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 assembly into $\alpha_2\beta\gamma\delta$ 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 (Wash. DC)*. 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)[1] 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 $\alpha_2\beta\gamma\delta$. 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

1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; CS, calf serum; LB, lysis buffer.

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 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-α_1, L6-DOL-α_2, L6-DOL-γ_1), C2 cells (C2-DOL-α_1), and NIH3T3 cells (3T3-DOJ-α_3), or a pool of several hundred colonies (3T3-DOL-αmass) expressing *Torpedo* AChR subunit cDNAs have been previously described (Claudio et al., 1989). 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$^-$aprt$^-$ fibroblasts (Claudio et al., 1987). The cells were maintained in DME plus 10% CS and 1 × 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, -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 5×10^5 L6 cells in 10-cm dishes. Cells 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% CO_2. Unless stated otherwise, experiments at lower temperatures were also carried out in incubators containing 5% CO_2. 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 [^{35}S]methionine (TRAN^{35}S; ICN Biochemical, Irvine, CA) in 2 ml of medium. Cells were then washed twice with 4°C PBS before lysis with 0.7 ml of a lysis buffer (LB) containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.02% NaN_3, and 10 μg/ml gelatin. 2 mM PMSF (Calbiochem-Behring Corp., San Diego, CA), 2 mM *N*-ethylmaleimide, and 10 μg/ml each of chymostatin, leupeptin, pepstatin, and tosyl-lysine chloromethyl ketone (Sigma Chemical Co.) were added to the LB immediately before use. At 4°C, lysates were scraped from the plates, vortexed several times over 10 min, and spun 10 min in an Eppendorf centrifuge (model No. 5414; Brinkman Instruments Co., Westbury, NY) to remove nuclear debris. The lysis procedure fully solubilized the sample: <3-5% of metabolically labeled subunits or AChR remained in the insoluble pellet. Lysates were then either directly immunoprecipitated (nondenaturing conditions) or immunoprecipitated after being subjected to a cycle of denaturation/renaturation (stringent conditions). Stringent conditions consisted of making lysates 1% in SDS, incubating 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. Gels 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.

Antisera and Immunoprecipitation

Anti-α, anti-β, anti-γ, and anti-δ antisera were generated in rabbits against SDS gel-purified *Torpedo* AChR α, β, γ, and δ subunits (Claudio and Raftery, 1977). α Antiserum cross-reacts with mammalian α subunit and can be used to quantitatively immunoprecipitate *Torpedo*, mammalian, or *Torpedo*/mammalian hybrid AChR. Five monoclonal antibodies were used that distinguish *Torpedo* and rat α subunits: mAb 147, recognizing *Torpedo* but not rat α subunit (Kordossi and Tzartos, 1987), and mAb 64, recognizing rat but not *Torpedo* α subunit (Tzartos et al., 1982), were kindly provided by Jon Lindstrom (the Salk Institute); mAbs 3A1, 3B1, and 14H110 (recognizing *Torpedo* but not mammalian α subunit) were the generous gift of Ed Hawrot (Yale University). Anti-BuTx antiserum and CH103, an anti-AChR antiserum, are polyclonal antisera produced in rabbits (T. Claudio, unpublished observations). CH103 was generated against native *Torpedo* AChR and recognizes each of the four *Torpedo* subunits.

Samples were incubated at 4°C for 3-6 h with saturating amounts of the appropriate antiserum, then with protein A-Sepharose for an additional 2-3 h. For immunoprecipitations with mAbs 64 and 147, goat anti-rat IgG (Organon Teknika-Cappel, Malvern, PA) coupled to cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co.) was used to precipitate the antibodies. Sepharose pellets were washed four times with 800 μl of LB. For samples that were SDS-denatured before immunoprecipitation, the LB contained 0.1% SDS in all four washes and 0.5 M NaCl in the first two washes.

Sucrose Gradients

500 μl of lysate was layered onto 12.5 ml 5-20% (wt/vol) continuous sucrose density gradients. Gradients were generated in LB containing 100 mM NaCl instead of 150 mM NaCl, and centrifuged in a rotor (SW41; Beckman Instruments Inc., Palo Alto, CA) at 5°C for a total ω^2t of 9.90 × 10^{11} to 1.20 × 10^{12}. Fractions were collected from the top of the gradient and $S_{20,w}$ values were calculated for the midpoint of each fraction according to McEwen (1967). Calculations were corroborated by sedimenting, in parallel, [^{125}I]BuTx-labeled purified *Torpedo* AChR (kindly provided by Arthur Karlin, Columbia University), which sediments as 9S AChR monomers and 13S disulfide-linked dimers.

