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A Muscle Acetylcholine Receptor Is Expressed in the Human Cerebellar Medulloblastoma Cell Line TE671

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The human neuromedulloblastoma cell line TE671 is shown by single-channel recordings to express nicotinic acetylcholine receptors (AChRs) that are blocked by α-bungarotoxin (αBgt). These AChRs do not react with antisera to the αBgt-binding protein of brain or with monoclonal antibodies (mAbs) to brain nicotinic AChRs that do not bind αBgt. TE671 AChRs do react with autoantibodies to muscle AChRs from myasthenia gravis patients and with mAbs to muscle AChRs, including mAbs specific for extrajunctional AChRs. AChRs purified from TE671 cells are composed of 4 kinds of subunits corresponding to those of muscle AChR. Sequences of cDNAs for the ACh-binding α subunit and the δ subunit of this AChR further identify it as muscle AChR. Expression of TE671 AChR can be up-regulated by nicotine and dexamethasone, and down-regulated by forskolin.

The nicotinic acetylcholine receptor (AChR) family includes muscle-type AChRs, neuronal AChRs, and neuronal α-bungarotoxin (αBgt)-binding proteins, all of which exhibit related but distinct biochemical properties (reviewed by Lindstrom et al., 1987b). AChRs from muscle and fish electric organs are composed of 4 kinds of homologous subunits, which form an ACh-regulated cation channel that can be competitively inhibited by αBgt. AChRs from neurons also contain an ACh-regulated cation channel but, in contrast, do not bind αBgt and are composed of only 2 kinds of homologous subunits. Neuronal αBgt-binding proteins do not have an ACh-regulated cation channel and may have 4 kinds of subunits. Despite little immunological cross-reaction between these proteins, the subunits have substantial sequence identity, suggesting a common ancestral origin.

Myasthenia gravis (MG) is an autoimmune disease mediated by antibodies to muscle AChRs (reviewed by Lindstrom et al., 1988). Research into the mechanisms responsible for this disease has been somewhat hindered by the lack of human muscle cell lines. Thus, despite the limited cross-reaction with rodent AChRs, the effects of MG patient sera antibodies are often studied on rodent muscle cell lines. Further, human muscle AChRs for diagnostic assays are usually obtained from leg amputations, a highly variable and unreliable source.

The human cell line TE671, established by McAllister et al. (1977) from an undifferentiated cerebellar tumor, expresses functional AChRs that bind αBgt. These receptors were originally characterized as neuronal AChRs equivalent to the αBgt-binding protein in brain (Syapin et al., 1982; Lukas et al., 1986a, b; Siegel and Lukas, 1988); however, studies from this laboratory suggest that TE671 cells, in fact, express musclelike AChRs. For example, MG patient autoantibodies immunoprecipitate ^{125}I-αBgt-labeled AChRs from TE671 cells as effectively as they do ^{125}I-αBgt-labeled AChRs from human muscle (Lindstrom et al., 1987a), while these autoantibodies do not immunoprecipitate ^{125}I-αBgt-labeled proteins from human brain (Whiting et al., 1987). Furthermore, monoclonal antibodies (mAbs) specific for rat brain AChRs, which do not immunoprecipitate AChRs from TE671 cells, will bind AChRs from human brain that exhibit a high affinity for nicotine and no affinity for αBgt (Whiting et al., 1987; Whiting and Lindstrom, 1988). These human brain AChRs also do not bind MG patient autoantibodies. Finally, a cloned cDNA for the ACh-binding α subunit of TE671 AChR (Schoepfer et al., 1988) has the identical sequence predicted from a genomic clone for the α subunits of human muscle AChRs (Noda et al., 1983a).

Here we report a multidisciplinary characterization of the TE671 AChR, establishing in detail that it is a muscle-type nicotinic AChR: (1) electrophysiological studies suggesting that AChRs from TE671 resemble those from muscle at the single-channel level; (2) immunological studies indicating that AChRs from TE671 resemble extrajunctional AChRs from muscle but not AChRs from brain; (3) biochemical studies demonstrating that AChRs purified from TE671 cells are composed of 4 kinds of subunits similar to those of muscle AChRs; (4) molecular genetic studies showing that the δ subunits of AChR from TE671 cells have the amino acid sequence expected of δ subunits from muscle AChRs; and (5) cell biology studies revealing that AChR expression is up-regulated by nicotine, human calcitonin gene-related peptide (hCGRP), or dexamethasone, and reduced by forskolin, which also inhibits cell division and promotes the development of extensive neuronlike processes.

Our identification of a human cell line that expresses a functional skeletal musclelike AChR should be beneficial for studies on the structure and function of human muscle AChRs at the protein, mRNA, and DNA levels, and also for studying the various mechanisms of regulation of AChR expression in this

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cell line. Further, it should also prove useful for studies of MG, both as a consistent and unlimited source of antigen for biochemical studies and for studying the effects of autoantibodies on AChR function and turnover.

Materials and Methods

TE671 cells. Cultures were grown at 37°C in 90% air/10% CO_2 in Iscove's modified Dulbecco's medium from Irvine Laboratories supplemented with either 10% fetal bovine serum or 5% bovine calf serum. For electrophysiological studies, 10^4 cells were plated per well in a 24 well plate on 12-mm-diameter glass coverslips in medium with 10% serum. One day later, serum was reduced to 0.01%, and 2 mM L-glutamine, 10 μg/ml insulin, and transferrin were added. Electrophysiological studies were done in 115 mM NaCl, 5 mM CsCl, 1 mM $MgCl_2$, 25 mM glucose, 25 mM HEPES, pH 7.4, 10 mM TEA, and 0.1 mM anthracene-9-carboxylic acid.

Fluorescent labeling. Cells on coverslips were incubated with 10^{-7} M mAb for 30 min, rinsed with 0.15 M NaCl, 15 mM Na phosphate, pH 7.4, incubated with rabbit anti-rat IgG (85 μg/ml), rinsed, incubated with rhodamine-labeled, affinity-purified goat anti-rabbit IgG (10 μg/ml), and rinsed again. A Zeiss photomicroscope equipped with Nomarski optics and a fluorescent attachment (556 nm bandpass excitation filter, 590 nm barrier filter) was used at 63× magnification.

Electrical recordings. Single-channel current electrical recordings were performed as previously described in detail (Sakmann and Neher, 1983). Recordings were obtained in both the cell attached and the excised patch configurations. The pipettes were fabricated from Kovar glass (Corning 7052, ID = 1.1 mm, OD = 1.5 mm, 70 mm long) using a vertical pipette puller (David Kopf 700 C, Tujunga, CA). The pipettes were coated with Sylgard-180 (Dow Corning) within 40 μm from the tip and fire-polished immediately before use under 320× magnification. The tip size was adjusted to yield 5–15 MΩ of open pipette resistance when filled and immersed in the buffer described before. The patch pipettes contained the indicated concentration of ACh diluted in the same solution. The cells were observed with an inverted microscope (Nikon -Diaphot) using a 40× objective (LWD DL 40XC, Nikon) equipped with Hoffman modulation contrast optics (Modulation Optics, Greenvale, NY). The microscope was mounted on a vibration isolation table (Micro g Technical Mfrg. Corp., Waltham, MA).

A commercially available extracellular patch-clamp system was used (LM EPC-5, List Electronics, Darmstadt, FRG, and Medical Systems Corporation, New York). The headstage of the amplifier was mounted on a hydraulic micromanipulator (MO-103N Narishige, Japan). The signal output from the clamp was recorded on FM tape (Racal 4DS, Hythe, Southhampton, England; bandwidth DC–5 kHz). All the records were filtered at 2 kHz on an 8-pole Bassel low-pass filter (Frequency Devices, 9028LPF, Haverhill, MA). The data were digitized at the sampling frequency of 10 kHz in an Indec-L-11/73-70 microcomputer system (Indec, Sunnyvale, CA). Conductance levels were discriminated as described previously (Labarca et al., 1984). Histograms of dwell times in the open and closed states of the AChR channel were analyzed as described in detail previously (Labarca et al., 1984, 1985; Montal et al., 1986). The results of at least 5 different experiments in each condition are presented. All experiments were done at room temperature (22°C).

Preparation of solubilized TE671 membrane extracts. TE671 cell cultures were grown in T-flasks for 6 d and then expanded to 2 liter (850 cm^2) roller bottles in 5% BCS in Iscove's modified DMEM medium (Irvine Laboratories) with 2.5 μM dexamethasone. After 10 d in culture the cells were harvested after aspiration of media by first rinsing with cold PBS, pH 7.5, containing 10 mM iodoacetamide (IAA), 10 mM aminobenzamidine, 1 mM phenylmethylsulfonylfluoride (PMSF) to remove the excess media, and second by shaking in 25 ml per bottle of 50 mM Tris, 150 mM NaCl, 100 mM KF, 5 mM EDTA, 5 mM EGTA, 5 mM IAA, 5 mM aminobenzamidine, 0.5 mM PMSF, bestatin (10 μg/ml), Trasylol (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), pH 7.5 (buffer A). The bottles were then rinsed with 4 volumes of buffer A to remove any remaining cells. The cells were then pelleted by centrifugation at 3000 × *g* for 30 min. The resulting cell pellet was resuspended in 400 ml of buffer A, lysed by homogenization using a Polytron for 30 sec, and centrifuged for 30 min at 10,000 × *g*. The membrane pellet was resuspended in 250 ml of buffer A, homogenized, and centrifuged as described in the previous step. The resulting pellet was then extracted for 30 min in 4 volumes of buffer A with 1% Thesit detergent (Boehringer) and 0.05% SDS, pH 7.5, centrifuged at 140,000 × *g* for 30 min, and the clarified supernatant was retained.

