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Structural and Pharmacological Characterization of the Major Brain Nicotinic Acetylcholine Receptor Subtype Stably Expressed in Mouse Fibroblasts

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SUMMARY

Previously, we purified the predominant subtype of brain nicotinic acetylcholine receptor (AChR), analyzed its structure, and found that it was composed of two kinds of subunit, with sequences encoded by cDNAs termed $\alpha 4$ and $\beta 2$. Here we express these cDNAs from chicken brain in stably transfected fibroblasts. We demonstrate by synthesis that these cDNAs encode subunit polypeptides of the expected sizes, which coassemble to form receptor macromolecules having the same size as native AChRs. Additionally, we demonstrate that the expressed AChRs exhibit the ligand-binding pharmacology of native brain AChRs and function as acetylcholine-gated ion channels.

Nicotinic AChRs from mammalian skeletal muscle and fish electric organs are thought to consist of five homologous subunits [two $\alpha 1$ and one each of β, ϵ (γ in fetal forms), and δ], organized like staves of a barrel around a central cation channel, whose opening is triggered by the binding of ACh (1, 2). The subunit composition, initially determined using biochemical approaches, has been confirmed by expressing functional AChRs transiently in *Xenopus* oocytes (3) and in permanently transfected fibroblasts (4, 5), and assembly of those AChRs has been studied by expressing various combinations of these subunits (6, 7).

Nicotnic AChRs immunoaffinity purified from neurons are thought to consist of only two kinds of homologous subunits, probably similarly arranged around a central cation channel (8–11). There are many candidate cDNAs for subunits of neuronal nicotinic AChR subtypes ($\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\beta 2$, $\beta 3$, $\beta 4$. . . .), and transient expression in *Xenopus* oocytes of combinations of these subunits results in ACh-gated cation channels ($\alpha 2\beta 2$, $\alpha 3\beta 2$, $\alpha 4\beta 2$, $\alpha 2\beta 4$, $\alpha 3\beta 4$, and $\alpha 4\beta 4$ but not $\beta 3$ or $\alpha 5$ combinations) (12–24). The predominant nicotinic AChR subtype purified from brains was shown by amino-terminal amino acid sequence determination to be composed of subunits corresponding to $\alpha 4$ and $\beta 2$ (18, 25). Their stoichiometry was shown to be $(\alpha 4)_2(\beta 2)_3$ by transient expression in *Xenopus* oocytes (26, 27).

In order to provide an expression system in which the biochemical, pharmacological, and electrophysiological properties of neuronal nicotinic AChRs expressed from subunit cDNAs can be most critically compared with the properties of native AChRs (and studied in greater detail than is usually possible in intact tissue), stably transfected cells would be especially useful. Here, for the first time, we describe such a cell line expressing AChRs synthesized from $\alpha 4$ and $\beta 2$ subunit cDNAs. This recombinant approach confirms our previous analyses of the structure of AChRs purified from brain, by showing that the expressed AChRs have biochemical, pharmacological, and electrophysiological properties expected of native AChRs.

Materials and Methods

Construction of Expression Vectors

pkOE expression vector was derived from pkOneo, the kind gift of Dr. Pam Mellon, Salk Institute, San Diego, CA. The *Eco*RI site in this vector was removed by digestion with *Eco*RI, removal of the 5′ overhang with Klenow polymerase, and religation. The neomycin resistance gene of pkOneo was then removed by digestion with *Hind*III, the 5′ overhang was removed with Klenow polymerase, and *Eco*RI linkers were ligated on, giving pkOE (Fig. 1). pMSGneo was derived from pMSG (Phar-

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ABBREVIATIONS: AChR, acetylcholine receptor; ACh, acetylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; MMTV, mouse mammary tumor virus; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; DHβE, dihydro-β-erythriodine; PAGE, polyacrylamide gel electrophoresis; bp, base pairs; SV40, simian virus 40; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; SSPE, standard saline-phosphate-EDTA; mAb, monoclonal antibody.

macia). The ~2500-bp *Hind*III-*Eco*RI fragment of pMSG, containing the *gpt* structural gene and SV40 polyadenylation signals, was replaced by the ~2800-bp fragment of pSV2-neo (28) containing the neomycin resistance gene and SV40 polyadenylation signals. The *Eco*RI site was then removed by restriction digestion, blunt ending with Klenow polymerase, and religation. An *Eco*RI cloning site was then inserted at the *Xho*I cloning site by restriction digestion, removal of the 5′ overhang with Klenow polymerase, and addition of *Eco*RI linkers (Fig. 1).

cDNA pCh 23.1, encoding the β2 structural subunit of chicken brain AChRs, has been previously described (18). The 3′ end of pCh 23.1 contains 192 bp of untranslated region, including a poly(A) tail. This 3′ untranslated region was removed using PCR (29), leaving only the stop codon (Fig. 1). Briefly, oligonucleotide primers around an internal *Bam*HI site (bp 1281 of pCh 23.1) and the stop codon of pCh 23.1 (bp 1545) were synthesized, and PCR was performed as previously described (30), using pCh 23.1 as template. The PCR product was purified on a 1% agarose gel, digested with *Bam*HI, and ligated into *Bam*HI (partial digestion)-*Eco*RV-digested pCh 23.1. The truncated pCh 23.1 was then removed from Bluescript SK⁻ (Stratagene) by *Eco*RI-*Hind*III (in the polylinker) digestion. The *Hind*III overhang was blunt ended with Klenow polymerase, *Eco*RI linkers were added, and the pCh 23.1 was subcloned into the *Eco*RI site of pkOE and pMSGneo.

cDNA pCh 26.1 (2450 bp) (31) encodes the α4 ACh-binding subunit of chicken brain AChRs. By complete digestion with *Ava*I and partial digestion with *Dra*III, a fragment of pCh 26.1 was isolated containing 17 bp of 5′ untranslated region and 16 bp of 3′ untranslated region. The fragment was blunt ended with Klenow polymerase and T4 polymerase, *Eco*RI linkers were added, and the fragment was subcloned into the *Eco*RI site of pkOE and pMSGneo (Fig. 1).

Transfections and Selection of Cell Lines

Mouse Ltk⁻ cells (obtained from Dr. Pam Mellon, Salk Institute), maintained in DMEM containing 10% fetal calf serum, were transfected by calcium phosphate precipitation (32, 33). Dishes (10 cm, containing about 5×10^5 cells) were transfected with 10 μg of pKOneo and 1.7 μg each of pkOE-23.1 and pkOE-26.1 or with 10 μg each of pMSGneo-23.1 and pMSGneo-26.1. Transfected cells were cultivated for 3 days in normal culture medium, split 1:4, and grown for 1 week in medium containing 1 mg/ml Geneticin (GIBCO) and then for 3 weeks in medium containing 2 mg/ml Geneticin. Cells were subsequently maintained in culture medium containing 0.5 mg/ml Geneticin. Resistant cells were cloned by limiting dilution (0.5 cells/well of a 96-well tissue culture plate). Ten cell lines (pkOE-AChR 1–10 and pMSGneo-AChR 1–10) were obtained from each transfection and subsequently analyzed by Northern blotting and ligand binding.

For induction of expression of AChRs, pkOE-AChR cell lines were grown for 2–4 days in culture medium containing 10 mM sodium butyrate, and pMSGneo-AChR cell lines were grown for 3–4 days in culture medium containing 1 μM dexamethasone.

