TE671 Cells Express an Abundance of a Partially Mature Acetylcholine Receptor α Subunit Which Has Characteristics of an Assembly Intermediate*

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A partially mature form of the nicotinic acetylcholine receptor α subunit was found to be expressed in the human cell line TE671. We found that 40–50% of the α-bungarotoxin-binding sites in detergent extracts of these cells correspond to this unassembled α subunit. These unassembled α subunits are not found in the surface membrane. The unassembled α subunits in extracts from TE671 cells appear, like mature receptors, to have a disulfide bond between Cys-192 and Cys-193 near the acetylcholine-binding site. The unassembled α subunit binds α-bungarotoxin with high affinity, but its dissociation constant is still 5-fold higher than the native assembled acetylcholine receptor. The cholinergic ligands d-tubocurarine and carbamylcholine have negligible affinity for the immature α subunit. Similarly, Xenopus oocytes injected with RNA transcripts for the TE671 α subunit express an α-bungarotoxin-binding component which is insensitive to carbamylcholine and has a sedimentation coefficient on sucrose gradients of 5.0 S. Oocytes injected with RNA for the Torpedo α subunit did not have α-bungarotoxin binding activity under similar conditions, suggesting a possible differential efficiency in the maturation of this α subunit.

We examined the binding of monoclonal antibodies specific to the main immunogenic region and found that this epitope on the unassembled α subunit was formed, but was not in a fully mature conformation because although these antibodies bound, they bound with lower affinity than to native acetylcholine receptors. Antibodies in myasthenia gravis patient sera also bound to the unassembled α subunits, but with an average 14-fold lower titer.

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The nicotinic acetylcholine receptor (AChR) found at the neuromuscular junction is an acetylcholine-activated cation channel composed of four structurally related subunits arranged as an αβγδ pentamer (reviewed in Karlin et al., 1986; Lindstrom et al., 1988; Unwin, 1989). The extracellular surface of the α subunit has the binding site for acetylcholine; other agonists and competitive antagonists, including α-bungarotoxin (α-Bgt), are presumed to bind at the same site. The disulfide-linked cysteine 192 and 193 of the α subunit are near this binding site (Kao et al., 1984; Kao and Karlin, 1986). An additional site on the extracellular surface of the α subunit is the main immunogenic region (MIR). Amino acids within the sequence α68–76 contribute to the structure of the MIR (Bellone et al., 1989; Tzartos et al., 1990; Das and Lindstrom, 1989; Saedi et al., 1990). The majority of antibodies produced in animals immunized with intact AChR bind to this epitope (Tzartos et al., 1981). Antibodies to the MIR have also been implicated in the pathology of myasthenia gravis (MG) (Lindstrom et al., 1988). About two-thirds of the anti-AChR antibodies in sera from MG patients bind to the MIR (Tzartos et al., 1982). Additionally, antibodies to the MIR can cause experimental autoimmune MG, and when added to cultured muscle cells can cause loss of AChRs (Tzartos et al., 1987; Tzartos and Starzinski-Powitz, 1986).

The ability of the α subunit to bind acetylcholinomimetic ligands, α-Bgt, and MIR-specific antibodies with high affinity is not an intrinsic property of the polypeptide chain. Cell-free translation systems produce full-length α subunit polypeptides which cannot bind α-Bgt (Anderson and Blobel, 1981; Sunsikawa et al., 1991). Similarly, α subunits of Torpedo AChR dissociated with sodium dodecyl sulfate and purified do not bind α-Bgt with high affinity (Haggarty and Froehner, 1981; Tzartos and Changeux, 1983). Merlie and co-workers (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983; Carlin et al., 1986) have shown that the α subunit, when initially synthesized, does not bind α-Bgt or MIR-specific monoclonal antibodies (mAbs). By means of [35S]methionine pulse-chase labeling of newly synthesized subunits, they showed that the α subunit acquires the ability to bind α-Bgt and MIR-specific mAbs 15–30 min after being synthesized. Even at this stage, the α subunit is not associated with β, γ, or δ subunits, thus indicating a maturation process in which the nascent α subunit undergoes post-translational modification or noncovalent conformational changes. Although the α subunits bound α-Bgt, they did not bind carbamylcholine.
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or d-tubucurarine. Similar results were obtained when the mouse α subunit was expressed in quail fibroblasts in the absence of β, γ, or δ subunits (Blount and Merlie, 1988).

When mouse α subunits were expressed in pairwise combinations with γ or δ subunits in fibroblasts, a fraction of the α subunits were associated with γ or δ subunits and could be differentiated from unassembled α subunits by their ability to bind d-tubucurarine and carbamylcholine with high affinity (Blount and Merlie, 1989). The combination of α and β subunits did not lead to efficient assembly and had binding properties indistinguishable from α subunits expressed alone. Similar results were obtained when combinations of Torpedo AChR subunits were expressed in Xenopus oocytes (Kurosky et al., 1987; Sumikawa and Miledi, 1989). Thus, the assembly of α with γ or δ subunits is the next step in the maturation of the α subunit. Merlie and Lindstrom (1983) showed in BCSH-1 cells that the assembly of α with other subunits proceeds from 30 to 90 min after synthesis.

