0026-895X/94/050964-06$03.00/0

MOLECULAR PHARMACOLOGY, 46:964-969, 1994.

Amino- and Carboxyl-Terminal Domains Specify the Identity of the δ Subunit in Assembly of the Mouse Muscle Nicotinic Acetylcholine Receptor

XIAO-MEI YU and ZACH W. HALL

Department of Physiology, University of California, San Francisco, San Francisco, California 94143

Received July 5, 1994; Accepted September 1, 1994

SUMMARY

We have used transient expression in COS cells of the subunits of the nicotinic acetylcholine receptor (AChR) from mouse skeletal muscle to investigate the role of transmembrane and cytoplasmic domains of the δ subunit in assembly of the AChR. When chimeric subunits whose extracellular amino- and carboxyl-terminal domains were from the δ subunit and whose transmembrane and cytoplasmic domains were from either the β, γ, or ε subunit were expressed with α, β, and ε subunits, α-bungarotoxin-binding activity appeared on the surface of the transfected cells. The resulting receptor complexes each had sedimentation constants resembling those of the native AChR, consistent with a pentameric structure. Further investigation of the δ_β chimeric subunit showed that it formed a heterodimer with the α subunit and that the resulting subunit bound *d*-tubocurarine with an affinity similar to that of the αδ heterodimer; δ_β also formed a heterodimer with a form of the α subunit that is truncated after the first transmembrane domain. A heterodimer formed from the ϵ_β and α subunits also bound *d*-tubocurarine with an affinity similar to that of the αε heterodimer. When both ϵ_β and δ_β subunits were substituted for the ε and δ subunits, respectively, a receptor complex was formed whose structure appeared to be $\alpha_2\beta(\epsilon_\beta)(\delta_\beta)$. These results show that, as with the ε subunit, the identity of the δ subunit in AChR assembly arises from the extracytoplasmic domains of the subunit.

The nicotinic AChR of adult skeletal muscle is a hetero-oligomer, of stoichiometry $\alpha_2\beta\epsilon\delta$, whose homologous transmembrane subunits surround a central aqueous pore (1–3). In muscle cells the AChR is assembled in the ER and subsequently transported to the cell surface (4, 5). Experiments in which combinations of AChR subunits were expressed in nonmuscle cells led to the idea that receptor assembly occurs according to a defined pathway, in which the first step is the formation of two heterodimers, αδ and αε (6–9) (see Ref. 10 for an alternate scheme for assembly). Each of these heterodimers forms a ligand binding site with characteristic pharmacological properties, corresponding to one of the two sites in the intact AChR (6, 7). When coexpressed with the α subunit, the β subunit does not form a heterodimer but can associate with each of the heterodimers to form a heterotrimer (8, 9). The heterotrimers then presumably associate with each other or with a heterodimer to yield the fully assembled AChR.

Correct assembly of the AChR requires that the α subunit recognize, and interact with, specific sequences in the δ or ε subunits that are not present in the β subunit. To determine the location of the sequences within the ε subunit that allow it to form a heterodimer with the α subunit, we previously investigated the behavior of a chimeric subunit whose amino- and carboxyl-terminal extracellular domains (luminal domains in the ER) were derived from the ε subunit and whose transmembrane and cytoplasmic domains were derived from the β subunit. We showed that this chimeric subunit can form a heterodimer with the α subunit and that it can replace the ε subunit but not the β subunit in AChR assembly (11). These results suggest that the sequences in the ε subunit that are specifically recognized by the α subunit are in the luminal domains of the protein. We now extend this analysis to the δ subunit and find that, as with the ε subunit, the sequences responsible for heterodimer formation are in the luminal domains. Furthermore, we show that a receptor complex can be formed in which the transmembrane and cytoplasmic domains of all of the subunits are derived from either the α or β subunit.

Materials and Methods

Antibodies. MAb 61 (12) and MAb 124 (13) were generous gifts from Dr. Jon Lindstrom (University of Pennsylvania), and MAb 88B

This work was supported by the National Institutes of Health (Grant NS13521) and the Muscular Dystrophy Association.

ABBREVIATIONS: AChR, acetylcholine receptor; α-BTX, α-bungarotoxin; ER, endoplasmic reticulum; MAb, monoclonal antibody; M1, transmembrane domain 1; M4, transmembrane domain 4; PBS, phosphate-buffered saline; dTC, *d*-tubocurarine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

(14) from Dr. Stanley C. Froehner (University of North Carolina). These antibodies recognize epitopes in the long cytoplasmic loops of the α, β, and δ subunits, respectively.

cDNAs. Full length cDNA clones coding for the α (15), β (16), γ (17), and δ (18) subunits of the mouse muscle nicotinic AChR were gifts from Drs. N. Davidson (California Institute of Technology), S. Froehner, P. Gardner (University of Texas, San Antonio), J. Lindstrom, and J. P. Merlie (Washington University). The full length cDNA for the mouse ε subunit was isolated as described (19). Each of the cDNAs was subcloned into the simian virus 40-based expression vector pSM (20) at the multiple cloning site. An M13 origin in this vector was used to generate uracil-containing single-stranded DNAs for site-directed mutagenesis (21, 22).