Toxin Binding to Surface and Internal AChR

For surface labeling, 10-cm dishes of cells were washed two times with 10 ml PBS, incubated 135 min at room temperature with 2.5 nM [^{125}I]BuTx (specific activity of 140-170 cpm/fmol; ICN Biochemicals) in PBS, washed 4× with 10 ml PBS over 3-5 min, then solubilized with 0.7 ml LB. In other experiments, mono-iodinated [^{125}I]BuTx 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-α_2 cells, cell lysates were incubated with 4 nM BuTx for 20-24 h at 4°C then immunoprecipitated with saturating amounts of α antiserum.

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 [^{125}I]BuTx in PBS at 22°C for 2 h, rapidly washed one time with 6 ml PBS and two times with 6 ml DME, fed with DME/2% horse serum preequilibrated to 26° or 37°C, and placed in incubators at 26° or 37°C. At various time points, duplicate plates were assayed to determine cell-associated counts versus counts released into the medium. "Counts released" includes the counts present in the medium and in a single 3-ml PBS wash of the cells; "cell-associated counts" are those present in the cell lysate after solubilization of the cells with LB.

Toxin Binding to α Subunit Expressed in Fibroblasts

Confluent dishes of 3T3-DOJ-α_3 or 3T3 cells were incubated 48 h at 26°C with sodium butyrate. Plates were lysed with 300 μl of LB containing pro-

tease inhibitors and 3% Triton X-100. Lysates were centrifuged to remove nuclear debris, and then pooled and precleared with protein A-Sepharose. 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 antiserum (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-DOJ-α_3 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 lysate were immunoprecipitated either with saturating amounts of α antiserum (which precipitates all AChR from these cells) or with a pool of three mAbs specific for *Torpedo* α subunit (mAbs 3A1, 3B1, 14H110). Counts precipitated by the *Torpedo* α-specific mAbs were then expressed as a percentage of total counts precipitated by the anti-α antiserum. Reimmunoprecipitations of the immunodepleted lysates were frequently done to insure 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% NaN_3. All subsequent steps were performed at room temperature. Filters were incubated for 2.5–4 h in heat-sealed bags containing α antiserum 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 × 10^6 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, and δ sequences was digested with Sty I, and DNA to be probed with γ sequences 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: a 1,090-bp Pvu II-Pst I fragment for α; a 700-bp Bgl II fragment for β; a 1,290-bp Bgl II-Eco RI fragment for γ; and a 450-bp Hind III fragment for δ. Each probe was radiolabeled with α[^{32}P]dCTP by the multiprime labeling system (Amersham Corp.) to a specific activity of ~10^9 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 mU/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° 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.

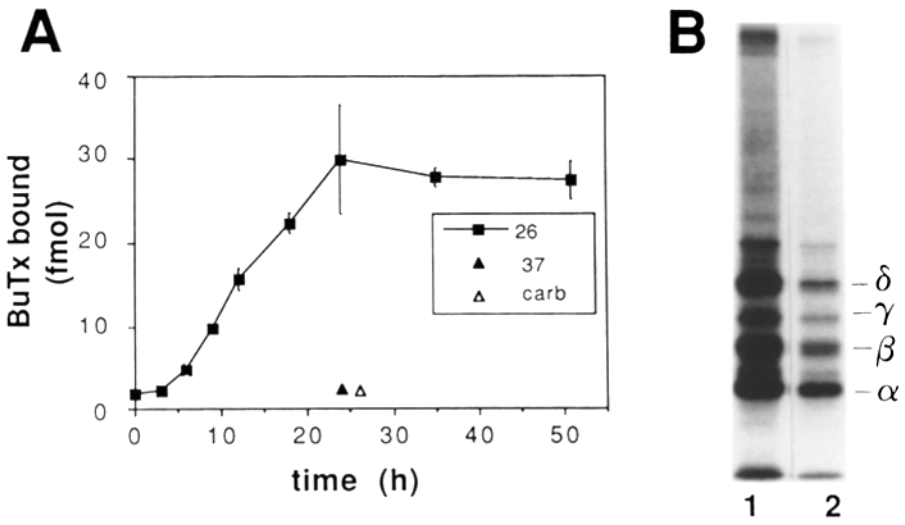


Figure 1. Temperature-sensitive expression of *Torpedo* AChR in all-11 fibroblasts. (*A*) Confluent 6-cm dishes of all-11 fibroblasts at 37°C were treated 24 h with 10 mM sodium butyrate, fluid changed with butyrate-containing medium preequilibrated to 26°C or 37°C and incubated at these two temperatures. At the indicated times, plates were labeled with [^{125}I]BuTx to quantify surface AChR (*solid squares*, 26°C; *solid triangle*, 37°C). Surface BuTx binding to cells at 26°C was fully blocked by 10 mM carbamylcholine (*open triangle*). Each value is the mean of duplicates ± SEM. (*B*) Expression of *Torpedo* subunits in all-11 fibroblasts at 37°C and 26°C. 10-cm dishes of all-11 cells were incubated with sodium butyrate at 37°C for 24 h, and then for an additional 12 h at 37°C (lane *1*) or 26°C (lane 2) before being labeled 20 min with [^{35}S]methionine at these temperatures. Subunits were immunoprecipitated from the solubilized lysate under stringent conditions (see Materials and Methods) with an anti-AChR antiserum that precipitates all four *Torpedo* subunits, and electrophoresed on a 7.5% polyacrylamide gel. Shown is a fluorograph of the gel (48-h exposure). The positions of native *Torpedo* AChR subunit standards are indicated. Relative TCA-precipitable counts in the lysates were 100% (lane *1*) and 44% (lane *2*).

