A C2 Muscle Cell Variant Defective in Transport of the Acetylcholine Receptor to the Cell Surface*

Yong Gu, Roy A. Black†, George Ring§, and Zach W. Hall¶

From the Neuroscience Program, Department of Physiology, School of Medicine, University of California, San Francisco, California 94143-0444

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The factors regulating assembly and transport to the cell surface of multimeric membrane proteins are poorly understood (Carlin and Merlie, 1987). One of the proteins whose synthesis and assembly has been studied most intensively is the nicotinic acetylcholine receptor (AChR), a pentameric transmembrane ion channel consisting of four different but highly homologous subunits that are assembled in the stoichiometric ratio α2β2γ (McCarthy et al., 1986). The α subunit carries the binding site for acetylcholine and also binds α-bungarotoxin (α-BuTx) (Karlin, 1980); the ion channel is thought to be formed by all five subunits (Popot and Changeux, 1984; McCarthy et al., 1986).

Each subunit of the AChR is synthesized as a separate polypeptide chain and is cotranslationally inserted into the endoplasmic reticulum (ER), where the signal sequence is removed and the polypeptide N-glycosylated (Anderson and Blobel, 1981; Merlie et al., 1981, 1982). The subunits are then assembled in the ER to form the oligomeric AChR (Smith et al., 1987). Assembled AChRs are transported to the Golgi apparatus (Pambrough and Devreotes, 1978) where the N-linked oligosaccharides on the γ and δ subunits are processed to complex forms (Gu and Hall, 1988). The receptor is then transported to the cell surface. Assembled AChR is first detected in the ER about 15 min after synthesis of α- and β-polypeptide chains (Merlie and Lindstrom, 1983; Smith et al., 1987), and most of the assembled AChR appears on the surface between 50 and 160 min after synthesis (Devreotes et al., 1977; Smith et al., 1987).

The α subunit undergoes an early maturation step before assembly occurs. Shortly after synthesis, the primary translation product of the α subunit undergoes a change in its conformation that is reflected by the acquisition of α-BuTx-binding activity and by changes in its immunological properties. This transition occurs in the ER, after the addition of N-linked oligosaccharides and the early trimming reactions, but before receptor assembly (Merlie et al., 1982; Smith et al., 1986). Only a small portion (30%) of the synthesized α subunit is assembled into oligomeric AChR; the remainder is rapidly degraded (Merlie and Lindstrom, 1983; Smith et al., 1987).

We have recently described genetic variants of the C2 mouse muscle cell line that are defective in expression of the AChR on the cell surface (Black and Hall, 1985). One of these variants, T−, appears to make a normal amount of the AChR but accumulates most of it in an intracellular pool. We report here experiments whose purpose is to explore the functional defect in this variant by characterizing AChR assembly and transport to the cell surface.

MATERIALS AND METHODS AND RESULTS*

**DISCUSSION**

The reduced binding of α-BuTx to intact T− myotubes (Black and Hall, 1985) is apparently the result of a decreased number of functionally normal AChRs in the surface membrane. Both the amount of surface α subunit detected by a monoclonal antibody and the rate of carbachol-stimulated...


$^{22}$Na influx into $T^-$ myotubes are reduced in proportion to the decrease in $\alpha$-BuTx binding. In addition, the kinetics of the $\alpha$-BuTx binding reaction and the half-life of surface AChR are similar in wild-type and $T^-$ cells.

Examination of detergent extracts of $T^-$ cells demonstrate that the cells contain toxin-binding activity in an internal compartment that is inaccessible to externally added $\alpha$-BuTx. This internal pool apparently consists of fully assembled $9S$ AChR that is accumulated to levels that are three to five times those in wild-type myotubes.

Two Functionally Distinct Pools of Internal AChR—Intra-
cellular AChR was first detected by Devreotes and Fambrough (1975) in primary cultures of chick myotubes, who found that it constituted about 30% of the total cellular AChR. They also showed that the internal AChR consists of two pools. The larger pool contains precursor for surface AChR; in the absence of protein synthesis continued transport of AChR to the surface depletes this pool within several hours. The second pool, which they termed the "hidden pool," constitutes about 25% of the total intracellular AChR, and is not depleted when protein synthesis is inhibited. Our experiments show that $C2$ cells behave similarly. In this case, approximately 80% of the internal AChR serves as precursor for surface AChR which is transported to the surface over a period of a few hours.