The question remains as to what happens to the nascent α subunit which causes it to change from an "immature" form to a "mature" form that can bind α-Bgt and MIR antibodies and assemble with other subunits. A number of possibilities have been suggested, in the form of covalent or noncovalent modification of the α subunit. These modifications include addition of carbohydrate, formation of disulfide bonds between amino acids Cys-128 and Cys-142 and between Cys-142 and Cys-193, fatty acid acylation, and phosphorylation (Merlie, 1984). These covalent modifications may initiate or accompany other noncovalent conformational changes of the α subunit, which may take place with the help of "molecular chaperonins" such as immunoglobulin heavy-chain binding protein and heat-shock proteins (reviewed in Ellis and Henningsen, 1989; Pelham, 1988).

Because of the role of AChRs in MG, another question which should also be addressed is whether these maturation processes also occur for AChRs in human muscle. Recently, our laboratory showed that the human cell line TE671 expresses functional muscle-type AChRs, and we also obtained our laboratory showed that the human cell line TE671 expresses functional muscle-type AChRs, and we also obtained our laboratory showed that the human cell line TE671 expresses functional muscle-type AChRs, and we also obtained our laboratory showed that the human cell line TE671 expresses functional muscle-type AChRs, and we also obtained.

MATERIALS AND METHODS

Triton X-100 Extracts of TE671 Cells—TE671 cells were grown to confluence in iscover's modified Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 37°C, 5% CO2. Typically 4–6 T-175 cm2 flasks were used to make an extract. The culture medium was removed and the cell layer washed with 10 mM sodium phosphate, 100 mM NaCl, pH 7.5 (PBS). The cells were removed from the flask using PBS containing 5 mM EDTA. The cells were pelleted at 800 × g for 10 min. The cell pellet was resuspended in 0.5% Triton X-100, 0.05% sodium deoxycholate, 50 mM Tris, 100 mM NaCl, 100 mM KF, 5 mM EDTA, 5 mM EGTA, 10 mM p-aminobenzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 40 units/ml Trypsol, pH 7.5 (extraction buffer), by homogenization with a Polytron for 20 s and then each tube for 50 s at a speed constant shaking. Insolubles were pelleted by centrifugation at 100,000 × g for 30 min.

Velocity Sedimentation on Sucrose Gradients—For analytical gradients 0.1 ml of extract was layered on a 5-mL 5–20% sucrose gradient containing 10 mM sodium phosphate, 100 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 5 mM EGTA, 10 mM p-aminobenzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM NaN3, pH 7.5. The gradients were centrifuged in a VTi 65.2 rotor (Beckman) at 65,000 rpm for 70 min at 4°C. The gradients were fractionated from the bottom of the tube with approximately 0.13 ml fraction directly into Immulon 1 microtiter plates (Dynatech) coated with mAb 210 (see solid-phase radioimmunoassay).

Crude extracts of Torpedo electric organ were similarly prepared and sedimented on identical gradients as markers for dimers and monomers of Torpedo AChRs. A standard curve for the determination of sedimentation coefficients was obtained by analyzing standards of Torpedo AChR monomers by velocity sedimentation. The peak fraction containing α-Bgt binding activity was pooled. The peak fractions containing α-Bgt binding activity were pooled.

Immunoadsorption of Native TE671 AChR—MAB 111 (β subunit specific; Tzartos et al., 1986; Luther et al., 1989) was purified on a Protein A-agarose column (Behring Diagnostics) and was then coupling to the immunoassay column (Chemical Dynamics Corp.) according to the manufacturer's instructions. Approx 3 mg of mAb 111 was coupled. A Triton X-100 extract of the TE671 cells (4 ml) was passed through the column four times by gravity flow. Samples of the original extract and the mAb 111 column effluent were analyzed by velocity sedimentation. Several identical gradients of each sample were fractionated and then assayed for 125I-labeled α-Bgt binding either in the absence or presence of 10 μM carbamylcholine.

Solid-Phase Radioimmunoassay—Immuno 1 microtiter remov-able strips were coated with Protein A-purified mAb 210. mAb 210 (40 μg/ml) in 10 mM carbonate/sodium bicarbonate buffer, pH 9.8, was incubated overnight at 4°C in Immulon 1 wells (0.05 ml/well). The mAb 210 solution was removed and the wells blocked with 2% bovine serum albumin in PBS containing 0.05% Tween 20 for 2 h at room temperature. The wells were washed three times with 0.05% PBS, 0.5% Triton X-100, pH 7.5. Gradient fractions (0.14 ml) were added and incubated with 125I-labeled α-Bgt (specific activity ~19,000 cpm/μg) at a final concentration of 2 μg. In some experiments the incubation mixture also contained 10 μM carbamylcholine chloride.

