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β3 Subunits Promote Expression and Nicotine-Induced Up-Regulation of Human Nicotinic α6* Nicotinic Acetylcholine Receptors Expressed in Transfected Cell Lines

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ABSTRACT

Nicotinic acetylcholine receptors (AChRs) containing α6 subunits are typically found at aminergic nerve endings where they play important roles in nicotine addiction and Parkinson's disease. α6* AChRs usually contain β3 subunits. β3 subunits are presumed to assemble only in the accessory subunit position within AChRs where they do not participate in forming acetylcholine binding sites. Assembly of subunits in the accessory position may be a critical final step in assembly of mature AChRs. Human α6 AChRs subtypes were permanently transfected into human tsA201 human embryonic kidney (HEK) cell lines. α6β2β3 and α6β4β3 cell lines were found to express much larger amounts of AChRs and were more sensitive to nicotine-induced increase in the amount of AChRs than were α6β2 or α6β4 cell lines. The increased sensitivity to nicotine-induced up-regulation was due not to a β3-induced increase in affinity for nicotine but probably to a direct effect on assembly of AChR subunits. HEK cells express only a small amount of mature α6β2 AChRs, but many of these subunits are on the cell surface. This contrasts with *Xenopus laevis* oocytes, which express a large amount of incorrectly assembled α6β2 subunits that bind cholinergic ligands but form large amorphous intracellular aggregates. Monoclonal antibodies (mAbs) were made to the α6 and β3 subunits to aid in the characterization of these AChRs. The α6 mAbs bind to epitopes C-terminal of the extracellular domain. These data demonstrate that both cell type and the accessory subunit β3 can play important roles in α6* AChR expression, stability, and up-regulation by nicotine.

Nicotinic acetylcholine receptors (AChRs) are composed of five homologous subunits (Lindstrom, 2000; Sine and Engel, 2006). Heteromeric neuronal AChRs contain two ACh binding sites formed by the interfaces of α2, α3, α4, or α6 subunits with β2 or β4 subunits. The fifth "accessory" subunit does not participate in forming an ACh binding site but contributes to the channel lining and influences assembly, transport, and function of AChRs. The accessory position is often occupied by β2 or β4 subunits, but α4 can also assemble there (Kuryatov et al., 2005). β3 and α5 can assemble only in this position.

AChRs containing α6 subunits (α6* AChRs) comprise minor subtypes selectively localized in the endings of aminergic neurons (Zoli et al., 2002; Champtiaux et al., 2003; Gotti et al., 2005b). α6* AChRs contribute to nicotine-stimulated dopamine release from striatal synaptosomes (Azam and McIntosh, 2005), are selectively lost in animal models of Parkinson's disease, and are potential targets for Parkinson's disease therapy (Quik and McIntosh, 2006). α6* AChRs are the major non-α4* AChR expressed in the optic tract (Gotti et al., 2005b). β3 subunits are usually found in α6* AChRs, and knockout of β3 reduces but does not eliminate expression of α6* AChRs (Gotti et al., 2005a).

Nicotine up-regulates the amount of brain AChRs (Flores et al., 1992). Nicotine treatment has been reported to both increase (Parker et al., 2004) and decrease the amount of brain α6* AChRs (Lai et al., 2005; McCallum et al., 2006; Mugnaini et al., 2006). In transfected HEK cell lines, nicotine applied overnight increases the amount of human α3β2 and α4β2 AChRs but not α3β4 or α4β4 AChRs, primarily by acting as a pharmacological chaperone to promote assembly of AChRs (Wang et al., 1998; Kuryatov at al., 2005; Sallette et al., 2005; Corringer et al., 2006). Putative assembly intermediates the size of α4β2α4β2 tetramers have been identified that could assemble with accessory subunits in a final step to produce mature AChRs (Kuryatov et al., 2005). Nic-

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ABBREVIATIONS: AChR, acetylcholine receptor; ACh, acetylcholine; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; mAb, monoclonal antibody; TPBS, 0.5% Triton X-100 in phosphate-buffered saline; MIR, main immunogenic region.

otine also contributes to up-regulation by increasing the lifetime of surface membrane AChRs (Kuryatov et al., 2005). Nicotine applied to transfected HEK cell lines for 5 days up-regulated rat AChRs containing α2, α3, or α4 in combination with β2 or β4 subunits (Xiao and Kellar, 2004).