This scheme offers two possible explanations for the increased proportion of internal receptor in the $T^-$ variant. One possibility is that a kinetic block slows the movement of the AChR to the cell surface, causing AChR to accumulate in the precursor pool. If this were the case, inhibition of protein synthesis should have little effect on the rate of AChR appearance on the surface as the large precursor pool slowly depletes over many hours. A second possibility is that the accumulated AChR is not in the precursor pool, but in another pool, perhaps resembling the hidden pool in its properties.

Our experiments clearly indicate that most of the accumulated AChR in $T^-$ myotubes does not serve as precursor for surface AChR. $T^-$ cells have a precursor pool, which is smaller than in normal cells, but which, in the absence of protein synthesis, is depleted by transport to the surface at normal rates. The remaining AChR, approximately 70% of the total internal AChR, remains in an internal pool that is unaffected by protein synthesis inhibitors.

Pulse-chase experiments confirmed the results obtained with cycloheximide. There was no difference between wild-type and $T^-$ cells in the kinetics of transport of newly assembled AChR to the surface. A striking difference was observed, however, between the proportion of newly assembled AChR appearing on the surface in the two cell types. Whereas almost all of the AChR in wild-type cells is transported to the surface, only a small proportion of the AChR that is made by $T^-$ cells is destined for the cell surface; the rest remains internal for many hours.

The decreased efficiency of transport of the AChR to the surface does not appear to be a general defect. Thus, $T^-$ myotubes contain a normal amount of insulin receptor and transferrin receptor on their surface and transport hemagglutinin to the surface with normal efficiency after infection with influenza virus.

AChR Assembly in $T^-$ Cells—Because of the availability of a monoclonal antibody that recognizes all forms of the $\alpha$ subunit (Smith et al., 1986, 1987), we were able to use pulse-chase experiments to follow its synthesis and assembly into intact receptor. These experiments showed that synthesis of $\alpha$ subunit, conversion to a toxin-binding form, and assembly into intact AChR occur at similar rates in wild-type and $T^-$ cells. A major difference, however, is the slower degradation of $\alpha$ subunit in $T^-$ cells. Thus, a larger amount of $\alpha$ subunit was found in all three forms: as $\alpha_0$, the primary translation product, as $\alpha_T$, the 5 $S$ toxin-binding form, and as assembled AChR. The $T^-$ cells are not completely lacking the ability to rapidly degrade $\alpha$ subunit, however, as $\alpha$ subunit lacking N-linked oligosaccharides, or with abnormal N-linked sugars, is quickly broken down (Fig. 7).

These experiments lead to two important insights related to AChR synthesis and assembly. First, although earlier experiments in BC3H-1 cells had shown that most newly synthesized $\alpha$ subunit was degraded, it was unclear whether extensive degradation of the $\alpha_0$ form, the $\alpha_T$ form, or both, occurred. The accumulation of $\alpha_0$ to a greater extent than either of the other forms in $T^-$ cells suggests that this is the form that is normally degraded.

Second, the accumulation of $\alpha$ subunit in $T^-$ cells to a level that is approximately 2-fold that found in normal cells results in increased formation of both $\alpha_T$ and assembled AChR. The proportion of total $\alpha$ that is converted to assembled AChR is in fact almost the same in wild-type and $T^-$ cells. These results suggest that the availability of $\alpha$ subunit is at least one limiting factor in the assembly of the AChR and that the degradation of the $\alpha$ subunit does not occur because it is in excess. Other subunits are probably also degraded in wild-type cells.

The Defect in $T^-$ Cells—The experiments reported here demonstrate that the $T^-$ variant of $C2$ muscle cells has two apparently paradoxical defects in AChR assembly and transport. The first is decreased degradation of newly synthesized $\alpha$ subunit, resulting in increased $\alpha$ levels and, consequently, higher levels of newly assembled AChR. In spite of the increased amount of newly assembled AChR, however, $T^-$ cells transport less AChR to the surface than do wild-type cells. Recent experiments indicate that the AChR that is not transported to the surface accumulates in a pre-Golgi compartment that is probably the endoplasmic reticulum (Gu et al., 1989).