RNA Analysis

Cells were grown in 10-cm dishes and, when cells were almost confluent, sodium butyrate (10 mM final concentration) was added to pkOE cells and dexamethasone (1 μM final concentration) was added

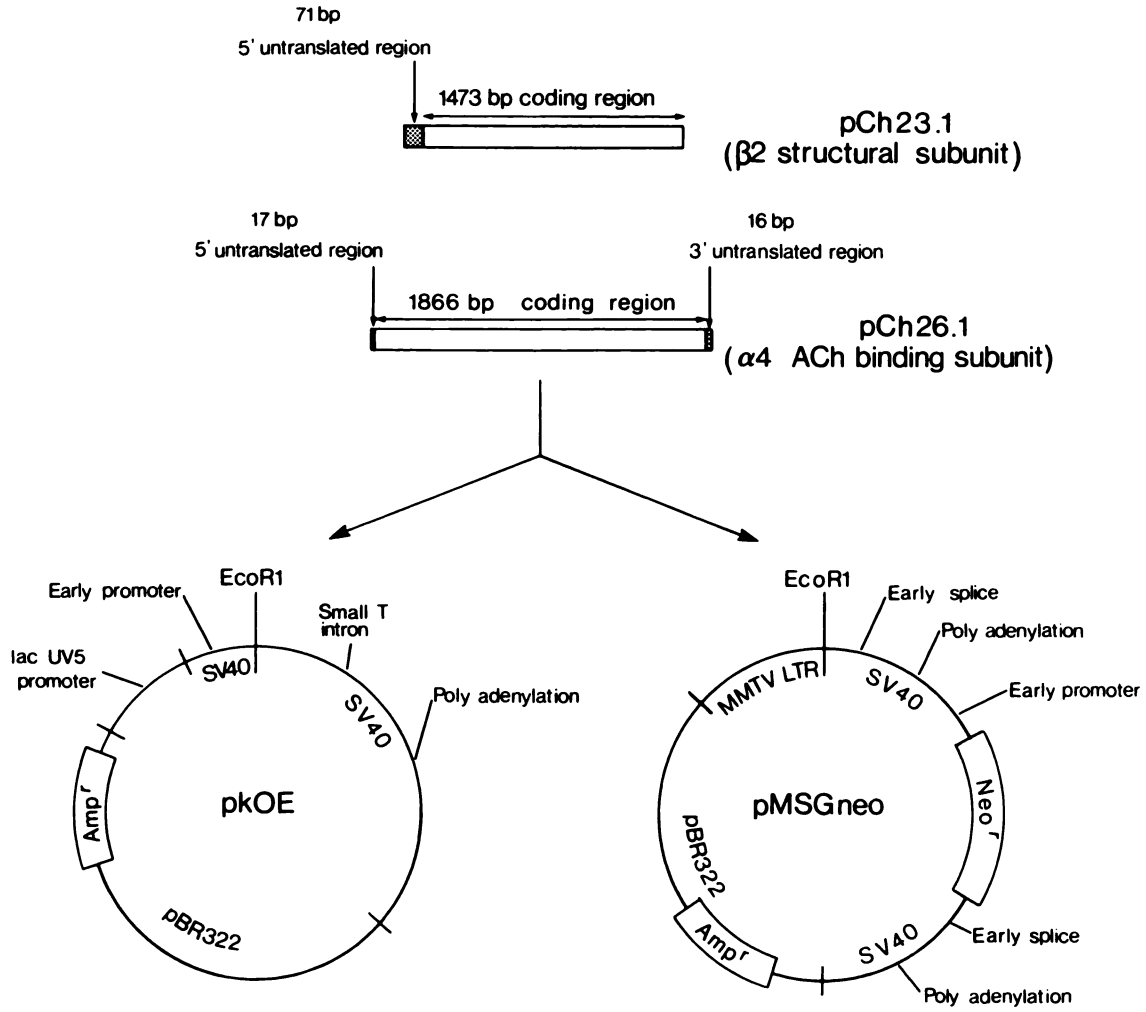

Fig. 1. Structure of vector constructs used for transfections.

to pMSGneo-AChR cells. After an additional 36 hr in culture, total cellular RNA was isolated according to the method of Gough (34). Half of the RNA from each cell line was analyzed by electrophoresis through a 1% agarose-formaldehyde gel and Northern blotting (35) onto Hybond-N (Amersham) membranes.

Hybridization of Northern blots was performed using random-primed ^{32}P-probes prepared from *Eco*RI inserts of pCh 23.1 and pCh 26.1 Hybridization was performed under high stringency in 5 × SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, pH 7.4, 1 mM EDTA), 50% formamide, at 42°. Filters were washed at 65° in 0.3 × SSPE and exposed to Kodak XAR film for 6 hr to 3 days, at −70°, using a Cronex QIII intensifying screen.

Preparation of Solubilized AChRs

Dishes (10 cm) or flasks (175 cm^2) were seeded with cell lines and grown until just confluent. Inducer (either sodium butyrate at a final concentration of 10 mM or dexamethasone at a final concentration of 1 μM) was added, and the cells were then generally grown for 3–4 days to allow expression of AChR. The cells were harvested by scraping into ice-cold 10 mM $NaPO_4$, pH 7.5, 100 mM NaCl (PBS), containing 10 mM EDTA, 10 mM EGTA, 5 mM iodoacetamide, and 1 mM PMSF, and were centrifuged (800 × *g*, 5 min) in a bench-top clinical centrifuge. The cell pellet was then solubilized in approximately 5–7.5 volumes of the aforementioned buffer, containing 0.5% Triton X-100, by agitation using a bench-top vortexer (small volumes) or by homogenization using a Semat Ultra-Turrax homogenizer (larger volumes). After gentle rotation for 30 min at 4°, particulate material was removed by centrifugation for 30 min at 4° in a bench-top microfuge or for 30 min at 4° at 80,000 × *g*, in a Beckman Ti 50.2 rotor. AChR was solubilized from chicken brains (obtained from Pel-Freez Biologicals) as previously described (8, 11, 36).

Ligand Binding

Ligand binding to AChRs solubilized from transfected cells was carried out using an immunoimmobilization assay, which has been described previously (36). Aliquots of detergent extract (150–500 μl, containing 15–50 fmol of L-[^{3}H]nicotine binding sites), in 1.5 ml microfuge tubes, were gently rotated overnight at 4° with 0.5 μl of mAb 290 (raised to rat brain AChRs and cross-reacting with chicken brain AChRs) (10) and 25 μl of a 1:1 slurry of goat anti-rat IgG coupled to Sepharose CL-4B. The Sepharose slurry was then centrifuged briefly (15–20 s) in a microfuge and washed with 1 ml of PBS, 0.5% Triton X-100, by resuspension and centrifugation as described above. The Sepharose was then resuspended in 100 μl of L-[^{3}H]nicotine (72 Ci/mmol; Amersham) in PBS, 0.5% Triton X-100, and was incubated for 15 min at room temperature. Free L-[^{3}H]nicotine was then removed by rapid washing at 4° with 3 × 1 ml of ice-cold PBS, 0.5% Triton X-100, with repeated centrifugation and resuspension of the Sepharose pellet. Bound L-[^{3}H]nicotine was then removed by addition of 150 μl of 2.5% SDS to the Sepharose pellet, incubation for 15 min, and then removal of the supernatant for scintillation counting. Nonspecific binding of L-[^{3}H]nicotine was determined by omission of the mAb from the incubation mixture and was subtracted from total binding to give specific binding. Nonspecific binding was always <150 dpm, whereas total binding was always >600 dpm, except where indicated. Saturation binding curves were obtained by incubating immunoimmobilized AChRs with various concentrations of L-[^{3}H]nicotine. Inhibition of L-[^{3}H]nicotine binding to AChRs by cholinergic ligands was determined by including various concentrations of the ligands in the presence of 3 nM L-[^{3}H]nicotine. Except where stated, all points on binding curves were derived from triplicate tubes. K_i values were determined from three or four independent experiments and were calculated using the equation $K_i = IC_{50}/1 + [\text{L-[}^3\text{H]nicotine}]/K_d$. Ligand binding to AChRs solubilized from chicken brain was carried out as described above, except that AChRs were immobilized upon mAb 299 (raised to rat brain AChRs and directed to the α4 subunit, cross-reacting with chicken brain AChRs) (10).

Sucrose Gradient Sedimentation Analysis of AChRs

Sucrose gradient sedimentation analysis of AChRs was performed as previously described (8). Briefly, 100 μl of M10 cell detergent extract, to which purified *Torpedo* AChR (the gift of Dr. A. Vincent, Institute for Molecular Medicine, Oxford) was added (approximately 50 nM final concentration) as an internal standard, were layered onto 4.9-ml sucrose gradients (5–20%, w/w, in PBS, 0.5% Triton X-100) and centrifuged for 70 min at 4° at 65,000 rpm, in a Beckman vTi 65.2 rotor. Fractions (14 drops) were collected through a 19-gauge needle. Aliquots (20 μl) of each fraction were assayed for *Torpedo* AChR; 20 μl of PBS, 0.5% Triton X-100, containing ^{125}I-labeled α-bungarotoxin (the gift of Dr. A. Vincent), 0.5 μl of mAb 35 (which binds to *Torpedo* AChR) (37), and 25 μl of goat anti-rat IgG-Sepharose were added to each 20-μl aliquot. After gentle rotation overnight at 4°, the Sepharose beads were washed with 3 × 1 ml of PBS, 0.5% Triton X-100, and the ^{125}I-α-bungarotoxin bound to the immunoimmobilized *Torpedo* AChRs was quantitated by γ counting. Neuronal nicotinic AChRs in each fraction were quantitated by L-[^{3}H]nicotine binding using mAb 290 (which does not bind to *Torpedo* AChRs) (10), as described above.