It has been challenging to express α6 AChRs (Gerzanich et al., 1997). In *Xenopus laevis* oocytes, human α6β4 AChRs were functional, and α6β4β3 AChRs were expressed at a higher level (Kuryatov et al., 2000). Although coexpression of α6 and β2 produced abundant ACh binding sites, they were on amorphous aggregates within the oocytes. Chimeric subunits with the extracellular domain of α6 and the remainder of either α3 or α4 subunits assembled efficiently with either β2 or β4 subunits in oocytes (Kuryatov et al., 2000). In human BOSC 23 cells, chicken α6β2 were AChRs expressed at a lower level than α4β4 (Fucile et al., 1998). Attempts to express human α6β2, α6β2β3, α6β4, and α6β4β3 AChRs in transfected SH-EPI cell lines were unsuccessful, but the α6β4β3α5 subunit combination exhibited cholinergic ligand binding (Grinevich et al., 2005). Chimeric subunits with the extracellular domain of α6 and the remainder of α4 formed functional AChRs in HEK cell lines (Evans et al., 2003). α6 subunits are closely related in sequence to α3 subunits, and β3 subunits are closely related to α5 subunits (Lindstrom, 2000; Le Novere et al., 2002). Permanently transfected HEK tsA201 cells have been used to express human α3* and α4* AChRs (Wang et al., 1998; Nelson et al., 2001; Kuryatov et al., 2005). α3β2 AChRs assembled efficiently in *X. laevis* oocytes (Wang et al., 1996; Gerzanich et al., 1998), but in HEK cells they do not assemble efficiently unless up-regulation is induced by nicotine or culture at low temperatures (Wang et al., 1998). Coexpression in HEK cells of α3β2 with α5 increases expression, but coexpression of α3β4 with α5 somewhat decreases expression (Wang et al., 1998). In oocytes, α5 subunits alter desensitization, pharmacology, and Ca^{2+} permeability of α3 AChRs (Gerzanich et al., 1998), so it might be expected that β3 subunits would similarly have substantial effects on α6 AChRs.

Here, we report the production of stably transfected tsA201 HEK lines expressing four subtypes of human α6* AChRs and their initial characterization. Expression of β3 with either α6β2 or α6β4 subunit combinations increased the amount of AChRs expressed and increased their sensitivity to up-regulation by nicotine. mAbs were made to human α6 and β3 subunits to aid in the characterization of these AChRs.

Materials and Methods

cDNAs. Human α6, β2, and β4 cDNAs were cloned in this laboratory as described previously (Anand and Lindstrom, 1990; Gerzanich et al., 1997; Kuryatov et al., 2000). Human β3 was obtained from Christopher Grantham (Janssen Research Foundation, Beerse, Belgium) and subcloned into pcDNA 3.1/Zeo(+) for transfection. α6 was subcloned into pEF6/blasticidin(+). β2 and β4 were separately cloned into pRc-CMV/Geneticin(+). All vectors were obtained from Invitrogen (Carlsbad, CA).

Cell Line Culture and Transfection. Human embryonic kidney tsA201 cell lines (Margolskee et al., 1993) were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose; Invitrogen) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37°C, 5% CO_2 at saturating humidity in a water jacket incubator. HEK cells were transfected using the FuGENE 6 DNA transfection kit (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instructions, with human α6 (pEF6/blasticidin), human β2 [pRc-CMV/Geneticin(+)], or human β4 [pRc-CMV/Geneticin(+)]. α6β2 was created by transfecting HEK cells with α6 and β2 cDNAs. α6β4 was created by transfecting HEK cells with α6 and β4 cDNAs. Selective pressure for cells containing α6 was applied with 5 μg/ml blasticidin starting 72 h after transfection. β2 and β4 cell lines were similarly selected with 600 μg/ml G418 (Geneticin). α6β2 and α6β4 AChRs were assayed for expression and screened by [^{3}H]epibatidine binding. Stably transfected α6β4 and α6β2 lines were cotransfected with human β3 [pcDNA 3.1/Zeocin(+)] to produce α6β4β3 and α6β2β3 AChRs. Selective pressure for cell lines containing β3 was applied using 500 μg/ml Zeocin starting 72 h after transfection. AChRs containing β3 were selected for high expression based on liquid phase radioimmunoassays. β3-transfected cell lines were plated in serial dilution on Costar 96-well plates (Corning Life Sciences, Acton, MA). Surviving colonies were plated individually to Costar 35-mm culture plates and then grown to confluence. Cells were detached with ice-cold DMEM and extracted as described above into a 1.5-ml microcentrifuge tube. AChRs were incubated with 2.5 nM [^{3}H]epibatidine and 5 μl of β3 antiserum, acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), for 1 to 2 h at room temperature. AChRs were precipitated by secondary antibody incubation with rabbit anti-goat antiserum for 1 to 2 h at room temperature and then pelleted at 13,000*g* for 10 min. Pellets were washed three times with 1 ml of 0.5% Triton X-100 in PBS + 10 mM NaN_3 to remove excess radioligand. Washing solution was aspirated from the pellet before adding 50 μl of 0.1 N NaOH to solubilize the pellet. One milliliter of Optiphase SuperMix (PerkinElmer Life and Analytical Sciences, Boston, MA) was added to each sample. Samples with scintillation fluid were set on rotation for 1 h at room temperature before counting in a 1450 Microbeta Trilux liquid scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences).

Membrane Fraction [^{3}H]Epibatidine Binding. To assess relative expression levels across α6 containing AChR types expressed in HEK cells, stably transfected HEK cells were grown to confluence on Costar 35-mm tissue culture plates (approximately 1×10^6 cells/plate) and then detached by ice-cold DMEM and collected by centrifugation at 500*g*. Cells were washed once with 1 ml of buffer A (50 mM $NaPO_4$, pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 15 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride), pelleted by centrifugation at 13,000*g*, and then resuspended in buffer A. Cells in buffer A were incubated with 2 nM [^{3}H]epibatidine for 30 min at room temperature with agitation. Cell membrane fractions were washed three times with PBS + 10 mM NaN_3 on glass filters (GF/F; Whatman, Maidstone, UK) pretreated with 1% polyethylenimine for 1 h and dried on blotting paper. Filters containing AChRs bound to radioligands were counted in 1.5-ml microcentrifuge tubes using 1 ml of Optiphase SuperMix scintillation fluid in a 1450 Microbeta Trilux liquid scintillation counter (PerkinElmer Life and Analytical Sciences). Results were standardized to the wet weight of the cells after the first wash in buffer A.