Purification of the TE671 AChR. αBgt was first coupled to Sepharose CL4B at 5.0 mg protein/ml of gel by a modified procedure of Kohn and Wilchek (1982) (D. Shelton, Y. Fujii, W. Knogge, and J. Lindstrom, unpublished data). The clarified, solubilized TE671 membrane extract (75–100 ml) from, typically, 12 roller bottles, was applied to a 20 ml column of Sepharose CL4B to adsorb any proteins that may nonspecifically adsorb to the column. The eluate was then applied to a 1 ml column of αBgt-affinity gel, and both columns were washed with 200 ml of the extraction buffer. The affinity column was consecutively washed with 200 ml of buffer A containing 1.0 M NaCl, 0.5% Thesit, 0.05% SDS, pH 7.5, followed by 150 ml of 10 mM Tris, 0.1% Thesit, 1 mM NaN_3, 10 mM KF, 1 mM IAA, 1 mM aminobenzamidine, 1 mM EDTA, and 1 mM EGTA pH 7.5 (buffer B). The affinity column was then coupled to a hydroxylapatite (HPT) column (1 ml) and the TE671 AChR eluted onto the HPT column by recirculating through both columns for 12 hr, 10 ml of buffer B containing 200 mM carbamylcholine, using a peristaltic pump. After displacement of the bound protein, the HPT column was washed with 200 ml of buffer B and then eluted with 150 mM sodium phosphate, 0.5% Thesit, 1 mM NaN_3, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 1 mM aminobenzamidine, and 1 mM IAA at pH 7.5.

Affinity labeling. TE671 AChR was immobilized on αBgt-Sepharose and then affinity-labeled with ^{3}H-MBTA (a gift from Dr. Mark McNamee) as previously described (Whiting and Lindstrom, 1987).

Electrophoresis. Electrophoresis was conducted on acrylamide slab gels in SDS using a Laemmli discontinuous buffer system (Laemmli, 1970). Polyacrylamide gels were silver-stained for protein according to the method of Oakley et al. (1980). Polyacrylamide gels of radiolabeled protein were autoradiographed for 4–24 hr at −70°C using preflashed Kodak X-Omat-AR film and an intensifying screen. Autoradiograms were standardized by using Sigma prestained low-molecular-weight standards resolved on the same gel. Electrophoretic transfer of proteins from gels to diazophenylthioether (DPT) paper and subsequent probing with antibodies were as described previously (Gullick and Lindstrom, 1982). After being probed, bound antibodies were detected by incubation with 0.5 nM ^{125}I-labeled mouse anti-rat IgG (1–3 × 10^{18} cpm/mol) and autoradiography.

Cloning and sequencing of TE671 AChR δ subunit cDNA. A cDNA library was prepared as previously described (Schoepfer et al., 1988). The filters were screened under high stringency with the ~450 base pair (bp) Eco RI-Ava I fragment of cDNA clone BMD451 (a gift of Dr. Jim Boulter) coding for the 114 N terminal amino acids of the mouse AChR δ subunit. A single positive clone was identified. Plasmid DNA was characterized by restriction enzyme digestion, followed by agarose gel electrophoresis and Southern blot analysis. From the ~3 kb insert, the 5′ ~1860 bp Eco-Ava fragment was subcloned into a plasmid vector. Nested deletions were produced by the Exo III/Mung Bean protocol provided by Stratagene. DNA sequencing was performed using a modification of the dideoxynucleotide chain termination method of Sanger et al. (1977).

Regulation of TE671 expression. Cultured cells grown in T-flasks were harvested and 1 × 10^5 cells were plated in 6-well tissue culture dishes in Iscove's medium containing 10% FCS. After 2 d, the media was removed and replaced with this medium containing the indicated concentrations of forskolin, nicotine, human CGRP (a gift from Dr. Jean Rivier), or dexamethasone. Forskolin and dexamethasone were dissolved in 95% ethanol, while CGRP was dissolved in PBS. Ethanol or PBS alone had no affect on cell growth or AChR expression. The cells were grown for 2 d and the number of α-Bgt binding sites, AChR function, and RNA encoding the α, β, γ, and δ subunits of the TE671 AChR were determined.

The number of αBgt binding sites was determined as follows. After 2 d the medium was removed, and the cells were washed 3 times with 2 ml of Iscove's media. The cells were then labeled for 1 hr with 0.5 ml of 20 nM ^{125}I-αBgt in Iscove's medium at 37°C. Nonspecific binding was determined by performing the experiments as described, in the presence of 1 mM carbamylcholine. After 1 hr, the cells were again washed 3 times with 2 ml Iscove's medium. The cells were solubilized with 1.5 ml of 0.5 N NaOH, removed, and bound ^{125}I-αBgt determined by gamma counting.

AChR function was measured by carbamylcholine-induced influx of $^{86}Rb^+$ using a modified procedure of Robinson and McGee (1985). Briefly, after 2 d of growth in the presence or absence of the various indicated effectors, the media was removed and the cells washed 3 times with 2.0

ml Iscove's. After the third wash, the cells were incubated for 1 hr in 0.5 ml Iscove's to allow recovery from desensitization of AChRs by the effectors. Media was removed and the cells washed 2 times with 2.0 ml 0.5 M sucrose, 5 mM KCl, 10 mM glucose, 1.8 mM $CaCl_2$, and 15 mM HEPES, pH 7.4. The cells were then washed with 0.5 ml of the same buffer with 2 mM ouabain for 20 sec to inhibit Na^+-K^+ ATPases. The buffer was removed and $^{86}Rb^+$ uptake was initiated by exposing cells to 0.5 ml of the ouabain buffer containing 5 μCi/ml of $^{86}Rb^+$ with 1 mM carbamylcholine. Control experiments were performed as described, in the absence of carbamylcholine. Uptake was terminated after 30 sec by aspirating the radioactive solution and rapidly washing 3 times with 3 ml of 0.3 M NaCl, 5 mM KCl, 1.8 nM $CaCl_2$, 10 mM glucose, and 15 mM HEPES, pH 7.5. The washed cells were solubilized with 1.5 ml 0.5 N NaOH to permit $^{86}Rb^+$ uptake and protein determination. Radioactivity was determined by liquid scintillation counting of the solubilized cells. Results were normalized as described for the determination of αBgt binding sites.

Total RNA was isolated by the guanidine thiocyanate-CsCl procedure of Chirgwin et al. (1979). The amount of RNA isolated was quantitated by OD_{260}, and equal amounts of RNA from each treatment were size-fractionated by agarose gel electrophoresis containing formaldehyde. The gel was transferred to Nylon membranes and probed (Fig. 8) with cloned cDNA inserts (gifts from Dr. Jim Boulter) for the α, β, and γ subunits of mouse muscle AChR (Heinemann et al., 1986), and the δ subunit probe was derived from the cDNA clone for TE671 δ. Hybridization was conducted under highly stringent conditions: 42°C, 50% formamide, 5 × SSPE, final washing at 65°C, 0.3 × SSPE (where 5 × SSPE is 0.9 M NaCl, 50 mM Na phosphate, pH 7.4, 5 mM EDTA). Autoradiography was performed as described above.

Poly A^+ RNA was prepared from total RNA by oligo-dT column chromatography. The mRNA species for α, β, γ, and δ was identified as above using mouse muscle cDNA probes (Heinemann et al., 1986).

Results and Discussion

AChRs are localized on the surface of some TE671 cells

Fluorescent labeling indicates that AChRs are present on the surface of some cells in a serum-starved TE671 culture but not others (Fig. 1). AChRs were identified using 3 mAbs directed at the main immunogenic region (MIR) on the extracellular surface of α subunits, raised against AChRs from *Electrophorus* (mAb 35), human muscle (mAb 203), and muscle of mice and cattle (mAb 210) (Tzartos et al., 1981, 1983, 1987). Each showed equivalent results. Under the serum starvation conditions used for electrophysiological studies, labeling of only about 60% of cells grown on coverslips is observed. From these results it is not evident whether several clonal types are present, or whether a pluripotent clonal type undergoes partial differentiation under these culture conditions.

AChRs from TE671 cells are not of the neuronal type

AChRs solubilized from TE671 cells and labeled with ^{125}I-αBgt are not immune precipitated by a 400-fold molar excess of antiserum to the αBgt-binding protein purified from chicken brain (data not shown). This high-titer antiserum precipitates 5 μmol of αBgt binding sites from chicken brain per liter of serum and cross-reacts 0.8% with the αBgt-binding protein from human brain. The antiserum also shows no reaction on Western blots of purified TE671 AChR under conditions where antisera to AChR purified from TE671 label corresponding subunits from AChRs of TE671 and *Torpedo* electric organ (M. Luther and J. Lindstrom, unpublished observations). This data, along with the fact that mAbs like 35, 203, and 210, and MG patient autoantibodies bind AChR from TE671 but not αBgt-binding proteins from human brain (Whiting et al., 1987), suggest that TE671 AChRs are not identical to the common αBgt-binding proteins from human brain.

