Temperature-sensitive Expression of All-Torpedo and Torpedo–Rat Hybrid AChR in Mammalian Muscle Cells

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Abstract. When the four subunits of the Torpedo californica nicotinic acetylcholine receptor (AChR) are expressed in mammalian fibroblasts, they properly assemble into α2βγδ pentamers only at temperatures lower than 37°C (Claudio, T., W. N. Green, D. S. Hartman, D. Hayden, H. L. Paulson, F. J. Sigworth, S. M. Sine, and A. Swedlund. 1987. Science (Washington, D.C.). 238:1688–1694). Experiments here with rat L6 myoblast cell lines indicate that this temperature sensitivity is not specific to fibroblasts, but is intrinsic to Torpedo subunits. A clonal isolate of L6 cells cotransfected with the four Torpedo subunit cDNAs synthesizes the exogenous AChR subunits at 37° and 26°C, but expresses Torpedo AChR complexes only at the lower temperature. When Torpedo α alone is expressed in L6 myotubes, hybrid AChRs are formed, again only at temperatures below 37°C. These hybrid AChRs can contain either two Torpedo α subunits or one each of rat and Torpedo α, proving that the two α subunits in an AChR pentamer need not derive from the same polysome. Further analysis of hybrid and all-Torpedo AChR established that there is no internally sequestered pool of AChR at the nonpermissive temperature, and that the AChR, once formed, is thermostable. Two lines of experimentation with α subunits expressed in fibroblasts indicate that α polypeptides exhibit different conformations at 26° and 37°C, favoring the hypothesis that the temperature-sensitive step occurs before assembly and reflects, at least in part, misfolding of subunits: at 37°C, there is a reduction in the fraction of α subunits that (a) bind the AChR antagonist α-bungarotoxin with high affinity; and (b) bind a monoclonal antibody that recognizes correctly folded and/or assembled α subunit.

The nicotinic acetylcholine receptor (AChR)† resides at the postsynaptic face of the vertebrate neuromuscular junction, where it mediates synaptic transmission between nerve and muscle. It is the best characterized member of a superfamily of ligand-gated ion channels that also includes neuronal AChRs, γ-aminobutyric acid, and glycine receptors (for review, see Claudio, 1989). An intrinsic membrane glycoprotein, the AChR is composed of four different subunit types with apparent molecular masses of ~40 (α), 50 (β), 60 (γ), and 65 (δ) kD. Together these assemble into a pentameric complex with the stoichiometry α2βγδ. The four subunit types show extensive sequence similarity at the amino acid level and are believed to possess similar tertiary structure and topology across the membrane (for reviews, see Popot and Changeux, 1984; Karlin et al., 1986; McCarthy et al., 1986; Maelicke, 1988; Claudio, 1989).

The AChR serves as an excellent model protein with which to examine the assembly of heteropolymeric membrane proteins. To study this and other cell biological aspects of the AChR, our laboratory has introduced combinations of AChR subunit cDNAs from the marine ray Torpedo californica into mammalian cells (Claudio et al., 1987, 1988, 1989). Using this approach, we have demonstrated that fully functional Torpedo AChRs can be expressed in mammalian fibroblasts (Claudio et al., 1987). However, the assembly of Torpedo AChR in fibroblasts appears to be profoundly temperature-sensitive. At 37°C all four subunits are synthesized and inserted into the membrane of the endoplasmic reticulum, but no functional AChR complexes are formed. Only at ~10° below 37°C are AChRs expressed at high levels.

Temperature-sensitive mutants have proved extremely useful for investigating the molecular events involved in many biosynthetic processes, including the assembly of multi-subunit membrane proteins (Kreis and Lodish, 1986; Doms et al., 1987). Because temperature-sensitive expression of Torpedo AChR in mammalian fibroblasts has the potential to be a similarly valuable tool for studying AChR assembly, in this study we investigate the temperature-sensitive phenomenon itself. To determine whether it is specific to expression in nonmuscle cells such as fibroblasts, or whether it is an intrinsic property of Torpedo subunits, we established and analyzed the expression of Torpedo subunits in mammalian muscle cells. We demonstrate that expression
in muscle cells is also highly sensitive to temperature and, further, determine which step in the biosynthetic pathway is temperature-sensitive. Using the Torpedo temperature-sensitive assembly phenomenon, we were able to establish that the two α subunits of an AChR pentamer need not derive from the same polysome. The implications of this finding relating to channel diversity among the superfamily of ligand-gated ion channels are discussed.

Materials and Methods

Cell Lines

Mouse NIH3T3 and L fibroblast cells were maintained in DME plus 10% calf serum (CS). Rat L6 and mouse C2 muscle cell lines were maintained in DME plus 10% FCS or DME plus 20% FCS and 0.5% chick embryo extract (Gibco Laboratories, Grand Island, NY), respectively. Muscle cell lines were induced to fuse and express endogenous AChR as follows: 70% confluent dishes of L6 myoblasts were changed to DME containing 2% horse serum and 70% confluent dishes of C2 myoblasts were given DME containing 5% FCS. L6 and C2 cells became maximally fused within 5-7 d; only dishes on which at least 75% of cells had fused were used for experiments.

Clonal isolates of L6 cells (L6-DOL-α1, L6-DOL-α2, L6-DOL-γ1), C2 cells (C2-DOL-α1), and NIH3T3 cells (3T3-DOL-α1), or a pool of several hundred colonies (3T3-DOL-omas) expressing Torpedo AChR subunit cDNAs have been previously described (Claudio et al., 1987). In these cell lines, the cDNAs were integrated into the host cell genome by viral infection and are under the control of the Moloney murine leukemia virus long terminal repeat.

The all-11 cell line is a Torpedo AChR expressing cell line produced by cotransfection of all four Torpedo subunit cDNAs (under the control of the SV-40 early promoter) and the thymidine kinase (tk) gene into Ltk- fibroblasts (Claudio et al., 1987). The cells were maintained in DME plus 10% CS and 1 X HAT (15 μg/ml hypoxanthine, 1 μg/ml aminopterin, 5 μg/ml thymidine). An L6 cell line containing the four Torpedo AChR subunit cDNAs was also established using the calcium phosphate precipitation procedure of Graham and van der Eb (1973) as modified by Wigler et al. (1979). Myoblasts were cotransfected with pSV2-α, -β, -γ, and -δ and a neomycin resistance (neo) gene using constructions and methods described previously (Claudio et al., 1987, 1989). 5 μg each of pSV2-α, pSV2-β, pSV2-γ, pSV2-δ, and 50 ng of pSV2-neo were introduced into 5 x 10^6 L6 cells in 10-cm dishes. Cultures were grown to confluency, then passaged at a 1:20 dilution into medium containing 0.6 mg/ml G418 (Gibco Laboratories). A single G418-resistant clone was obtained and characterized (termed L6-all). All cell lines were maintained in incubators at 37°C, 5% CO2. Unless stated otherwise, experiments at lower temperatures were also carried out in incubators containing 5% CO2. Expression of subunits in cell lines containing pSV2-subunit constructs was greatly enhanced by adding 10 mM sodium butyrate (butyric acid titrated with NaOH; Sigma Chemical Co., St. Louis, MO) to the medium (Claudio et al., 1987). Butyrate-containing medium was changed every 2 d.