Purification and SDS-PAGE of [^{35}S]methionine-labeled AChRs

[^{35}S]Methionine labeling of cells and immunoaffinity purification. A confluent 10-cm dish of M10 cells was induced for 16 hr with 1 μM dexamethasone, washed with 3 × 10 ml of methionine-free DMEM, and incubated for 6 hr at 37° in 2 ml of methionine-free DMEM containing 500 μCi of [^{35}S]methionine (71,000 Ci/mmol; Amersham). The cells were scraped off in 1 ml of PBS, pelleted, and then resuspended in 500 μl of lysis buffer (50 mM Tris, pH 7.5, 200 mM NaF, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 5 mM iodoacetamide, 1 mM PMSF) containing 5 mg/ml bovine serum albumin (from a 50 mg/ml stock in 1% SDS), 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml antipain. After 15 min of gentle rotation at 4°, insoluble material was removed by centrifugation for 30 min at 4° in a microfuge. The extract was preabsorbed by gentle rotation at 4° for 15 min with 50 μl of goat anti-rat IgG coupled to Sepharose. This was removed by centrifugation, and the extract was then gently rotated for 4 hr at 4° with 25 μl of mAb 295 coupled to AFC resin (New Brunswick Sceintific) (10). The cell extract was then removed by centrifugation, and the resin was washed with 5 × 1 ml of lysis buffer, 5 × 1 ml of lysis buffer containing 800 mM NaCl, and 2 × 1 ml of lysis buffer, by repeated centrifugation and resuspension. AChRs were then eluted by incubation of the resin for 2 × 10 min in 50 μl of 50 mM citrate, pH 3.0, containing 0.01% Triton X-100, and 2 min in 100 μl of H_2O. The eluate was neutralized with 1 M Tris, pH 7.5, concentrated using a Centricon (Amicon) to approximately 50 μl, diluted with 1 ml of H_2O, concentrated, and then lyophilized.

Glycopeptidase F treatment and SDS-PAGE analysis. Lyophilized immunoaffinity-purified AChRs were resuspended in H_2O, and a 10-μl aliquot was adjusted to contain 0.75% SDS and 1.5% β-mercaptoethanol and was then heated to 90° for 3 min. Buffer (35 μl of 10 mM $NaPO_4$, pH 7.5, 0.75% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, pepstatin, and antipain, containing 2.5 units of glycopeptidase F; Boehringer) was added, followed by incubation overnight at 37°. The AChR was then analyzed by SDS-9% PAGE and fluorography, using prestained protein standards for apparent molecular weight determination.

Electrophysiological Recordings

For electrophysiological recordings, M10 cells were subcultured onto uncoated glass coverslips. Coverslips were transferred to a glass-bottomed Perspex recording chamber mounted on the stage of a Nikon Diaphot inverted microscope. Cultures were observed using phase contrast optics and were continuously perfused (approximately 1 ml/min) with a salt solution of the following composition (in mM): NaCl, 124; KCl, 3.25; $MgCl_2$, 2; $CaCl_2$, 2; HEPES, 10; D-glucose, 11; pH 7.4 using NaOH. In a number of experiments, cell cultures were briefly treated

with trypsin/EDTA solution (GIBCO) before transfer to the recording chamber. This had the effect of changing the M10 cell morphology from the usual flat sheet-like appearance to a more rounded form and facilitated both seal formation and viability. The enzyme treatment had no discernable effect on agonist-evoked nicotinic responses.

Patch pipettes with an approximate tip diameter of 1 μm were pulled from boroscilicate glass (Clark Electromedical) using a Mechanex BBCH puller. No additional fire-polishing was performed, and pipettes had a resistance of 5.8 ± 1 MΩ (mean ± standard error; $n = 5$) when filled with the following solution (in mM): CsF, 120; CsCl, 10; HEPES, 10; EGTA, 10; $CaCl_2$, 0.0005; pH adjusted to 7.3 with CsOH.

Whole-cell currents were recorded from voltage-clamped M10 cells using a LIST EPC-7 amplifier, 3–5 days after plating and induction with 1 μM dexamethasone. After formation of a high resistance seal with the cell under investigation, capacitance transients were minimized using the C-Fast facility on the EPC-7. Mean seal resistance measured from five cells was 5.7 ± 0.8 GΩ. No additional capacitance compensation was applied, and no compensation was made for series resistance. All experiments were performed at a holding potential of −75 mV, unless otherwise indicated.

Drugs were applied by gravity perfusion from a large bore (approximately 100 μm) double-barrelled pipette assembly positioned 100–150 μm from the cell. The pipette assembly was positioned using a motorized micromanipulator. Fast on-off drug applications were made by stepping the arrangement laterally such that the cell under investigation experienced a rapid change from control solution flowing from one of the barrels to drug solution flowing from the adjacent barrel.

Results

Preparation of expression system. To allow expression of a multisubunit protein such as a neuronal nicotinic AChR in transfected cells, both stable integration and subsequent expression of cDNAs encoding the AChR must be achieved. Two different mammalian expression systems were used, one utilizing a SV40 promoter (pkOE vector) and the other utilizing the dexamethasone-inducible MMTV promoter (pMSGneo vector) (Fig. 1). An expression vector with an inducible promoter was used to circumvent the possibility that constitutive expression of neuronal AChRs with functional ion channels may be in some way toxic to the transfected cell.

The cDNAs encoding the β2 structural subunit (pCh 23.1) and the α4 ACh-binding subunit (pCh 26.1) were engineered to remove the majority of the untranslated regions (Fig. 1). As such, the expression of these cDNAs should be totally under the control of the transcription signals present in the expression vector.

Mouse fibroblast L cells were co-transfected with either pMSGneo-Ch 23.1 and pMSGneo-Ch 26.1 or pkOE-Ch 23.1, pkOE-Ch26.1, and pkOE-neo (Fig. 1). Cells were selected for neomycin resistance, and resistant cells were subcloned by limiting dilution. Clonal cell lines were subsequently analyzed for expression of subunit mRNAs and expression of L-[^{3}H] nicotine binding sites.

RNA analysis of transfected cell lines. Ten stable cell lines transfected with either pkOE vectors (pkOE-AChR 1–10) or pMSGneo vectors (pMSGneo-AChR 10–10) were obtained. Cell lines pMSGneo-AChR 1–3 were subsequently lost. Each clonal cell line was analyzed for expression of AChR subunit mRNAs. To induce the transcription of RNAs driven by the SV40 promoter of pkOE-AChR-transfected cells, cultures were grown for 36 hr in the presence of 10 mM sodium butyrate, which is known to increase expression of transcription from the SV40 early promoter (38). To induce transcription of RNAs driven by the MMTV promoter of pMSGneo-AChR-transfected cells, cultures were grown for 36 hr in the presence of 1 μM dexamethasone. RNA was isolated and subjected to Northern blot analysis (Fig. 2). Less than half of the cell lines expressed detectable amounts of subunit RNAs. With the exception of the expression of pCh23.1 RNA in pkOE-AChR 7, a single species of RNA was expressed, which was considerably larger than the size of the cDNAs (Fig. 1), indicating addition of other sequences and probably a poly(A) tail.