To test for subunit expression, undifferentiated L6-all myoblasts were grown in the presence or absence of butyrate, metabolically labeled with [^{35}S]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° 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° or 26°C in the presence or absence of butyrate, and then measured surface AChR by [^{125}I]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-α_2, 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-α_2 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-γ_1, which contains an integrated retroviral γ-recombinant but does not express detectable *Torpedo* γ subunit. A second L6 clone, L6-DOL-α_1, and an α subunit–expressing clone derived from the mouse C2 muscle cell line, C2-DOL-α_1, 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-α_2 cells is incorporated into AChR complexes and not simply expressed

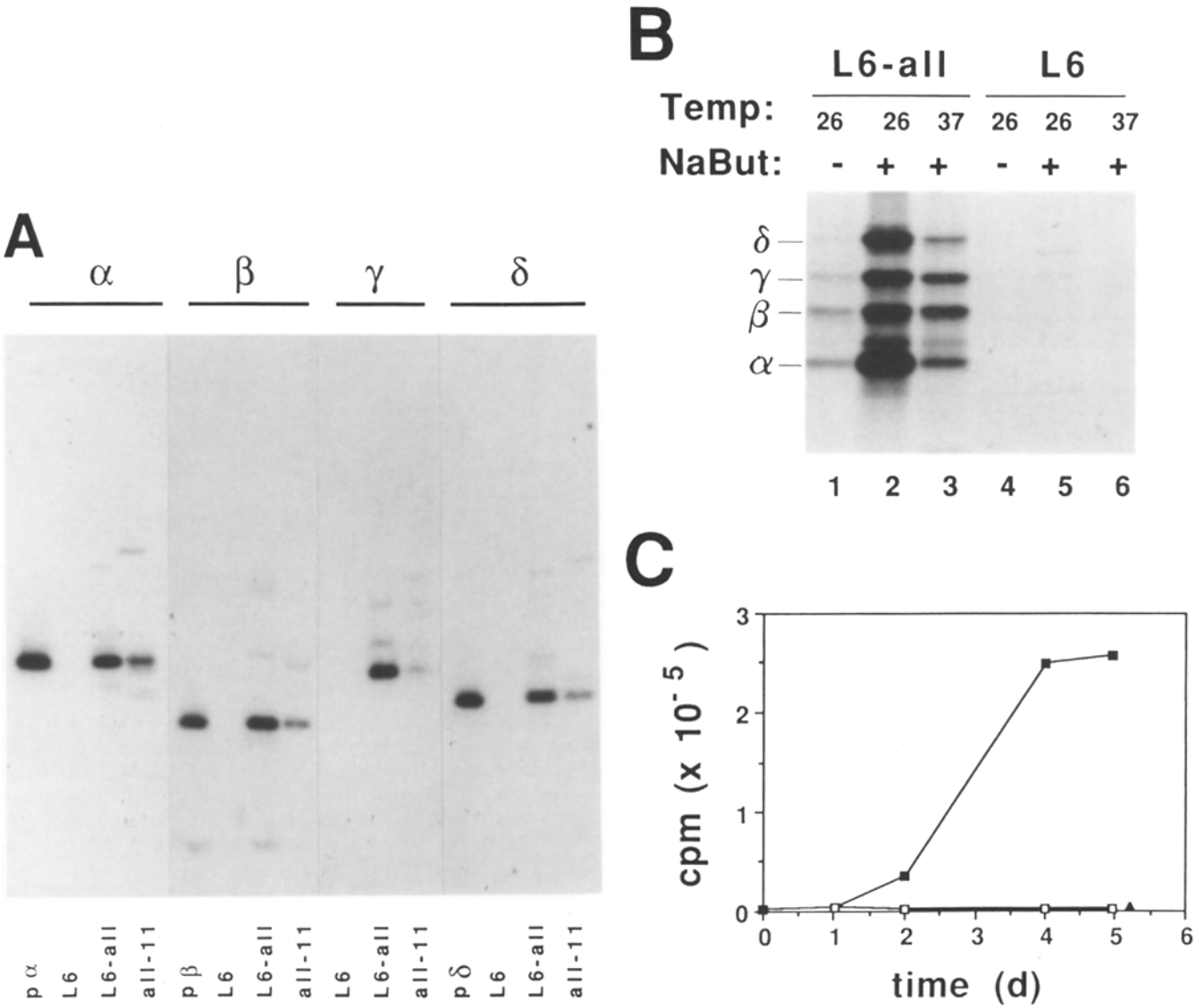


Figure 2. Torpedo AChR subunits expressed in rat L6 myoblasts. (*A*) DNA blots of the L6-all cell line. DNA from L6 and L6-all myoblasts, and from all-11 fibroblasts, was digested with restriction enzymes, electrophoresed on agarose gels, blotted to nitrocellulose and hybridized with ^{32}P-labeled probes 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° or 37°C in the presence or absence of sodium butyrate, and then labeled 20 min with [^{35}S]methionine at 26° 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° or 26°C for the indicated times, and then labeled with [^{125}I]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	*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). ^{125}IButTx-labeled AChR from L6-DOL-α_2 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