Site-directed mutagenesis. Site-directed mutagenesis was conducted according to previously described methods (21, 22), using synthetic oligodeoxyribonucleotides prepared with an automated DNA synthesizer. Uracil-containing single-stranded DNAs, prepared by infecting plasmid-transformed *Escherichia coli* CJ236 (*dut-ung-F'*) cultures with M13 helper phages, were used as the templates. Each of the constructed cDNAs was analyzed with restriction enzymes. In some cases, plasmid sequences were confirmed by DNA sequencing.

For construction of the plasmids encoding the chimeric subunits, two novel restriction enzyme sites, for *Sna*BI and *Spe*I, were introduced into pSMβ, pSMγ, pSMδ, and pSMε plasmids, with the *Sna*BI site at the beginning of M1 and the *Spe*I site at the end of M4 of each subunit. The plasmids with the introduced sites were then digested with the two restriction enzymes. The plasmids for the chimeric subunits β_δ, δ_β, δ_γ, and δ_ε were constructed by purifying the corresponding fragments and ligating them to either pSMδ or pSMβ vectors that had been digested with the same restriction enzymes. pSMϵ_β was constructed as described previously (11). The plasmids were analyzed and mapped by restriction enzymes to verify their identity.

Transfection. Transfection was performed in COS cells by a modified DEAE-dextran transfection procedure (23), as described previously (19). Cells grown to 30–50% confluence in a 60-mm dish were incubated for 3–5 hr at 37° with transfection mixture containing plasmid cDNA in Dulbecco's modified Eagle's medium-H21 supplemented with 1.0% heat-inactivated fetal bovine serum, 0.10 mM chloroquine diphosphate, and 0.4 mg/ml DEAE-dextran. The amount of plasmid used for each subunit in the transfection mixture was empirically determined to produce maximal cell surface AChR expression. For analysis of cell surface AChR expression, 3 ml of the transfection mixture were added to a 60-mm dish containing cDNAs for α (1.32 μg), β (0.66 μg), γ or ε (1.0 μg), and δ (0.26 μg) subunits. For immunoprecipitation of heterodimers, 5 ml of the transfection mixture were added to a 100-mm dish containing 2.0 μg of each cDNA for the α and δ or δ_β subunits. After 3–4 hr of incubation, the transfection solution was removed and the cells were treated for 2 min at room temperature with 10% dimethylsulfoxide/PBS, which was replaced with 5 ml (60-mm dish) or 10 ml (100-mm dish) of growth medium (10% fetal bovine serum in Dulbecco's modified Eagle's medium-H21 medium supplemented with 100 units/ml penicillin and 100 units/ml streptomycin). After 24 hr at 37°, the cells were trypsinized and distributed into three wells of 24-well plates (surface AChR assay) or into 60-mm dishes (heterodimer immunoprecipitation assay). The surface AChR assay and heterodimer immunoprecipitation analysis were carried out after an additional 24 hr.

Surface AChR assay. Surface AChR expression was determined by incubating intact transfected cells for 90 min at 37° with 10 nM ^{125}I-α-BTX (Amersham Corp., Arlington Heights, IL). Nonspecific binding was measured by addition of >100-fold excess unlabeled α-BTX to the 10 nM ^{125}I-α-BTX-containing medium or by sham transfection. Unbound α-BTX was removed by washing the cells with PBS. The amount of bound toxin was measured by solubilizing the cells in 0.1 M NaOH and counting the radioactivity in a γ counter.

Immunoprecipitation of heterodimers. Immunoprecipitation with subunit-specific antibodies was performed as described previously (24). The COS cells transfected with the α and β, α and δ_β, αM1 and β, or αM1 and δ_β cDNAs were lysed in a solubilizing buffer containing 50 mM Tris·HCl, pH 7.4, 50 mM NaCl, 1.0% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM sodium tetrathionate, 1 mM *N*-ethylmaleimide, 0.4 mM phenylmethylsulfonyl fluoride, 10 units/ml aprotinin, and 20 μg/ml leupeptin. ^{125}I-α-BTX was then added to the lysates, to a final concentration of 10 nM, to label all toxin binding sites. Eighty microliters of the cell lysate samples were incubated with MAb 124 in the presence of 10 nM ^{125}I-α-BTX for 2 hr at 4°. Samples of all reaction mixtures were then added to a 50-μl slurry of rabbit anti-rat IgG-conjugated Sepharose 4B and were incubated at 4°, with rocking, for an additional 2 hr.

Immunoprecipitations of the αδ or αM1δ heterodimers were performed by incubating the lysate samples with MAb 88B-coupled Sepharose in the presence of 10 nM ^{125}I-α-BTX for 2 hr at 4°. All precipitates were washed three times with washing buffer (50 mM Tris·HCl, pH 7.4, 1 M NaCl, 1% Triton X-100) before being counted in a γ counter. Control immunoprecipitations were carried out using sham-transfected COS cells.