AChRs solubilized from TE671 cells and labeled with ^{125}I-αBgt were also not immune precipitated by a 100-fold molar excess of mAbs 290, 293, or 299, which react with AChRs from human brain that have high affinity for nicotine but do not bind αBgt (Whiting et al., 1987; Whiting and Lindstrom, 1988). Thus, TE671 AChRs are also different from AChRs detected in adult human brain.

Functional AChRs are detected on TE671 cells

The observation of Syapin et al. (1982) that these AChRs could be blocked by αBgt by measuring carbamylcholine-induced $^{86}Rb^+$ influx was confirmed (data not shown). We next used the patch-clamp technique to study AChR activity electrophysiologically at the single-channel level. To record only ACh-activated channels, several other channel types present in these cells are blocked pharmacologically: K^+ channels are eliminated by adding tetraethylammoniumchloride (TEA) and removing K^+ from the medium; Ca^{2+} channels are eliminated by removing Ca^{2+} from the medium; and Cl^- channels are blocked with 0.1 mM anthracene-9-carboxylic acid.

ACh induces bursts of AChR channel openings (Figs. 2, 3). At 0.5 μM ACh the channels are open 3.3% of the time, whereas at 10 μM ACh this increases to 8.8% (Fig. 2). Opening of TE671 AChR channels induced by ACh is blocked by αBgt (Fig. 3*A*). This is expected for muscle AChRs but not for neuronal nicotinic AChRs (reviewed in Lindstrom et al., 1988). TE671 AChR channels exhibit a linear current–voltage relationship in the range 10–100 mV (Fig. 3*B*) with a single-channel conductance (γ) of 44–45 pS (Fig. 2). The majority of channel openings are brief (65% have a time constant [τ] of 0.82 msec), whereas a minority of the openings are more prolonged (for 35% τ = 3.3 msec) (Fig. 3*C*). The duration of opening and magnitude of conductance are not affected by the concentration of ACh in the range 0.5–20 μM or by voltage in the range 50–100 mV. These properties are all consistent with those of muscle AChRs (Neher and Sakmann, 1976). Blockage of function by αBgt is the critical characteristic distinguishing AChRs on TE671 from those on neurons, since other electrophysiological properties are similar for neuronal AChRs which do not bind αBgt (Lipton et al., 1987). Sine (1988) has recently observed electrophysiological properties of AChRs from TE671 cells similar to those reported here, and he has characterized their pharmacology in more detail. He finds that AChRs in TE671 cells are similar in many respects to those from *Torpedo* electric organ or mouse BC3H-1 muscle cells but differ from these most prominently in having longer closed-channel times, slower desensitization onset and offset, symmetry in binding reversible nicotinic antagonists, and much more rapid dissociation of bound αBgt.

AChRs at mature neuromuscular junctions typically have shorter open channel lifetimes and greater conductances than do extrajunctional AChRs (Schuetze and Role, 1987). Analysis of ACh-induced noise at human neuromuscular junctions suggested that τ = 1.3 msec (Cull-Candy et al., 1979; Albuquerque et al., 1981), whereas human myotubes in culture, which would be expected to have extrajunctional AChRs, exhibit τ = 2.4 msec at room temperature (Bevan et al., 1978; Adams and Bevan, 1985). The observation that TE671 AChRs exhibit τ = 0.82 and 3.3 msec may suggest that the cells synthesize both junctional and extrajunctional forms of AChRs. However, AChRs from both *Torpedo* electric organ (Labarca et al., 1984, 1985) and muscle (Sine and Steinbach, 1984; Colquhoun and Sakmann, 1985) open into either a short- or long-duration state.

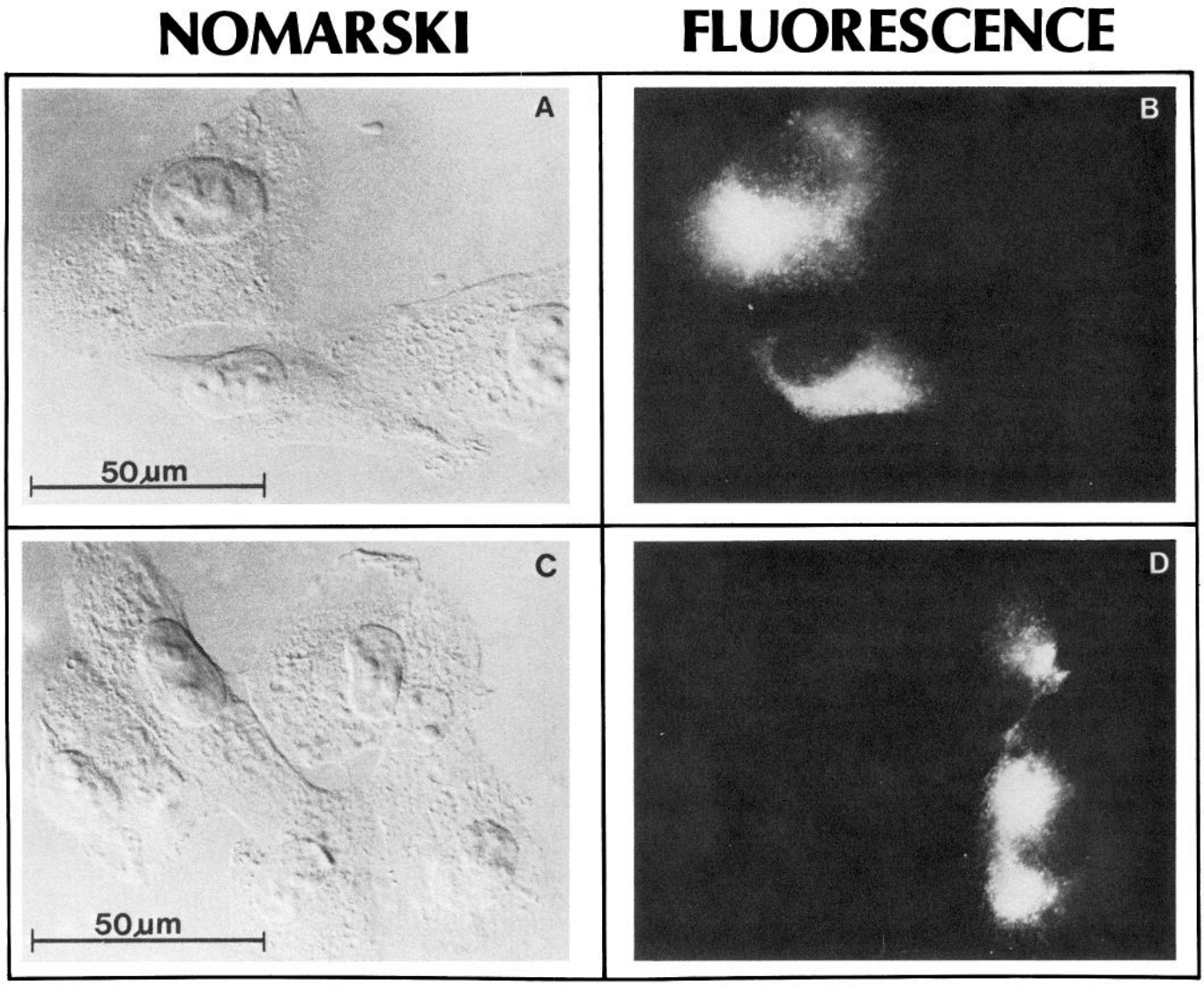

Figure 1. AChRs are not localized on the surface of all cells in a serum-starved TE671 culture. AChRs were labeled with mAb 210, followed by rabbit anti-rat IgG, and then rhodamine-labeled goat anti-rabbit IgG. The same fields are examined in the pairs *A, B* and *C, D.*

Junctional AChRs are thought to contain ϵ subunits instead of the γ subunits characteristic of extrajunctional AChRs (Mishina et al., 1986; Witzemann et al., 1987). Determining whether the AChRs from TE671 cells are of the junctional or extrajunctional type is relevant not only to their electrophysiological properties and biochemical structure, but also to their antigenic structure, as some MG patient autoantibodies react exclusively with extrajunctional AChRs (Weinberg and Hall, 1979; Schuetze et al., 1985).

AChRs on TE671 cells have the antigenic structure of extrajunctional muscle AChRs

TE671 AChRs cross-react with antisera and mAbs specific for the 4 kinds of subunits in muscle AChR (Table 1). These data, in conjunction with the evidence that MG patient autoantibodies bind TE671 AChRs equally as well as muscle AChRs (Lindstrom et al., 1987a; Whiting et al., 1987), indicate that AChRs from TE671 cells appear to have the basic antigenic structure expected of human muscle AChRs.