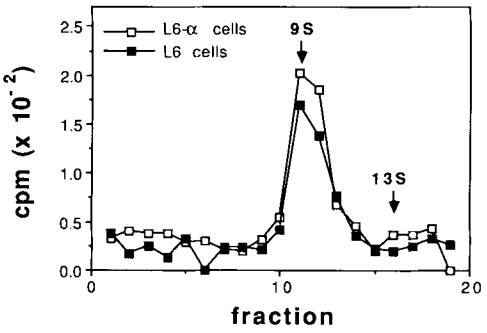


Figure 3. Sedimentation profiles of *Torpedo*, rat, and *Torpedo*–rat hybrid AChRs. [^{125}I]BuTx-labeled surface AChR was solubilized from L6-DOL-α_2 myotubes (at 26°C) and sedimented on a 5–20% sucrose density gradient (*open squares*). Parallel gradients were run which contained rat AChR isolated from L6 myotubes (*solid squares*) or *Torpedo* AChR isolated from electroplax (arrows indicate the positions of AChR 9S monomers and 13S dimers).

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° 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

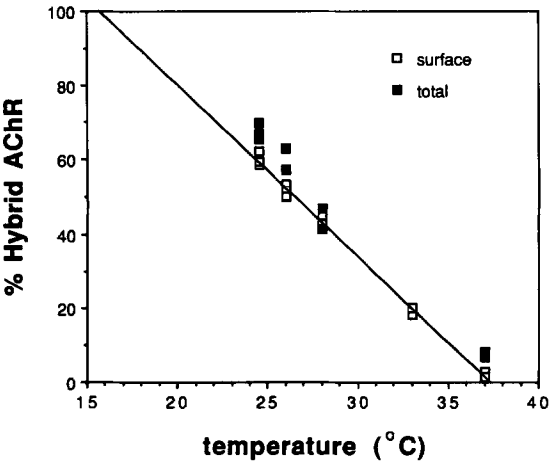
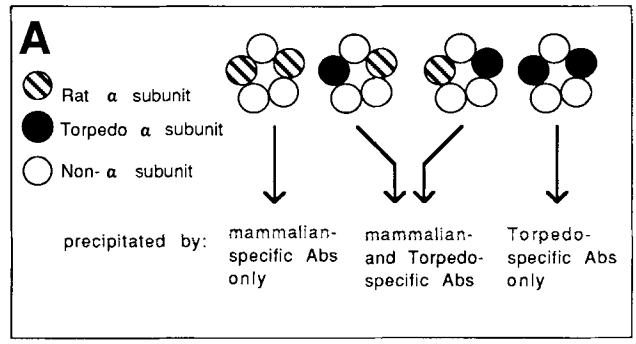


Figure 4. Temperature-sensitive profile of hybrid AChR expression. Parallel plates of L6-DOL-α_2 myotubes were incubated 5 d at the specified temperature, and the percentage of surface AChR that was hybrid was then determined by immunoprecipitation (*open squares*). Cell lysates were also incubated with additional [^{125}I]BuTx to determine the percentage of total BuTx sites (internal plus surface) that contained *Torpedo* α (*solid squares*); at all temperatures, internal binding sites were less than half of total. The line represents a first order polynomial fit of the surface data (r = .996), suggesting that hybrid AChR expression increased linearly with a decrease in temperature. Plates at 37°, 33°, and 28°C were maintained in incubators with 5% CO_2; plates at 26° and 24.5°C were sealed with parafilm and kept in atmospheric CO_2.