Sucrose gradient sedimentation. To label intracellular intermediates, transfected COS cells in 60-mm dishes were incubated for 1–2 hr on ice with 0.5% saponin buffer containing 10 nM ^{125}I-α-BTX, 10 mM HEPES, pH 7.4, 0.1% bovine serum albumin, and 0.5% saponin and were then washed three times in the same buffer without ^{125}I-α-BTX. Surface AChRs were labeled with 10 nM ^{125}I-α-BTX in growth medium for 90 min at 37°. The cells were scraped off the plate with a rubber policeman, pelleted, and solubilized in extraction buffer. The cell lysates, mixed with gradient markers [bovine alkaline phosphatase (6.3 S) and catalase (11.4 S)], were applied to 5–20% sucrose gradients. The gradients were centrifuged at 36,000 rpm in a Beckman SW 50.1 rotor for 15–16 hr at 4° and were collected in 100-μl aliquots. Fractions were counted in a γ counter.

dTC inhibition. COS cells (in 100-mm dishes) expressing either the AChR or the heterodimers were trypsinized after the transfections and split into 24-well plates. Duplicate wells were used for each concentration of dTC. After 20 hr, the cells expressing surface AChRs were preincubated for 1 hr with 200 μl of growth medium containing the final concentration of dTC; 10 μl of 100 nM ^{125}I-α-BTX were then added, to yield a final concentration of about 5 nM ^{125}I-α-BTX. After incubation for another 30 min, the cells were washed three times with PBS, dissolved in 0.5 ml of 0.1 N NaOH, and counted in a γ counter. For cells expressing heterodimers, the cells were preincubated, 20 hr after transfection, for 1 hr with 200 μl of 0.5% saponin buffer containing the indicated final concentration of dTC, 10 μl of 100 nM ^{125}I-α-BTX were added to produce a final concentration of 5 nM ^{125}I-α-BTX, and the cells were then incubated for another 30 min. The cells were washed three times with the saponin buffer, dissolved in 0.5 ml of 0.1 N NaOH, and counted. Nonspecific binding was defined as that obtained by adding 1 μM unlabeled α-BTX with the ^{125}I-α-BTX. Specific binding was defined as total binding minus nonspecific binding. Apparent inhibition constants (IC_{50}) were obtained by computer fitting of the data to a one-component binding curve, using a conventional, least-mean squares program.

Results

Chimeric subunits bearing the amino- and carboxyl-terminal domains of the [delta] subunit can substitute for [delta] in supporting AChR assembly. When all four subunits of the adult AChR (α, β, ε, and δ) are expressed in COS cells, AChRs appear on the cell surface, as measured by the binding of α-BTX (Table 1). The physiological and pharmacological properties of the AChR are similar to those of the AChR at adult endplates (19). When either the β, δ, or ε subunit cDNA is omitted from the transfection mixture, little or no toxin-binding activity is detected on the cell surface, but assembly intermediates are found intracellularly (8, 11). We showed previously that a

TABLE 1

Chimeric subunits bearing the amino- and carboxyl-terminal domains of the δ subunit can substitute for the δ subunit in AChR assembly

COS cells were transfected with various combinations of AChR subunit cDNAs as shown, and the amount of ^{125}I-BTX binding to the intact cells was determined as described in Materials and Methods. Each value is the mean ± standard error of three determinations.

Subunits expressed	Surface toxin bound
	fmol/well
$\delta\beta\epsilon\delta$	60.9 ± 1.6
$\delta\beta\epsilon$	<0.3
$\delta\beta\epsilon(\delta_\beta)$	12.4 ± 0.4
$\alpha\beta\epsilon(\delta_\gamma)$	41.6 ± 1.1
$\alpha\beta\epsilon(\delta_\epsilon)$	4.6 ± 1.5
$\alpha\epsilon\delta$	<0.3
$\alpha(\delta_\beta)\epsilon\delta$	0.4 ± 0.1

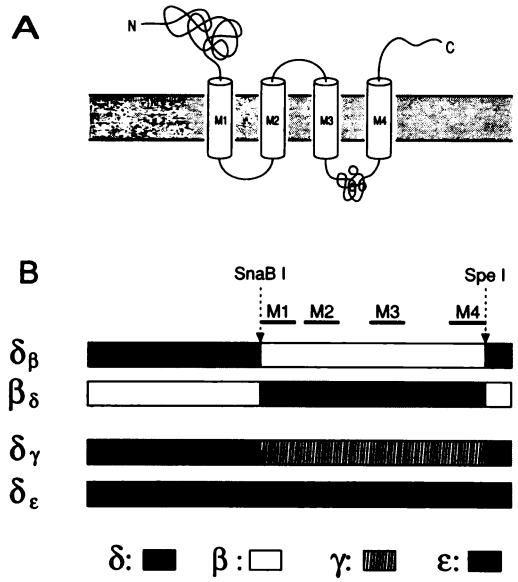

Fig. 1. Diagrammatic model of the chimeric AChR subunit used. (A) Diagrammatic representation of the transmembrane topology of AChR subunits. (B) Structures of the chimeric subunits with M1–M4 aligned. The chimeric subunits [delta][inf][gamma][r] and [delta][inf][silon][r] have structures similar to that of [delta][inf][beta][r]. Each of the chimeric subunit cDNAs was constructed as described in Materials and Methods. The locations of the introduced *Sna*BL and *Spe*L sites are indicated.

chimeric subunit bearing specific sequences that mediate association with other subunits can substitute for an intact subunit in supporting surface AChR expression (11). The transient expression of chimeric subunits in COS cells is thus a convenient experimental system for identifying these sequences.

To locate the domains that confer identity upon the δ subunit during AChR assembly, chimeric subunit cDNAs were constructed in which the region from M1 to M4 was exchanged between the δ subunit and other subunits (Fig. 1). These cDNAs were then tested for their ability to substitute for each of the two parental subunit cDNAs in the COS cell transfection system.