Previously Used Antisera and Monoclonal Antibodies. A rat antiserum to bacterially expressed α6 subunit sequences (excluding the transmembrane domains) was raised as described by Kuryatov et al. (2000). The rat mAb 210 binds to the main immunogenic region on native α1, α3, and α5 (Lindstrom, 2000) and β3 (A. Kuryatov and J. Lindstrom, unpublished data). The rat mAb 295 binds to the extracellular surface of AChR β2 subunits when they are assembled with α subunits (Lindstrom, 2000; Kuryatov et al., 2005). The mouse mAb 337 was raised to bacterially expressed human β4 subunit large cytoplasmic domain (Nelson et al., 2001).

Preparation of New mAbs. Human α6 and β3 subunits lacking the transmembrane domains of these subunits were constructed in the pET-26b(+) vector (Novagen, Madison, WI) and expressed in bacteria. α6 subunits were expressed in oocytes and extracted with

2% Triton X-100 as described in Kuryatov et al. (2000). The extracts were incubated with mAb 295 coupled to CH-Sepharose (GE Healthcare) for purifying AChRs for use in immunization or with cell culture supernatants in protein A-coated microtiter wells for solid phase radioimmunoassay.

Female BALB/c mice, 3 to 4 weeks of age, were obtained from Charles River Laboratories, Inc. (Wilmington, MA). All animals were handled in accordance with guidelines set forth by the Institutional Animal Care and Use Committee at the University of Pennsylvania under an approved protocol on file with that office. Institutional Animal Care and Use Committee operates under an institutional Animal Welfare Assurance (A3079-01) on file with the Office for Protection from Research Risks at the National Institutes of Health.

BALB/c mice were immunized and then boosted at 3-week intervals with 40 μg per mouse of bacterially expressed subunit constructs lacking the transmembrane domains of the subunits in TiterMax (TiterMax USA, Inc., Norcross, GA). The titers were monitored by test bleeds against corresponding subunits in enzyme-linked immunosorbent assay. Five days after a final boost with antigen, the harvested splenic lymphocytes ($\sim 10^8$) from the animal with the highest titers were fused with SP2/0 myeloma cells (1×10^8) using 50% polyethylene glycol (Eastman Kodak, Rochester, NY) and inoculated into 24 96-well plates (Costar 3595; Corning Life Sciences, Acton, MA).

Initial screening was done by enzyme-linked immunosorbent assay as described previously (Kuryatov et al., 2005). To eliminate mAbs that cross-reacted with closely related subunits, the cross-reaction of mAbs with a subunit that has the highest homology with the immunogen was tested (α3 in the case of α6 and α5 in the case of β3). mAbs that could bind both denatured subunits and native AChRs were selected using [^{3}H]epibatidine-labeled native AChRs in radioimmunoassays and then cloned by a limiting dilution method.

To determine titers of antibody to native AChRs, various concentrations of mAbs were incubated in 100 μl of buffer C containing a 0.5 nM concentration of AChRs and 1.5 nM [^{3}H]epibatidine. After overnight at 4°C, 25 μl of zysorbin (Invitrogen) was added into each tube, followed by a 30-min incubation at room temperature. The material in the tubes was diluted into 1 ml with 0.5% Triton X-100 in PBS (TPBS). After a 5-min centrifugation at 4000*g*, the supernatant was removed by aspiration. The pellet was washed with 1 ml of TPBS and then suspended in 100 μl of 0.1 N NaOH. The suspension was mixed with 900 μl of Optiphase SuperMix scintillation fluid (PerkinElmer Life and Analytical Sciences). The amount of bound [^{3}H]epibatidine was determined by liquid scintillation counting for 5 min. Background was determined by substituting normal mouse serum for the mAb.

To determine cross-reactivity of mAbs to related subunits with dot blot immunoassay, 167 ng/well of various bacterially expressed human AChR subunits was gravity-blotted onto prewetted nitrocellulose membranes in 100 μl of TPBS (20 mM Tris-HCl and 500 mM NaCl, pH 7.5) using a Bio-Dot apparatus (Bio-Rad, Hercules, CA). Then, 300 μl of TPBS containing 1% bovine serum albumin was applied into each well to block nonspecific-antibody binding in subsequent steps. After blocking, the membranes were incubated for 1 h with a 1 μg/ml mAb or a 1:500 dilution of rat antiserum specific for the different subunits as controls. The membranes were rinsed three times with TPBS containing 0.05% Tween 20 and then probed for 1 h with a 1:2000 dilution of biotinylated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD), followed by a 1-h incubation with peroxidase-labeled streptavidin (Kirkegaard and Perry Laboratories), and a final incubation in peroxidase substrate to visualize antibody binding. A 1:2000 dilution of biotinylated goat anti-rat IgG (Kirkegaard and Perry Laboratories) was used under conditions where rat antiserum was used as the first antibody instead of a mAb. Membranes were washed five times with TPBS containing 0.05% Tween 20 and then two times with TPBS before substrate addition.