The AChRs on TE671 cells are extrajunctional. This is shown by their ability to react with mAbs specific for extrajunctional AChRs. Two mouse mAbs, C_9 and F_8, raised against AChR from human muscle have previously been shown to react with AChRs extracted from human denervated or fetal muscle but not with junctional AChRs in sections of human muscle (Whiting et al., 1986). The titers of mAbs C_9 and F_8 are quite similar for AChRs extracted from the muscles of a leg amputated for diabetic neuropathy (5.3×10^{-4} and 6.8×10^{-5} M, respectively) and for AChRs extracted from TE671 cells (6.8×10^{-4} and 8.3×10^{-5} M, respectively). This suggests that AChRs from TE671 cells should have γ rather than ϵ subunits.

AChRs purified from TE671 cells have the biochemical structure of muscle AChRs

AChRs affinity-purified from TE671 cells on an αBgt affinity column consist of 4 polypeptides corresponding to α, β, γ, and δ subunits of AChR from *Torpedo* electric organ by apparent molecular weights (42,000; 52,500; 55,000; and 62,000)

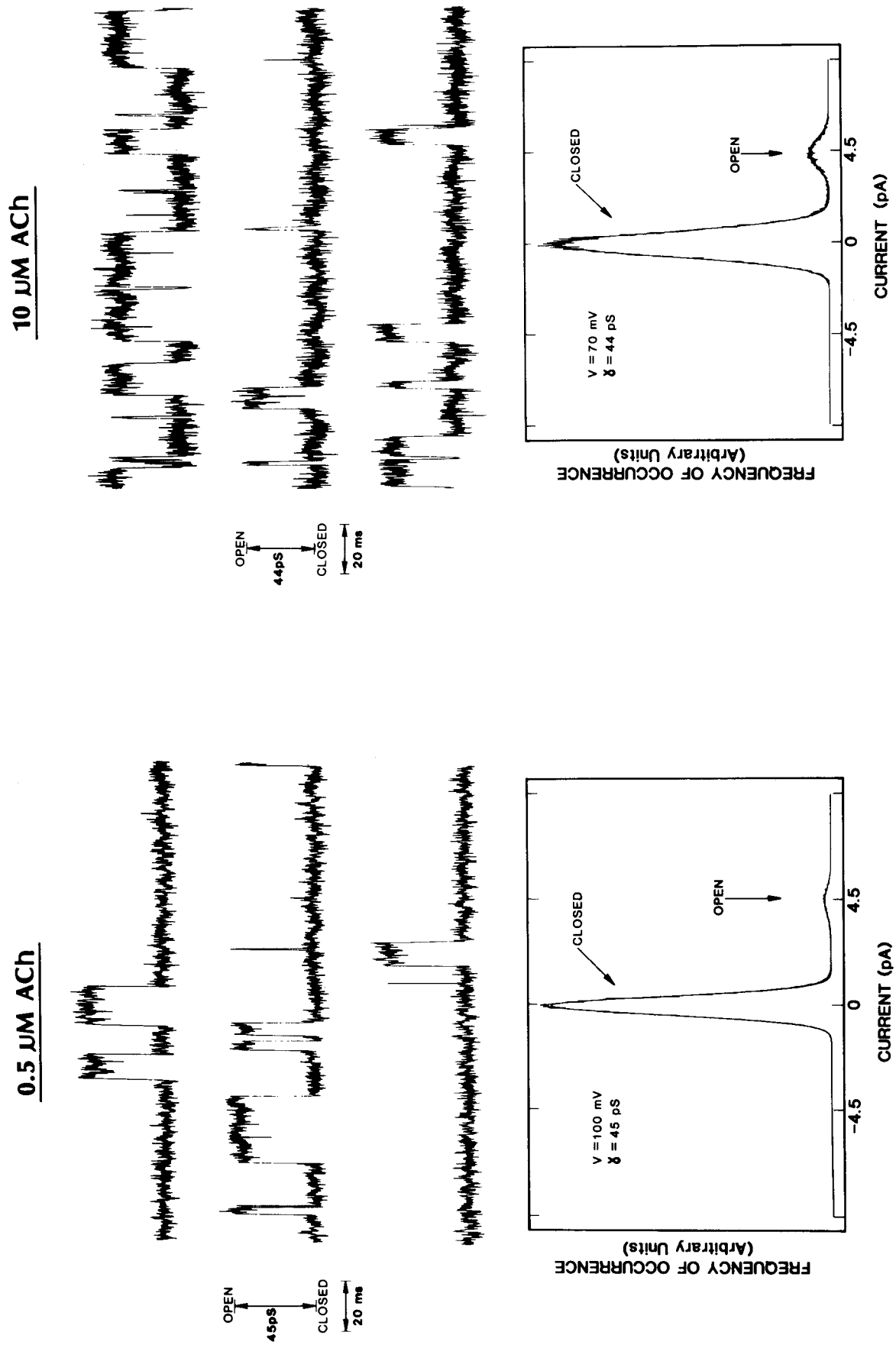

Figure 2. ACh induces openings of single AChR channels. Openings were recorded at an applied voltage of 100 mV at 0.5 μM ACh or 70 mV at 10 μM ACh.

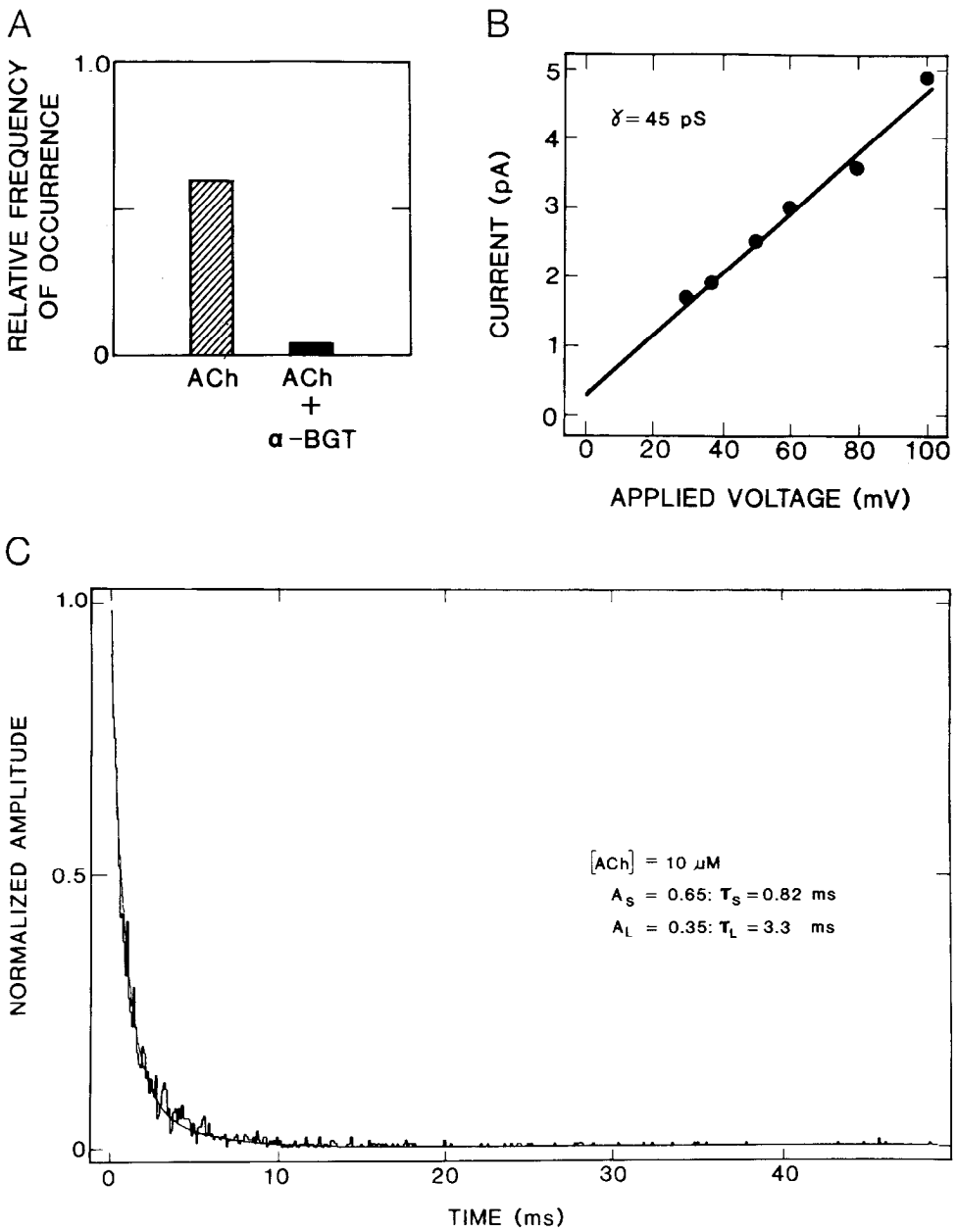

Figure 3. Analysis of single AChR channel currents. *A*, αBgt blocks ACh-induced channel openings. Results from several experiments are summed. ACh was used at 0.5–50 μM. αBgt was used at 0.04–0.15 μM. Membrane voltage was 70–100 mV. *B*, Linear current–voltage characteristics indicate ohmic channels. The slope conductance was 45 pS. *C*, Analysis of open-channel duration suggests that there are frequent short openings and less frequent longer openings. ACh was used at 10 μM at a membrane potential of 100 mV. The data (noisy curve) were well fitted by a sum of 2 exponentials (smooth curve) ($p > 0.05$). Total number of events analyzed, 2035.