When the chimeras δ_ϵ, δ_γ, and δ_β, in which the sequence from M1 to M4 was replaced by the corresponding sequence from the ε, γ, or β subunit, respectively, were expressed in COS cells along with α, β, and ε subunits, each was able to support AChR assembly. Values for cell surface ^{125}I-α-BTX binding obtained with the chimeric subunits were compared with those seen with the intact δ subunit and ranged from 8% in the case of δ_ϵ to 68% in the case of δ_γ. When δ was completely omitted from the reaction, <0.3% of the normal toxin-binding activity was seen. None of the chimeras was able to substitute for the β subunit when its cDNA was omitted from the transfection mixture (Table 1).[1] The chimeric subunit βδ was unable to substitute for either the β or δ subunit.

To determine whether the cell surface toxin-binding activity seen when the chimeric subunits replaced the δ subunit corresponds to a fully formed receptor complex, toxin-bound complexes were solubilized from transfected cells and sedimented in a sucrose gradient. All gave values close to 9.5 S (data not shown), similar to values seen for native AChRs expressed in COS cells (19) and for the rodent muscle nicotinic AChR (25).

The chimeric δ_β subunit stably associates with the α subunit to form a heterodimer. The δ subunit normally forms a heterodimer with the α subunit, whereas the β subunit does not (6, 9, 19). We used immunoprecipitation and sucrose gradient sedimentation to determine whether δ_β, like δ, forms a heterodimer with the α subunit. COS cells were transfected with α cDNA alone, with α plus δ cDNAs, or with α plus δ_β cDNAs. Lysates from the transfected cells were then incubated with ^{125}I-α-BTX and, in separate samples from each lysate, the abilities of the δ subunit-specific antibody MAb 88B (Fig. 2A) and the β subunit-specific antibody MAb 124 (Fig. 2B) to

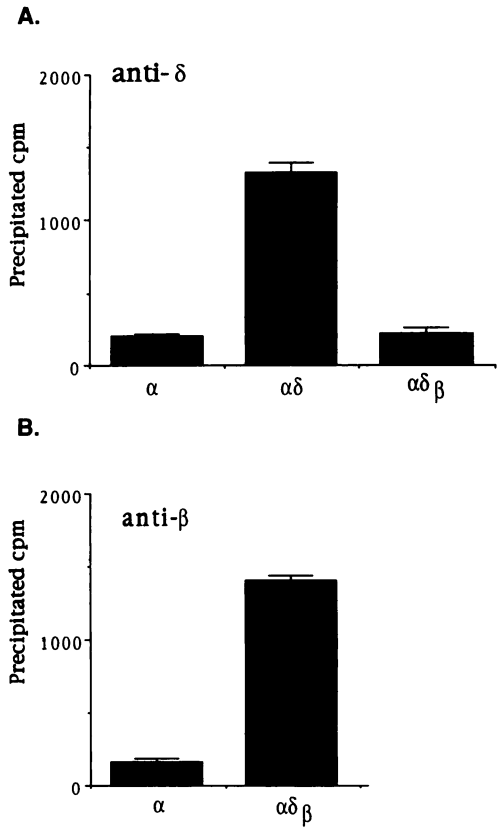

Fig. 2. Formation of a heterodimer between the chimeric subunit δ_β and the α subunit. COS cells were transfected with cDNAs for the α, the α plus δ, or the α plus δ_β subunits. Forty-eight hours later, extracts of the cells were immuniprecipitated with an antibody to the cytoplasmic loop of the δ subunit (MAb 88b) (A) or with an antibody to the β subunit (MAb 124) (B), as described in Materials and Methods. Each value represents the mean ± standard deviation of three determinations.

[1] X.-M. Yu. and Z. Hall, unpublished observations.

immunoprecipitate toxin were tested. In extracts of cells coexpressing α and δ subunits, MAb 88B (which recognizes the cytoplasmic loop of δ), but not MAb 124 (which recognizes the cytoplasmic loop of β), precipitated toxin-binding activity. Conversely, in extracts of cells coexpressing α and δ_β subunits MAb 124, but not MAb 88B, precipitated toxin-binding activity. Thus, the δ_β subunit, like δ, can associate with the α subunit. Sedimentation analysis of extracts of the transfected COS cells showed that the complex formed with α and δ_β subunits is similar in size to the αδ heterodimer (Fig. 3).

The δ_β subunit can associate with a truncated α subunit. We then used immunoprecipitation experiments to determine whether the δ_β subunit can associate with αM1, a fragment of the α subunit that is truncated after M1. We first used immunoprecipitation with MAb 88B to demonstrate that αM1 forms a heterodimer with the δ subunit (Fig. 4A), thus confirming previous experiments (26). When COS cells were transfected with cDNAs for the αM1 plus δ_β subunits, MAb 124, which recognizes the δ_β chimeric subunit, was able to immunoprecipitate toxin-binding activity from the cell extracts (Fig. 4B). No toxin-binding activity was immunoprecipitated from cells transfected with cDNAs for α plus β or αM1 plus β subunits. These experiments demonstrate that δ_β, like the δ subunit, forms a heterodimer with αM1, providing strong evidence that the amino- and carboxyl-terminal domains of the δ subunit contain all of the subunit-specific information necessary for heterodimer formation, the first step in AChR assembly.