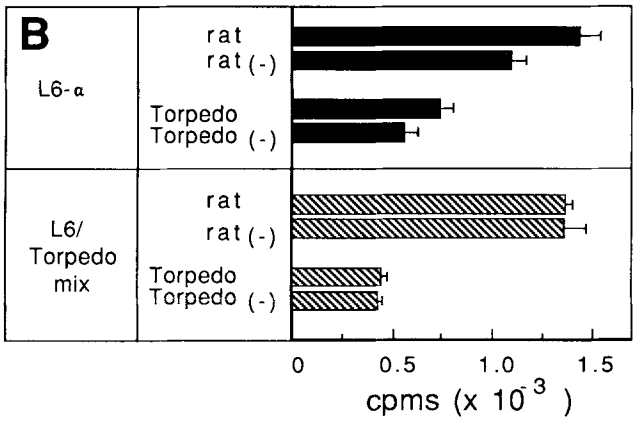


Figure 5. Composition of α subunits in *Torpedo*–rat hybrid AChRs. (*A*) Diagram illustrating the immunoprecipitation strategy used to determine the composition of α subunits in a given hybrid AChR pentamer. (*B*) Solid bars show the results of an experiment in which hybrid AChR-expressing L6-DOL-α_2 myotubes were labeled with [^{125}I]BuTx, solubilized in LB, and equal aliquots of the lysate immunoprecipitated with rat α-specific ("rat") mAb 64 or a pool of three *Torpedo* α-specific mAbs. Half of each sample was first immunodepleted with the opposite species-specific mAb. The immunodepleted samples are indicated by the minus signs. A control sample, containing a mixture of *Torpedo* AChR from electroplax membranes and endogenous AChR from L6 myotubes, was analyzed in parallel (*hatched bars*). All values represent the mean of 2–3 samples ± SEM.

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° 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-α_2 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-α_2 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-α_2 myotubes expressing hybrid AChR were labeled with [^{125}I]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-α_2 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.

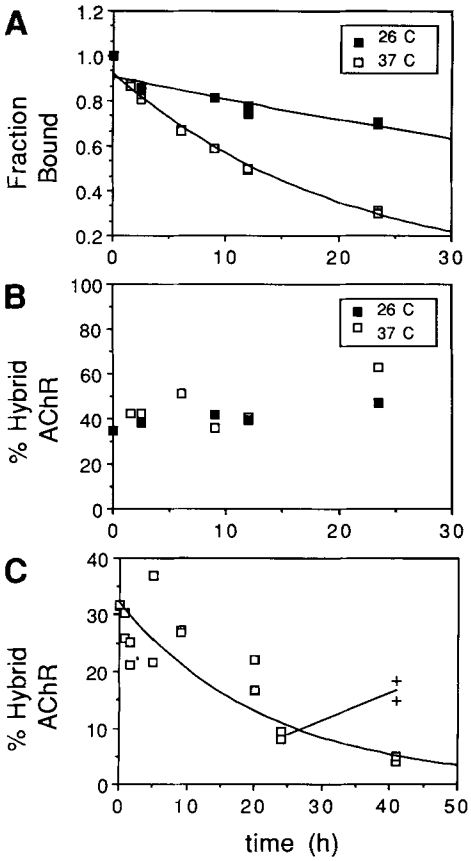


Figure 6. Thermostability of surface hybrid AChR. (*A*) Disappearance of surface BuTx binding material in L6-DOL-α_2 myotubes at 26° and 37°C. 6-cm plates of cells expressing steady-state levels of hybrid AChR were labeled with [^{125}I]BuTx, washed free of unbound BuTx, fed with preequilibrated medium and placed in incubators at 26°C (*solid squares*) or 37°C (*open squares*). At the indicated times, counts associated with cells (*Fraction Bound*) were determined for duplicate plates and plotted versus time. Curves represent single exponential best fits of the data. (*B*) The solubilized samples from each time point in *A* were immunoprecipitated to determine how much of the remaining AChR was hybrid. Values represent the mean of duplicates, and are expressed as percents hybrid AChR versus time at 26°C (*solid squares*) and 37°C (*open squares*). (*C*) In a separate experiment, plates of myotubes expressing steady-state levels of hybrid AChR were shifted to 37°C. At the indicated times, duplicate plates were labeled with [^{125}I]BuTx, solubilized in LB, and immunoprecipitated to determine the percentage hybrid AChR (*open squares*). The crosses demonstrate the reversibility of temperature-sensitive expression: two plates that had been incubated at 37°C for 24 h were shifted again to 26°C for 24 h before analysis.

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 polypeptide into the endoplasmic reticulum and co-translocational 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-

tinguish between two of the remaining possibilities: (*a*) thermolability of assembled AChR; and (*b*) misfolding of subunits before assembly.