Are these two defects linked? The best evidence for such a relationship would be the demonstration that phenotypic reversion of the $T^-$ strain results in the loss of both defects. In the absence of such evidence, the relationship of these properties must remain speculative. One possibility is that degradation normally removes $\alpha$ subunit polypeptides that have folded incorrectly and thus prevents their incorporation into the oligomeric AChR. A defect in degradation might then lead to the production of AChRs that are not competent for transport to the surface. It should be noted, however, that primary myotubes do not break down unassembled $\alpha$ subunit and have no apparent defect in transporting AChR to the surface (Carlin et al., 1986). Because the defect in transport appears to be specific for the AChR, it most likely arises from a mutation in the structural gene for one of the subunits. If this proves to be the case, it will be of interest to know what distinguishes the two pools of AChR, one that is competent for transport to the surface and one that is not.

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Antibodies -- Four monoclonal antibodies (mAbs) were used to immunoprecipitate the AChR: mAbs 6A1, 25 and 21D (Tzartos et al., 1981; Ratafia et al., 1986). Detailed characterization of the antibodies is published elsewhere (K. S. Matlin and K. Simons, unpublished observations, 1985). The cell culture-secreted (C2) mouse muscle cell line, isolated originally by Yaffe and Roche (1977), was maintained in DME media supplemented with 20% fetal bovine serum and 0.1% chick embryo extract as described in Dumont et al. (1981) and Gu et al. (1983). Differentiation of myoblasts into myotubes was stimulated by transferring cells into medium containing 0.1% horse serum in DME (13% C2 cells) and 82% were used 4 days after transfer to culture medium.

Materials and Methods

Immunoprecipitation of pulse-chase labeled AChR -- Labeled cells were extracted with Buffer A and the solubilized receptors precipitated using one of these procedures. For immunoprecipitation with mAb 6A1, which recognizes all species of the n-subunit (Tzartos et al., 1981, 1985), cell extracts were incubated with 2 µl of the antibody C25 at 4°C, followed by the addition of protease inhibitors (Salk Institute for Biological Studies, San Diego, CA; generously supplied by Dr. J. B. Sleat). The extract was then incubated with 3 µl of Protein A-Sepharose for 2 hr. The precipitate was washed three times in Buffer A and then solubilized in sodium dodecyl sulfate (SDS) buffer for analysis on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Biological assays -- Binding of 125I-labeled to intact myotubes was measured by the method of Tzartos et al. (1981). The bound receptor was determined by dissociating the cells in 0.1 M NaOH and measuring the radioactivity in a counter. Surface binding of the 125I-labeled toxin was blocked by washing the membrane with the toxin-binding activity. The extracts were then incubated with 2 µl of the antibody for 2 hr at 4°C, followed by the addition of 10 µl of Protein A-Sepharose for 2 hr. The precipitate was washed three times in Buffer A and then solubilized in sodium dodecyl sulfate (SDS) buffer for analysis on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Results

Immunoprecipitation of pulse-chase labeled AChR -- Labeled cells were extracted with Buffer A and the solubilized receptors precipitated using one of these procedures. For immunoprecipitation with mAb 6A1, which recognizes all species of the n-subunit (Tzartos et al., 1981, 1985), cell extracts were incubated with 2 µl of the antibody C25 at 4°C, followed by the addition of protease inhibitors (Salk Institute for Biological Studies, San Diego, CA; generously supplied by Dr. J. B. Sleat). The extract was then incubated with 3 µl of Protein A-Sepharose for 2 hr. The precipitate was washed three times in Buffer A and then solubilized in sodium dodecyl sulfate (SDS) buffer for analysis on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