2 M. Saedi, W. G. Conroy, and J. Lindstrom, unpublished results.
Expression of TE671 α Subunits in Xenopus Oocytes—Full-length cDNA clones of Torpedo AChR subunits cloned under the control of SP6 promoter were generously provided by Dr. T. Claudio (Yale University). A full-length cDNA insert of TE671 α subunit isolated previously (Schoepfer et al., 1988) was subcloned into the BglII site of the plasmid vector pSP64T (Krieg and Melton, 1984) under the control of SP6 promoter, and the insertion verified by DNA sequencing. Plasmids were linearized by digestion with XbaI and used for in vitro transcription (Krieg and Melton, 1984). Oocytes were prepared from Xenopus laevis (Sham, 1984) and injected with 1.5 ng of each subunit RNA synthesized in vitro. Oocytes were incubated at 19°C for 2 days before analysis. Subunit expression was assessed by homogenizing the oocytes in extraction buffer, incubating the lysate at 4°C for 30 min, centrifuging the lysate in a microfuge for 30 min at 4°C, and sedimenting the resulting supernatant on a 5-ml 5-20% sucrose gradient and analyzing the fractions for 125I-labeled α-Bgt, as described above.

Antigenic Modulation of TE671 Surface AChRs—Several control experiments were conducted to show that 125I-labeled α-Bgt binding sites were lost from the cell surface after mAb 210 treatment. TE671 cells were grown on 6-well plates (Costar) in the medium described above until confluent. Various concentrations of mAb 210, mAb 60, or PBS (also the diluent for the mAbs) in a volume of 10 μl was added to the TE671 cells which had 1 ml of medium. The cells were incubated for 24 h at 37°C. 125I-labeled α-Bgt binding to the cell surface was assayed after washing the cells twice with 2 ml of serum-free medium. 125I-labeled α-Bgt (10 nm) was added in serum-free medium, and the cells incubated for 60 min at room temperature. After the incubation the medium was aspirated and the cell layer washed three times with media. The cell layer was solubilized with 0.5 N NaOH (0.5 ml) and counted in a γ-counter. Nonspecific binding was determined in the presence of 1 μM α-Bgt or 1 mM carbamylcholine chloride. All binding measurements were determined from 3-6 identically treated wells.

Extracts of cells treated with mAb 210 or PBS were also examined by velocity sedimentation. For these experiments, TE671 cells were grown to confluence in T-175 cm² flasks. mAb 210 (2 nm) or PBS (200 μl) was added to flasks which had 100 ml of medium. The flasks were incubated for 24 h at 37°C. Extracts of the cells were made and analyzed by velocity sedimentation. The fractionated material was assayed for 125I-labeled α-Bgt in the solid-phase radioimmunoassay. The cells from three separate flasks for each treatment (PBS or mAb 210) were examined independently.

RESULTS

Detergent extracts of TE671 cells when analyzed by velocity sedimentation in sucrose gradients shows the presence of two α-Bgt-binding components with very different molecular weights (Fig. 2). The larger component (9.5 S) cosediments with the monomer of Torpedo AChRs (9.5 S) and is probably the TE671 AChR monomer as characterized by Luther et al. (1989). The smaller component sediments with a size of 5.0 S. The 5.0 S component could go unnoticed if these analyses were conducted using 125I-labeled α-Bgt labeled extracts to determine the AChR, because excess free 125I-labeled α-Bgt might not be resolved from the 5.0 S component. The size of this component and the ability to bind 125I-labeled α-Bgt is similar to the unassembled α subunit described by Merlie-Lindstrom (1983).

Although not completely resolved from the 9.5 S component, the 5.0 S component consistently represents 40-50% of the total binding sites for α-Bgt in extracts of the TE671 cells.

Consistent with the assumption that the 9.5 S component is the intact TE671 AChR is the fact that the binding of 125I-labeled α-Bgt is inhibited by the acetylcholinomimetic agent carbamylcholine (Fig. 2). However, the binding of 125I-labeled α-Bgt to the 5.0 S component is not inhibited by carbamylcholine, even at the high concentration of 10 mM. Cold α-Bgt in excess (1 μM) completely inhibited this binding, indicating that this is not just nonspecific.

More direct evidence that the 5.0 S component is composed of unassembled α subunits comes from an adsorption experiment using a specific mAb against the β subunit. Extracts
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FIG. 2. Velocity sedimentation profile of 125I-labeled α-Bgt binding activity in extracts of TE671 cells. Triton X-100 extracts of TE671 cells were sedimented on linear 5-20% sucrose gradients. The gradients were fractionated into microtiter wells coated with mAb 210 (α subunit specific) and analyzed for 125I-labeled α-Bgt binding (■). The fractions are numbered from the bottom of the gradient. Identical gradients were assayed for 125I-labeled α-Bgt binding in the presence of 10 mM carbamylcholine (□) or 1 μM α-Bgt (△). The values reported are means of two gradients. Crude extracts of Torpedo electric organ were analyzed similarly as standards for the position of AChR monomers (9.5 S) and dimers (11.5 S).

were passed through an immunoadsorbent Zetaffinity-10 column that has mAb 111 (specific for the β subunit of TE671 AChR; Luther et al., 1989; with an epitope that maps within amino acids 370-410 of Torpedo β subunits; Ratnam et al., 1986b) coupled to it, and then the unbound material was analyzed by velocity sedimentation. α-Bgt binding in the fractionated material was assayed in the presence and absence of carbamylcholine. As shown in Fig. 3, the 9.5 S component can be completely adsorbed from the extract, leaving behind the 6.0 S component which is unable to bind carbamylcholine. A small peak observed at a position slightly smaller than the size of the 9.5 S component after mAb 111 adsorption also does not bind carbamylcholine and may represent aggregates of α as seen in oocytes injected with TE671 α RNA (described below).