If AChR is absent at 37°C because the correctly assembled pentameric complexes are thermolabile, then preformed all-*Torpedo* or hybrid AChR should rapidly dissociate after a shift from 26° to 37°C. To test this, L6-DOL-α_2 myotubes were incubated at 26°C until steady-state levels of hybrid AChR were present on the cell surface, and then incubated with [^{125}I]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 temperatures. 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 $t_{1/2}$ ~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 myotubes expressing only mammalian AChR ($t_{1/2}$ ~14 h), thus demonstrating that hybrid AChR is as stable as mammalian AChR. This was confirmed by determining the amount of hybrid 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 constant, indicating that both species were degraded with similar kinetics. In a second test of hybrid AChR thermostability (Fig. 6 *C*), L6-DOL-α_2 myotubes expressing steady-state levels of hybrid AChR were shifted to 37°C and, at the indicated times, labeled with [^{125}I]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 ($t_{1/2}$ ~15 h) similar to the rate of degradation determined in the preceding experiment. From these experiments, we conclude that hybrid AChR, once formed and expressed on the cell surface, is thermostable.

At 37°C, all-*Torpedo* AChR on the cell surface of all-11 fibroblasts also was degraded slowly, $t_{1/2}$ ~11 h (not shown). Because this rate is similar to the rate of AChR degradation in the nonfusing mouse BC_3H-1 myocyte line (Hyman and Froehner, 1983), we conclude that all-*Torpedo* AChR is stable 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 temperature-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 unassembled (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-α_3 cells. Specific binding was detectable at

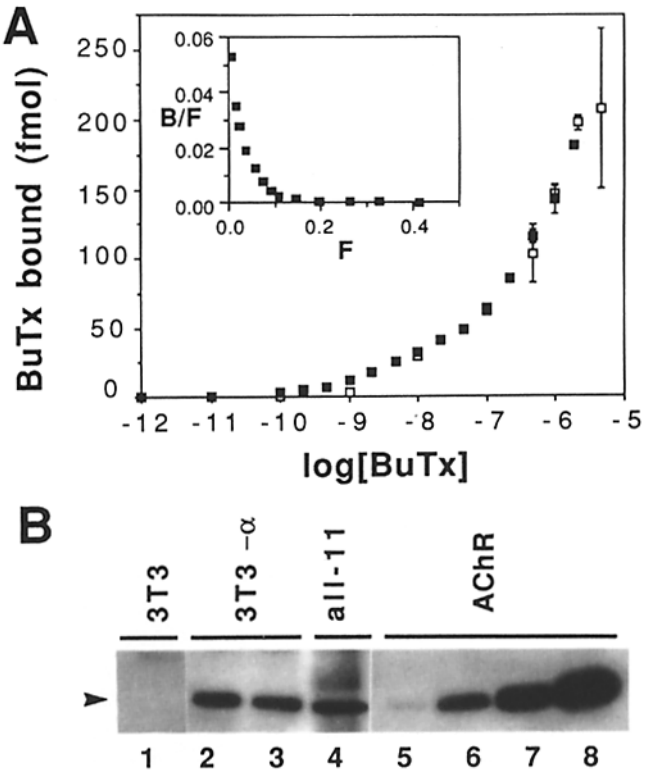


Figure 7. [^{125}I]BuTx binding to α subunit expressed in fibroblasts. 3T3-DOJ-α_3 cells at 26°C were solubilized with LB, aliquots of the lysate incubated with the specified concentrations of [^{125}I]BuTx, and binding determined by immunoprecipitation with α antiserum. (*A*) Specific binding of BuTx over the concentration range of 10^{-12} to 4.7×10^{-6} M. Backgrounds, determined by immunoprecipitating parallel samples containing an excess of unlabeled BuTx or immunoprecipitating parallel samples of 3T3 lysates, have been subtracted 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^{-6} M. In experiment No. 2 (*solid squares*), the means of duplicates ± SD were determined for the concentration range of 10^{-12} to 1.8×10^{-6} M. The inset shows a Scatchard analysis of the binding data from experiment 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 quantitative immunoblot shown in *B*). Since the absolute binding in experiments Nos. 1 and 2 differed, values were normalized to superimpose 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 (*a*) AChR purified from all-11 fibroblasts after incubation with BuTx and immunoprecipitation with anti-BuTx antiserum; and (*b*) *Torpedo* AChR purified from electroplax. (Lane *1*) 3T3 lysate; (lanes *2* and *3*) 3T3-DOJ-α_3 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 electroplax AChR BuTx binding sites; The results of this immunoblot 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 K_d 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-α_{mass} cells 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 [^{125}I]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-DOJ-α_3 fibroblasts at 26°C were either kept at 26°C or shifted for 3 h to 37°C. Cells were then solubilized and [^{125}I]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° 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.