Receptor assembly can occur with both δ_β and ϵ_β. Because each of the chimeric subunits δ_β and ϵ_β (11) can subsitute for its respective parental subunit, we tested whether a complete receptor complex can be made with both chimeric subunits. COS cells were transfected with α, β, ϵ_β, and δ_β subunit cDNAs and toxin binding to the intact cells was measured. Toxin-binding activity was detected at a level of about 20% of that seen when all four parental subunits were used (Table 2). This is approximately the same level of surface AChR expression seen when only one of the chimeric subunit cDNAs is substituted into the transfection mixture. The reduced efficiency seen with only one chimeric subunit is thus not additive when both are used.

When either of the chimeric subunit cDNAs was omitted,

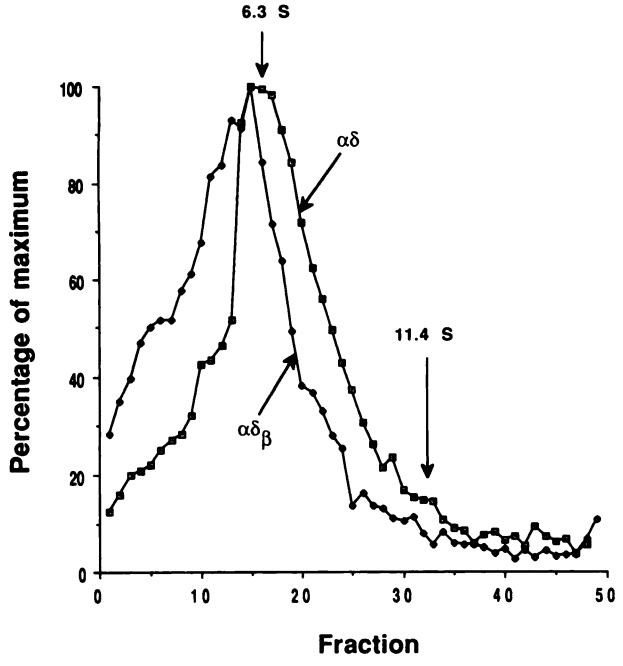

Fig. 3. Sucrose gradient sedimentation of αδ and α(δ_β) heterodimers. COS cells transfected with cDNAs for the α plus δ or the α plus δ_β subunits were extracted with detergent. The extracts were labeled with ^{125}I-α-BTX and sedimented on a 5–20% sucrose gradient as described in Materials and Methods. The positions of the two gradient markers, alkaline phosphatase (6.3 S) and catalase (11.4 S), are indicated. The counts were plotted as percentages of the cpm of the fraction with the highest value.

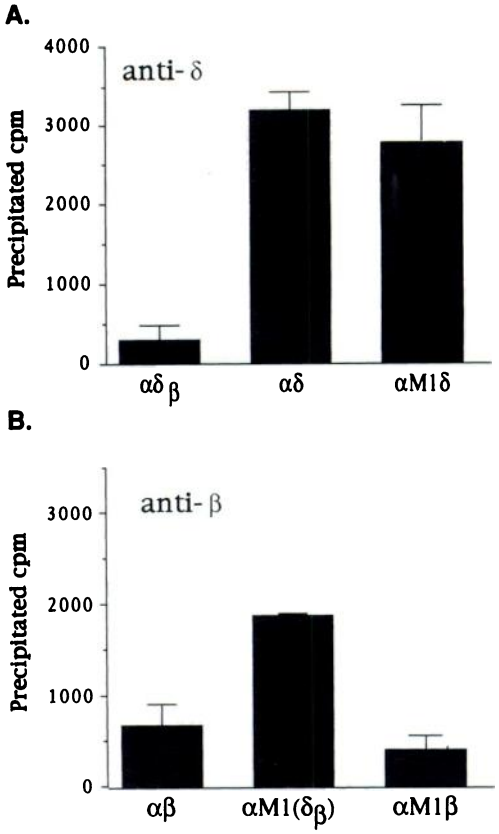

Fig. 4. Association of the chimeric subunit δ_β with a truncated α subunit containing only the amino-terminal and M1 domains. COS cells were transfected with normal and chimeric subunit cDNAs as indicated. Extracts of the transfected cells were then labeled with ^{125}I-α-BTX and immunoprecipitated either with an antibody to the δ subunit (MAb 88B) (A) or with an antibody to the β subunit (B), as described in Materials and Methods. Each value represents the mean ± standard deviation of three determinations.

TABLE 2

A receptor complex containing only α and β transmembrane and cytoplasmic domains is expressed in COS cells

COS cells were transfected with various combinations of normal and chimeric AChR subunit cDNAs as shown, and the amount of ^{125}I-α-BTX binding to the intact cells was determined as described in Materials and Methods. Each value is the mean ± standard error of three determinations.