(Fig. 4*A*) and also by antibody labeling on Western blots (Fig. 4*B*). The ACh binding site is formed by α subunits, as shown by affinity labeling with 4-(*N*-maleimido)benzyltrimethylammoniumiodide (MBTA) (Fig. 4*C*), an agonist that specifically blocks ACh binding and labels cysteines 192,193 on the α subunit (Kao et al., 1984, 1986). A typical purification is reported in Table 2. AChRs are present in crude extracts at twice the specific activity reported for BC3H1 cells (Boulter and Patrick, 1977) and can be purified at 3 times the yield. The specific activity of unpurified AChR from TE671 cells (0.09 nmol/gm tissue) is 5% that of *Torpedo* electric organ and 27 times that of fetal calf muscle (Einarson et al., 1982). Immunoaffinity chromatography on mAb 210 yields a preparation of similar purity which is unable to bind αBgt efficiently due to the use of a denaturing rather than a competitive elution step.

AChRs are highly susceptible to proteolysis (Lindstrom et al., 1980), and prevention of proteolysis requires rigorous use of protease inhibitors, rapid preparation, and extensive washing. AChR purified from human muscle by affinity chromatography using αBgt (Turnbull et al., 1985) or mAbs (Momoi and Lennon, 1982) has been reported to contain, respectively, 4 or 5 polypeptide chains of apparent molecular weights ranging from 44,000 to 66,000. The 44,000-molecular-weight polypeptides are identified as α subunits by affinity labeling, but the other polypeptides are not identified. In these preparations, only AChR monomers are observed on sucrose gradients.

Sequences of cDNAs for the subunits of AChRs from TE671 show that these are muscle AChRs

TE671 cells contain poly A^+ mRNAs corresponding to the 4 kinds of subunits of muscle AChR detectable under high-stringency hybridization conditions with probes for the α, β, γ, and δ subunits of AChR from mouse muscle (Fig. 4*D*). α Subunit mRNA is present in 3- to 5-fold higher concentration than the other subunit mRNAs.

The sequence of a cDNA for the α subunits of AChR from

Table 1. mAb cross-reactivity between human muscle and TE671 receptor

Specificity	Immunogen	mAb	Titer (μM) Human muscle	Titer (μM) TE671
MIR	Human muscle AChR	192	12.0	22.5
MIR	Human muscle AChR	196	0.24	0.74
MIR, α	BC3H1 and fetal bovine muscle	210	7.1	7.0
MIR, α	Human muscle AChR	203	1.4	2.2
MIR, α	Human muscle AChR	207	7.3	5.1
α	*Torpedo* α	Antisera	0.005	0.008
β	*Torpedo* β	111	0.70	1.2
β	*Torpedo* β	Antisera	0.004	0.005
γ	Bovine muscle AChR	66	0.89	0.72
γ	*Torpedo* γ	Antisera	0.0015	0.001
δ	*Torpedo* δ	137	0.52	0.74
δ	*Torpedo* δ	Antisera	0.002	0.001

TE671 (Fig. 5; Schoepfer et al., 1988) is identical to that expected from a human genomic clone (Noda et al., 1983a). It exhibits 97% sequence identity with α subunits from bovine muscle (Noda et al, 1983a), 95% sequence identity with α subunits from mouse (Boulter et al., 1985), 82% identity with α subunit for *Xenopus* (Baldwin et al., 1988), and 81% identity with α subunits from *Torpedo* (Noda et al., 1982). In particular, α subunits from TE671 resemble those from other species in exhibiting 4 hydrophobic sequences, an N-glycosylation site, and cysteines at positions 192,193, which, in the case of *Torpedo,* have been shown to react with affinity-labeling reagents for the ACh binding site (Kao et al., 1984).

The sequence of a cDNA for the δ subunits of AChR from TE671 is that also expected for δ subunits of muscle AChRs (Figs. 6, 7). Comparison of the amino acid sequence of TE671 AChR δ with that of δ subunits from other species reveals 91% identity to calf (Kubo et al., 1985), 90% identity to mouse (La Polla et al., 1984), 72% identity to chicken (Nef et al., 1984), 68% identity to *Xenopus* (Baldwin et al., 1988), and 61% to *Torpedo* (Noda et al., 1983c). It exhibits 30% sequence identity in the mature protein to α subunits from TE671 (Schoepfer et al., 1988), showing that the subunits forming the AChR are homologous, as expected from previous studies (Raftery et al., 1980; Noda et al., 1983b). In comparison with δ subunits of other species, the δ subunits from TE671 show conservation of 4 hydrophobic sequences, 3 putative N-glycosylation sites, and 3 putative phosphorylation sites. Like δ subunits from muscle of other species, δ subunits from TE671 lack a cysteine penultimate to the C-terminus found in δ subunits of AChRs from *Torpedo* electric organ (Noda et al., 1983c). It is suspected that AChR dimers from *Torpedo* are formed by a disulfide bond between the δ subunits of adjacent AChR monomers which involves this penultimate cysteine (Wennogle et al., 1981; Noda et al., 1983c).

Expression of AChR in TE671 cells can be regulated by neurotransmitters, hormones, and neurotrophic factors

Treatment of TE671 cells with nicotine, dexamethasone, or hCGRP results in an increase in the observed αBgt binding to AChRs on the cell's surface (Fig. 8). Nicotine and dexamethasone result in an elevation in the number of functional AChRs, while only dexamethasone causes an increase in the amounts of most AChR subunit RNAs. Curiously, although hCGRP up-regulates αBgt binding, AChR function is reduced. It is apparent that regulation of AChRs in TE671 cells appears to occur by more than one mechanism. Both transcriptional regulation of synthesis (in the case of dexamethasone) and other mechanisms (in the cases of nicotine and hCGRP) seem to be involved.

The means by which AChR synthesis is regulated in TE671 cells and in muscle cells may differ because, whereas nicotine up-regulates the amount of AChR in TE671 cells, agonists down-regulate the number of AChR in muscle cells (Ashizawa et al., 1982). We have observed that agonists up-regulate the amount of ganglionic-type AChR in the rat PC12 cell line (P. Whiting and J. Lindstrom, unpublished observations), whereas in chicken ciliary ganglion cultures agonists down-regulate the number of AChRs (Smith et al., 1986). Thus, mechanisms of AChR regulation may vary greatly in different cell types. For example, it is possible that in TE671 the AChR is under a "neuronal" type of regulation that may differ from that observed in muscle. Interestingly, the nonfusing mouse muscle-like cell line BC3H1, like TE671, was also obtained from the CNS (Schubert et al., 1974) but exhibits muscle-like regulation. In ciliary ganglion cells, growth conditions can also alter the ratio of functional to nonfunctional AChRs in the surface membrane (Margiotta et al., 1987). Similarly, in TE671 cells, hCGRP seems to increase the total number of AChRs while decreasing the number of functional AChRs. These preliminary observations on the regulation of AChR expression in TE671 cells merit more detailed study. The effects of CGRP are especially interesting in view of its postulated role as a neurotrophic factor in chicken myotubes, where it increases both AChR α subunit mRNA and AChR protein, perhaps via an increase in cyclic AMP concentration (Fontaine et al., 1986, 1987).

Forskolin, an adenylate cyclase activator (Seaman et al., 1981), is reported to induce AChR phosphorylation (Grassi et al., 1987;

Table 2. Purification of AChR from 20 gm (12 roller bottles) of TE671 cells

Fraction	Volume (ml)	Protein (mg)	AChR αBgt binding sites pmol	AChR αBgt binding sites %	^{125}I-αBgt specific activity (pmol/mg protein)
Initial extract	100	570	1804	100	3.16
Unbound to αBgt affinity column	100	380	702	39	—
Wash steps	650	180	342	19	—
Affinity column eluate	6	0.100	776	43	7800