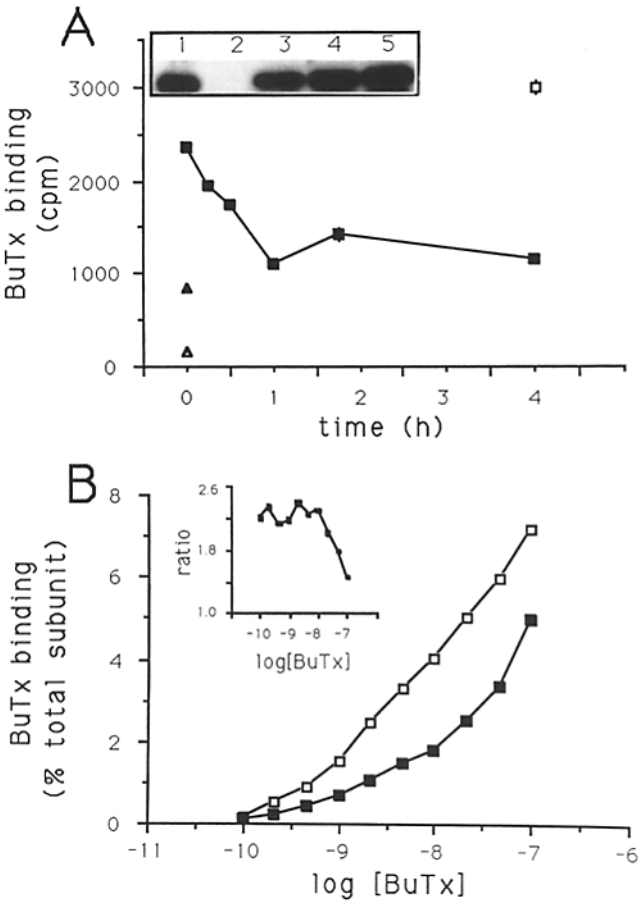


Figure 8. Temperature dependence of BuTx binding to α subunit. (*A*) A time course showing changes in BuTx binding at 37°C. Confluent plates of 3T3-DOL-α_{mass} were incubated 16 h at 37°C (*solid triangle*) or 26°C (*solid squares*). Plates at 26°C were then fed media preequilibrated to 37°C (*solid squares*) or 26°C (*open square*), and incubated at 26° or 37°C for the indicated times. Cells were solubilized in LB, and [^{125}I]BuTx binding (at 10 nM) determined by immunoprecipitation with α antiserum. Background counts precipitated from a parallel lysate of 3T3 cells (at 26°C) are shown (*open triangle*). Each point is the mean of triplicates ± SEM. The inset shows an immunoblot quantitating the amount of α subunit in selected samples: (lane *1*) purified *Torpedo* AChR run as a standard; (lane *2*) 3T3 cells, 26°C; (lane *3*) 3T3-α cells, 26°C at time 0; (lane *4*) 3T3-α, 37°C at time 0; (lane *5*) 3T3-α, shifted from 26° to 37°C for 4 h. (*B*) BuTx binding curves at 26° and 37°C. Parallel dishes of 3T3DOJ-α_3 cells were incubated at 26°C for 48 h, then either shifted for 3 h to 37°C (*solid squares*) or maintained at 26°C (*open squares*). Cells were solubilized and BuTx binding determined over the indicated concentration range. Background counts, determined by incubating parallel samples in an excess of unlabeled BuTx, have been subtracted at each point. Binding is expressed as percentage of total α subunit, determined by quantitative immunoblot as in Fig. 7 *B* (not shown). Values are the mean of duplicates (except the lowest concentration at 26°C and the highest concentration at 37°C, which are single data points). The inset shows the ratio of binding (26°C:37°C) versus the concentration of BuTx.

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° 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-α_{mass} and 3T3DOJ-α_3. 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 antiserum 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,

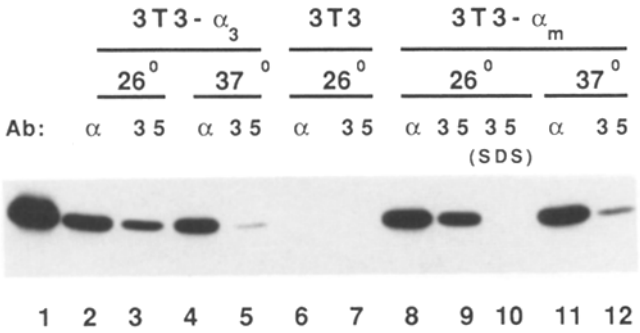


Figure 9. mAb 35 binding to α expressed at 37°C and 26°C. 3T3-DOJ-α_3, 3T3DOL-α_{mass}, or 3T3 cells were incubated 48 h at 26°C, then 3.5 h at 26° or 37°C before solubilization with LB. Equal aliquots of the lysate were immunoprecipitated with α antiserum (lanes *2*, *4*, *6*, *8*, and *11*) or mAb 35 (lanes *3*, *5*, *7*, *9*, *10*, and *12*), and the immunoprecipitates quantitated by immunoblot using α antiserum as the probe. Shown is an autoradiograph of the immunoblot exposed 6 h. The cell type, temperature, and antibody used are indicated above the blot (α = anti-α; 35 = mAb 35). To demonstrate that mAb 35 binding was conformation-specific, an aliquot of 3T3DOL-α_{mass} 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.

lane *10*). The polyclonal antiserum, in contrast, quantitatively recognized even denatured subunit (Fig. 7 *B*). Although there was essentially no change in total α subunit levels 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-incompetent.

