica technique in which intact cells were screened for a reduced amount of binding of α-BuTx. The two variants, 1R$^-$ and 2R$^-$, are characterized by a deficiency of toxin-binding activity, both in cell extracts and on the cell surface. They are similar in all aspects that we have investigated. Because the cells fuse to form myotubes and express levels of creatine phosphokinase and acetylcholinesterase that are equal to or above those of wild-type cells, the defect is not a simple failure of differentiation, but appears to be specific to the AChR.

One possibility is that the toxin-binding function of the AChR is aberrant, but that the receptor is otherwise normal. This does not seem to be the case, as the ability of the variants to take up ^{22}Na was reduced in proportion to the reduction in toxin-binding activity. Moreover, the kinetics of toxin binding associated with the residual activity is similar to that found in wild-type cells, as is the sedimentation constant. We conclude that the deficiency in toxin binding is due to a reduced amount of functional receptor.

Analysis of cell extracts of both 1R$^-$ and 2R$^-$ with subunit-specific antibodies showed that neither of the variants accumulated detectable amounts of the α-subunit. One possible explanation of the failure to accumulate the α-subunit is the production of a polypeptide chain that is abnormally suceptible to proteolytic degradation. In our experiments, however, the amount of labeled α-subunit detected after a 5-min pulse with [^{35}S]methionine was reduced by over 90%. Because of the short time of labeling, this decrease is unlikely to be due simply to an increase in the rate of α degradation.

Our results are most easily explained by a defect in synthesis of the α-subunit or by the failure of our antibodies to recognize a polypeptide chain that is altered or that is not properly modified. The specificities of the antibodies that we have used makes it unlikely that an intact α-chain or any substantial fragment of it is produced in normal amounts by the variant cells. Both the serum and mAb 61, the antibodies used in the short-term labeling experiments, recognize the α-chain produced after a 5-min pulse with [^{35}S]methionine in wild-type C2 cells. In BC3H-1 cells Merlie and his colleagues have previously shown that mAb 61 recognizes the α-subunit after 5 min of labeling, and that this corresponds to a time before a substantial fraction of the α-subunit has

undergone the modifications that confer toxin-binding activity on it (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983; Smith et al., 1987). mAb 61 has been also shown to recognize proteolytic fragments of the *Torpedo* α-subunit (Ratnam et al., 1986*a*) and forms of the α-subunit in BC3H-1 cells in which the NH_2-linked sugars are altered or missing (Merlie et al., 1982; Smith et al., 1986). In other experiments, we used mAb 210 which recognizes a portion of the α-chain that is near the NH_2-terminus, between amino acid residues 46 and 127. This antibody binds to denatured α-subunit and to proteolytic fragments of α on immunoblots (Ratnam et al., 1986*a*). Finally, we have used in experiments not reported here an mAb (14-3-F7) that binds to the α-chain on immunoblots and that recognizes an unidentified cytoplasmic portion of the α-chain (Black, R. A., A. Dowding, and Z. W. Hall, unpublished results). None of these antibodies detects a polypeptide chain made in substantial amounts in the variant cells.

In contrast to α, the β-chain was clearly detected in immunoblots of extracts from both variants, although in reduced amounts. In quantitative radioimmunoassay experiments, the β-subunit in $1R^-$ cells has been estimated to be 35% that of the wild-type (LaRochelle, W. S., S. Froehner, and Z. W. Hall, unpublished results). The β-subunit in extracts of the variant myotubes was not complexed with α-subunits, since it could not be precipitated by antibodies to α. These results are consistent with the virtual absence of an immunologically competent α-chain in the extracts. The lower amounts of β found in variant as compared with wild-type cells could result from a reduced rate of synthesis of β or from a greater susceptibility of unassembled β-subunit to proteolytic degradation. Because of difficulties in reliably identifying the β-band in radiolabeling experiments, we did not pursue this question. Whether γ- and δ-chains are also made by these cells is unknown. Because both are highly susceptible to proteolysis in extracts, and fewer specific antibodies are available for their detection, we did not test for their presence.

Although α-chains were found at barely detectable levels, α-mRNA was present in both variants at approximately normal levels. Moreover, an S1 analysis using three mouse cDNA probes that covered the entire coding region for the α-subunit revealed no differences between variant and wild-type mRNAs. These results suggest that if there are differences in the regions covered by the probes, they are subtle and probably consist of single base pair changes. Since the original cDNA clone lacks part of the leader sequence and the 5′-untranslated region, there could be differences in these regions between variant and wild-type mRNAs that we do not detect.

What is the molecular basis of the difference between the variants and the wild-type cells? One possibility is that there is a defect in the mRNA for the α-subunit that escaped detection by S1 nuclease analysis. Such a defect might occur either in the coding region leading to a truncated polypeptide chain, or in the 5′-untranslated region leading to defective chain initiation. A second, more remote possibility is that the defect is outside the structural gene for the α-subunit, and is expressed as the absence of some function that is required for α-chain synthesis. Experiments with expression vectors containing an intact cDNA for the α-subunit should distinguish these possibilities.

Whatever the cause of the defect, the availability of a muscle cell line that makes reduced amounts of α-subunit, and therefore of the AChR, should be a useful tool in investigating assembly of the receptor and construction of the specialized postsynaptic membrane.

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