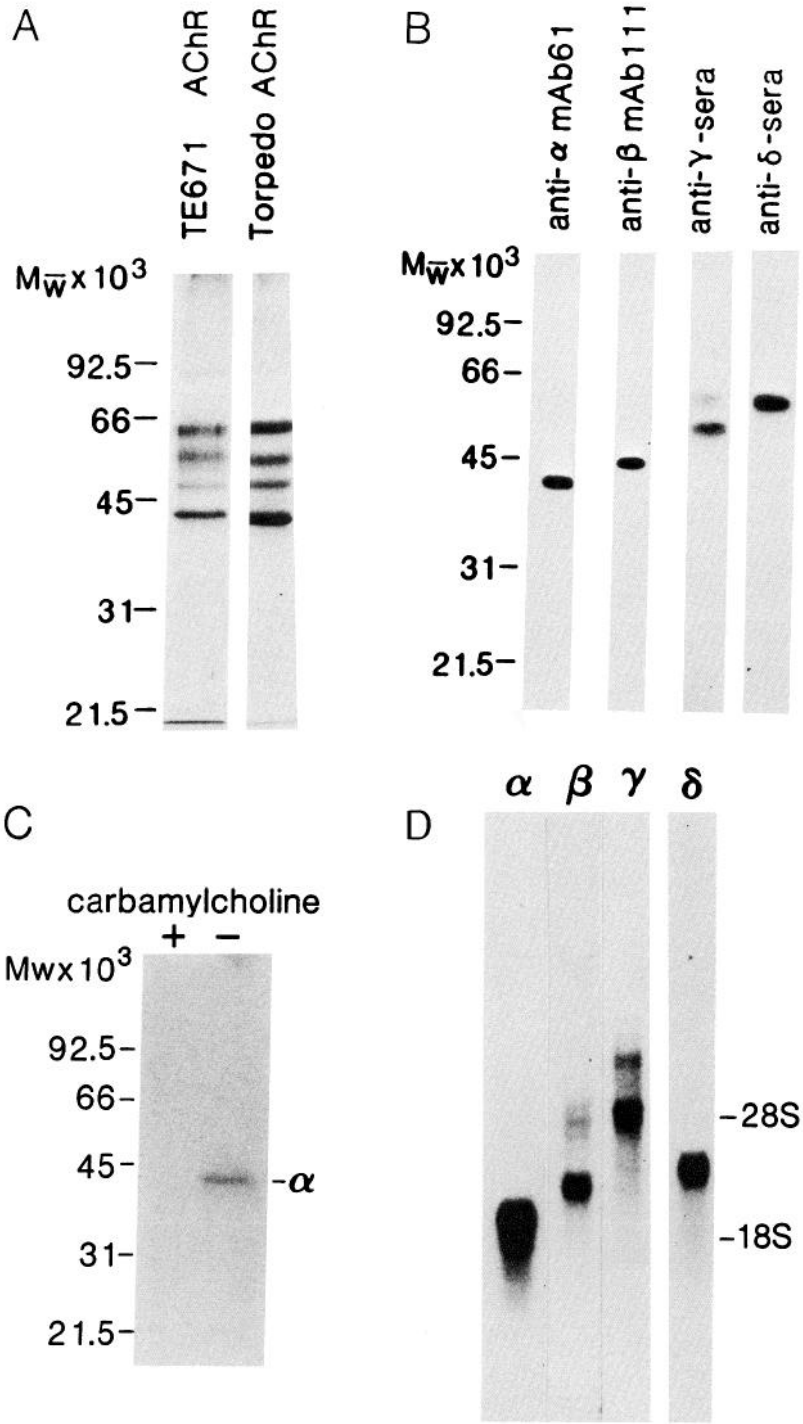

Figure 4. Subunits of AChRs from TE671. *A,* AChRs affinity-purified from TE671 and *Torpedo* eletric organ have similar subunits. AChRs (10 μg) were resolved into their subunits by electrophoresis on a 10% acrylamide gel in SDS under reducing conditions and stained with Coomassie blue. *B,* Subunits from TE671 AChRs correspond to those of electric organ AChRs by Western blotting. Purified TE671 AChR (50 ng/lane) was resolved into subunits by electrophoresis and then blotted onto DPT paper. Each lane was incubated with the indicated antibody, mAb 61 (Tzartos et al., 1981) and mAb 111 (Tzartos et al., 1986) at 10 nM, and γ and δ anti-subunit sera (Lindstrom et al., 1979) at 1 nM. Bound antibodies were localized by autoradiography using ^{125}I mouse anti-rat IgG. *C,* Affinity labeling with ^{3}H-MBTA identifies the α subunits of AChR from TE671 as forming the ACh binding site. *D,* Poly A$^+$ mRNAs for the 4 subunits of TE671 AChR are detected by high-stringency hybridization with cDNAs for mouse muscle AChR α, β, γ, and δ subunits. The cDNA probes used were described by Heinemann et al. (1986).

Miles et al., 1987; Smith et al., 1987) and to enhance AChR desensitization (Albuquerque et al., 1986; Huganir et al., 1986; Middleton et al., 1986). Treatment of TE671 cells with forskolin causes a decrease in the number of functional AChRs to background levels, which is accounted for by the reduction of αBgt binding and AChR subunit mRNAs (Fig. 8). Forskolin also appears to inhibit cell division and result in the formation of extensive neuron-like projections (Fig. 9). This effect, however, is reversible. These results suggest that if the effects of forskolin are mediated by an elevation in cAMP concentration, then the increase in AChR induced by hCGRP in TE671 must not be mediated by increased cAMP and may involve some other second messenger. However, a recent report by Wagoner and Pallotta (1988) indicates that although forskolin may stimulate cAMP-dependent phosphorylation in intact muscle and facilitate AChR desensitization, nonetheless, in intact muscle cAMP-dependent phosphorylation does not modulate desensitization. cAMP-induced phosphorylation and desensitization of AChRs could explain the lower number of active AChRs on the surface of hCGRP-treated TE671 cells. Forskolin treatment of TE671 cells does not induce synthesis of neuronal nicotinic AChRs since treated cells do not bind ^{3}H-nicotine with high

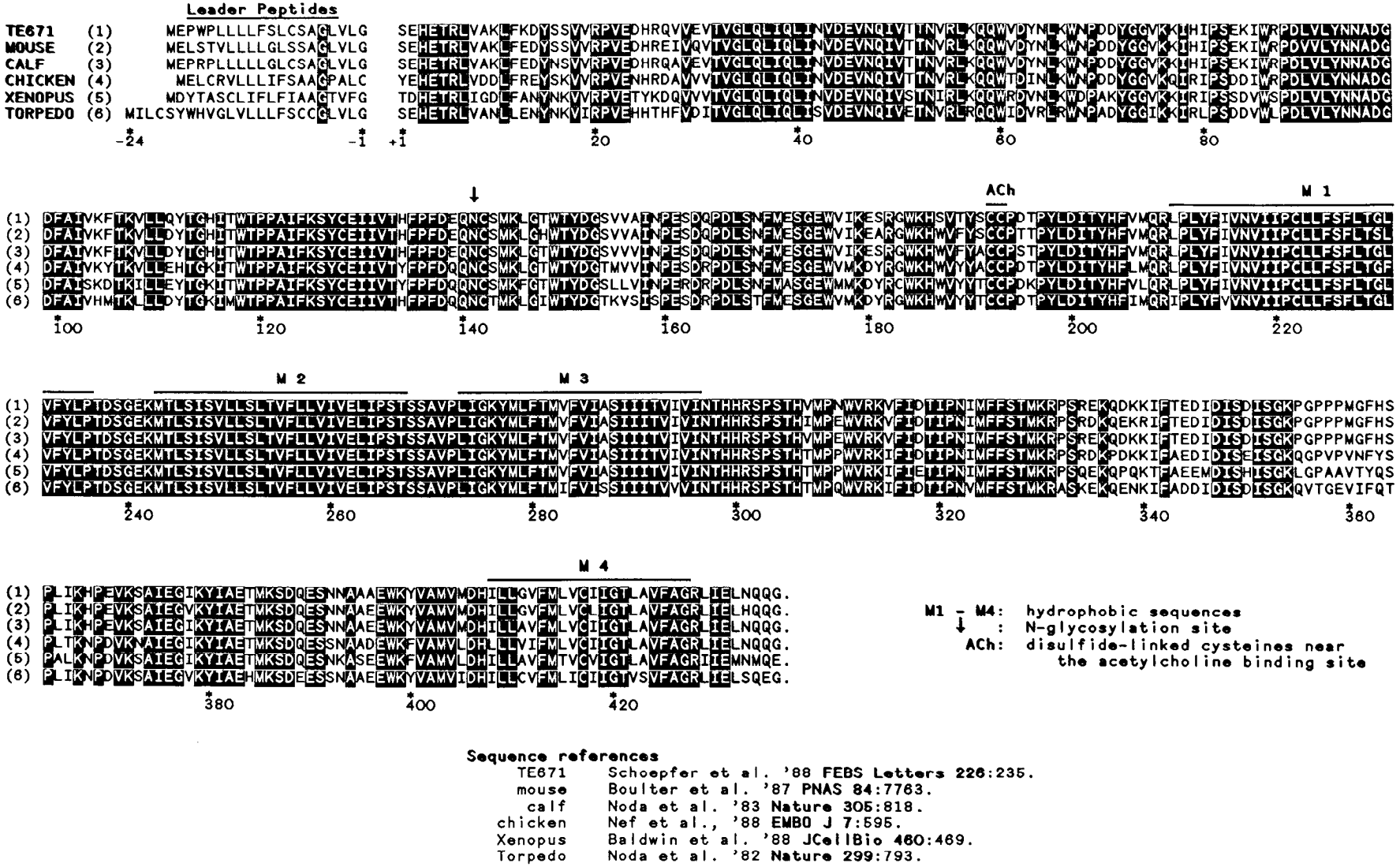

Figure 5. Comparison of deduced amino acid sequences for AChR α subunits among species. Amino acids conserved in all 5 species are highlighted. *M1–M4* indicate hydrophobic sequences. The N-glycosylation site on α subunits is indicated by the *downward arrow* (↓). Cysteines 192 and 193, which are the site of affinity labeling by MBTA (Kao et al., 1984), are marked by ACh.