Cell Labeling and Lysis

10-cm dishes of cells were incubated 15 min with methionine-free medium, then labeled 20 min with 330 μCi of [35S]methionine (TRAN35S, ICN Biochemical, Irvine, CA) in 2 ml of medium. Cells were then labeled 20 min with 330 μCi of [35S]methionine (TRAN35S; ICN Biochemicals) and are under the control of the Moloney murine leukemia virus long terminal repeat.

In the experiment in Fig. 10 A, cells were surface-labeled as above but with the following modifications: 6-cm dishes were incubated with 2.5 nM [125I]BuTx (specific activity of 140-170 cpm/fmol; ICN Biochemicals) in PBS, washed 4 x with 10 ml PBS over 3-5 min, then solubilized with 0.7 ml LB. In other experiments, mono-iodinated [125I]BuTx was prepared according to the method of Wang and Schmidt (1980) was used (sp act, 450-850 cpm/fmol). To determine total high affinity BuTx sites in all-11 or L6-DOL-α1 cells, cells lysed were incubated with 4 nM BuTx for 20-24 h at 4°C then immunoprecipitated with saturating amounts of α antisera. In the experiments involving the determination of high affinity BuTx sites in all-11 or L6-DOL-α1 cells, the cells were incubated with 4 nM BuTx for 20-24 h at 4°C then immunoprecipitated with saturating amounts of α antisera.

At room temperature 45 min, adding Triton X-100 to 5%, cooling to 4°C, and preclearing with protein A-Sepharose (Sigma Chemical Co.). After immunoprecipitation, samples were prepared for SDS-PAGE as described (Claudio et al., 1987), except samples were not heated before loading onto the gel. Cells were fixed for 30 min in 25% methanol and 10% acetic acid, then soaked 30 min in water and 30 min in Fluorohance (Research Products International Corp., Mt. Prospect, IL), dried on a gel dryer, and put on X-Omat AR film at -70°C with an intensifying screen. Quantitation of gel bands was done by densitometric scanning with a Visage 2000 digital scanner.
Cells were labeled with $^{125}$I BuTx and solubilized in LB. Duplicate aliquots of unlabeled BuTx.

In a total volume of 160 μl, 130 μl of lysate was incubated with the indicated concentrations of $^{125}$I BuTx for 21 h (previous experiments indicated that equilibrium binding was reached in 8-10 h with 4.7 nM BuTx). Samples were next incubated 150 min with α antisemur (25 μl of a 10-fold diluted stock), and then 70 min with 50 μl of protein A-Sepharose (diluted twofold in LB). Samples were washed four times with 800 μl LB with a total wash time of 8-9 min for each sample. Background values were determined using the counts precipitated from parallel lysates of 3T3 cells or from parallel lysates of 3T3-DQ1-α cells that had been incubated with a 40- to 250-fold excess of unlabeled BuTx.

Binding experiments were also performed on permeabilized vesicle preparations using a rapid filtration technique; however, high background values made this approach impractical for BuTx concentrations higher than 100 nM. Briefly, cells from a 10-cm dish were harvested in ice-cold 10 mM phosphate buffer, homogenized by 12 strokes in a 1-ml Dounce homogenizer (Wheaton Instruments Div., Millville, NJ) with a tight fitting pestle, and centrifuged 5 min at 500 g to remove nuclei. In final concentrations of 10 mM EDTA, 10 mM phosphate, 0.5% saponin, and 5 mg/ml BSA, aliquots of the supernatant were incubated 20 h with varying concentrations of $^{125}$I BuTx, and passed rapidly through filters (Millipore Continental Water Systems, Bedford, MA) with two 10 ml washes (elapsed time less than 45 s). Backgrounds were determined in the presence of a 200-fold excess of unlabeled BuTx.

**Determination of Hybrid AChR**

Cells were labeled with $^{125}$I BuTx and solubilized in LB. Duplicate aliquots of the lysates were immunoprecipitated either with saturating amounts of α antisemur (which precipitates all AChR from these cells) or with a pool of three mAbs specific for Torpedo α subunit (mAbs 3A1, 3B1, 14H10). Counts precipitated by the Torpedo α-specific mAbs were then expressed as a percentage of total counts precipitated by the anti-α antisemur. Reimmunoprecipitations of the immunodepleted lysates were frequently done to ensure that the initial immunoprecipitations were quantitative. In all cases, this second incubation showed the first to be ≥95% quantitative.

**Immunoblots**

Cell extracts or immunoprecipitates were run on 7.5% SDS-polyacrylamide gels then transferred to Zetabind filters (American Laboratory Supply, Natick, MA) in a Transblot apparatus (Bio-Rad Laboratories, Richmond, CA) for 5-7 h at 4°C according to the manufacturer's instructions. Zetabind filters were quenched overnight at 45°C with 5% Carnation nonfat milk in PBS containing 0.05% NaN₃. All subsequent steps were performed at room temperature. Filters were incubated for 2.5-4 h in heat-sealed bags containing α antisemur diluted 1:500 in 5% milk/PBS. After washes of 1 × 15 min in PBS containing 0.1% Triton X-100, 2 × 15 min in PBS, and 1 × 15 min in 5% milk/PBS, filters were incubated with $^{125}$I protein A (Amersham Corp., Arlington Heights, IL) for 120 min in 5% milk/PBS, 1.0 × 10⁶ cpm of $^{125}$I protein A (sp act, 30 mCi/mg) were added per lane. Filters were washed in PBS plus 0.1% Triton X-100 for 1 × 15 min, 3-12 h with PBS, and 1 × 15 min with PBS, and then exposed to Kodak XAR film at −70°C with an intensifying screen. The α signals were quantitated by counting excised bands in a gamma counter.

**DNA Blots**

DNA was isolated from cell lines and subjected to Southern blot analysis (Southern, 1975) as described previously (Claudio et al., 1987). Cellular or plasmid DNA that was to be probed with α, β, γ, and δ sequences was digested with SstI, and DNA to be probed with γ sequences was digested with Pvu II and Eco RI. DNA from L6-all and all-11 cells, or Torpedo whole nerve, was digested with Pvu II and Eco RI (enzymes were from New England Biolabs, Beverly, MA). 5 μg of digested cellular DNA and 2.5 pg of digested plasmid DNA were electrophoresed on 1% agarose gels, transferred to nitrocellulose, and hybridized to probes specific for each cDNA. The four probes were: 1.30 kb Pvu II-Bgl II fragment for α; a 700-bp Bgl II-Bgl II fragment for β; a 1.2 kb Bgl II-Eco RI fragment for γ; and a 450-bp Hind III fragment for δ. Each probe was labeled with $^{32}$P dCTP by the multiprime labeling system (Amersham Corp.) to a specific activity of ~10⁶ cpm/μg DNA.