This analysis indicates that only the 9.5 S component is associated with the β subunit. To further test whether the 5.0 S component was associated with γ or δ subunits, we separated the 9.5 S and 5.0 S components of TE671 extracts by velocity sedimentation on preparative sucrose gradients. The peak fractions of α-Bgt binding activity were pooled and analyzed for associated subunits by immunoprecipitation using subunit-specific mAbs and antisera. Fig. 4 shows that mAbs to α and β subunits and antisera to γ and δ subunits can readily immunoprecipitate the 9.5 S component. The quantitative difference in the ability of the mAbs and antisera to immunoprecipitate the TE671 AChR probably reflects differences in affinity or titer, not the absence or presence of subunits in the AChR. In contrast, only the α-specific mAb (mAb 210) effectively precipitates the 5.0 S component. The small effects of the antisera to γ and δ (12-22% of maximum) may indicate that a small percentage of the binding sites are composed of trimers of α with γ or δ. When pairs of Torpedo AChR subunits are expressed in Xenopus oocytes and then analyzed, it is found that complexes of α with γ or α and δ subunits, but not α and β subunits, efficiently form, sediment at 6.1 S, and bind α-Bgt. Contamination of the 5.0 S with 9.5 S material can be ruled out since mAb 111 (anti-β) did not precipitate any 5.0 S component, but was almost as efficient as mAb 210 (anti-α) in precipitating the 9.5 S component. In addition, we reanalyzed the pooled 9.5 S and 5.0 S components by velocity sedimentation and found that both the 9.5 S and 6.0 S components were essentially devoid of the other component.
when proteins from Triton X-100 extracts of oocytes injected with TE671 α subunit RNA were sedimented on sucrose gradients, a 5.0 S peak of 125I-labeled α-Bgt binding activity was found (Fig. 5B). In addition, a smaller amount of an 8.5 S component was found. Binding of 125I-labeled α-Bgt to neither the 5.0 S nor the 8.5 S component was inhibited by 10 μM carbamylcholine. The 9.5 S component in these oocyte extracts could be oligomers of the α subunit or α subunits associated with accessory proteins from the oocytes. A similar peak of α-Bgt binding activity was also found when TE671 extracts were depleted of native AChR and analyzed by velocity sedimentation (Fig. 3). These results suggest that, in Xenopus oocytes, Torpedo α subunits do not efficiently mature in conformation from nascent chains to synthetic intermediates able to bind α-Bgt, but that human α subunits do efficiently make this conformation change. Paulson and Claudio (1990) have shown that when expressed in mammalian cells, Torpedo α subunits fail to efficiently make a conformation change needed for assembly with other subunits, except at temperatures below 37 °C. This temperature-sensitive conformation change may be the maturation step from nascent chains to assembly intermediates which is efficiently achieved by human α subunits.

The TE671 α subunit that is expressed in oocytes can assemble with the β, γ, and δ subunits of the Torpedo AChR and forms a 9.5 S species the size of native Torpedo AChRs which is able to bind carbamylcholine (Fig. 5C). The smaller (6.1 S) peak of α-Bgt binding activity corresponds in size and carbamylcholine affinity to an αγ or αδ pair.

Even though we were unable to show significant amounts of αn α subunit with affinity for α-Bgt typical of the putative assembly intermediate when Torpedo α subunit RNA is injected alone (Fig. 5A), 9.5 S AChRs can be assembled when the Torpedo α, β, γ, and δ subunits are co-injected into oocytes (Saedi et al., 1990). However, the total amount of 125I-labeled α-Bgt binding activity in extracts and on the oocyte surface is 5–10-fold greater (90 fmol/oocyte in extracts and 11 fmol/oocyte on the surface) when human α subunits replace the Torpedo α subunits. The greater efficiency of assembly of hybrid AChRs probably results from the greater efficiency with which human α subunits mature to the conformation of an assembly intermediate as compared to Torpedo α subunits.