Subunits expressed	Surface toxin bound
	%
αβεδ	100 ± 9.9
αβ(ϵ_β)	<0.5
αβ(δ_β)	<0.5
αβε(δ_β)	15.1 ± 3.8
αβ(ϵ_β)δ	23.8 ± 0.3
αβ(ϵ_β)(δ_β)	12.7 ± 2.5

toxin binding on the surface was not detected (Table 2). Because the toxin-binding activity is provided by the α subunit, and the β subunit is required for surface toxin-binding activity (data not shown), the complex must thus contain all four subunits. Also, the surface toxin-binding activity increases as a function of the concentration of either δ_β or ϵ_β cDNA (data not shown). As with the other complexes, the sedimentation constant of the complex (9.8 S) is consistent with the formation of a pentamer. Thus, AChR assembly can occur when three of the five transmembrane and cytoplasmic regions in the fully assembled receptor complex are derived from the β subunit, with the remaining two from the α subunit.

dTC can inhibit toxin binding both to the heterodimers and to the complete receptor complexes formed by δ_β and ϵ_β subunits. Association of the α and δ subunits to form the heterodimer $\alpha\delta$ creates a low affinity binding site for dTC, whereas association of the α and γ subunits generates a site with higher affinity (6). These affinities correspond to the two nonequivalent dTC binding sites seen in the fully assembled AChR (6, 27, 28). We have determined the binding of dTC both to intracellular heterodimers and to surface receptor complexes formed from the chimeric subunits δ_β and ϵ_β. For the heterodimers, COS cells were transiently transfected with α subunit cDNA plus cDNA for either the δ, δ_β, ϵ, or ϵ_β subunit. The transfected cells were then permeabilized with saponin and the effects of different concentrations of dTC on the rate of α-BTX binding were determined (6). For both δ_β and ϵ_β subunits, the heterodimers formed by the chimeric subunit had apparent inhibition constants that were approximately the same as those seen with the intact subunit. Thus, the IC_{50} for $\alpha(\epsilon_\beta)$ was 1.0×10^{-7} M, compared with 4.3×10^{-8} M for $\alpha\epsilon$, and the IC_{50} for $\alpha(\delta_\beta)$ was 8.5×10^{-7} M, compared with 8.9×10^{-7} M seen for $\alpha\delta$ (Table 3).

We also examined dTC inhibition for chimeric subunit-containing receptor complexes expressed on the surface of transfected COS cells. Inhibition by dTC of the rate of binding of α-BTX to intact cells was measured, and the results in each case were expressed by a single apparent inhibition constant. Again, substitution of the chimeric δ_β or ϵ_β subunit for the intact subunit had little, if any, effect on dTC binding. The results we obtained for receptor complexes with intact δ or ϵ subunits are consistent with those found for the AChR in earlier investigations (6, 19, 29, 30). Our result for the receptor complex containing the ϵ_β subunit, however, is different from what we reported previously (11) based on a less complete analysis. The reason for this difference is not known. The finding that substitution of the transmembrane and cytoplasmic regions of the δ and ϵ subunits with the corresponding regions of the β subunit has little effect on dTC binding suggests, in agreement with other findings (reviewed in Ref. 2; see also Ref. 31), that the contribution of these subunits to the ligand binding site is chiefly through their extracellular or luminal domains.

TABLE 3

dTC inhibits binding to heterodimers and AChR complexes containing chimeric subunits

COS cells were transfected with combinations of subunit cDNAs as indicated, and the inhibition by dTC of the rate of binding of ^{125}I-BTX was determined as described in Materials and Methods.

Subunits expressed	Apparent inhibition constant
	M
Heterodimers	
$\alpha\epsilon$	1.0×10^{-7}
$\alpha(\epsilon_\beta)$	4.3×10^{-8}
$\alpha\delta$	8.9×10^{-7}
$\alpha(\delta_\beta)$	8.5×10^{-7}
AChR complexes	
$\alpha\beta\epsilon\delta$	9.2×10^{-7}
$\alpha\beta\epsilon(\delta_\beta)$	3.0×10^{-6}
$\alpha\beta(\epsilon_\beta)\delta$	5.1×10^{-7}
$\alpha\beta(\epsilon_\beta)(\delta_\beta)$	3.4×10^{-7}

Discussion

The experiments reported here show that the identity of the δ subunit in the assembly pathway of the AChR is determined by its amino- and carboxyl-terminal sequences. Thus, if chimeric subunits bear these domains, their transmembrane and cytoplasmic domains can be replaced by the corresponding regions of the β, γ, or δ subunits without affecting their ability to replace the δ subunit in formation of an AChR complex. Each of the complexes formed with the chimeric subunits has the size expected of a pentameric complex and is transported to the cell surface. Because assembly intermediates and complexes with the incorrect number of subunits are retained in the ER (8),[2] these results suggest that the oligomers formed with the chimeric subunits are recognized by the COS cells as completely assembled receptor complexes. Although we do not know the exact composition or stoichiometry of the receptor complexes, formation of the complex containing the δ_β subunit requires each of the other subunits (α, β, and δ) to be expressed, suggesting that all four subunits are present in the complex.

The ability of the chimeric subunits to substitute for the δ subunit in forming a receptor complex presumably depends upon their ability to form a heterodimer with the α subunit (8, 11). This was shown directly for the δ_β subunit, which, when coexpressed with the α subunit, was able to increase the amount of toxin binding of the α subunit and which could be immunoprecipitated with the α subunit, using an antibody to the cytoplasmic loop of the β subunit. The ability of the δ_β subunit to increase toxin binding has been shown previously to arise from the decrease in α subunit degradation that results from its association to form a heterodimer (32, 33).