**Glycosidase Treatment**

Subunits were digested with endoglycosidase H (endo H) (Boehringer Mannheim, Indianapolis, IN) at 26°C for 20 h as previously described (Claudio et al., 1989). Digestions with peptidoglycase F (Boehringer Mannheim) were performed as follows: immunoprecipitated subunits were first dissociated from the protein A-Sepharose pellet in a solution containing 20 mM potassium phosphate, 25 mM EDTA, 0.66% SDS (pH 7.5), then buffer was added to give final concentrations of 20 mM potassium phosphate, 25 mM EDTA, 0.5% NP-40, 0.13% SDS (pH 7.5), and the sample was incubated 20 h at 37°C with peptidoglycase F (10 μg/ml).

**Results**

We have previously shown that mouse fibroblasts express functional Torpedo AChR when transfected with the four AChR subunit cDNAs (Claudio et al., 1987). This expression, however, is profoundly temperature-sensitive; only at temperatures ~10°C below 37°C are high levels of functional AChR produced. At 37°C each of the four subunits is abundantly synthesized, inserted into the membrane of the endoplasmic reticulum, and acquires the appropriate number of N-linked oligosaccharides, yet these subunits do not form pentameric complexes that bind the receptor antagonist BuTx (Claudio et al., 1987, 1989). This temperature-sensitive expression in the fibroblast line, all-11, is demonstrated in Fig. 1. All-11 cells at 37°C were treated with sodium butyrate for 24 h to induce expression of the Torpedo subunit cDNAs. Butyrate greatly increases cDNA expression in our SV-40 constructs (Claudio et al., 1987, 1989), probably because of an enhancer-dependent increase in transcription from the SV-40 early promoter (Gorman et al., 1983). After induction with butyrate, cells shifted to 26°C undergo a burst of AChR production, detected by the appearance on the cell surface of high affinity BuTx binding sites (Fig. 1 A). Cells maintained at 37°C do not express AChR either on the cell surface (Fig. 1 A) or internally (not shown), despite the fact that all four subunits in all-11 cells are synthesized at greater levels at 37°C (Fig. 1 B). To test directly whether this temperature sensitivity is due to an inability of fibroblasts to assemble AChR at 37°C or to temperature-sensitive properties intrinsic to one or more Torpedo subunits, we analyzed Torpedo AChR expression in mammalian muscle cells that normally express their endogenous AChR at 37°C.

**L6-all Myoblasts Express Torpedo AChR in a Temperature-sensitive Manner**

The four Torpedo subunit cDNAs were co-transfected with neo into L6 myoblasts using a calcium phosphate precipitation procedure, and a single clone was obtained after selection in G418. To determine whether this clone, termed L6-all, had integrated each of the four subunit cDNAs, we analyzed its DNA by Southern blot hybridization. As shown in Fig. 2 A, α, β, γ, and δ subunit cDNA probes hybridize to DNA from L6-all and all-11 cells, but not from L6 cells. With each probe, there is a single predominant hybridizing band that coelectrophoreses with the appropriate restriction fragment from the plasmid DNA and/or the integrated cDNA in all-11 cells. L6-all thus contains correctly integrated cDNAs for each of the four subunits. We have previously estimated the number of integrated cDNAs in all-11 cells to be 4:2:2:8 for α, β, γ, δ, respectively (Claudio et al., 1987). By comparing the band intensities between all-11 and L6-all DNAs, we estimate the copy number in L6-all to be ~5:5:10:20 for α, β, γ, δ.
To test for subunit expression, undifferentiated L6-all myoblasts were grown in the presence or absence of butyrate, metabolically labeled with [35S]methionine, solubilized in LB, and immunoprecipitated with anti-AChR antiserum. After incubation with butyrate, L6-all myoblasts synthesize all four Torpedo AChR subunits at both 26°C and 37°C (Fig. 2 B). The α, β, and δ subunits display apparent molecular weights identical to those of the respective native Torpedo subunits. The γ subunit shows slightly faster electrophoretic mobility than native Torpedo γ, but we and others have consistently observed this when γ is expressed in mammalian cells (Claudio et al., 1987, 1989) or Xenopus oocytes (Mishina et al., 1984; Claudio et al., 1988).

To determine whether Torpedo AChR is produced in L6-all myoblasts in a temperature-sensitive manner, we incubated cells at 37°C or 26°C in the presence or absence of butyrate, and then measured surface AChR by [125I]BuTx binding. The results (Fig. 2 C) show that L6-all myoblasts produce surface BuTx binding sites at 26°C, but not at 37°C, and that binding is fully blocked by the AChR agonist carbamylcholine. At 26°C, surface expression plateaus after ~5 d of butyrate treatment and is maintained at this level for ~4 d (data not shown). The maximum level of expression obtained thus far is ~19,000 surface-expressed AChRs per cell.

Several experimental observations indicate that the surface BuTx sites in L6-all myoblasts represent fully assembled AChR pentamers composed only of Torpedo subunits. First, endogenous AChRs are undetectable in L6 myoblasts, and thus it is unlikely that endogenous AChR subunits contribute to the surface AChRs. Second, the expression of surface AChRs is dependent on the presence of butyrate, correlating with butyrate's enhancement of Torpedo subunit cDNA expression. Finally, immunoprecipitations with Torpedo-specific monoclonal and polyclonal antisera, together with velocity sedimentation experiments, have confirmed that L6-all myoblasts express 9S AChR complexes that are composed of all four Torpedo subunits (data not shown).

Temperature-sensitive Incorporation of Torpedo α into Hybrid AChR

The preceding results indicate that Torpedo AChR expression is temperature-sensitive even in rat myoblasts. This implies that the temperature sensitivity is not a fibroblast-specific phenomenon, but is intrinsic to Torpedo subunits. This conclusion is confirmed and extended by the following results obtained with a second L6 cell line expressing only the Torpedo α subunit cDNA. This clonal cell line, L6-DOL-α2, is one in which the cDNA has been integrated into the genome using a packaged retroviral α-recombinant and viral infection (Claudio et al., 1989). Torpedo α is constitutively expressed at high levels in these cells, whether grown as myoblasts or as differentiated myotubes.