**Binding Site Characteristics of the Unassembled α Subunits**—Because of the abundance of the unassembled α subunits (5.0 S component) in extracts of TE671 cells, we were able to isolate them from the native TE671 AChR (9.5 S component) and characterize the binding of α-Bgt, d-tubocurarine, and carbamylcholine. For analysis of 125I-labeled α-Bgt, pooled fractions of native AChR and unassembled α were incubated overnight with various concentrations of 125I-labeled α-Bgt and immunoprecipitated with an excess of mAb 210 (which binds to the MIR). The data from this experiment are shown in a Scatchard plot in Fig. 6. The binding of 125I-labeled α-Bgt to both native AChR and unassembled α is concentration dependent, saturable, and of high affinity. For native AChR solubilized in Triton X-100 and labeled with mAb 210, 125I-labeled α-Bgt bound to a single class of sites with a dissociation constant (KD) of 1.3 × 10−10 M. 125I-labeled α-Bgt similarly bound to unassembled α subunits with a slightly lower affinity having a KD of 6.2 × 10−10 M. These values agree with a kinetically derived KD of 5.6 × 10−10 M for 125I-labeled α-Bgt binding to membrane fragments of TE671 cells (Lukas, 1986) and a KD of 2.6 × 10−10 M for binding of α-Bgt to TE671 AChRs on intact cells (Sine, 1988).

The ability of d-tubocurarine and carbamylcholine to inhibit the binding of 125I-labeled α-Bgt was also studied in...
Various concentrations of d-tubocurarine or carbamylcholine were incubated with detergent-solubilized native TE671 AChR and unassembled α subunits in the presence of 0.1 nM 125I-labeled α-Bgt. MBTA 210 was included in the incubation mixture to precipitate the AChR. d-Tubocurarine and carbamylcholine displace 125I-labeled α-Bgt from native TE671 AChRs with moderate to high affinity with inhibition constants (K_i) of 2.4 x 10^-9 M and 9.5 x 10^-8 M, respectively (Fig. 7A). The inhibition of 125I-labeled α-Bgt binding to unassembled α by these ligands is more complex. Approximately 25% of the α-Bgt-binding sites have high affinity for d-tubocurarine and carbamylcholine, whereas the majority of the α-Bgt-binding sites have negligible affinity (Fig. 7B). The K_i values for the high-affinity site are 3.8 x 10^-9 M for d-tubocurarine and 2.5 x 10^-8 M for carbamylcholine, values comparable to the K_i values for native TE671 AChR. The K_i values for the low-affinity site are greater than 10^-8 M. Because reanalysis of the unassembled α fraction did not show evidence of contamination with native AChR, it is possible that the carbamylcholine-inhibitable α-Bgt binding is due to α-β or α-γ dimers, as described by Blount and Merlie (1989), Kuroskas et al. (1987), and Sumikawa and Miell (1989). Fig. 4 suggests that αγ and αβ pairs could collectively account for 25% of the α-Bgt binding in the unassembled α fraction. The formation of disulfide bonds is one of the post-translational modifications that may be involved in the maturation of the α subunit (Merlie, 1984). The disulfide between cysteines 192 and 193 is presumed to be in or near the ACh-binding site and can be irreversibly labeled with the affinity reagent MBTA only after reduction of the disulfide (Kao and Karlin, 1986; Kao et al., 1984). The presence of the disulfide between Cys-192 and Cys-193 was tested using MBTA on the unassembled α subunits isolated from sucrose gradients after velocity sedimentation. Native AChR, also isolated from sucrose gradients, was used as a positive control. As seen in Table I, MBTA had no effect on the binding of 125I-labeled α-Bgt to either native AChR or unassembled α unless the samples were initially reduced with dithiothreitol, suggesting that a disulfide bond between Cys-192 and Cys-193 is present in unassembled α subunits from TE671 cells.
bled α subunits isolated from sucrose gradients. MAb 210, which was raised against mammalian AChR, binds to native TE671 AChR (Luther et al., 1989), denatured TE671 α subunit on Western blots, and to the synthetic LY subunit peptide sequence a68-76, which forms part or all of the human MIR epitope (Merlie and Lindstrom, 1983), does not bind to denatured α subunit on Western blots unless very high concentrations are used, and does not bind to any peptides corresponding to a MIR epitope (Tzartos et al., 1990; Das and Lindstrom, 1989). MAb 35 raises against Electrophorus AChR binds to the MIR on TE671 AChR, but is highly conformation dependent, does not bind nascent BC3H-1 α subunits prior to a conformational maturation (Merlie and Lindstrom, 1983), does not bind to denatured α subunit on Western blots unless very high concentrations are used, and does not bind to any peptides corresponding to a MIR epitope (Tzartos et al., 1990; Das and Lindstrom, 1989). MAb 35 binds to the same basic region as does mAb 210, since in vitro mutagenesis of Torpedo Asn a68 to Asp or Asp a71 to Lys inhibits the binding of both mAb 210 and mAb 35 to Torpedo AChRs expressed in oocytes (Saedi et al., 1990).