Our finding that the δ subunit associates with the α subunit via luminal sequences is consistent with previous experiments from our laboratory showing that the amino-terminal domain of the δ subunit is able to exert a dominant negative effect on AChR assembly in COS cells and that this effect appears to arise from the ability of the amino-terminal fragment to interfere with heterodimer formation (26). Thus, in the chimeric subunits used here, it is presumably sequences in the amino-terminal domain that are responsible for association with the α subunit. As with the ϵ subunit,[3] chimeric subunits containing only the amino-terminal domain from δ and the remainder from the β subunit were unable to substitute for either δ or β subunits in AChR assembly. Presumably the carboxyl-terminal domains or interactions between the amino- and carboxyl-terminal domains are necessary for steps of assembly that are subsequent to heterodimer formation.

Because we reported earlier that the receptor complex formed with the ϵ_β subunit had an anomalously high affinity (10^{-10} M) for dTC when expressed on the surface of COS cells (11), we

[2] J. Forsayeth and Z. Hall, unpublished observations.
[3] X.-M. Yu and Z. Hall, unpublished observations.

wished to investigate the affinity for dTC of the heterodimers and the receptor complexes formed with either δ_β or ϵ_β subunits. The results reported here show no difference, in the case of either the heterodimers or the complete complexes, between the affinities for dTC that are found when chimeric subunits are used and those found when the intact δ or ε subunits are used. These results provide support for the idea that the dTC binding sites are formed exclusively from the extracellular domains of the δ and ε (and presumably γ) subunits (31). In recent experiments, Sine (31) has used chimeric subunits and mutational analysis to identify the residues in the amino termini of the δ and γ subunits that are responsible for the different dTC affinities of the αδ and αγ heterodimers. We have no explanation for the results that we obtained earlier with the ϵ_β subunit (11) but, in light of the more detailed studies reported here, we now believe them to be in error.

The results reported in this paper for the δ subunit, along with those reported earlier (11, 26), suggest that heterodimer formation between the α subunit and either the δ or ε subunit depends almost entirely on interactions that occur between the amino-terminal domains of these subunits within the lumen of the ER. By extension, we believe that this mechanism is also responsible for αγ heterodimer formation. In other experiments we have investigated the later steps of AChR assembly and have found evidence for the participation of subunit-specific sequences in the cytoplasmic loops of the α and β subunits (34).[4] The experiments reported here demonstrate that subunit-specific sequences in the δ and ε subunits are not required at any step of the assembly pathway. The dependence of interactions between subunits in the later steps of AChR assembly on prior interactions (8, 35) suggests that interactions in the luminal domain must affect the ability of sequences on the other side of the ER membrane to interact with their neighbors. How interactions in the luminal domain can alter the conformation of cytoplasmic domains is a topic for future investigation.

Acknowledgments

We thank Drs. N. Davidson, S. Froehner, P. Gardner, J. Lindstrom, and J. Merlie for antibodies and cDNAs. We are grateful to members of the Hall laboratory for their helpful suggestions.