L6-DOL-α2 cells were induced to terminally differentiate into AChR-expressing myotubes at 37°C, and then either maintained at 37°C or shifted to 27°C for 4 d (a time course of expression indicated that hybrid AChRs were maximally expressed 3–4 d after a shift to 26°C). As shown in Table I, AChR expressed at 37°C in myotubes is not precipitable by Torpedo α-specific mAbs. In contrast, at 27°C, 40% of the surface BuTx binding sites contain Torpedo α, presumably in hybrid AChR complexes. From nine similar experiments, the mean percentage (+ SEM) of hybrid AChR at 27–28°C and 37°C was 39 ± 2.2% and 1.6 ± 0.3%, respectively. In all experiments, the values at 37°C were not statistically significantly greater than those seen in a control muscle cell line, L6-DOL-γ1, which contains an integrated retroviral γ-recombinant but does not express detectable Torpedo γ subunit. A second L6 clone, L6-DOL-α1, and an α subunit–expressing clone derived from the mouse C2 muscle cell line, C2-DOL-α1, were also tested for hybrid AChR production. Both cell lines displayed similar temperature-sensitive expression of Torpedo α in surface AChR (data not shown).

To demonstrate that Torpedo α in L6-DOL-α2 cells is incorporated into AChR complexes and not simply expressed
Figure 2. Torpedo AChR subunits expressed in rat L6 myoblasts. (A) DNA blots of the L6-all cell line. DNA from L6 and L6-all myoblasts, and from all-I 1 fibroblasts, was digested with restriction enzymes, electrophoresed on agarose gels, blotted to nitrocellulose and hybridized with 32P-labeled probes specific for the four subunit cDNAs. The probe used is indicated above the blot, and the source of the DNA below each lane. pSV2 plasmid DNAs, digested with the same restriction enzymes, are marked p. (B) Expression of Torpedo subunits in undifferentiated L6-all myoblasts. L6 and L6-all myoblasts were incubated 2 d at 26°C or 37°C in the presence or absence of sodium butyrate, and then labeled 20 min with [35S]methionine at 26°C or 37°C. Subunits were immunoprecipitated with anti-AChR antiserum and analyzed by SDS-PAGE and fluorography (28-h exposure): lanes 1-3, L6-all cells; lanes 4-6, L6 cells. The positions of native Torpedo subunits are shown. Relative TCA-precipitable counts in the lysates corresponding to lanes 1-6 were respectively 48, 31, 72, 71, 58, and 100%.

(C) Temperature-sensitive expression of surface BuTx binding sites in L6-all myoblasts. 10-cm dishes of L6-all myoblasts were incubated with butyrate-containing media at 37°C or 26°C for the indicated times, and then labeled with [125I]BuTx. Total cpm/dish are plotted versus days at 37°C (open squares) or 26°C (solid squares). BuTx binding to cells (at 26°C) was fully blocked by 10 mM carbamylcholine (solid triangle). Each point is the mean of two samples that differed by <10%.

Table I. Temperature-sensitive Expression of Hybrid AChRs in L6 cells

| Cell | Temperature | Total AChR | Hybrid AChR |
|------|-------------|------------|-------------|
|      | cpm         | %          |             |
| L6-α | 37°C        | 2,752 ± 264 | 59 ± 38     | 2.1 ± 1.5 |
| L6-α | 27°C        | 2,526 ± 526 | 993 ± 153   | 39.6 ± 2.7 |
| L6-γ | 27°C        | 1,585 ± 102 | 24 ± 8      | 1.5 ± 0.5 |

Values represent the mean of triplicates ± SEM.

on the cell surface as monomeric α, we performed a velocity sedimentation experiment (Fig. 3). [125I]BuTx-labeled AChR from L6-DOL-α myotubes at 26°C was solubilized in LB and centrifuged on a sucrose density gradient. The BuTx-binding material sedimented as a 9S complex, consistent with the sedimentation of a fully assembled pentameric AChR (Fig. 3). When the 9S fractions were incubated with Torpedo α-specific mAbs, ~30% of the counts were immunoprecipitated, which is consistent with the data in Table I.

Paulson and Claudio Temperature-sensitive AChR 1709
and implies that the BuTx binding material containing Torpedo α subunits is indeed hybrid AChR.

To determine the optimal temperature for hybrid AChR production, we incubated identical plates of cells for 5 d at various temperatures between 37°C and 24°C, and then measured hybrid AChR levels. As shown in Fig. 4, the percentage of surface hybrid AChR increases as the incubation temperature is lowered. This represents an absolute increase in hybrid AChR, since the surface expression of total AChR (endogenous plus hybrid) is similar at 37°C and at the lower temperatures (mean cpm ± SEM of 8,546 ± 256 at 37°C versus 8,660 ± 1,249 at 24-33°C). The data indicate a nearly linear inverse correlation between temperature and the percent hybrid AChR, and predict that this percentage would approach 100% at 16°C. Unfortunately, surface expression is markedly inhibited at temperatures below 20°C; hence, it has not been possible for us to test this prediction.

The results obtained between 37°C and 24°C nevertheless imply that hybrid AChR expression, as a function of temperature, does not follow a sharp transition between nonpermissive and permissive states, as has been shown for some temperature-sensitive assembly systems (Haase-Pettingell and King, 1988).

We also measured the level of total (internal plus surface) hybrid AChR (Fig. 4) and did not find a large pool of internally sequestered hybrid AChR at 37°C. This result demonstrates that the temperature sensitivity is not due to a block in the cell surface delivery of AChR at 37°C. Because we observe temperature-sensitive Torpedo AChR assembly in muscle cells as well as in fibroblasts, we can further conclude that the phenomenon is a property intrinsic to Torpedo AChR and that minimally, one subunit (α) is temperature-sensitive.
Hybrid AChR Can Contain One or Two Torpedo α Subunits

The presence of hybrid AChR in L6-DOL-α2 cells allowed us to answer a significant question regarding the biogenesis of AChR: must the two α subunits residing in a given AChR pentamer derive from the same polysome? The existence of hybrid AChR containing one rat and one Torpedo α subunit would directly demonstrate that the two α subunits can derive from independent polysomes. To test whether such hybrid AChR existed, we performed a series of immunoprecipitation and immunodepletion experiments.

The strategy underlying these experiments is diagrammed in Fig. 5 A. There are potentially four classes of AChR in L6-DOL-α2 myotubes: fully mammalian AChR, and three types of hybrid AChR containing either two Torpedo α subunits, or a single Torpedo α in either of the two α positions. Hybrid AChR with two Torpedo α subunits should be precipitable by Torpedo α-specific, but not rat α-specific, mAbs. The two remaining hybrid AChR classes, each containing one Torpedo and one rat α, should be precipitable by both types of mAbs. Our experiments were designed to see whether these latter two classes existed.