Immunoprecipitation of native TE671 AChR and unassembled α subunits by mAb 210 gives titers of 5.4 and 1.1 nM, respectively (Fig. 8). Because the assay contained equal concentrations of α-Bgt-binding sites for the native AChR and unassembled α subunits, the differences in the titers may reflect a difference in the affinity for mAb binding to native and unassembled α subunits, as well as the ability of one antibody molecule to precipitate two α subunits/α-Bgt-binding sites in native AChRs and only one α-Bgt-binding site in unassembled α subunits. In contrast to the 5-fold difference in the titers for mAb 210, we found a 20-fold difference in titers for mAb 35 binding to native TE671 AChR and unassembled α subunits. This difference in titers for mAb 35 probably reflects a significant difference in affinity for binding, which suggests that the unassembled α subunit has a MIR that has an immature conformation. Furthermore, this conformation of the MIR is probably closer to the native AChR than denatured α subunits because mAb 35 can bind, although with low affinity. MAb 35, when added in sufficient quantity, can precipitate all of the α-Bgt-binding sites of the unassembled α subunit, which means that the difference in titers is because of a difference in affinity and not because it binds to only a fraction of the α subunits present.

Reaction with MG Patient Autoantibodies—Because the MIR has been proposed as a major epitope for the binding of immunoglobulins in MG patient sera (Tzartos et al., 1982; Lindstrom et al., 1988), and the unassembled α subunits have a near native conformation of the MIR in the absence of other subunits, we had the unique opportunity to test MG patient sera for the contribution of α-specific antibodies to the total complement of anti-AChR antibodies. The titers of 45 different MG patients' sera with a wide range of titers were determined for native TE671 AChR and unassembled α subunits (Fig. 9). Of the 45 sera tested, only one did not have a titer for either the native or unassembled α subunits. Two sera had moderate titers for native AChR (18.7 and 19.9 nM), but did not immunoprecipitate the unassembled α subunits. There was a strong linear correlation between the titers for native AChR and unassembled α subunits (r = 0.92; p ≤ 0.0001), the slope of the regression line was 13.6 (regression of native on unassembled α). The high degree of correlation, but a slope very much greater than 1, may indicate that antibodies to other subunits make a significant contribution to the titer for native AChR, or it may indicate that anti-MIR antibodies make a major contribution to the titer, but bind with lower affinity to the unassembled α subunits, as was seen with mAb 35. Since there is a close correlation between titers

FIG. 8. Analysis of MAb binding to the MIR on native AChR and unassembled α subunits. The binding of the MIR-specific mAbs 210 and 35 to native AChR and unassembled α subunits was determined in a radioimmunoassay. The volume of mAb stock solution was titrated against equivalent concentrations of α-Bgt-binding sites on native TE671 AChR or unassembled α subunits. Samples (0.1-pmol binding sites) with 125I-labeled α-Bgt (2 nM) and mAb were incubated overnight at 4 °C in a total volume of 0.5 ml. The maximum precipitated was determined using mAb 210 (5 μl of stock solution). The apparent titer is reported with units of moles of 125I-labeled α-Bgt bound per liter of serum.

FIG. 9. Binding of MG patients' sera to native TE671 AChR and unassembled α subunits. MG patients' sera (45 independent samples) were assayed against native TE671 AChR and unassembled α subunits. Native AChR or unassembled α subunits (0.1-pmol binding sites) were incubated with MG sera and 2 nM 125I-labeled α-Bgt overnight and then precipitated with goat anti-human immunoglobulin. The apparent titer was determined from the 125I-labeled α-Bgt precipitated and is reported as nanomoles of 125I-labeled α-Bgt-binding sites bound per liter of serum. A linear regression line was fitted to the data with the following parameters: r = 0.92 and p ≤ 0.0001 and a slope of 13.6.

4 W. G. Conroy and J. Lindstrom, unpublished data.
cells. A, titers, and since the titers against unassembled cy are uni-

60, or PBS (control) for 24 h and were then assayed for \(^{125}\text{I}-\)labeled cu-Bgt bound was determined for three

1989), and thereby a loss of surface ^{125}\text{I}-labeled \(\alpha\)-Bgt binding sites. TE671 cells treated with mAb 210 (0.2-10 nM) for 24 h show a concentration-dependent reduction in surface \(^{125}\text{I}-\)labeled \(\alpha\)-Bgt binding which reaches a maximum of \(\sim 80\%\) (Fig. 10A). An antibody which does not bind TE671 AChRs (mAb 60) has no effect. When extracts of TE671 cells simi-

Failure of Surface Expression of Unassembled \(\alpha\) Subunits—To test if the unassembled \(\alpha\) subunits were on the cell surface, we analyzed the surface \(^{125}\text{I}-\)labeled \(\alpha\)-Bgt binding and velocity sedimentation of extracts after exposure of the cells to extern-

acicetylcholine binding site-blocking antibodies in MG pa-

FIG. 10. Antigenic modulation of surface AChRs on TE671 cells. A, confluent TE671 cells in 6-well plates were incubated with various concentrations (calculated from the titer) of mAb 210, mAb 60, or PBS (control) for 24 h and were then assayed for \(^{125}\text{I}-\)labeled \(\alpha\)-Bgt binding. The \(^{125}\text{I}-\)labeled \(\alpha\)-Bgt bound was determined for three

against native AChR and unassembled \(\alpha\) over a wide range of

\(\neq 0.13\) nM, but still slightly lower affinity than does the fully

DISCUSSION

Several recent papers from our laboratory have shown that

the human cell line TE671 expresses muscle-type AChRs

and we have found that the immature form is unassembled \(\gamma\)

mature \(\alpha\) subunit in the assembled AChR \(\neq 0.13\) nM,

In mouse BC3H-1 cells, the acetylcholine-binding site on

\(\sim 80\%\) decrease in the amount of \(\alpha\)-Bgt-binding activity was accounted for by a component which sedimented in the 5.0 S region in sucrose gradients; we found that the TE671 cells typically have 40-