AChR Extraction from HEK Cells with Triton X-100. HEK cells expressing AChRs were grown in Costar 10- or 15-cm plates and then up-regulated by exposure to 100 μM nicotine overnight at 37°C. Cells were detached using 5 or 10 ml of ice-cold DMEM and then collected via centrifugation at 500*g*. DMEM was aspirated from the pellet. Cells were suspended with 1 ml of buffer A and then transferred to a 1.5-ml Eppendorf microcentrifuge tube and collected via centrifugation at 13,000*g* for 15 min at 4°C. Buffer A was aspirated from the pellets, which were then weighed. AChRs were extracted with 3 volumes of the pellet weight using buffer C (buffer A with 2% Triton X-100). The suspension was gently rotated for 1 h at room temperature. Cell debris was pelleted by centrifugation at 13,000*g* for 15 min at 4°C. Supernatant containing AChRs in Triton X-100 was removed to new 1.5-ml centrifuge tubes and used immediately, keeping samples at 0–4°C.

Western Blots. AChRs were extracted with 2% Triton X-100 as described above from either HEK cell lines or *X. laevis* oocytes expressing either $\alpha 6\beta 2$, chimeric $\alpha 6/\alpha 3\ \beta 2$, or chimeric $\alpha 3/\alpha 6\ \beta 2$ AChRs. Samples were electrophoresed in precast 10% polyacrylamide Tris-glycine gels (Novex, San Diego, CA) under reducing conditions. Western transfer was done using a semidry electroblotting chamber (Semi-Phor; Hoeffer, San Francisco, CA) to Trans-Blot Medium polyvinylidene difluoride membrane (Bio-Rad). Blots were quenched with 5% Carnation dried milk in 0.5% Triton X-100 in PBS, 10 mM NaN_3. mAbs were used as indicated at 1:1000 dilution in milk blocking solution. Blots were probed overnight at 4°C on a shaker followed by three washes with 0.5% Triton X-100 in PBS, 10 mM NaN_3. Blots were then incubated with 2 nM ^{125}I-goat anti-mouse IgG (specific activity 2.5×10^{18} cpm/mol) overnight on a shaker at 4°C followed by three washes with TPBS. Autoradiography was done at −80°C with Kodak BioMax film using a Kodak MS screen (Eastman Kodak) for the indicated time periods.

Agonist Binding on Fixed Cells. Cells were plated onto Costar 96-well white with clear bottom plates and grown to 70% confluence. Nicotine was added to induce up-regulation of AChRs. Cells were incubated overnight at 37°C. Cells grown to confluence were exposed to 1 volume of 10% formalin added directly to the growth medium for 1 h at room temperature to fix the cells to the wells. Cells were washed free of agonist and formalin with 200 μl of PBS + 10 mM NaN_3 three times and stored at 4°C with 1 volume of PBS-NaN_3 until use. Agonists were applied at indicated concentrations with [^{3}H]epibatidine at 2 nM. Binding was conducted at room temperature for 2 h with gentle agitation. Plates were again washed to remove unbound ligands as described above. [^{3}H]Epibatidine was eluted from the AChRs by 50 μl of 0.1 N NaOH. Then, 200 μl of Optiphase SuperMix (PerkinElmer Life and Analytical Sciences) scintillation fluid was added to each well. Plates were then shaken at room temperature for 1 to 2 h before scintillation counting. Comparison of the maximum number of epibatidine binding sites in all four cell lines after up-regulation with nicotine revealed that in fixed cells 93 ± 7% of the binding sites observed in membrane fragments were detected on fixed cells. Binding to fixed cells was faster and easier than using membrane fragments and avoided variation as a result of cells detaching during washing, which occurred without fixation.

Sucrose Density Gradients. A linear gradient maker was loaded with 5.7 ml each of 5 and 20% sucrose in 0.5% Triton X-100 to construct an 11.4-ml, linear 5 to 20% sucrose gradient. Gradients were built in Quick-Seal centrifuge tubes (Beckman Coulter, Inc., Fullerton, CA) (13 × 51 mm). Then, 400 μl of cell extract was combined with 2 μl of extract of the electric organ from *Torpedo californica* ($\sim$1 μM α-bungarotoxin binding sites) for an internal size marker and loaded onto the top of each gradient. Gradients were centrifuged at 40,000 rpm for 16 h at 4°C in an XL-90 ultracentrifuge using an SW-41 rotor (Beckman Coulter, Inc.). After ultracentrifugation, the tubes were punctured, and fractions were collected from the bottom into mAb-coated Immulon flat-bottomed 4HBX wells (Thermo Electron Corporation, Waltham, MA) using a fraction collector set to collect 10 drops per well (approximately 130 μl/well). The

96-well Immulon 4HBX plates were coated with mAb 295 to bind AChRs containing β2 or with mAb 338 to bind AChRs containing β4. Fractions were bound to their respective antibody overnight at 4°C with gentle agitation. After incubation, 30 μl from each fraction was transferred onto an Immulon plate coated with mAb 210 to bind *Torpedo californica* AChRs, and 70 μl of 2% Triton X-100 in buffer A was added. Solid phase radioimmunoassays were probed for 2 h at room temperature with agitation with 2 nM ^{125}I-α-bungarotoxin to detect *T. californica* AChR binding on mAb 210 plates, or with 2 nM [^{3}H]epibatidine to detect α6* AChR binding on mAb 295 or mAb 338 plates. Then, plates were washed three times with TPBS. Radioligand was dissociated by denaturing the samples with 50 μl of 0.1 N NaOH and transferred for counting into Costar 96-well white-walled plates containing 200 μl of Optiphase SuperMix.