Expression of L-[^{3}H]nicotine binding sites. To determine which cell lines expressed assembled AChRs capable of binding ligand, a confluent 10-cm dish of each cell line was cultured for 3 days in the presence of the appropriate inducer (either 10 mM sodium dutyrate or 1 μM dexamethasone), the cells were harvested and solubilized, and L-[^{3}H]nicotine binding was determined by an immunoimmobilization assay, as described in Materials and Methods. mAb 290 was used in all the binding assays described below. This mAb is directed to the β2 structural subunit encoded by cDNA pCh 23.1 but binds only to native AChRs, having little or no affinity for denatured

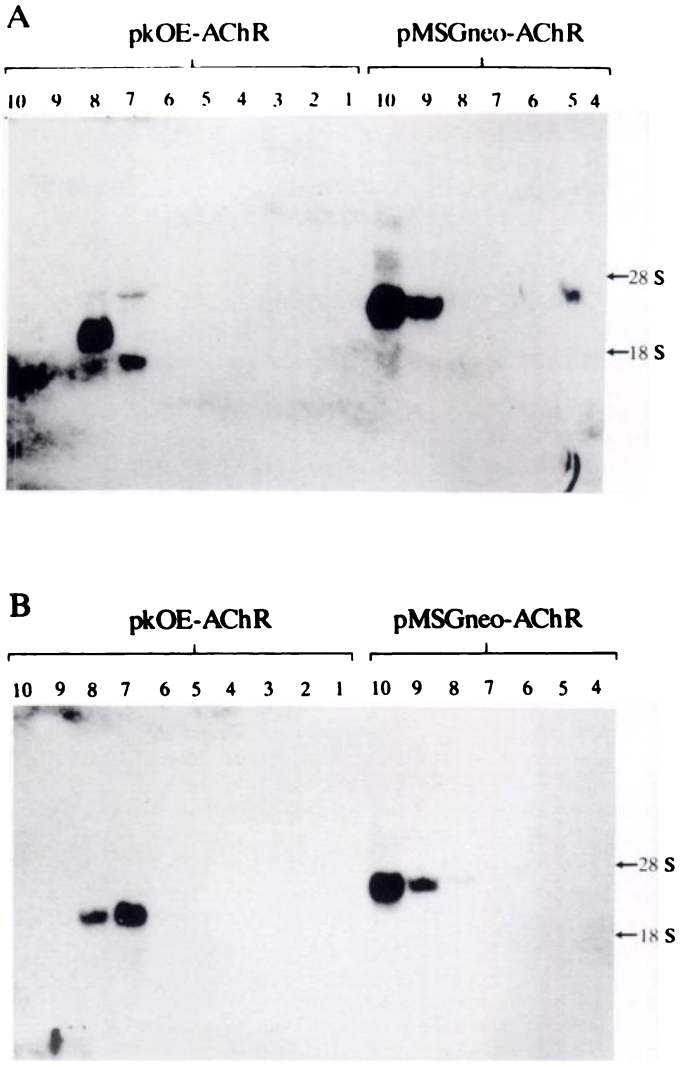

Fig. 2. Northern blot analysis of transfected cell lines. RNA from transfected cell lines (pMSGneo-AChR 4–10 and pkOE-AChR 1–10) was analyzed as described in Materials and Methods. Blots were hybridized under high stringency conditions with random-primed ^{32}P-probes prepared from the *Eco*RI inserts of pCH 23.1 (A) and pCh 26.1 (B).

subunits (10). We have obtained similar results using other mAbs directed to $\beta 2$ (mAbs 270 and 295) and to $\alpha 4$ (mAbs 286 and 299). Only two cell lines, pkOE-AChR 8 (P8) and pMSGneo-AChR 10 (M10), expressed L-[^{3}H]nicotine binding sites significantly above background nonspecific binding (data not shown).

The time course of expression of AChRs by P8 and M10 cells was then determined (Fig. 3). Uninduced cells had no detectable L-[^{3}H]nicotine binding sites. After addition of 1 μM dexamethasone to M10 cells, there was a relatively rapid induction of AChR expression, as measured by L-[^{3}H]nicotine binding sites, reaching maximal levels of about 30,000 dpm (190 fmol) of L-[^{3}H]nicotine binding sites/10-cm culture dish by 48–72 hr. After addition of 10 mM sodium butyrate to P8 cells, there was a somewhat slower induction of AChRs, reaching maximal levels of about 3000 dpm (19 fmol) of L-[^{3}H]nicotine binding sites/10-cm culture dish by 80–120 hr. Thus, approximately 10-fold higher expression of AChRs by M10 cells was found, compared with P8 cells, reaching levels of 10,700 AChR molecules/cell (mean of three determinations), assuming two L-[^{3}H]nicotine binding sites/AChR molecule. Similar levels of expression have been reported for L cells transfected with *Torpedo* AChRs (4). The specific activity of detergent extracts of pMSGneo-AChR 10 cells was 0.4–0.5 pmol/mg of protein. This is approximately 16-fold higher than the specific activity of AChRs in detergent extracts of chicken brains (about 0.028 pmol mg of protein) (8). Subsequent studies thus focused upon M10. This cell line has been maintained in continuous culture for time periods of up to 5 months, and has been retrieved from frozen storage, without any obvious loss of ability to express AChRs.

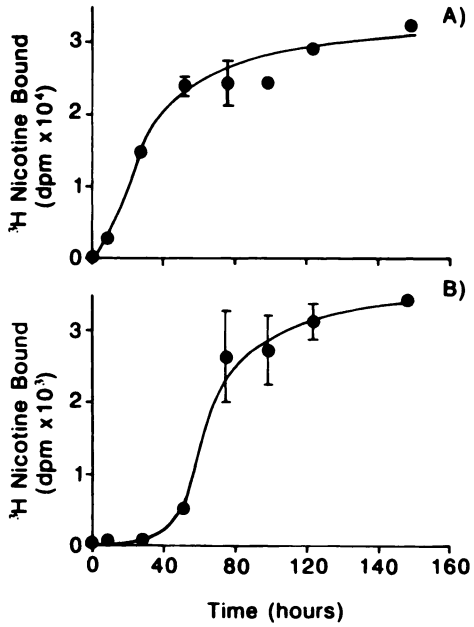

Fig. 3. Time course of induction of AChR expression in M10 cells (A) and P8 cells (B). Transfected cell lines were seeded into 10-cm tissue culture dishes and, when cells were semiconfluent (time zero), fresh culture medium containing 1 μM dexamethasone (A) or 10 mM sodium butyrate (B) was added to the dishes. At various time intervals thereafter, cells were harvested by washing the monolayer in 10 ml of PBS, scraping off the cells in 10 ml of PBS, pelleting, and freezing the cell pellet. When the cells at each time point had been harvested, the cell pellets were solubilized in 500 μl of PBS containing 0.5% Triton X-100, 10 mM EDTA, 10 mM EGTA, 5 mM iodoacetamide, and 1 mM PMSF, and L-[^{3}H]nicotine binding was determined as described in Materials and Methods. Each data point (shown as mean ± standard deviation) is the mean of values determined from three tissue culture dishes, except for the 122-hr and 144-hr time points, where two tissue culture dishes were used. L-[^{3}H] Nicotine binding sites are expressed as dpm/tissue culture dish.

Pharmacological characterization of recombinant and native $\alpha 4\beta 2$ AChRs. Radioligand binding studies were performed using AChRs solubilized with Triton X-100 detergent, from either M10 cells or chicken brains and then immobilized upon a mAb, as described above. AChRs from chicken brain were immobilized upon mAb 299 (10). This mAb is directed to the $\alpha 4$ subunit and only binds the $\alpha 4\beta 2$ AChR subtype from chicken brain, thus allowing direct comparison of the pharmacology of the native AChR with that of the $\alpha 4\beta 2$ AChR expressed by M10 cells. L-[^{3}H]Nicotine binding to the immunoimmobilized M10 AChRs was both saturable and of high affinity ($K_d = 3.2 \pm 1 \times 10^{-9}$ M; mean ± standard deviation of four determinations) (Fig. 4). An almost identical value was obtained for L-nicotine binding to $\alpha 4\beta 2$ AChRs from chicken brain ($K_d = 3.6 \pm 0.3 \times 10^{-9}$ M; mean ± standard deviation of three determinations; data not shown). The L-[^{3}H]nicotine binding to both AChRs from M10 cells and AChRs from chicken brain was displaced by competing cholinergic agonists and antagonists, with very similar K_i values (Table 1). The correlation of these values was excellent (Fig. 5), with a correlation coefficient of 0.998. These K_i values are also in good agreement with values determined for AChRs from rat brain (39–41).