50% of the \(^{125}\text{I}-\)labeled \(\alpha\)-Bgt-binding sites in the 5.0 S region and sometimes even more binding sites than in the 9.5 S fractions (see Fig. 3). Only mAbs specific for the \(\alpha\) subunit were able to immunoprecipitate this 5.0 S component, con-

the presence of this component in extracts of the cells. For example, Carlin et al. (1986) found that 10% or less of the \(\sim 80\%\) decrease in the amount of the unassembled \(\alpha\) subunit component (Fig. 10B).

the BC3H-1 cell line (Merlie and Lindstrom, 1983) and cultured rat (Carlin et al., 1986) and chick myotubes (Ross et al., 1987), the TE671 cells produce such an abundance of the unassembled \(\alpha\) subunit that we did not have to resort to metabolic labeling to show the presence of this component in extracts of the cells. For example, Carlin et al. (1986) indicated that TE671 cell line has been suggested
to do extensive biochemical and pharmacological characterization. Unlike the BC3H-1 cell line (Merlie and Lindstrom, 1983) and cultured rat (Carlin et al., 1986) and chick myotubes (Ross et al., 1987), the TE671 cells showed that mAbs to different regions of the AChR immu-

has been studied by Lang et al. (1988) and Walker et al. (1988). The fact that this cell line, originally identified as a medulloblastoma (McAllister et al., 1977), expressed a muscle-type AChR was at first perplexing, but a recent report has suggested that this cell line was misidentified and may indeed

mAbs, and since the titers against unassembled cy are uni-

Again, there is a 32% decrease in the amount of the unassembled \(\alpha\) over a wide range of titers, and since the titers against unassembled \(\alpha\) are uniform-

with 40-50% of the \(^{125}\text{I}-\)labeled \(\alpha\)-Bgt-binding sites in the 5.0 S region and sometimes even more binding sites than in the 9.5 S fractions (see Fig. 3). Only mAbs specific for the \(\alpha\) subunit were able to immunoprecipitate this 5.0 S component, confirm-

Our findings in this report have confirmed this, and we have found that the immature form is unassembled \(\alpha\) subunits which have the characteristics of an assembly inter-

leave the confusion (Stratton et al., 1989). Nevertheless, TE671 cell line has been suggested

as a source of human AChR for diagnostic radioimmunoassays for detection of anti-AChR antibodies in MG (Lindstrom et al., 1987; Luther et al., 1980) and has been used to detect acetylcholine binding site-blocking antibodies in MG pa-

in MG patients (Pachner, 1989) and to show that MG patient sera can modulate surface AChRs (Sophianos and Tzartos, 1989). However, Walker et al. (1988) indicated that TE671 cells had a heterogeneous population of \(\alpha\)-Bgt-binding sites. They showed that mAbs to different regions of the AChR immu-

noprincipated different fractions of the total \(^{125}\text{I}-\)labeled \(\alpha\)-Bgt binding activity and that a similar fraction of these binding sites were insensitive to \(\alpha\)-tubocurarine. They sus-

The abundance of the unassembled \(\alpha\) subunits in the TE671 cells has allowed us to do extensive biochemical and pharmacological characterization. Unlike the BC3H-1 cell line (Merlie and Lindstrom, 1983) and cultured rat (Carlin et al., 1986) and chick myotubes (Ross et al., 1987), the TE671 cells showed that mAbs to different regions of the AChR immu-

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Failure of Surface Expression of Unassembled \(\alpha\) Subunits—To test if the unassembled \(\alpha\) subunits were on the cell surface, we analyzed the surface \(^{125}\text{I}-\)labeled \(\alpha\)-Bgt binding and velocity sedimentation of extracts after exposure of the cells to extern-

mAb 210 (W) or PBS (C) for 24 h were extracted with Triton X-100 and the extract analyzed by velocity sedimentation and \(^{125}\text{I}-\)labeled \(\alpha\)-Bgt binding.
Unassembled α Subunits in TE671 Cells

(Blount and Merlie, 1989). This may result from species differences, or methodological differences because they studied the binding in crude membranes using low ionic strength buffers, whereas we studied the binding in Triton X-100 extracts using buffers with higher ionic strength. The dissociation constants we obtained (native, 0.13 nM; unassembled α, 0.62 nM) are similar to those reported for α-Bgt binding to membrane fragments of TE671 cells (Kd = 0.56 nM; Lukas, 1986) and binding to the TE671 surface AChRs (Kd = 0.26 nM; Sine, 1988).