Determining β3 Incorporation. α6* and β3* cell lines were extracted as described above using Triton X-100 in buffer A. Extracts were aliquoted into different sets for liquid phase immunoprecipitation, total [^{3}H]epibatidine binding, and mAb 210 agarose resin depletion. Sets aliquoted for mAb 210 agarose resin depletion were incubated with 20 μl of agarose resin coated with mAb 210 in a total volume of 100 μl along with 2 nM [^{3}H]epibatidine. These samples were incubated overnight at 4°C with constant agitation. Supernatant fluid was collected from samples after 5000*g* centrifugation for 5 min, and then AChRs were precipitated with mAb 338. All samples were denatured with 50 μl of 0.1 N NaOH for 5 min and then shaken with 1 ml of Optiphase SuperMix for 2 h. β3 incorporation was calculated by the difference between the total and depleted samples over the total binding.

Binding of [^{3}H]Epibatidine to Cells. Cells expressing α6* AChRs were grown in media as described above on 35-mm dishes. One day before assay, nicotine was added into the growth media at a final concentration of 10 μM for β3-containing cells and 100 μM for other cells. Binding to living cells attached to 35-mm plates was done in DMEM at 4°C for only 15 min with 1 nM [^{3}H]epibatidine to minimize ongoing up-regulation and penetration of quaternary amines inside the cells. To determine the internal pool of epibatidine binding sites, 1 mM of the membrane-impermeable quaternary amine methylcarbamylcholine was added together with 1 nM [^{3}H]epibatidine to inhibit binding to cell surface AChRs. Nonspecific labeling (around 1% of total) was determined by incubation with 100 μM nicotine and subtracted from total binding. After incubation, the cells were detached using 1 ml of ice-cold PBS with 5 mM EDTA and washed three times with 1 ml of ice-cold PBS by centrifugation (5 min at 500*g*) in Eppendorf tubes. The washed pellets were dissociated with 100 μl of 0.1 M NaOH, and bound radioactivity was determined in the same tubes using the scintillation counter with 1 ml per tube of scintillation fluid.

Results

Construction of Stably Transfected tsA201 HEK Cell Lines Expressing α6β2, α6β2β3, α6β4, and α6β4β3 AChRs. First α6β2 and α6β4 lines were established. Then, these lines were transfected with β3 to produce lines expressing α6β2β3 and α6β4β3 AChRs.

Total amounts of AChRs expressed were measured by [^{3}H]epibatidine binding to cell membrane fractions (Fig. 1A). Expression of α6β2 AChRs (45 fmol/mg protein) was very low compared with α3β2 (200 fmol/mg; Wang et al., 1998) or α4β2 AChR HEK cell lines (900 fmol/mg; Kuryatov et al., 2005). Coexpression with β3 doubled the amount of AChR, much as coexpression with α5 doubled the amount of α3β2 AChRs (Wang et al., 1998). α6β4 expression was twice that of α6β2. Likewise, α3β4 expression in a HEK line exceeded α3β2 expression by 4-fold (Wang et al., 1998). The increased expression of β4-containing compared with β2-containing AChRs reflects less efficient assembly of β2 subunits as a result of a microdomain near the ACh binding site (Sallette et al., 2004) and results in increased sensitivity of β2-con-