Structure of AChRs expressed by M10 cells. Both the macromolecular size and the subunit structure of AChRs expressed by M10 were determined. The macromolecular size was analyzed by sucrose gradient sedimentation analysis of detergent-solubilized AChRs. As previously described (8, 10), *Torpedo* AChR was used as an internal size marker, sedimenting at 9 S (monomer) and 13 S (dimer) (42). By resolving *Torpedo* AChR on the same gradient as the transfected cell detergent extract, any possible variation between gradients was eliminated. AChRs from the transfected cells reproducibly sedimented as a single peak, which ran slightly ahead (about 10 S) of *Torpedo* AChR monomers (Fig. 6). Thus, the expressed AChR behaves the same as native brain AChRs upon sucrose gradient analysis (8–10). The subunit structure of the recombinant AChRs was defined by [^{35}S]methionine labeling of M10 cells and immunoaffinity purification of radiolabeled AChRs using mAb295. Like mAb290, mAb295 binds to native AChRs, showing little binding to denatured subunits (10). Thus, it probably binds with highest affinity to receptor macromolecules that have been correctly assembled to achieve a native conformation. SDS-PAGE and autoradiography of recombinant AChRs revealed two closely migrating doublets of apparent M_r 50,000 and 75,000 (Fig. 7). This corresponds to the expected size of the $\beta 2$ and $\alpha 4$ subunits, respectively (11). By excising the part of the polyacrylamide gel corresponding to the $\alpha 4$ and $\beta 2$ subunit bands, quantitating the radioactivity in each band by scintillation counting, and normalizing the radioactivity for the number of methionine residues in each subunit (see Ref. 26 for details), a subunit stoichiometry of 2:3.55 ± 0.22 (mean ± standard deviation of two determinations) $\alpha 4$:$\beta 2$ was found. This value is in reasonable agreement with a stoichiometry of $(\alpha 4)_2(\beta 2)_3$ previously obtained in more detailed studies of AChRs expressed in *Xenopus* oocytes, using either metabolic labeling techniques (26), as above, or an electrophysiological approach (27).

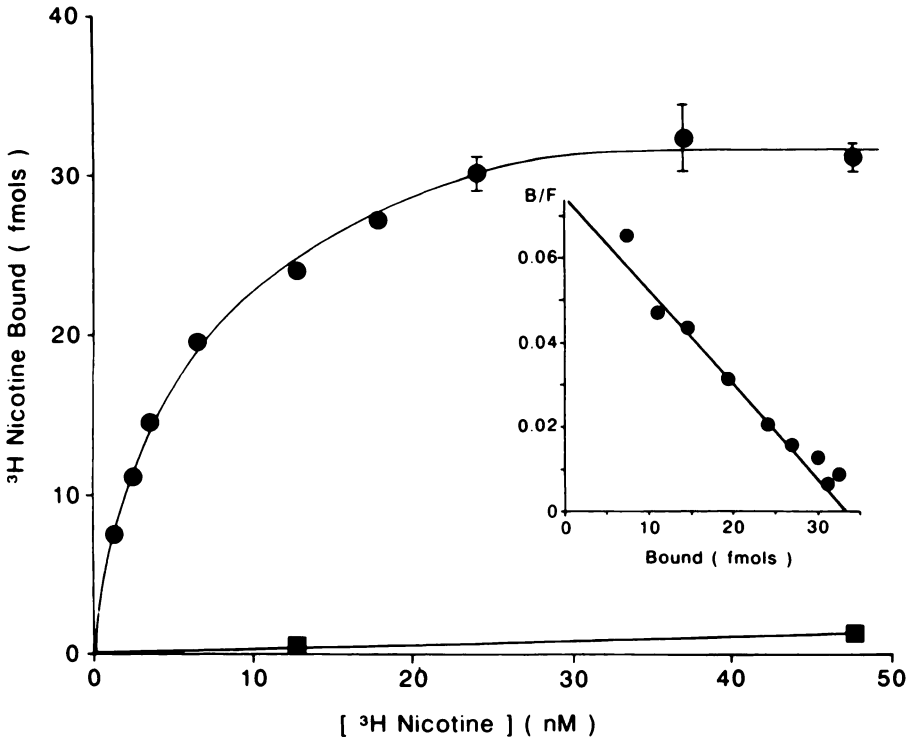

Fig. 4. Binding of L-[^{3}H]nicotine to immunoimmobilized AChRs solubilized from M10 cells. Binding assays were performed exactly as described in Materials and Methods. Each point is the mean ± standard deviation of three values. ●, Specific binding; ■, nonspecific binding, determined by omission of mAb 290. *Inset*, Scatchard analysis of the data, displayed as bound/free (*B*/*F*) (fmol/fmol) versus bound (fmol).

TABLE 1

Inhibition by cholinergic ligands of L-[^{3}H]nicotine binding to AChRs solubilized from transfected cells and chicken brains

K_i values are the mean ± standard deviation of three or four determinations.

Competing ligand	K_i	
	α4β2 AChR from transfected cells	α4β2 AChR from chicken brain
	M	
Cytisine	$1.4 \pm 0.3 \times 10^{-10}$	$1.4 \pm 0.4 \times 10^{-10}$
L-Nicotine	$3.9 \pm 2.1 \times 10^{-9}$	$2.4 \pm 0.5 \times 10^{-9}$
Carbachol	$3.6 \pm 1.3 \times 10^{-7}$	$4.5 \pm 1.2 \times 10^{-7}$
D-Tubocurarine	$2.5 \pm 1.4 \times 10^{-5}$	$7.7 \pm 1.5 \times 10^{-6}$
Hexamethonium	$3.0 \pm 0.6 \times 10^{-4}$	$1.6 \pm 0.5 \times 10^{-4}$
Mecamylamine	$>10^{-3}$	$>10^{-3}$

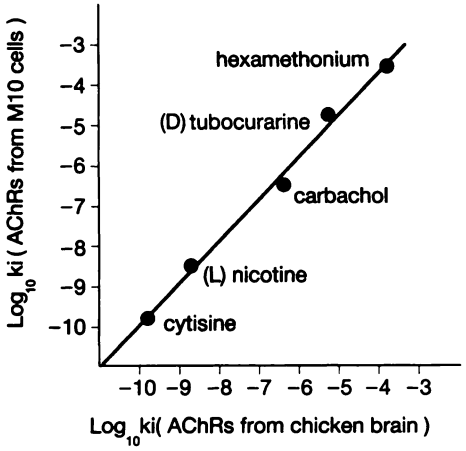

Fig. 5. Correlation between the affinities for nicotinic cholinergic ligands of α4β2 AChRs expressed by M10 cells and α4β2 AChRs from chicken brain.

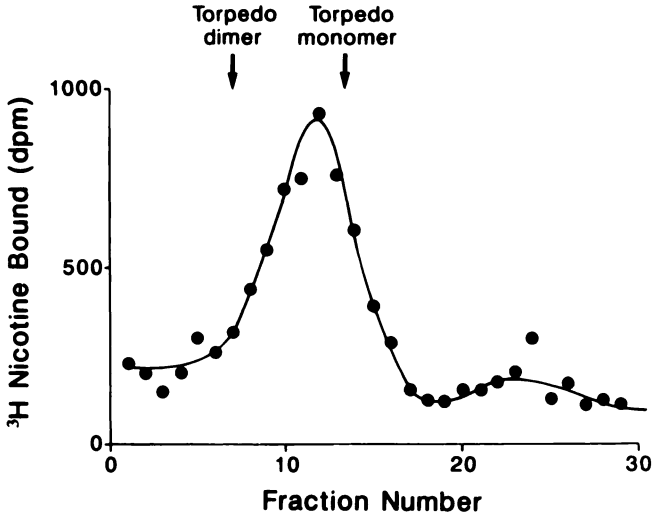

Fig. 6. Sucrose gradient sedimentation analysis of AChRs expressed in M10 cells. *Arrows*, positions of *Torpedo* AChR monomer (9 S) and dimer (13 S), which were resolved on the same gradient, as internal standards.