References

1. Karlin, A. Explorations of the nicotinic acetylcholine receptor. *Harvey Lect.* **85**:71–107 (1991).
2. Galzi, J. L., F. Revah, A. Bessis, and J. P. Changeux. Functional architecture of the nicotinic acetylcholine receptor: from electric organ to brain. *Annu. Rev. Pharmacol.* **31**:37–72 (1991).
3. Unwin, N. Nicotinic acetylcholine receptor at 9 Å resolution. *J. Mol. Biol.* **229**:1101–1124 (1993).
4. Smith, M. M., J. Lindstrom, and J. P. Merlie. Formation of the α-bungarotoxin binding site and assembly of the nicotinic acetylcholine receptor subunits occur in the endoplasmic reticulum. *J. Biol. Chem.* **262**:4367–4376 (1987).
5. Gu, Y., E. Ralston, R. A. Black, C. Murphy-Erdosh, and Z. W. Hall. Acetylcholine receptor in a C2 muscle cell variant is retained in the endoplasmic reticulum. *J. Cell Biol.* **109**:729–738 (1989).
6. Blount, P., and J. P. Merlie. Molecular basis of the two nonequivalent ligand binding sites of the muscle nicotinic acetylcholine receptor. *Neuron* **3**:349–357 (1989).
7. Blount, P., M. S. McHardy, and J. P. Merlie. Assembly intermediates of the mouse muscle nicotinic acetylcholine receptor in stably transfected fibroblasts. *J. Cell Biol.* **111**:2601–2611 (1990).
8. Gu, Y., J. R. Forsayeth, S. Verrall, X.-M. Yu, and Z. W. Hall. Assembly of the mammalian muscle acetylcholine receptor in transfected COS cells. *J. Cell Biol.* **114**:799–807 (1991).
9. Saedi, M. S., W. G. Conroy, and J. Lindstrom. Assembly of *Torpedo* acetylcholine receptors in *Xenopus*. *J. Cell Biol.* **112**:1007–1015 (1991).
10. Green, W. N., and T. Claudio. Acetylcholine receptor assembly: subunit folding and oligomerization occur sequentially. *Cell* **74**:57–70 (1993).
11. Yu, X.-M., and Z. W. Hall. Extracellular domains mediate subunit interactions of the muscle acetylcholine receptor. *Nature (Lond.)* **352**:64–67 (1991).
12. Tzartos, S. J., D. E. Rand, B. L. Einarson, and J. M. Lindstrom. Mapping of surface structures of *Electrophorus* acetylcholine receptor using monoclonal antibodies. *J. Biol. Chem.* **256**:8635–8645 (1981).
13. Gullick, W., and J. Lindstrom. Mapping the binding of monoclonal antibodies to the acetylcholine receptor from *Torpedo californica*. *Biochemistry* **22**:3312–3320 (1983).
14. Froehner, S. C., K. Douville, S. Klink, and W. J. Culp. Monoclonal antibodies to cytoplasmic domains of the acetylcholine receptor. *J. Biol. Chem.* **258**:7112–7120 (1983).
15. Isenberg, K. E., J. Mudd, V. Shah, and J. P. Merlie. Nucleotide sequence of the mouse muscle nicotinic acetylcholine receptor α subunit. *Nucleic Acids Res.* **14**:5111–5119 (1986).
16. Buonanno, A., J. Mudd, V. Shah, and J. P. Merlie. A universal oligonucleotide probe for acetylcholine receptor genes: selection and sequencing of cDNA clones for the mouse muscle β subunit. *J. Biol. Chem.* **266**:15532–15538 (1986).
17. Yu, L., R. J. LaPolla, and N. Davidson. Mouse muscle nicotinic acetylcholine receptor γ subunit: cDNA sequence and gene expression. *Nucleic Acids Res.* **14**:3539–3555 (1986).
18. LaPolla, R. J., K. M. Mayne, and N. Davidson. Isolation and characterization of a cDNA clone for the complete protein coding region of the δ subunit of the mouse acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **81**:7970–7974 (1984).
19. Gu, Y., P. Camacho, J. B. Gardner, and Z. W. Hall. Properties of embryonic and adult muscle acetylcholine receptors transiently expressed in COS cells. *Neuron* **5**:147–157 (1990).
20. Brodsky, M. H., M. Warton, R. M. Meyers, and D. R. Littman. Analysis of the site in CD4 that binds to the HIV envelope glycoprotein. *J. Immunol.* **144**:3078–3086 (1990).
21. Geisselsoder, J., F. Witney, and P. Yuckenberg. Efficient site-directed *in vitro* mutagenesis. *Biotechniques* **5**:786–791 (1987).
22. Kunkel, T. A. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**:488–492 (1985).
23. Seed, B., and A. Aruffo. Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA* **84**:3365–3369 (1987).
24. Gu, Y., and Z. W. Hall. Immunological evidence for a change in subunits of the acetylcholine receptor in developing and denervated rat muscle. *Neuron* **1**:117–125 (1988).
25. Berg, D. K., R. B. Kelly, P. B. Sargent, P. Williamson, and Z. W. Hall. Binding of α-bungarotoxin to acetylcholine receptors in mammalian muscle. *Proc. Natl. Acad. Sci. USA* **69**:147–151 (1972).
26. Verrall, S., and Z. W. Hall. The N-terminal domains of acetylcholine receptor subunits contain recognition signals for the initial steps of receptor assembly. *Cell* **68**:22–31 (1992).
27. Dowding, A. J., and Z. W. Hall. Monoclonal antibodies specific for each of the two toxin-binding sites of the *Torpedo* acetylcholine receptor. *Biochemistry* **26**:6372–6381 (1987).
28. Pederson, S. E., and J. B. Cohen. *d*-Tubocurarine binding sites are located at *alpha-gamma* and *alpha-delta* subunit interfaces of the nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **87**:2785–2789 (1990).
29. Sine, S. M., and P. Taylor. Relationship between reversible antagonist occupancy and the functional capacity of the acetylcholine receptor. *J. Biol. Chem.* **256**:6692–6699 (1981).
30. Gu, Y., L. Silberstein, and Z. W. Hall. The effects of a myasthenic serum on the acetylcholine receptors of C2 myotubes. *J. Neurosci.* **5**:1909–1916 (1985).
31. Sine, S. M. Molecular dissection of subunit interfaces in the acetylcholine receptor: identification of residues that determine curare selectivity. *Proc. Natl. Acad. Sci. USA* **90**:9436–9440 (1993).
32. Blount, P., and J. P. Merlie. Mutational analysis of muscle nicotinic acetylcholine receptor subunit assembly. *J. Cell Biol.* **111**:2613–2622 (1990).
33. Chavez, R. A., and Z. W. Hall. The transmembrane topology of the amino terminus of the α subunit of the nicotinic acetylcholine receptor. *J. Biol. Chem.* **266**:15532–15538 (1991).
34. Yu, X.-M., and Z. W. Hall. A sequence in the main cytoplasmic loop of the α subunit is required for assembly of the mouse muscle nicotinic acetylcholine receptor. *Neuron* **13**:247–255 (1994).
35. Hall, Z. W. Recognition domains in assembly of oligomeric membrane proteins. *Trends Cell Biol.* **2**:66–68 (1992).

[4] X.-M. Yu, Z.-Z. Wang, and Z. Hall, unpublished observations.

Send reprint requests to: Zach W. Hall, NINDS, Building 31, Room 8A52, 9000 Rockville Pike, Bethesda, MD 20892.