L6-DOL-α2 myotubes expressing hybrid AChR were labeled with [125I]BuTx, solubilized in LB and immunoprecipitated with three different antisera: a polyclonal antibody that precipitates both rat and Torpedo α, a monoclonal antibody (mAb 64) that precipitates rat α, and a pool of three mAbs that precipitate Torpedo α. In the experiment shown in Fig. 5 B, 36% and 77% of the AChR was precipitable by Torpedo α-specific and rat α-specific mAbs, respectively. The sum, 113%, is statistically significantly greater (P < 0.02) than the total AChR in the sample, implying that the two antisera have precipitated a common pool of AChR. This was confirmed by immunoprecipitating equal aliquots of the lysate with species-specific mAbs after first immunodepleting the sample with the opposite species-specific mAb (indicated by the minus signs in Fig. 5 B). Torpedo α-specific mAbs depleted the lysate of 24% of the AChR that contained rat α, and the rat α-specific mAb depleted the sample of 25% of the AChR that contained Torpedo α. A mixture of AChR from Torpedo electroplax and endogenous AChR from L6 myotubes showed no such immunodepletion, demonstrating that the antisera are species-specific. In a second immunodepletion experiment, the rat α-specific mAb depleted a L6-DOL-α2 lysate of 45% of the AChR containing Torpedo α (not shown). The results therefore indicate that a significant fraction (24–45%) of hybrid AChR contains one each of rat and Torpedo α subunit, proving that the two α subunits in a given AChR complex need not derive from the same polysome or be encoded by the same gene.

AChR Complexes Are Stable to Increases in Temperature

Experiments described in the preceding sections demonstrate that the temperature sensitivity is directly attributable to Torpedo subunits, including at least the α subunit. Points in the biosynthetic pathway where the temperature-sensitive step(s) might occur include: (a) translocation of the subunit polyepitope into the endoplasmic reticulum and co-translational addition of N-linked oligosaccharides; (b) initial folding of unassembled subunits; (c) assembly with heterologous subunits; (d) intracellular transport of assembled AChR; and (e) thermostability of AChR complexes. Since at 37°C in fibroblasts (Fig. 1 B) and myoblasts (Fig. 2 B), Torpedo subunits acquire the correct number of N-linked oligosaccharides, the temperature-sensitive step does not appear to affect translocation into the endoplasmic reticulum or the addition of N-linked oligosaccharides. Since, moreover, at 37°C Torpedo AChR (not shown) and hybrid AChR (Fig. 4) are not sequestered within the cell, the temperature sensitivity cannot be due to an inhibition of intracellular AChR transport. We performed several experiments to dis-
tistinguish between two of the remaining possibilities: (a) ther-

molarity of assembled AChR; and (b) misfolding of sub-

units before assembly.

If AChR is absent at 37°C because the correctly assembled
pentameric complexes are thermostable, then preformed al-
Torpedo or hybrid AChR should rapidly dissociate after a
shift from 26° to 37°C. To test this, L6-DOL-α2 myotubes
were incubated at 26°C until steady-state levels of hybrid
AChR were present on the cell surface, and then incubated
with [125I]BuTx. The washed cells were then incubated at
37° or 26°C and the fraction of counts still bound at different
time points was determined as a function of the two tem-
peratures. The fall in cell-associated counts over time provides
an estimate of the rate of AChR degradation (Devreotes and
Fambrough, 1975). Total surface AChR was degraded with
a t½ ~13 and 50 h at 37°C and 26°C, respectively (Fig. 6
A), displaying a temperature dependence consistent with the
results from an earlier study of AChR degradation in chick
muscle (Devreotes and Fambrough, 1975). The rate of
degradation at 37°C did not differ from that in control myo-
tubes expressing only mammalian AChR (t½ ~14 h), thus
demonstrating that hybrid AChR is as stable as mammalian
AChR. This was confirmed by determining the amount of hy-
brid and mammalian AChR at each time point (Fig. 6 B).
We found that at every time point after a shift to 37°C, the
ratio of hybrid to mammalian AChR was essentially con-
stant, indicating that both species were degraded with simi-
lar kinetics. In a second test of hybrid AChR thermostability
(Fig. 6 C), L6-DOL-α2 myotubes expressing steady-state
levels of hybrid AChR were shifted to 37°C and, at the indi-
cated times, labeled with [125I]BuTx, solubilized in LB, and
immunoprecipitated to determine hybrid AChR levels. At
37°C, surface hybrid AChR in these cells disappeared with
a time course (t½ ~15 h) similar to the rate of degradation
determined in the preceding experiment. From these experi-
ments, we conclude that hybrid AChR, once formed and ex-
pressed on the cell surface, is thermostable.

At 37°C, all-Torpedo AChR on the cell surface of all-11
fibroblasts also was degraded slowly, t½ ~11 h (not shown).
Because this rate is similar to the rate of AChR degrada-
tion in the nonfusing mouse BC3H-1 myocyte line (Hyman and
Froehner, 1983), we conclude that all-Torpedo AChR is sta-
ble to 37°C once it is formed and expressed on the cell
surface. Temperature-sensitive expression of Torpedo AChR
and Torpedo-mammalian hybrid AChR therefore is not due to
thermolability of the assembled complex.

Conformational Changes Detected by BuTx Binding

The results presented thus far indicate that the tempera-
ture-sensitive step occurs before subunit assembly and suggest
that misfolding of newly synthesized subunits at 37°C may
render them incompetent to assemble. Using two different
probes of the α subunit's tertiary structure, BuTx and mAb
35 binding, we tested whether the subunit exists in an altered
conformation at 37°C.

In studies of AChR assembly in muscle cells, newly
synthesized α is thought to acquire high affinity BuTx binding
before assembling into stable AChR pentamers (Merlie and
Lindstrom, 1983). Isolated (Haggerty and Froehner, 1981;
Tzartos and Changeux, 1983; Wilson et al., 1984) or unas-
sembled (Sweet et al., 1988; Claudio et al., 1989) Torpedo
α polypeptides also have been shown to bind BuTx, but with
low affinity. To avoid possible interference from endogenous
α subunits in myoblasts (Claudio et al., 1989), we analyzed
BuTx binding to Torpedo α in α fibroblast cell lines. Fig. 7
shows the results of BuTx binding to α synthesized at 26°C
in 3T3-DOJ-α1 cells. Specific binding was detectable at

\[ \text{BuTx bound (fmol)} = \frac{B/F}{c} \times \text{BuTx} \]

\[ \log[\text{BuTx}] \]

\[ F = \text{the concentration of free ligand} \]

\[ B = \text{fraction of total AChR that bound BuTx} \]

The Journal of Cell Biology, Volume 110, 1990 1712

Figure 7. [125I]BuTx binding to α subunit expressed in fibroblasts.

3T3-DOJ-α1 cells at 26°C were solubilized with LB, aliquots of the
lysate incubated with the specified concentrations of [125I]BuTx,
and binding determined by immunoprecipitation with α antiserum.