Efficiency of Assembly

The observations that there are fewer high affinity BuTx binding sites and mAb 35 epitopes on α polypeptides expressed 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 temperature 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 determining the percentage of α subunits in AChR-fibroblasts that acquire high affinity BuTx binding (Fig. 10 *A*). All-11 fibroblasts shifted to the permissive temperature were pulse-labeled 30 min with [^{35}S]methionine and chased for up to 24 h. Cells were then solubilized in LB and three equal aliquots were immunoprecipitated with the following antisera: (*a*) α antiserum to measure total α; (*b*) anti-BuTx antiserum

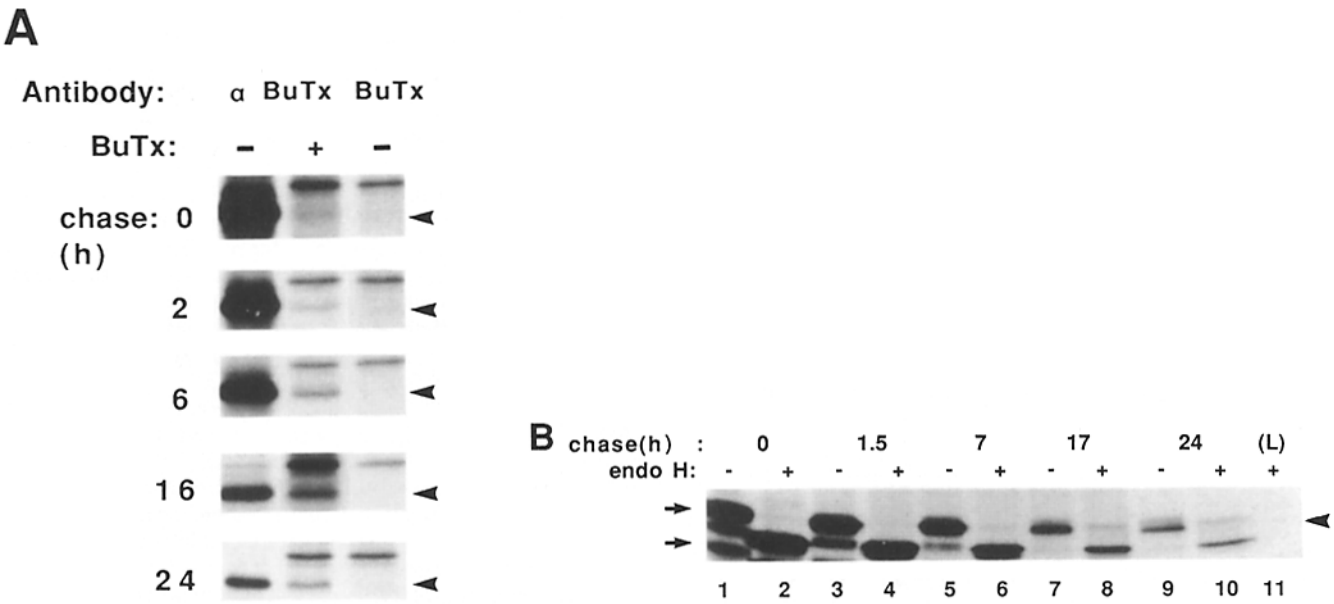


Figure 10. Efficiency of assembly of α and δ subunits in all-11 fibroblasts. (*A*) Assembly assayed by the acquisition of high affinity BuTx binding. Butyrate-treated cells were shifted to 26°C for 3 h, labeled 30 min with [^{35}S]methionine, chased for the indicated times, and solubilized in LB. Lysates were split into three equal aliquots that were then immunoprecipitated with the following antisera: (*a*) α antiserum (α, −); (*b*) anti-BuTx antiserum after a 21-h incubation with 3 nM BuTx (BuTx, +); and (*c*) anti-BuTx antiserum in the absence of BuTx (BuTx, −). The immunoprecipitated samples were analyzed by SDS-PAGE and flourography (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 [^{35}S]methionine and chased for the indicated times. Immunoprecipitated δ subunits were divided into two equal aliquots 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 antiserum, 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.

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 $t_{1/2}$ ~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 ($t_{1/2}$ ~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 immunoblot, 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 [^{35}S]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° 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 (eg., 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 as α subunits assemble with heterologous subunits, they apparently do not do so from a homo-oligomeric precursor derived from a single polysome 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 (α_1, α_2, α_3) 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 polysome, then a mosaic of different oligomeric receptors with functionally distinct channel properties could be generated.

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