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**Results**

Surface AChR — The T-variant was originally isolated from transgenic C2 cells on the basis of reduced binding of a-wraptotoxin to the surface of differentiated myotubes (Ishikawa et al., 1986; Gu et al., 1991). The T-variant half-life of surface AChR as determined by labeling surface AChR with 125I-a-BuTx and following the loss of surface AChR site on the cells as described in Gu et al. (1986; 1991) was 1.9 ± 0.5 min. When calculated quantitatively, the amount of surface AChR bound to intact T- myotubes was about one-fifth that bound to wild-type myotubes (Table III). Thus, the amount of surface bound AChR was due to a reduced amount of surface AChR was confirmed by the observation that intact myotubes n-100 was about 50% of the internal 125I-a-BuTx amount of intact myotubes determined by binding assay were not different in wild-type and T- myotubes. Although 125I-labeled surface proteins may have altered levels, it is unlikely from these results that the defect is a general one. The fact that T- cells grow and fuse normally (Shack and Hall, 1985) also argues against a general defect that reduces the levels of cell surface proteins.

**Table II.** Properties of Surface AChR

| Phenotype | T- | ACHE |
|-----------|----|------|
| Association constant of hemicholinium reaction (10^6 M⁻¹ sec⁻¹) | 2.43 | 2.94 |
| Carbamylated-125I uptake (nM/mg membrane) | 160 | 164 |
| Half-life of the membrane 125I | 10.3 + 0.3 | 13.9 + 0.4 |
| Segmentation coefficient | 6.6 | 6.8 |

**Table III.** Activities of Surface Proteins in wild-type and T- myotubes.

| Myotubes | T- | ACHE |
|----------|----|------|
| Surface AChR | 51 | 60 |
| Surface ACHE | 51 | 60 |

**Table IV.** Expression of surface AChR in wild-type and T- myotubes.

| Wild-type | T- |
|-----------|----|
| M-H-11.3 | 31 |

**Discussion**

Surface AChR in T- myotubes behaved normally by several criteria. Thus, the rate constant of the association reaction with a-wraptotoxin and carbamylated-125I uptake was similar to that seen in wild-type cells (Table I). The morphologic half-life of the AChR was of particular interest because a defective AChR might be degraded more quickly, resulting in a shorter halftime. No change was detectable in the AChR half-life of T- cells, however, which is slightly longer than that of normal cells, suggesting that the defect is not related to a deficiency in the cellular compartmentalization of AChR. In both wild-type and T- myotubes, the AChR on the surface of T- cells were found to have a reduced amount of functionally normal AChR on their cell surface.

**Table V.** Velocity sedimentation analysis of a-wraptotoxin-binding activities in wild-type and T- myotubes.

| Myotubes | T- |
|----------|----|
| Velocity sedimentation analysis of a-BuTx-binding activity | 0.24 ± 0.03 |

**Conclusion**

The internal pool as a reservoir of surface AChR. Although the internal AChR in T- cells are not apparent in a quantitatively normal pool as shown in chick transgenic T- cells (Ishikawa et al., 1986) and in chick embryonic T- cells (Gu et al., 1991), it is clear that these two pools of AChR are significantly different and that the internal pool of AChR in T- cells is not detectable by labeling with surface proteins. In summary, the results of these experiments suggest that the wild-type mouse variant phenotype is dominant and that the products of each mouse behave independently.
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In the case of T-cells, the results were similar to those obtained in the wild-type AChR, although the rate of AChR disappearance was slower, approximately 10% of the rate observed in wild-type AChR. The percentage of internal AChR in T-cells was slightly lower than in wild-type AChR, in agreement with previous reports (Merlie et al., 1983). The results suggest that the rate of appearance of newly synthesized AChR in wild-type cells is similar to that observed in T-cells, indicating that there is no significant difference in the rate of AChR transport between wild-type and T-cell lines.

In summary, the results of these experiments indicate that the rate of AChR transport is similar between wild-type and T-cell lines, suggesting that the defect in AChR transport in T-cells is not due to a difference in the rate of AChR transport itself. Further studies will be needed to determine the specific mechanisms underlying this defect.

In wild-type myotubes, the amount of radiolabeled α-subunit immunoprecipitated by mAb 61 was at a maximum immediately after the pulse with labeled amino acids and then declined rapidly to less than 5% of the initial value 60 min after the pulse. These results are consistent with those of Merlie and colleagues, who found that T-cells that approximately 10% of the α-subunit does not assemble into AChR, but is rapidly degraded in the presence of mAb 61, and that wild-type myotubes that express T-cells do not have a significant amount of labeled α-subunit synthesized by T-cells during the pulse. However, the amount of labeled α-subunit in wild-type myotubes was substantially higher than in T-cells, indicating that the defect in AChR transport in T-cells is not due to a difference in the synthesis of labeled α-subunit by wild-type and T-cell lines.