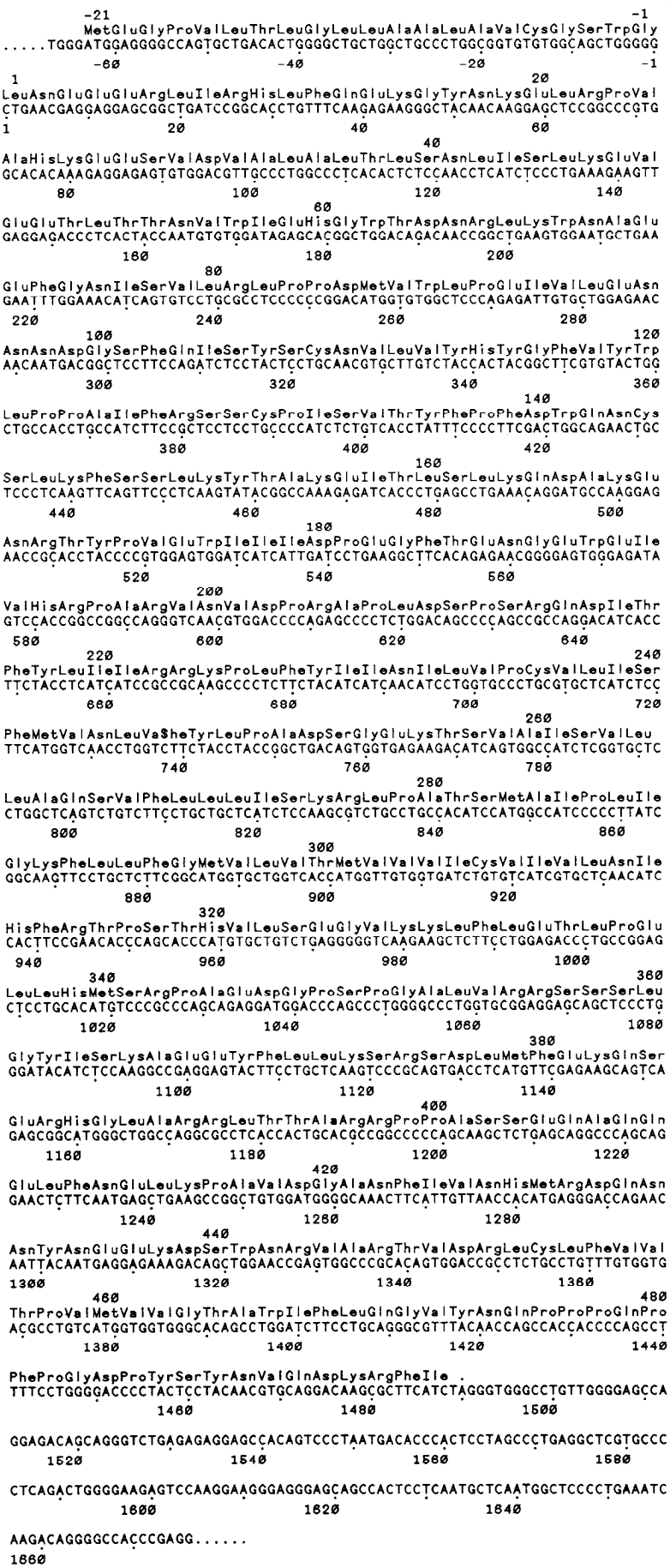

Figure 6. Nucleotide sequence and deduced amino acid sequence of a TE671 cDNA clone coding for the AChR δ subunit. The mature protein starts at position +1. The cDNA clone 6.4 untranslated region extends 124 nucleotides further upstream. This portion of the sequence contains unrelated sequences revealed by Northern blot analysis (data not shown).

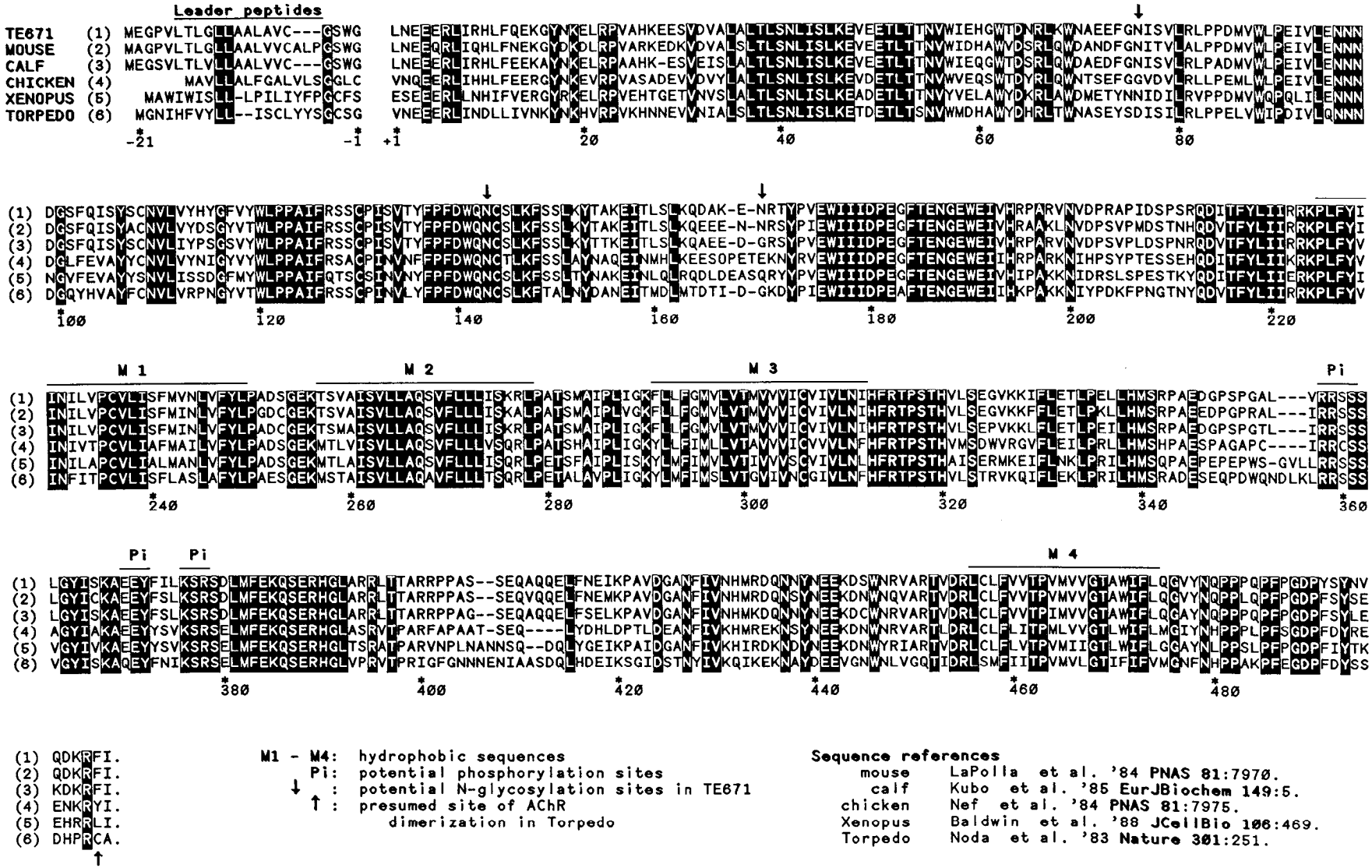

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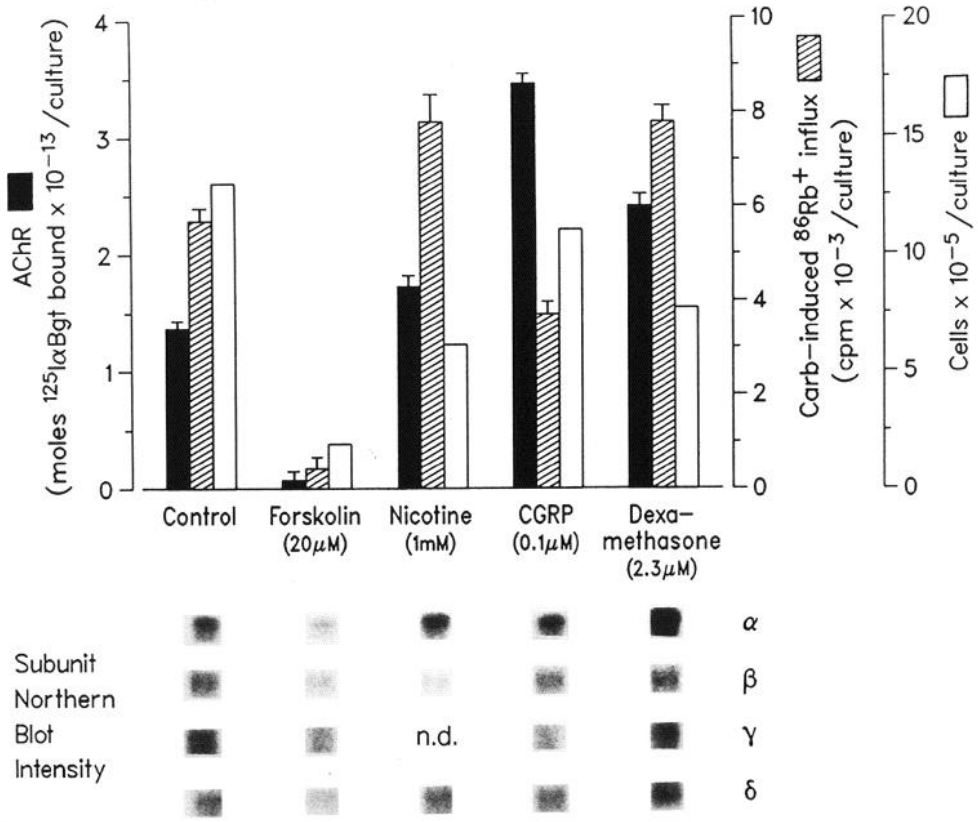
Figure 7. Comparison of deduced amino acid sequences for AChR δ subunits among various species. Numbers indicate amino acid position within *Torpedo* sequence. Amino acids conserved in all 6 species are highlighted. *M1–M4* indicate hydrophobic sequences; potential phosphorylation sites are indicated by *Pi*; potential N-glycosylation sites are indicated by the *downward arrow* (↓). The penultimate cysteine, which is thought to be the site of AChR dimerization in *Torpedo,* is pointed out by the *upward arrow* (↑).