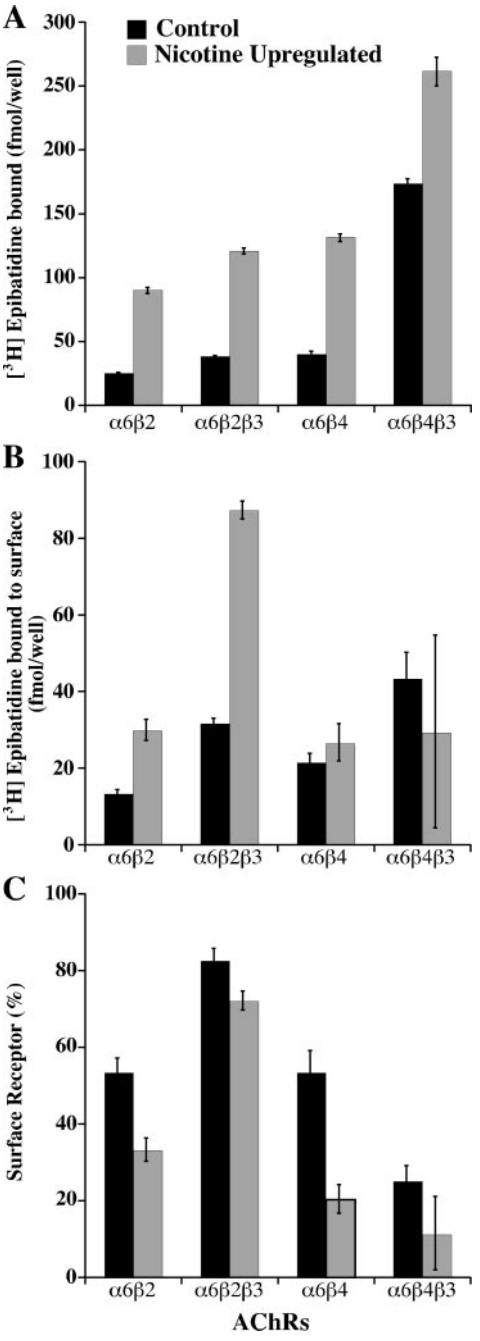

Fig. 1. Extent of α6* AChR expression in transfected cell lines. A, total AChRs were determined by 2 nM [^{3}H]epibatidine binding to membrane fragments of the four cell lines expressing different α6* AChR subtypes. Binding was assayed under control conditions and after culture overnight in medium containing 100 μM nicotine to maximally up-regulate the amount of AChR. B, cell surface AChRs were determined by measuring the fraction of 2 nM [^{3}H]epibatidine binding to intact cells which was inhibited by the presence of 10 mM methylcarbamylcholine. The tertiary amine epibatidine rapidly crosses the cell membrane to label AChRs both on the surface and interior of cells, but the quaternary amine methylcarbamylcholine crosses the membrane so slowly that during the 1-h assay it binds almost exclusively to AChRs on the cell surface (Kuryatov et al., 2005). C, data were plotted to indicate the fraction of AChRs on the cell surface under control conditions and after up-regulation overnight by 100 μM nicotine. Bars show measured values ± S.E.M. for quadruplicate samples.

taining AChRs to the molecular chaperone effects of nicotine, which selectively promotes increased assembly of β2-containing AChRs (Wang et al., 1998; Kuryatov et al., 2005; Fig. 1). Coexpression with β3 increased α6β4 expression a further 6-fold. By contrast, coexpression of α5 with α3β4 decreased expression by 30% (Wang et al., 1998).

Incubation with 100 μM nicotine overnight increased the amount of α6* AChRs (Fig. 1A). This increased α6β2 AChRs 4-fold, α6β2β3 AChRs 3.3-fold, α6β4 AChRs 3.2-fold, and α6β4β3 AChRs 1.5-fold. At the same time, up-regulation significantly increased surface expression in β2-containing cells (Fig. 1B). After up-regulation a greater fraction of the AChRs was found inside the cells (Fig. 1C). This was also observed after nicotine-induced up-regulation of α4β2 AChRs (Kuryatov et al., 2005). Nicotine acts as a molecular chaperone to rapidly promote assembly of new α4β2 AChRs in the endoplasmic reticulum (Kuryatov et al., 2005; Sallette et al., 2005; Corringer et al., 2006). Transport of α4β2 AChRs to the surface through the Golgi apparatus for modification of glycosylation may be a rate-limiting step in surface expression. Similar processes may occur with α6* AChRs.

The time course of up-regulation by 100 μM nicotine was similar for all of the cell lines (Fig. 2). Up-regulation was half complete within 3 h and close to maximal by 24 h. This is similar to the kinetics of up-regulation of α3β2 and α4β2 AChRs in tsA201HEK cell lines (Wang et al., 1998; Kuryatov et al., 2005).

Raising mAbs to α6 and β3 Subunits. Mouse mAbs were raised to bacterially expressed human α6 and β3 subunits. Those mAbs that cross-reacted between subunits by dot blots of bacterially expressed subunits were eliminated, leaving six mAbs to α6 and one to β3, which were subunit-specific by this criterion (Fig. 3A). mAb 376 reacted only on blots with denatured β3, not with native AChRs. The mAbs to α6 also reacted on Western blots with α6 subunits from AChRs expressed in HEK cell lines and showed no cross-reaction with α3, α4, β2, or β4 subunits of AChRs expressed in these cell lines (Fig. 3B). mAb 349 was the best for use on Western blots.

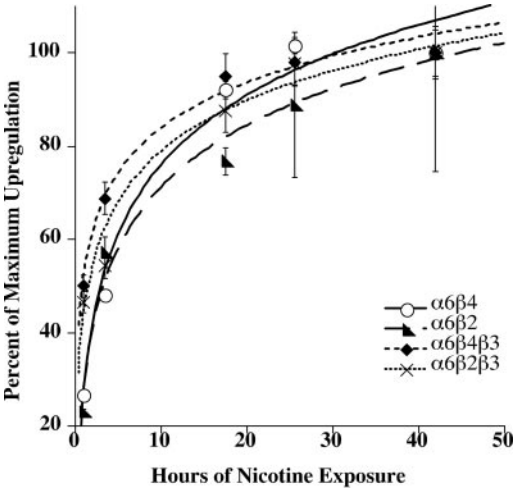

Fig. 2. Time course of nicotine-induced up-regulation of α6* AChRs. Cells were grown on 24-well tissue culture plates. All cells were grown for the same total time and fixed at the same time before assay. Nicotine (100 μM) was added at various times before fixation. The specific binding of 2 nM [^{3}H]epibatidine was determined. Specific binding for parallel culture not treated with nicotine was subtracted. Then, the amount of nicotine-induced binding for each line was expressed as a percentage of the maximum obtained for each line. Figure 1A shows the different base lines and maximum extents of up-regulation for each line. Expressing the data for all lines as a percentage of the maximum up-regulation allows the kinetics of up-regulation in all lines to be compared. Although the baseline and extent of up-regulation differed with each line, the time courses of up-regulation for all were the same. The values shown are mean ± S.E.M. for triplicate samples.