The polypeptide doublets observed for [^{35}S]methionine-labeled AChRs probably result from differences in processing of carbohydrate groups. This was confirmed by treatment of purified AChRs with glycopeptidase F; the doublets were resolved to single deglycosylated polypeptides, of apparent M_r 43,000 and 64,000. The deduced molecular weights of β2 and α4 are 54,000 and 68,400, respectively (15, 18, 31), somewhat larger than the apparent molecular weight of the deglycosylated polypeptides. This discrepancy between apparent and deduced molecular weight has been reported for other polypeptides; for instance, *Torpedo* electric organ AChR α subunits have an apparent molecular weight of 40,000 (2) but a deduced molecular weight of 50,116 (43).

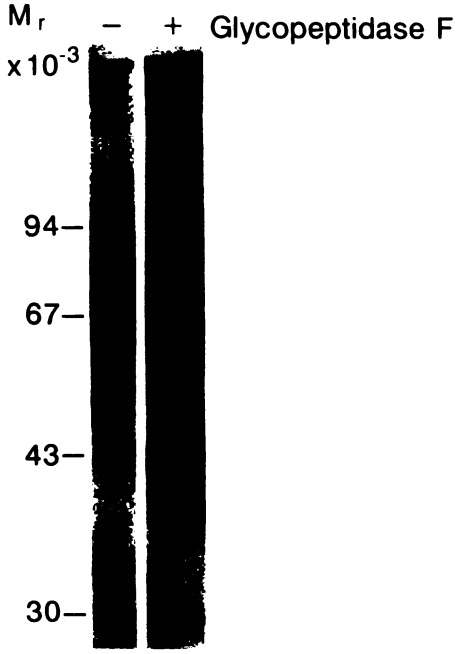

Fig. 7. Subunit structure of AChRs expressed by M10 cells. AChRs were immunoaffinity purified from [^{35}S]methionine-labeled cells and then analyzed by SDS-9% PAGE and fluorography. AChRs that had been treated with glycopeptidase F are also shown. The positions of prestained molecular weight standards are shown to the *left*.

Expression of ACh-gated ion channels. The electrophysiological effects of ACh were studied on approximately 50 M10 cells in which the expression of the AChR had been induced by the addition of dexamethasone (1 μM) to the culture medium for 3–5 days. In all cases, ACh evoked an inward current at negative pipette potentials. ACh responses were not observed in cells that had not been induced by dexamethasone. Muscarine (100 μM) failed to elicit a response in induced cells ($n = 3$), and responses to ACh were not modified by the addition of atropine (1 μM) to the perfusate, confirming that the responses were not due to endogenous muscarinic AChRs.

Brief applications (0.5–1 sec) of either ACh (30 μM) or the selective nicotonic agonist DMPP (30 nM) (data not shown) characteristically evoked responses that were rapid in onset but desensitized in the presence of the agonist (Fig. 8). Responses to ACh were antagonized by hexamethonium, D-tubocurarine, and DHβE (Fig. 8). The effects of hexamethonium (0.1–10 μM) were studied on six cells. In all cases, the responses to ACh were reduced and were not readily reversible, despite extensive washing. D-Tubocurarine (10 μM) reduced the response to ACh (30 μM) to 34 ± 2% (mean ± standard error; $n = 4$) of control. DHβE, at concentrations of 300 nM, significantly antagonized the responses to ACh, indicating that DHβE is a potent antagonist at these AChRs.

The amplitude of the current response to ACh was found to be dependent not only on the agonist concentration but also on the pipette potential. The current-voltage (I-V) relationship was studied more fully on five cells. The ACh response consistently showed a marked inward rectification, such that outward membrane currents at positive pipette potentials were not apparent (Fig. 9). The inward current response was approximately linear over the pipette potential range of −50 to −100 mV and, when measured over this range, current amplitude was found to change e-fold (2.718) with a mean 5.5 ± 1.3-mV ($n = 5$) change in potential.

Discussion

Here we have reported the stable expression in mouse fibroblasts of a neuronal nicotinic AChR composed of α4 and β2 subunits. Initially, two systems of expression vectors were explored, an SV40 promoter-driven system and an MMTV promoter-driven system. In what should be considered to be a very limited comparison, the latter system appeared more suitable, giving more rapid induction of expression and significantly higher levels of expression. In the absence of the inducer, expression of AChRs was below detectable levels (Fig. 4). Overall, we found that only a small proportion of transfected cell lines (2 of 17) expressed detectable amounts of AChR.

The macromolecular size and the subunit structure of AChRs expressed by M10 cells were indistinguishable from those of native brain $\alpha4\beta2$ AChRs. Sucrose gradient sedimentation analysis (Fig. 6) demonstrated that, like native brain AChRs, recombinant $\alpha4\beta2$ AChRs sediment as a 10 S species (8, 10). [^{35}S]Methionine labeling and immunoaffinity purification using a subunit-specific mAb demonstrated that these recombinant AChRs are formed by coassembly of $\beta2$ and $\alpha4$ subunits. Previous studies using oocytes have assumed that this occurs (21–24). The apparent molecular weights of the subunits were essentially identical to those of $\alpha4\beta2$ AChRs purified from chicken brain (11). Because recombinant AChR subunits were very similar in size to subunits of AChRs purified from brain, the glycosylation performed by the transfected cell must be almost equivalent to that performed normally by a neuron. The doublets observed upon SDS-PAGE analysis of recombinant AChRs represent incomplete processing of the added carbohydrate moieties (Fig. 7). It remains to be determined whether this incompletely glycosylated form of the subunits is present in the native AChR expressed on the cell surface or whether it is a glycosylation intermediate found in the Golgi apparatus that we have been able to visualize by virtue of the very rapid rate of subunit synthesis achieved at 20 hr after induction (Fig. 3). Quantitation of the [^{35}S]methionine incorporated into the AChR subunits allowed determination of a subunit stoichiometry for $\alpha4$:$\beta2$ of 2:3.5. In a more detailed investigation of stoichiometry, using the same approach for AChRs expressed in *Xenopus* oocytes, we have determined the subunit arrangement to be a pentamer of $(\alpha4)_2(\beta2)_3$ (26). The reasonable agreement between these two values indicates that the subunit stoichiometry is independent of the expression system.

Pharmacological analysis indicated that the ligand-binding properties of $\alpha4\beta2$ AChRs expressed by M10 cells and native $\alpha4\beta2$ AChRs from chicken brain were approximately identical. Clearly, the very similar macromolecular size and pharmacological profile of these recombinant $\alpha4\beta2$ AChRs, compared with those of the major AChR subtype solubilized from brain, which has been proposed to consist of $\alpha4$ and $\beta2$ subunits (9, 11, 18), is very strong evidence that this subunit composition is correct.

The ability of ACh to evoke membrane current responses in M10 cells that had been induced by dexamethasone, but not in uninduced cells, indicates that the binding sites labeled by L-[^{3}H]nicotine constitute AChRs with functional ion channels. The responses to ACh were blocked by hexamethonium, D-tubocurarine, and DHβE, confirming the nicotinic cholinergic