In order to determine whether the rate of appearance of newly synthesized AChR is similar in wild-type and T-cell lines, the experiments were repeated with a longer incubation period. The results showed that the amount of labeled AChRs synthesized in wild-type cells is approximately 10% higher than in T-cells, indicating that there is a difference in the synthesis of AChR in these two cell lines.

In conclusion, the results of these experiments indicate that the rate of appearance of newly synthesized AChR is similar in wild-type and T-cell lines, suggesting that the defect in AChR transport in T-cells is not due to a difference in the rate of AChR transport itself. Further studies will be needed to determine the specific mechanisms underlying this defect.
The assembly of newly-synthesized α and β subunits was more quantitatively measured by isolating the AChR with mAb 88B-Sepharose. Quantitation of the labeled α subunit assembled into AChR in wild-type cells showed that assembly was complete by about two hr (Fig. 5D). Similar results were seen for T' myotubes (Fig. 5F). The total amount of newly-synthesized α subunit in assembled AChR was greater, however, in T' myotubes than in wild-type myotubes. The proportion of total labeled α subunit that acquired toxin-binding activity and that was assembled into intact AChR was about the same in T' and wild-type cells (Fig. 6). Thus the steps from synthesis of the α subunit to assembly of the AChR all appear to occur normally in T' cells with one exception: α subunit is broken down less rapidly.

α Subunit degradation in the absence of glycosylation -- Merlie et al. (1982) have shown that a subunit synthesized in the presence of tunicamycin (TM) does not acquire the ability to bind α-Btx with high affinity and is rapidly degraded. Inhibitors of trimming of glycosyl residues on N-linked oligosaccharides with deoxynojirimycin (DNJ) also increases the degradation of the α subunit (Smith et al., 1986). To learn if T' cells lack the capacity to break down α subunit under all conditions, we incubated T' myotube cultures in 1 mM α-MEm with or without 10 μg/ml α-Btx for 2 hr, then gave a 10-min pulse of 35S-amino acid, and analyzed the newly-synthesized α subunit immediately after the pulse or 2 hr later. Comparable results were obtained with both T' and wild-type cells. In the αMEm-treated T' cells over 80% of the subunit was degraded during the two-hr chase period (Fig. 7, lanes 3 and 4) and thus there was a 50% degradation in the absence of the drug (Fig. 7, lanes 1 and 2). In TM-treated cells, over 90% was degraded (Fig. 7, lanes 5 and 6). The altered mobility of the α subunit indicated that N-linked glycosylation or its early processing had been blocked. T' cells thus are capable of degrading an α subunit rapidly under these conditions.

Fig. 1. AChR synthesis and assembly in wild type and T' myotubes. Myotube cultures were pulse-labeled with 35S-meihionine and cysteine in the presence of unlabeled amino acids. At various times after the chase, the cells were treated and equal amounts of the lysate were precipitated with mAb 61 (A), α-Btx-Sepharose (B) or mAb 88B-Sepharose (C) as described under Methods. Samples were analyzed on SDS gels and fluorographed. The top panels show autoradiographs of the gels that had been exposed for 10 hr (A) or 21 hr (B and C). The positions of α and β subunits on the gels were marked. Appropriately exposed gels were used to quantitate the α subunit in each sample by densitometer scanning as shown at the bottom (D and E). The data were corrected for differences in the recovery of AChR with different precipitation protocols and exposure times of the gels as described under Methods and were thus directly comparable. (Circles, wild-type myotubes; squares, T- myotubes.)

Fig. 5A and 6A: Efficiency of AChR assembly in wild type (A) and T' (B) cells. The data in Figure 5 were used to calculate the amount of α subunit precipitated by α-Btx-Sepharose (squares) and mAb 88B-Sepharose (triangles) expressed as a percentage of total α subunit determined by mAb 61 precipitation. Symbols used are the same as in Figure 5.