Treatment	Subunit mRNA α	β	γ	δ	AChR Protein (αBgt/cell)	AChR Function (flux/cell)	Specific Activity (flux/αBgt)	Potential Primary Regulatory Mechanism
forskolin	↓	↓	↓	↓	↓	↓	→	transcription ↓
nicotine	→	↓	?	→	↑	↑	→	post transcription ↑
CGRP	→	→	↓	→	↑	↓	↓	post transcription ↑ post translation ↓
dexa-methasone	↑	→	→	↑	↑	↑	↓	transcription ↑ post translation ↓

Figure 8. Nicotine, dexamethasone, CGRP, and forskolin affect AChR expression in TE671 cells. First, 1×10^5 cells were plated in each 3.5 cm dish on day 0. On day 2, media was supplemented as indicated. On day 4, carbamylcholine-induced $^{86}Rb^+$ influx was measured on sister triplicate cultures. Background was determined for each culture conditions and subtracted to give the values shown (average background, 500 cpm). Northern blots using equal amounts of total RNA from other sister cultures were probed successively with ^{32}P-labeled mouse α, mouse β, mouse γ, and human δ cDNAs. ^{125}I-αBgt binding to cell surfaces was measured in a series of cultures.

affinity or mAbs specific for human brain AChRs. Also, hybridization signals are not detected when northern blots of TE671 RNA are hybridized at high stringency with the cDNA probe α4 (Goldman et al., 1987); this probe codes for the ACh-binding subunit of the AChR from rat brain, which has high affinity for nicotine. It is possible that forskolin may induce the synthesis of some other neuronal receptors or AChRs, but these have not yet been detected.

Concluding remarks

We have proven that extrajunctional muscle-like AChRs are expressed in relatively large amounts by the human neuronal cell line TE671. This cell line is a valuable system for studying the structure of human muscle AChR. AChR expression in these cells can be regulated by several factors, which can be employed to study in detail the mechanisms regulating AChR expression in TE671 cells.

This cell line may prove valuable for studies of MG because (1) it provides a much larger and more uniform source of human AChR for use in diagnostic immunoassays than does amputated leg muscle; (2) it can be used more effectively to study antigenic modulation or inhibition of AChR function by MG autoantibodies than can primary cultures of human fetal muscle (which are difficult to obtain) or rodent muscle cell lines (which cross-react poorly); and (3) it is a good source of human muscle AChR subunit cDNAs, which in suitable expression systems may prove valuable for mapping the antigenic structure of human AChR and providing antigen for studies of specific immunosuppression of MG.

Why should muscle-like AChRs be expressed in a neuronal cell line? We have considered 3 possibilities. First, expression of muscle AChR may be an aberration induced by the transformation events that produced this tumor line. Second, TE671 cells may be derived from a neuronal cell type that transiently expresses muscle-like AChRs during development. This would explain why muscle-like AChRs are not detected in extracts of adult human brains, and one could imagine that some developmental inducer could normally affect the development of this

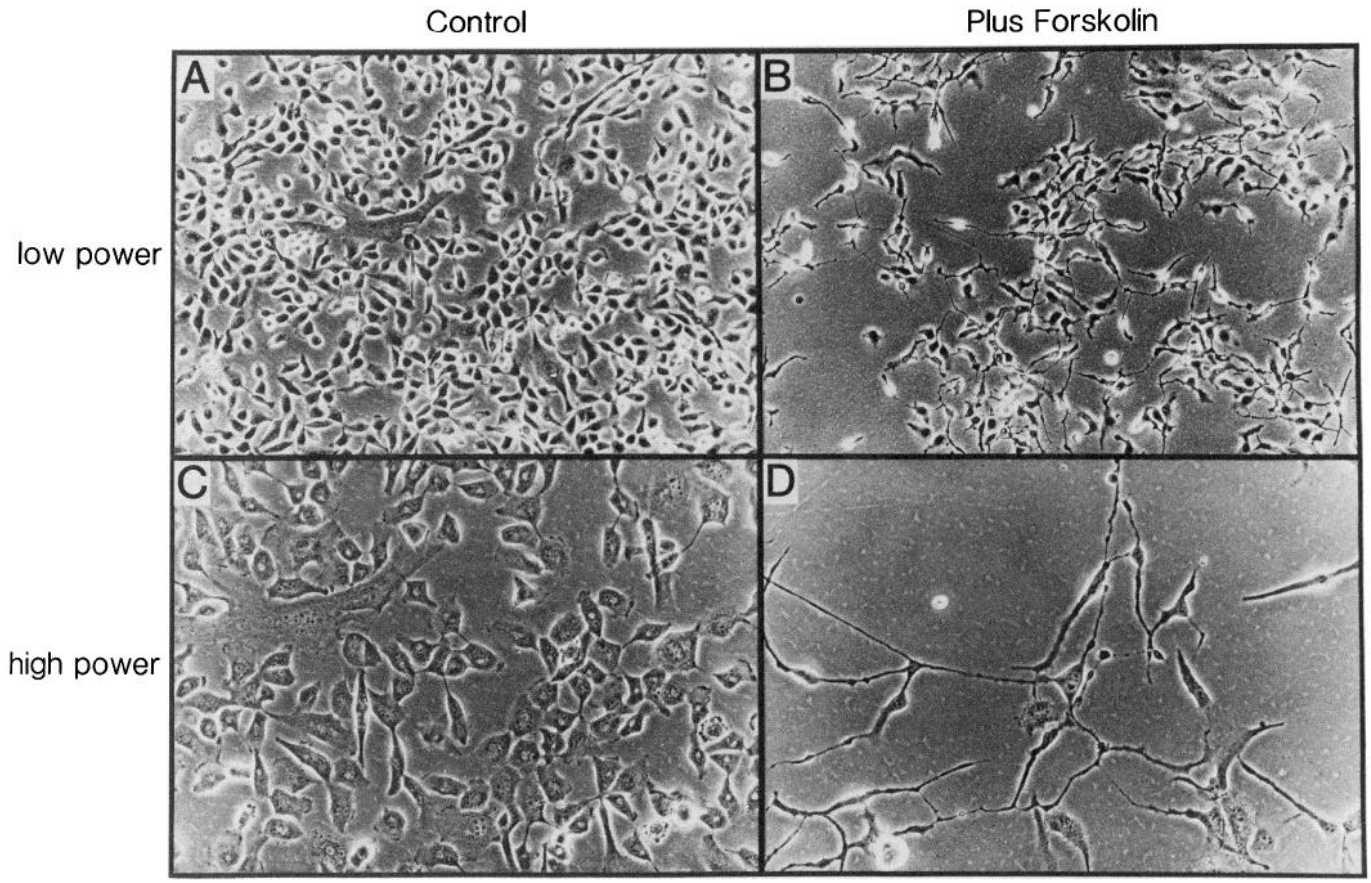


Figure 9. Forskolin affects cell division and morphology of TE671 cells. Equal amounts of cells were grown for 48 hr in Iscove's medium plus 5% fetal bovine serum plus (*B, D*) or minus (*A, C*) 20 μM forskolin. Note that in the presence of forskolin, cell density is reduced and extension of processes by the cells is greatly increased.

neuronal cell type in the same way that forskolin affects TE671 cells, terminating AChR synthesis and cell division. And third, TE671 cells may be derived from a cell type normally present in adult cerebellum. In this case, to escape detection in brain extracts the amount of muscle-like AChRs expressed on these cells would have to comprise a very small fraction of the total αBgt-binding proteins in the brain. This is a possibility. Interestingly, it has been reported that there are interneurons in rat cerebellar cortex with AChRs that can be activated by nicotine and blocked by αBgt (De la Garza et al., 1987).

Note added in proof: Stratton et al. (1989) have recently reported several lines of evidence suggesting that TE671 was misidentified when initially reported by McAllister et al. (1977), and that it is in fact identical to the rhabdomyosarcoma cell line RD, previously reported by McAllister et al. (1969). This would explain the presence of muscle AChR.

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