(A) Specific binding of BuTx over the concentration range of 10^{-12}
to 4.7 × 10^{-8} M. Backgrounds, determined by immunoprecipit-
ating parallel samples containing an excess of unlabeled BuTx or im-
umoprecipitating parallel samples of 3T3 lysates, have been sub-
tracted from each point. Open and solid squares represent the
results of two separate experiments. In experiment No. 1 (open
squares), the means of duplicates ± SD were determined over the
concentration range of 10^{-10} to 4.7 × 10^{-8} M. In experiment
No. 2 (solid squares), the means of duplicates ± SD were deter-
mined for the concentration range of 10^{-12} to 1.8 × 10^{-8} M. The
inset shows a Scatchard analysis of the binding data from experi-
ment No. 2 (only concentrations of 2.1 × 10^{-10} M and greater are
plotted). F = the concentration of free ligand, B = fraction of α
that bound BuTx (the fraction of total α was determined by quan-
titative immunoblot shown in B). Since the absolute binding in ex-
periments Nos. 1 and 2 differed, values were normalized to super-
impose the binding curves. (B) The amount of α subunit in
representative immunoprecipitated samples from experiment No. 2
was quantitated by immunoblot and signals were compared to
known amounts of AChR standards. AChR standards used were (α)
AChR purified from all-11 fibroblasts after incubation with BuTx and
immunoprecipitation with α antiserum; and (β) Torpedo AChR purified from electricorg. (Lane 1) 3T3 lysate; (lanes
2 and 3) 3T3-DOJ-α1 lysate; (lane 4) 0.39 pmol of all-11 AChR
BuTx binding sites; lanes 5–8 contain, respectively, 0.11, 0.44, 1.76,
and 7.04 pmol of electrophor AChR BuTx binding sites; The results
of this immuno blot indicated that ~40% of α subunit (arrow)
bound BuTx at 1.9 μM (the highest concentration tested).
BuTx concentrations lower than 1 nM and increased over a broad range, saturating at ~2 μM (Fig. 7 A). Half-maximal binding was achieved at ~4 × 10⁻⁷ M. The broad concentration dependence of BuTx binding implies that not all α subunits bind BuTx with the same affinity. Indeed a Scatchard plot was nonlinear (Fig. 7 A, inset), indicating multiple classes of BuTx binding sites. The results suggest that even at the permissive temperature, α subunits exist in several different conformations. Of these, only a small percentage bind BuTx with moderately high affinity. Quantitation of the α signal by immunoblot (Fig. 7 B) revealed that 2.6% and 7.3% of α bound BuTx at 1 and 10 nM, respectively. This is in striking contrast to the binding properties of Torpedo α subunit incorporated into AChR pentamers. Whereas Torpedo AChR expressed in AChR-fibroblasts has a Kᵦ for BuTx of 7.8 × 10⁻¹¹ M (Claudio et al., 1987), <0.6% of the α subunit expressed in α-fibroblasts binds BuTx at this low concentration.

Because the high affinity BuTx-binding subset of α subunits may represent the assembly-competent pool, we compared this fraction of subunits at the permissive and nonpermissive temperatures. Fig. 8 A displays the results of an experiment in which 3T3-DOL-α₅₃ fibroblasts were incubated at 26°C for 16 h, shifted to 37°C, or left at 26°C for the indicated lengths of time after which cells were solubilized and [³²P]BuTx binding (at 10 nM) measured. After 1 h at 37°C, BuTx binding fell nearly to the level seen in cells maintained at 37°C throughout the initial 16 h incubation, even though total α polypeptide levels actually increased at 37°C (quantitative immunoblot, Fig. 8 A, inset).

The results of a related experiment (Fig. 8 B) illustrate that this reduction in BuTx binding may represent a preferential loss of higher affinity BuTx binding. 3T3-DOL-α₅₃ fibroblasts at 26°C were either kept at 26°C or shifted for 3 h to 37°C. Cells were then solubilized and [³²P]BuTx binding was measured from 100 pM to 100 nM. At each temperature, BuTx binding was normalized to the levels of subunit by quantitative immunoblot (not shown). There is more than twofold greater binding at 26°C than at 37°C with BuTx concentrations of 200 pM to 10 nM, whereas this ratio begins to fall (Fig. 8 B, inset) at higher BuTx concentrations. The results from these experiments demonstrate that at the nonpermissive temperature, fewer α subunits are able to bind BuTx with high affinity, which may indicate that fewer subunits have an assembly-competent conformation at 37°C.

**Conformational Changes Detected by mAb 35 Binding**

As a second test of conformational changes in α, we looked for changes in mAb 35 binding to α subunit expressed at 37°C and 26°C (Fig. 9). mAb 35 is a conformation-specific antibody that recognizes the main immunogenic region of α (Tzartos et al., 1981). Acquisition of the mAb 35 epitope occurs early in α subunit maturation, soon after α polypeptides are translocated into the endoplasmic reticulum but before they acquire the ability to bind BuTx or assemble with heterologous subunits (Merlie and Lindstrom, 1983). We tested binding of mAb 35 to α subunit synthesized in two fibroblast lines, 3T3DOL-α₅₃₅ and 3T3DOL-α₅. Confluent plates of cells at 26°C were either maintained at 26°C or shifted to 37°C for 3.5 h. Cells were then solubilized in LB, and equal aliquots of the lysate incubated with either mAb 35 or a polyclonal α antisemur that quantitatively immunoprecipitates α subunits. At 26°C, 30–45% of α subunit expressed in the two α-fibroblast cell lines was precipitable by mAb 35 (Fig. 9, lanes 3 and 9). Consistent with its being a conformation-specific antibody, mAb 35 was unable to precipitate any α subunit if the lysate was first denatured with SDS (Fig. 9, lanes 4 and 5).
Figure 9. mAb 35 binding to α expressed at 37°C and 26°C. 3T3
- DOJ-α_3, 3T3DOL-α_3m, or 3T3 cells were incubated 48 h at
26°C, then 3.5 h at 26°C or 37°C before solubilization with LB.
Equal aliquots of the lysate were immunoprecipitated with α antisema
(lanes 2, 4, 6, 8, and 11) or mAb 35 (lanes 3, 5, 7, 9, 10, and 12),
and the immunoprecipitates quantitated by immunoblot using
α antisera as the probe. Shown is an autoradiograph of the immu-
noblot exposed 6 h. The cell type, temperature, and antibody used
are indicated above the blot (α = anti-α; 35 = mAb 35). To demon-
strate that mAb 35 binding was conformation-specific, an aliquot
of 3T3DOL-α_3m lysate was denatured with SDS and renatured
with Triton X-100 before immunoprecipitation with mAb 35 (lane
10). Lane 1 contains purified Torpedo AChR run as a standard.