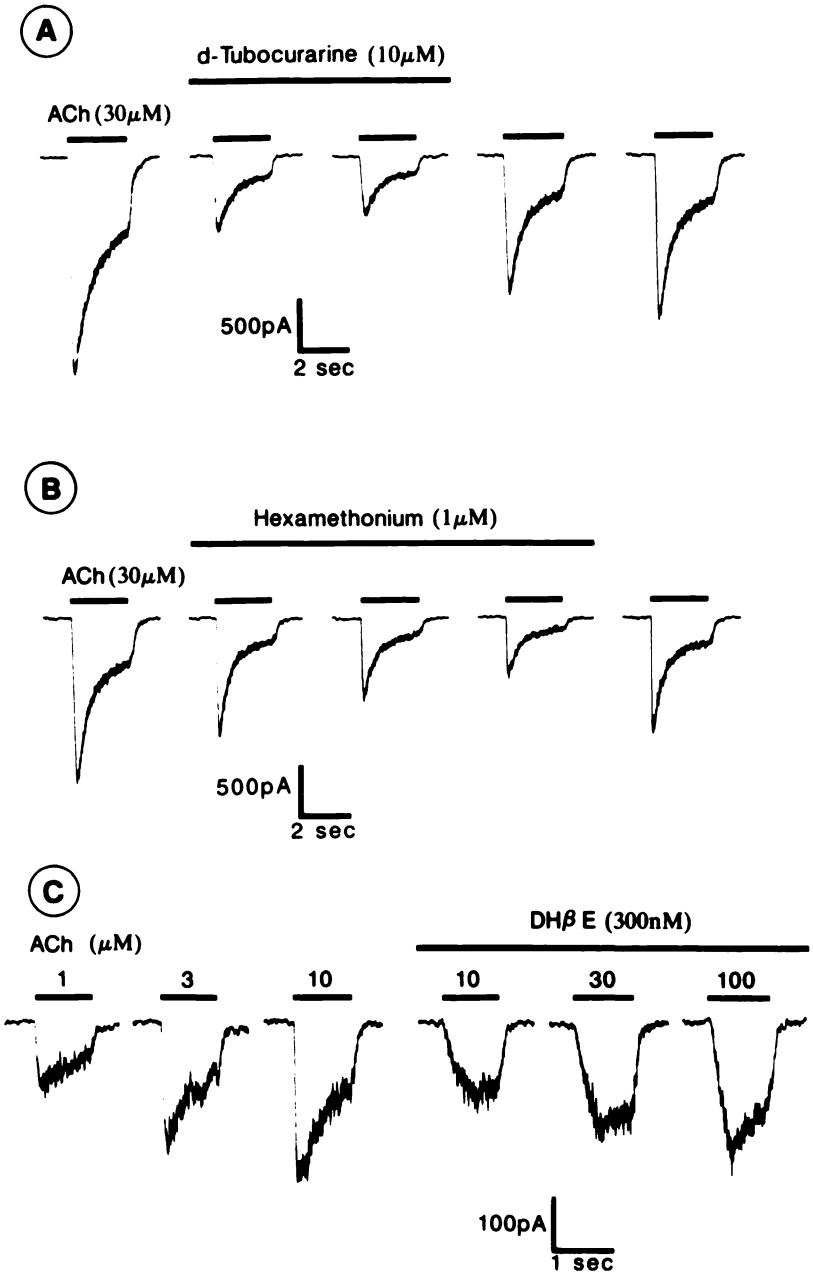

Fig. 8. Membrane current responses induced by ACh are antagonized by D-tubocurarine, hexamethonium, and DHβE. A and B, responses to 2 sec applications of ACh were obtained at approximately 30-sec intervals, using the fast perfusion system described in Materials and Methods. After stable control ACh responses were obtained, either D-tubocurarine (10 μM) (A), hexamethonium (1 μM) (B), or DHβE (C) was applied. D-Tubocurarine produced a rapid antagonism, which was readily reversible. Hexamethonium provided a more slowly developing block, which appeared to be use dependent and was often only poorly reversible. The final response in B was obtained after washing of the cell in antagonist-free solution for approximately 2 min. C, Inward currents were elicited in response to increasing concentrations of ACh. The cell was then continuously perfused with DHβE (300 nM); higher concentrations of ACh were required to evoke similar inward currents in the presence of the antagonist. Note the slower on-rate kinetics of the ACh response in the presence of DHβE; this is most likely to be the consequence of a slow rate of dissociation of the antagonist from the receptor during the approach to equilibrium. Cells were voltage clamped at −75 mV.

nature of the response. In the future, a more detailed and extensive study will define the electrophysiological properties of these α4β2 AChRs. A significant characteristic of the functional response we did observe was a strong inward rectification. The phenomenon has been observed in whole-cell recordings of nicotinic AChRs in neurons dissociated from rat sympathetic ganglia (44) and in cultured PC12 pheochromocytoma cells (45). Thus, inward rectification may be a common property of neuronal AChR subtypes. Its physiological role, however, remains open to speculation.

We have previously proposed that the major subtype of nicotinic AChRs in the brain is composed of two types of subunits, structural (encoded by cDNA β2) and ACh-binding (encoded by cDNA α4) (18, 25). The expression of functional ACh-gated cation channels composed of α4 and β2 subunits in oocytes (21–24) and mouse fibroblasts (Fig. 8) supports this hypothesis, as does the essentially identical pharmacology and structure of recombinant α4β2 AChRs expressed in fibroblasts, compared with those of native AChRs (Table 1). However, it is still possible that some subpopulations of α4β2 AChRs in the brain do contain an additional subunit that has eluded detection. A role for the putative structural subunits β3 and β4 has yet to be determined. They certainly appear to be of lower abundance and more limited distribution than β2 and α4 (19, 20). It is possible that in certain regions of the brain they may associate with an α subunit in addition to, or in place of, β2.

We have shown here, by synthesis in a stably transfected cell line, that AChRs with the subunit composition α4β2, determined initially by analysis of AChRs purified from brain, in fact exhibit the properties expected of the predominant brain AChR subtype *in vivo*. Such stable cell lines may provide a useful system for study of the function and pharmacology of a

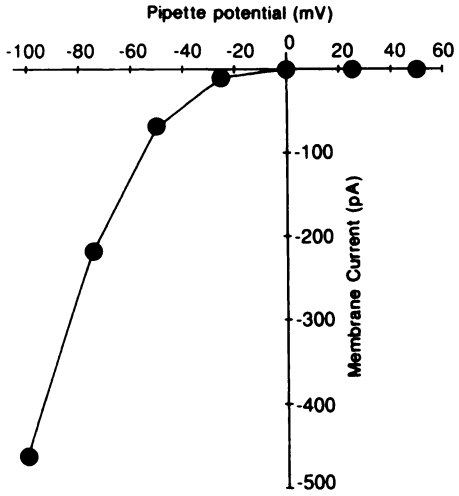

Fig. 9. Current-voltage relationship for ACh-evoked whole-cell currents. The amplitude of the membrane current evoked by brief applications of ACh (30 μM) is plotted as a function of pipette potential. M10 cells characteristically showed strong inward current rectification. Outward currents at positive pipette potentials were not observed. Inward currents increased in amplitude with membrane hyperpolarization, with the relationship being approximately linear over the range of −50 to −100 mV.

single AChR subtype of known subunit composition. Future studies will attempt to utilize this expression system for more detailed study of AChR subunit topology, assembly, function, and regulation.