The relative amounts of α6 subunits in the four cell lines were compared by Western blotting (Fig. 3C). The α6β2 cell line expressed few native AChRs (Fig. 1A) and correspondingly contained few α6 subunits (Fig. 3C). Thus, there were not large pools of unassembled α6 subunits in the α6β2 line. The fact that transfection with β3 resulted in a greatly increased amount of α6 as seen in the α6β2β3 cell line suggests that unassembled α6 subunits and α6β2 AChRs turn over rapidly and that the presence of β3 subunits permits the formation of α6β2β3 AChRs, which are much more stable and therefore much more α6 accumulates.

The transmembrane orientation of the epitopes of the α6 mAbs was determined by Western blots (Fig. 3D). All six mAbs to α6 on Western blots recognized a chimera (α3/6) with the extracellular domain of α3 and the remainder of α6, but not a chimera (α6/3) with the extracellular domain of α6 and the remainder of α3 (Fig. 3D). This indicates that the epitopes recognized by the mAbs are located C-terminal of the extracellular domain in the α6 subunit.

The six mAbs to bacterially expressed (denatured) α6 also immunoprecipitate native human α6 AChRs, with mAbs 338, 350, and 351 being the most potent (Table 1). Three of these mAbs also cross-reacted weakly with AChRs from rat brains. This was tested using high (25 μg) amounts of mAbs in a 200-μl reaction mix containing 0.2 nM [^{3}H]epibatidine-labeled rat brain AChRs precipitable by mAb 295 to β2 subunits. Of these AChRs, 4% were bound by mAb 350, and approximately 0.25% was bound by mAbs 338 and 351.

Immunoadsorption demonstrated that β3 subunits were incorporated into α6 AChRs (Fig. 4), as would be expected from the large increase of expression in the presence of β3 (Fig. 1). The putative main immunogenic region (MIR) sequence 66-76 of human α1 subunits (KWNPDDYGGVK) is closely related to sequences on human α3 (KWNPSDYGGAE), α5 (RWNPDDYGGIK), and β3 (RWNPDDYGGIH). mAb 210 was made to muscle type AChRs, competes for binding to them with other mAbs to the MIR, and also binds to denatured α1 (Lindstrom, 2000). In addition, it binds very well to native human α3 AChRs (Wang et al., 1998), but it does not bind well to denatured α3. mAb 210 also binds to native but not denatured human α5 (Kuryatov et al., 1997). mAb 210 exhibited low affinity for direct immunoprecipitation of α6β2β3 or α6β4β3 AChRs, but mAb 210 coupled to agarose efficiently adsorbed α6β2β3 and α6β4β3 AChRs. It did not bind α6β2 or α6β4 AChRs. Figure 4 shows that 40% of α6β2β3 and 60% of α6β4β3 AChRs precipitated by mAb 338 could be adsorbed by mAb 210 coupled to agarose. This probably underestimates the actual percentage of these AChRs, which contain β3 because of the low affinity of mAb 210 for β3 in these α6 AChRs. Because mAb 210 binds efficiently only to native α3, α5, and β3, but to both native and denatured α1, probably both the sequence, and, especially, the conformation of this MIR epitope are important for binding of mAb210 to neuronal AChRs.

Sedimentation of α6*AChRs on Sucrose Gradients. We have observed previously that, when expressed in *X.*

laevis oocytes, α3β2 AChRs sedimented on sucrose gradients as a uniform 11S component intermediate in size between monopentamers and dipentamers of *T. californica* electric organ α1 AChRs, whereas α6β2 AChRs assembled efficiently to form epibatidine binding sites within the oocytes, but these sedimented as large amorphous aggregates (Kuryatov et al., 2000). When expressed in HEK cells, the α6β2 combination results in only small amounts of epibatidine binding sites, but most of these are on the cell surface (Fig. 1). Most of these α6β2 AChRs sediment as components larger than 11S (Fig. 5), and substantial amounts pelleted in the centrifuge tube. Monopentamers were not clearly resolved from aggregates. Thus, α6β2 AChRs do not efficiently form stable pentamers and may partially dissociate and aggregate. This disruption probably occurs during solubilization in Triton X-100, because the efficient expression of α6β2 AChRs on the cell surface (Fig. 1C) suggests that in the membrane most were pentamers able to pass quality control analysis for exit from the endoplasmic reticulum and transport through the Golgi apparatus to the cell surface. Incorporation of the β3 subunit to form α6β2β3 AChRs not only results in assembly of many more AChRs (Fig. 1) but also most of these sediment as a component that is nearly the size of 9.5S *T. californica* AChR monopentamers (Fig. 5). Thus, the presence of β3 accessory subunits permits the efficient assembly of stable pentameric AChRs. Both α6β4 and α6β4β3 AChRs expressed in the cell lines sediment as a mixture of components corresponding to monopentamers and larger aggregates (Fig. 5). The larger proportion of aggregates may bias the monopentamer peak to seem to sediment more rapidly than in the case of α6β2β3 AChRs. The formation of aggregates on sucrose gradients that is prominent with α6* AChRs is not observed with α1*, α3β2, or α4β2 AChRs (Kuryatov et al., 2000, 2005).