The polyclonal antisera, in contrast, quantita-
tively recognized even denatured subunit (Fig. 7 B). Al-
though there was essentially no change in total α subunit lev-
els when cells were incubated at 37°C for 4 h (Fig. 9, lanes
4 and 11), the percentage of α subunit precipitable by mAb
35 was only 7% at this temperature (Fig. 9, lanes 5 and 12)
compared with 30–45% at 26°C. Taken together with the
BuTx binding results, the four- to sixfold reduction in mAb
35 binding indicates that newly synthesized α subunits at the
nonpermissive temperature adopt a conformation different
from those synthesized at the permissive temperature, and
suggests that the former are misfolded and assembly-incom-
petent.

Efficiency of Assembly

The observations that there are fewer high affinity BuTx
binding sites and mAb 35 epitopes on α polypeptides ex-
pressed at 37°C versus 26°C compare favorably with our
finding that there is no subunit assembly at 37°C. If the 2.6% α
subunits that are capable of binding BuTx with high affinity
(i.e., 1 nM) at 26°C are indeed assembly competent, then
one would expect an efficiency of assembly at this tempera-
ture of ≤ 2.6%. Two approaches were taken to measure the
efficiency of subunit assembly at 26°C.

In the first approach, assembly was measured by determi-
ning the percentage of α subunits in AChR-fibroblasts that
acquire high affinity BuTx binding (Fig. 10 A). All-11 fibro-
blasts shifted to the permissive temperature were pulse-la-
beled 30 min with [35S]methionine and chased for up to
24 h. Cells were then solubilized in LB and three equal ali-
quots were immunoprecipitated with the following antisera:
(a) α antisera to measure total α; (b) anti-BuTx antisera

\[
\text{Antibody: } \begin{array}{ccc}
\alpha & \text{BuTx} & \text{BuTx} \\
\text{BuTx:} & - & + & - \\
\text{chase:} & 0 & - & - \\
\text{(h)} & 2 & 6 & 16 \\
& 24 & & \\
\end{array}
\]

(b) anti-BuTx antisera after a 21-h incubation with 3 nM BuTx (BuTx, +); and (c) anti-BuTx antisera in the absence of BuTx
(BuTx, -). The immunoprecipitated samples were analyzed by SDS-PAGE and fluorography (a 48-h exposure is shown). An arrowhead
marks the α band in each sample. Reimmunoprecipitations of the supernatants indicated that the initial immunoprecipitations were >90%
quantitative. (B) Assembly assayed by acquisition of endo H resistance by the δ subunit. 5 h after shiftdown to 26°C, all-11 cells were
labeled 30 min with [35S]methionine and chased for the indicated times. Immunoprecipitated δ subunits were divided into two equal ali-
quots that were digested with endo H (even lanes 2-10) or mock-digested (odd lanes 1-9). As a control, untransfected L cells were labeled
in parallel, chased 7 h, solubilized, immunoprecipitated with δ antisera, and the immunoprecipitate digested with endo H (lane 11).
Arrows (left) indicate the positions of the fully glycosylated (upper) and deglycosylated (lower) forms of δ subunit, and the arrowhead
(right) indicates endo-H resistant δ subunit.

\[
\text{B chase(h): } \begin{array}{cccccccccc}
0 & 1.5 & 7 & 17 & 24 & (L) \\
\text{endo H:} & - & + & - & + & - & + & + & \\
\end{array}
\]

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after first incubating the sample with 3 nM BuTx; and (c) anti-BuTx antiserum in the absence of BuTx. Although the BuTx-anti-BuTx procedure immunoprecipitates unassembled α subunits that bind BuTx with high affinity as well as correctly assembled subunits, the signal seen in Fig. 10 A represents only assembled α subunits. This is because α subunits assembled into AChR pentamers have a surface t,~ 65 h at 26°C (Green, W. N., A. F. Ross, T. Claudio, manuscript submitted for publication) while unassembled α subunits are degraded quickly at this temperature (t,~ 70 min at 28°C; Claudio et al., 1989). Because high affinity BuTx binding subunits comprise only ~2.6% of this pool of shortlived unassembled subunits, their contribution to the signal would be negligible at chase times >4 or 5 h. Scanning densitometry of the bands in Fig. 10 A indicated that at 6, 16, or 24 h of chase, only 1-2% of α was assembled into AChR complexes.

In the second approach, we measured assembly by monitoring changes in the N-linked oligosaccharides of the δ subunit. As in native Torpedo AChR (Nomoto et al., 1987), at least two of the three N-linked glycans of δ in AChR-fibroblasts are modified to complex forms that are resistant to digestion with endo H. This was determined by incubating purified surface AChR from all-11 cells with endo H and observing, by immuno blot, that all of the surface δ subunit was endo H resistant. To use this assay to measure assembly, all-11 fibroblasts were shifted to the permissive temperature, pulse-labeled 30 min with [35S]methionine, and chased up to 24 h at 26°C. δ Subunit was immunoprecipitated from the solubilized cells, and equal aliquots were digested with endo H or mock-digested. In the experiment shown in Fig. 10 B, as well as in two similar experiments, densitometric scanning of the endo H-resistant bands at the 16 and 24 h time points indicated that only ~1-2% of δ subunit acquired endo H resistance. This endo H-resistant population could be fully deglycosylated by peptidoglycase F (not shown), an enzyme that cleaves both simple and complex N-linked oligosaccharides from glycoproteins (Tarentino, 1985).

Our two independent measurements show an efficiency of assembly of only 1-2% for Torpedo subunits at 26°C, agreeing well with the value we obtained for the percentage of α subunits that are capable of binding BuTx with high affinity (2.6%). Although not direct evidence, the results favor the hypothesis that the small subset of α subunits that bind BuTx with high affinity comprise the pool of assembly-competent subunits. The lack of assembly at 37°C could be accounted for by the reduction in size of this BuTx-binding subset.

Discussion

To address several questions concerning the cell biology of the AChR, we used the strategy of expressing one or more Torpedo AChR subunit cDNAs in nonmuscle cells. When all four subunits are present in the same cell, fully functional AChRs are expressed in mammalian fibroblasts (Claudio et al., 1987, 1989). An intriguing feature of Torpedo AChR expression in these cells is that it is profoundly sensitive to temperature. Temperature-sensitive synthesis mutants, particularly from viruses, have proved useful in dissecting the molecular events involved in the folding and oligomerization of membrane protein complexes (Hurtley and Helenius, 1989). The availability now of a temperature-sensitive expression system for the nicotinic AChR should facilitate studies of heterooligomeric membrane proteins. To exploit this phenomenon for the study of AChR biogenesis, it was necessary to first investigate the nature of the temperature-sensitive expression.