Acknowledgments

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References

1. Unwin, N. The structure of ion channels in membranes of excitable cells. *Neuron* **3**:665–676 (1989).
2. Changeux, J. P. Functional architecture and dynamics of the nicotinic acetylcholine receptor: an allosteric ligand gated ion channel. *Fidia Research Foundation Neuroscience Award Lectures* (J. P. Changeux, R. Llines, D. Purves, and F. Bloom, eds.), Vol. 4. Raven Press, New York, 21–168 (1990).
3. Mishina, M., T. Tobimatsu, K. Imoto, K. Tanaka, Y. Fujita, K. Fukuda, M. Kurasaki, H. Takahashi, Y. Morimoto, T. Hirose, S. Inayama, T. Takahashi, M. Kuno, and S. Numa. Localization of functional regions of acetylcholine receptor α subunit by site directed mutagenesis. *Nature (Lond.)* **313**:364–369 (1985).
4. Claudio, T., W. N. Green, D. S. Hartman, D. Hayden, H. L. Paulson, F. J. Sigworth, S. M. Sine, and A. Swedlund. Genetic reconstitution of functional acetylcholine receptor channels in mouse fibroblasts. *Science (Washington D. C.)* **238**:1688–1694 (1987).
5. Forsyth, J., A. Franco, A. Rossi, J. Lansman, and Z. Hall. Expression of functional mouse muscle acetylcholine receptors in Chinese hamster ovary cells. *J. Neurosci.* **10**:2771–2779 (1990).
6. Blount, P., M. Smith, and J. P. Merlie. Assembly intermediates of the mouse muscle nicotinic acetylcholine receptor in stably transfected fibroblasts. *J. Cell. Biol.* **111**:2601–2611 (1990).
7. Saedi, M., W. Conroy, and J. Lindstrom. Assembly of *Torpedo* acetylcholine receptor in *Xenopus* oocytes. *J. Cell. Biol.* **112**:1007–1015 (1991).
8. Whiting, P. J., and J. M. Lindstrom. Purification and characterization of a nicotinic acetylcholine receptor from chick brain. *Biochemistry* **25**:2082–2093 (1986).
9. Whiting, P. J., and J. M. Lindstrom. Purification and characterization of a nicotinic acetylcholine receptor from rat brain. *Proc. Natl. Acad. Sci. USA* **84**:595–599 (1987).
10. Whiting, P. J., and J. M. Lindstrom. Characterization of bovine and human neuronal nicotinic acetylcholine receptors using monoclonal antibodies. *J. Neurosci.* **8**:3395–3404 (1988).
11. Whiting, P. J., R. Liu, B. J. Morley, and J. M. Lindstrom. Structurally different neuronal nicotinic acetylcholine receptor subtypes purified and characterized using monoclonal antibodies. *J. Neurosci.* **7**:405–416 (1987).
12. Boulter, J., K. Evans, D. Goldman, G. Martin, D. Treco, S. Heinemann, and J. Patrick. Isolation of a cDNA clone coding for a possible neuronal nicotinic acetylcholine receptor *alpha* subunit. *Nature (Lond.)* **319**:368–374 (1986).
13. Goldman, D., E. Deneris, W. Luyten, A. Kochhar, J. Patrick, and S. Heinemann. Members of a nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian central nervous system. *Cell* **48**:965–973 (1987).
14. Wada, K., M. Ballivet, J. Boulter, J. Connolly, E. Wada, E. S. Deneris, L. W. Swanson, S. Heinemann, and J. Patrick. Functional expression of a new pharmacological subtype of brain nicotinic acetylcholine receptor. *Science (Washington D. C.)* **240**:330–334 (1988).
15. Nef, P., C. Oneyser, C. Alliod, S. Couturier, and M. Ballivet. Genes expressed in the brain define three distinct neuronal acetylcholine receptors. *EMBO J.* **7**:595–601 (1988).
16. Boulter, J., A. O'Shea-Greenfield, R. M. Duvoisin, J. G. Connolly, E. Wada, A. Jensen, P. D. Gardner, M. Ballivet, E. S. Deneris, D. McKinnon, S. Heinemann, and J. Patrick. α2, α5 and β4: three members of the rat neuronal nicotinic acetylcholine receptor-related gene family form a gene cluster. *J. Biol. Chem.* **265**:4472–4482 (1990).
17. Deneris, E. S., J. Connolly, J. Boulter, E. Wada, K. Wada, L. W. Swanson, J. Patrick, and S. Heinemann. Primary structure and expression of β2: a novel subunit of neuronal nicotinic acetylcholine receptors. *Neuron* **1**:45–54 (1988).
18. Schoepfer, R., P. Whiting, F. Esch, R. Blacher, S. Shimasaki, and J. Lindstrom. cDNA clones coding for the structural subunit of a chicken brain nicotinic acetylcholine receptor. *Neuron* **1**:241–248 (1988).
19. Deneris, E. S., J. Boulter, L. W. Swanson, J. Patrick, and S. Heinemann. β3: a new member of nicotinic acetylcholine receptor gene family is expressed in brain. *J. Biol. Chem.* **264**:6268–6272 (1989).
20. Duvoisin, R. M., E. S. Deneris, J. Patrick, and S. Heinemann. The functional diversity of the neuronal nicotinic acetylcholine receptors is increased by a novel subunit: β4. *Neuron* **3**:487–496 (1989).
21. Boulter, J., J. Connolly, E. Deneris, D. Goldman, S. Heinemann, and J. Patrick. Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identifies a gene family. *Proc. Natl. Acad. Sci. USA* **84**:7763–7767 (1987).
22. Ballivet, M., P. Nef, S. Couturier, D. Rungger, C. R. Bader, D. Bertrand, and E. Cooper. Electrophysiology of a chick neuronal nicotinic acetylcholine receptor expressed in *Xenopus* oocytes after cDNA injection. *Neuron* **1**:847–852 (1988).
23. Papke, R. L., J. Boulter, J. Patrick, and S. Heinemann. Single channel currents of rat neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. *Neuron* **3**:589–596 (1989).
24. Bertrand, D., M. Ballivet, and D. Rungger. Activation and blocking of neuronal nicotinic acetylcholine receptor reconstituted in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **87**:1993–1997 (1990).
25. Whiting, P. J., F. Esch, S. Shimasaki, and J. Lindstrom. Neuronal nicotinic acetylcholine receptor β subunit is coded by the cDNA α4. *FEBS Lett.* **219**:459–463 (1987).
26. Anand, R., W. G. Conroy, R. Schoepfer, P. Whiting, and J. Linstrom. Neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes have a pentameric quaternary structure. *J. Biol. Chem.* **266**:11192–11198 (1991).
27. Cooper, E., S. Couturier, and M. Ballivet. Pentameric structure and subunit stoichiometry of a neuronal nicotinic acetylcholine receptor. *Nature (Lond.)* **350**:235–238 (1991).
28. Southern, P. J., and P. Berg. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. App. Genet.* **1**:327–341 (1982).
29. Saiki, R. H., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. S. Mullis, and H. A. Erlich. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase *Science (Washington D. C.)* **239**:487–491 (1988).
30. Diehl, R. E., P. Whiting, J. Potter, N. Gee, C. I. Ragan, D. Linemeyer, R. Schoepfer, C. Bennett, and R. A. F. Dixon. Cloning and expression of bovine brain inositol monophosphatase. *J. Biol. Chem.* **265**:5946–5949 (1990).
31. Whiting, P. J., R. Schoepfer, W. G. Conroy, M. J. Gore, K. T. Keyser, S. Shimasaki, F. Esch, and J. Lindstrom. Expression of nicotinic acetylcholine receptor subtypes in brain and retina. *Mol. Brain Res.* **10**:61–70 (1991).
32. Graham, F. L., and A. J. van der Eb. Transformation of rat cells by DNA of human adenovirus 5. *Virology* **52**:456–467 (1973).
33. Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlabu, and L. Chasin. DNA mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA* **76**:1373–1376 (1979).
34. Gough, N. M. Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. *Anal. Biochem.* **173**:93–95 (1988).
35. Maniatis, T., E. Fritsch, and J. Sambrook. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
36. Whiting, P. J., and J. M. Lindstrom. Pharmacological properties of immunoisolated neuronal nicotine receptors. *J. Neurosci.* **6**:3061–3069 (1986).
37. Tzartos, S., and J. M. Lindstrom. Monoclonal antibodies used to probe acetylcholine receptor structure: localization of the main immunogenic region and detection of similarities between subunits. *Proc. Natl. Acad. Sci. USA* **77**:755–759 (1980).
38. Gorman, C. M., and B. H. Howard. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* **11**:7631–7648 (1983).
39. Lipiello, P. M., and K. G. Fernandes. The binding of L-^{3}H-nicotine to a single

class of high affinity sites in rat brain membranes. *Mol. Pharmacol.* **29**:448–454 (1986).
40. Martino-Barrows, A. M., and K. J. Kellar. ^{3}H-Acetylcholine and ^{3}H-nicotine label the same recognition site in rat brain. *Mol. Pharmacol.* **31**:169–174 (1987).
41. Marks, M. J., J. A. Stitzel, E. Romm, J. M. Wehner, and A. C. Collins. Nicotine binding sites in rat and mouse brain: comparison of acetylcholine, nicotine and β-bungarotoxin. *Mol. Pharmacol.* **30**:427–436 (1986).
42. Reynolds, J. A., and A. Karlin. Molecular weight in detergent solution of acetylcholine receptor from *Torpedo californica*. *Biochemistry* **17**:2035–2038 (1978).
43. Noda, N., H. Takahashi, T. Tanabe, M. Toyosato, Y. Furutani, T. Hirose, M. Asai, S. Inayama, T. Miyata, and S. Numa. Primary structure of a α subunit precursor of *Torpedo california* acetylcholine receptor deduced from cDNA sequence. *Nature (Lond.)* **299**:793–797 (1983).
44. Mathie, A., D. Colquhoun, and S. G. Cull-Candy. Rectification of currents activated by nicotinic acetylcholine receptors in rat sympathetic ganglion neurones. *J. Physiol. (Lond.)* **427**:625–655 (1990).
45. Ifune, C. K., and J. H. Steinbach. Rectification of acetylcholine elicited currents in PC12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA* **87**:4794–4798 (1990).

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