The binding of α-Bgt to the unassembled α subunit indicates that the acetylcholine-binding site has matured, but further maturation steps are still needed. This is especially true when the binding of small ligands to the unassembled α subunits is studied. We found that the majority of the α-Bgt-binding sites were not able to bind either d-tubocurarine or carbamylcholine. Native AChRs bound these ligands with high affinity. Similar results were found in rat myotubes (Carlin, 1986) and for α subunits of BC3H-1 AChRs expressed in fibroblasts (Blount and Merlie, 1988) and Torpedo AChR α subunits expressed in Xenopus oocytes (Kuroskaki et al., 1987).

We know from expression of αγ and αδ subunit pairs in fibroblasts (Blount and Merlie, 1989) and oocytes (Kuroskaki et al., 1987; Sunikawa and Miledi, 1999) that the high affinity binding of d-tubocurarine and carbamylcholine is acquired when the α subunit is associated with either the γ or the δ subunits. Approximately 25% of the α-Bgt-binding sites of the TE671 unassembled α subunits were displaced by d-tubocurarine and carbamylcholine with high affinity. These binding sites may be on αγ or αδ pairs, which were not adequately resolved from the α subunit monomer on preparative sucrose gradients.

When we expressed the TE671 α subunit in Xenopus oocytes, we found that the α subunits had the expected size, by velocity sedimentation, and bound α-Bgt, but not carbamylcholine. Oocytes injected with Torpedo α subunit RNA and analyzed in the same way had no α-Bgt-binding component, but had α subunit protein as detected by the binding of an α subunit-specific mAb (mAb 142). Other groups have shown that oocytes injected with Torpedo α subunit RNA do express α subunits which bind α-Bgt (Kuroskaki et al., 1987; Sunikawa and Miledi, 1999), however, the binding is sufficiently low and variable that we may not detect it under our assay conditions. Although differences in the amounts of α subunit synthesized or differences in protein stability may explain the differences between Torpedo and human α expression in oocytes, these data might also indicate that the TE671 α subunit forms a mature conformation competent to bind α-Bgt, with greater efficiency than does the Torpedo α subunit. This may also explain the 5- to 10-fold difference in α-Bgt binding observed when the TE671 α subunit is substituted for the Torpedo α subunit and co-expressed in oocytes with the Torpedo β, γ, and δ subunit RNA.

The human TE671 α subunit may be unique in this ability to efficiently form a more mature conformation. A low efficiency of formation of the mature conformation was also found for the mouse α subunit. Only 30% of the α subunit synthesized in BC3H-1 cells acquires the ability to bind α-Bgt (Merlie and Lindstrom, 1983). Similarly, the proportion of α subunit synthesized that binds α-Bgt in fibroblasts transfected with mouse α subunit DNA is approximately 20% (Blount and Merlie, 1988). The differences in the efficiency with which the nascent α chain matures in conformation to an assembly intermediate may be due, in part, to the ability to form a disulfide bridge between the cysteines at positions 192 and 193 near the acetylcholine-binding site. We found that the unassembled α subunit in detergent extracts from the TE671 cells had this disulfide intact. The binding of α-Bgt to the DTT-reduced unassembled α subunits was considerably decreased; thus if these cysteines were in the reduced state within TE671 cells, there would be a significant fraction of α subunit which does not bind α-Bgt, as seen in BC3H-1 cells and rat myotubes, as well as myoblasts expressing Torpedo or fibroblasts expressing mouse α subunits (Paulson and Claudio, 1990; Blount and Merlie, 1988). Conformation of the α subunit is important for binding of α-Bgt; although the intermediate conformation of unassembled human α subunits has relatively higher affinity for α-Bgt than do Torpedo α subunits expressed in Xenopus, synthetic α subunit peptides from human α have much lower affinity for α-Bgt than do synthetic Torpedo α subunit peptides (Neumann et al., 1986; Wilson and Lentz, 1988), and fully assembled human AChR has lower affinity for α-Bgt than does fully assembled Torpedo or mouse AChR (Sine, 1988).

The MIR is another extracellular region which is thought to change conformation as the α subunit undergoes maturation. We found that the MIR of the TE671 unassembled α subunit bound Torpedo-specific mAbs, even the highly conformation-dependent mAb 35. Again, as we found for the acetylcholine-binding site, the MIR was still in an immature conformation. The maturation of the MIR has not been extensively studied, but there is evidence that the maturation of the MIR is coincident with the maturation of the acetylcholine-binding site (Merlie and Lindstrom, 1983). Further conformational changes in the MIR also take place when the Torpedo α subunit associates with β, γ, or δ subunits. Because the unassembled α subunits from TE671 cells had a near native conformation of the MIR, we were able to test MG patient sera for the titer of α subunit-specific antibodies. We found that there was a 14-fold difference in the titers for MG patient sera for the titer of LY subunit-specific antibodies. This suggests that the amount of a subunit is not rate-limiting for assembly of AChRs in TE671 cells. In transfected fibroblasts, Claudio et al. (1990) found that Torpedo δ subunits have the shortest half-life of the subunits and suggested that δ subunits may be rate-limiting in assembly of AChRs. Our data are consistent with this suggestion.

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