Large quantities of Torpedo subunits are synthesized at permissive (26°C) and nonpermissive (37°C) temperatures in fibroblasts; however, no AChR complexes are formed at the nonpermissive temperature. In this report, the question we addressed first was whether the lack of complex formation at 37°C is a property intrinsic to Torpedo subunits or due to an inability of fibroblasts to assemble a muscle-specific protein. To answer this question we analyzed Torpedo AChR expression in mammalian muscle cells, which normally express endogenous AChR at 37°C. We established two L6 myoblast cell lines: L6-all, which expresses all four Torpedo subunits, and L6-α, which expresses Torpedo α. Both cell lines express exogenous Torpedo subunits at 37°C and 26°C, but neither expresses an assembled Torpedo subunit at 37°C. At 26°C, however, the L6-all line expresses all-Torpedo AChR complexes and the L6-α line expresses Torpedo-rat hybrid AChRs. These results demonstrate that the temperature sensitivity is directly attributable to Torpedo subunits, including but not necessarily limited to the α subunit.

We next investigated where in the biosynthetic pathway the temperature-sensitive step was occurring. At the nonpermissive temperature in both myoblasts and fibroblasts, Torpedo subunits are synthesized, translocated into the endoplasmic reticulum, and acquire the correct number of N-linked oligosaccharides. These results show that the temperature-sensitive step is not due to incorrect translocation or core oligosaccharide addition. We then determined that there is not a large pool of internally sequestered AChR, which indicates that the temperature sensitivity is not due to a block in intracellular transport of AChR. Finally, we established that once formed and expressed on the cell surface, all-Torpedo or hybrid AChR is thermostable. These results all strongly indicate that the temperature-sensitive step occurs after synthesis but before assembly with heterologous subunits, which may reflect changes in polypeptide folding. We were able to look for such conformational changes in the α subunit at 37°C and 26°C using two separate probes: high affinity BuTx binding and binding by a conformation-specific monoclonal antibody, mAb 35. Both probes revealed changes in Torpedo α at the nonpermissive temperature. At 37°C, four- to sixfold fewer α subunits expressed the mAb 35 epitope and two- to threefold fewer displayed high-affinity BuTx binding. The simplest explanation for these results is that the temperature-sensitive block in AChR assembly is due to misfolding of minimally the α subunit.

Although no probes have been developed to monitor the assembly competence of the β, γ, and δ subunits, it might be possible to predict the efficiency of AChR pentamer assembly based on the efficiency with which α subunits acquire an assembly-competent conformation. Both BuTx and mAb 35 have been used to monitor unassembled and assembled α subunits (Merlie and Lindstrom, 1983; Ross et al., 1987). Using two separate assays, we calculated the efficiency of assembly of the α and δ subunits in AChR-fibroblasts. We determined that only 1-2% of these subunits are correctly assembled and transported to the cell surface. These numbers are in agreement with the small percentage of α polypeptides...
that acquire high-affinity BuTx binding (e.g., in Fig. 7, 2.6% binding at 1 nM). The results are consistent with those of mammalian AChR, in which it is believed that only the subset of α polypeptides that acquire a BuTx-binding conformation are competent to assemble. However, at 37°C, there is still a small pool of subunits that acquire the mAb 35 epitope and display moderately high affinity BuTx binding, yet no hybrid or all-Torpedo AChR is expressed at this temperature. One possible explanation for this observation is that the three non-α subunits contribute to the temperature sensitivity, misfolding to a greater extent at 37°C than at 26°C. Alternatively, incubations at 37°C may not eliminate correctly folded subunits but simply reduce their numbers to a level where, coupled with the faster rate of subunit degradation at 37°C, assembly is unlikely to occur. Although our analysis in this paper has focused almost entirely on assembly of the α subunit, other subunits may play equal or more important roles in assembly. For example, the unique and extremely short half-life of the β subunit (12 min at 37°C) suggests that it may play a key regulatory role in AChR assembly (Claudio et al., 1989).

One question still remains: why is assembly so inefficient in these cells even at 26°C? Though Torpedo α subunit’s ability to achieve the appropriate tertiary structure may be facilitated by contacts with heterologous subunits (Kurosaki et al., 1987), the most likely explanation for inefficient assembly is that 26°C is not the optimal temperature for Torpedo AChR. This is shown by the finding (Fig. 4) that hybrid AChR production continues to increase at temperatures below 26°C, even though biosynthetic processes in mammalian cells are markedly slowed at these lower temperatures. 26°C may serve as a compromise temperature situated ~10°C below the host cell optimum and 10°C above the Torpedo optimum. Analysis of the efficiency of Torpedo AChR assembly in a fish cell might clarify this point.

Microinjection studies in Xenopus oocytes have shown that Torpedo α and mammalian β, γ, δ subunit can assemble into hybrid AChRs (Sakmann et al., 1985; Mayne et al., 1987). The production of Torpedo-rat hybrid AChR in L6-α myotubes extends these studies, as it indicates that Torpedo α is able to compete directly with endogenous α for incorporation into mammalian AChR. This implies that both the tertiary structure of α and its specific sites of interactions with heterologous subunits are highly conserved between Torpedo and mammalian α subunits. The expression of hybrid AChR also allowed us to demonstrate that Torpedo α and mammalian α subunits can coexist in the same AChR pentamer. This finding indicates that α subunits assemble with heterologous subunits, they apparently do not do so from a homo-oligomeric precursor derived from a single polypepide as proposed by Anderson and Blobel (1983). It also demonstrates that the two α subunits need not derive from the same gene. This latter result, together with the recent discovery of two muscle-specific α subunits expressed at the same time in development in Xenopus laevis, suggests a new mechanism of channel diversity for AChRs not previously observed (Hartman and Claudio, 1990). It may also suggest a similar type of channel diversity for other heterooligomeric proteins, especially other members of the superfAMILY of ligand-gated channels. The composition and stoichiometry of the GABA A receptor(s) has not been determined. However, at least four different subunit types (α, β, γ, δ) have been identified and multiple subunits of each type (α1, α2, α3) have also been isolated (Pritchett et al., 1989). If different members of one subunit type can combine to form functional channels with other subunit types, then a variety of different oligomeric receptors could result. The glycine receptor has been shown to be a pentamer composed of three 48- and two 58-kD polypeptides (Langosch et al., 1988). If multiple 48 and 58 kD subunits are found with this receptor as well and if like subunits need not derive from the same polypepide, then a mosaic of different oligomeric receptors with functionally distinct channel properties could be generated.

We thank Ed Hawrot (Yale University) and Jon Lindstrom (the Salk Institute) for monoclonal antibodies, and Arthur Karlin (Columbia University) for Torpedo californica AChR.

This work was supported by National Institutes of Health grants NS21714 and HL38156.

Received for publication 16 November 1989 and in revised form 23 January 1990.

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