Agonist Binding to α6 AChRs. Figure 6 shows the concentration dependence of [^{3}H]epibatidine binding to all four α6* AChR subtypes. The K_D for binding of epibatidine to α6β2 AChRs (0.154 nM) was basically the same as the K_D for binding to α6β2β3 AChRs (0.198 nM), α6β4 AChRs (0.162 nM), or α6β4β3 AChRs (0.130 nM). This compares to the K_D for binding of epibatidine to human α4β2 AChRs similarly expressed and measured (0.017 nM) (Kuryatov et al., 2005). Thus, the presence of β3 does not alter equilibrium binding affinity of α6β2* or α6β4* AChRs for epibatidine. All four α6* AChR subtypes exhibit much lower affinity for epibatidine than do α4β2 AChRs.

Agonists were tested for their ability to inhibit the binding of [^{3}H]epibatidine to fixed cells (Table 2). In general, the

TABLE 1

Immunoprecipitation of native [^{3}H]epibatidine-labeled human AChRs by mAbs to α6 subunits

The cell lines expressing α3β2 and α4β2 AChRs were described previously (Wang et al., 1998; Kuryatov et al., 2005). A 0.5 nM concentration of AChRs and 1.5 nM [^{3}H]epibatidine were used in these assays using triplicate samples.

AChR	Titer of mAb					
	mAb 338	mAb 339	mAb 349	mAb 350	mAb 351	mAb 353
	μmol [^{3}H]epibatidine binding sites/l mAb					
α3β2	0	0	0	0	0	0
α4β2	0	0	0	0	0	0
α6β2	4.1	0.09	0.3	5.7	6	0

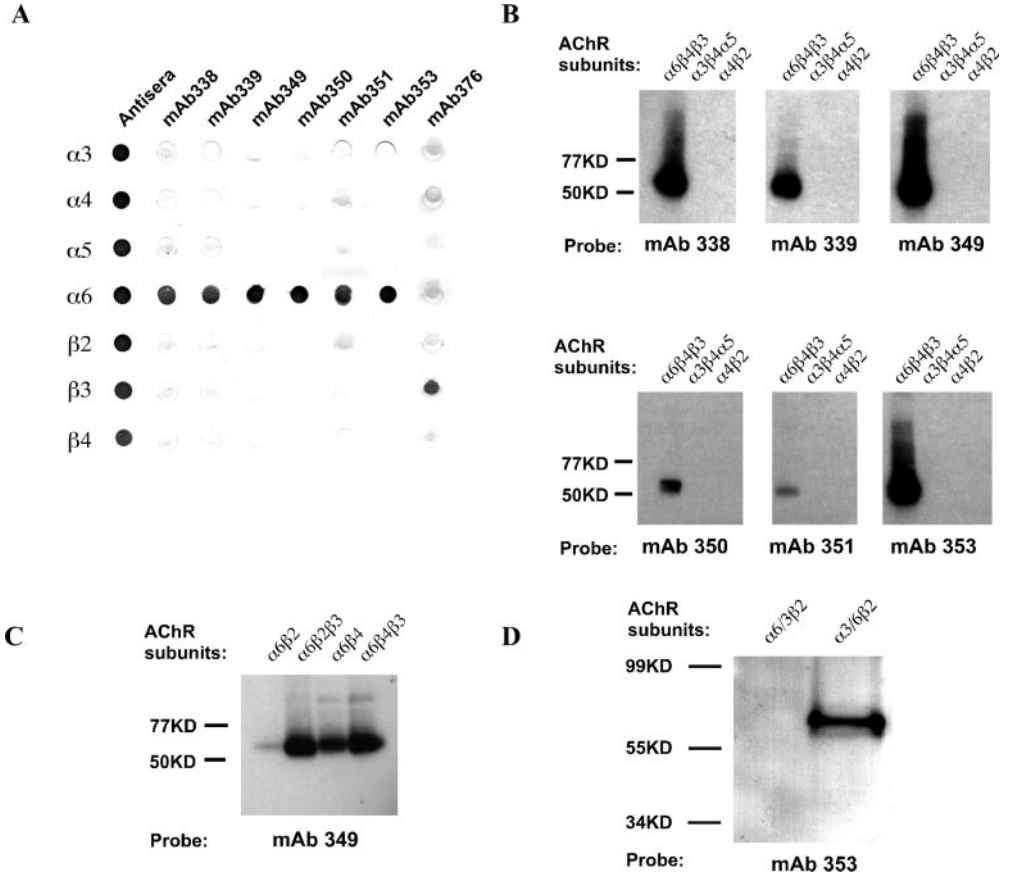

Fig. 3. Reaction of mAbs to α6 and β3 with denatured subunits on blots. A, subunit specificity of the mAbs was demonstrated by dot blot assays using bacterially expressed human AChR subunit constructs. Reaction is compared with that of antiserum to each of the subunit constructs to demonstrate equal loading of the blots of all the subunits and thus the high specificity of the mAbs. B, specificity of the mAbs was further evaluated using Western blots of equal amounts of AChRs from HEK cell lines transfected with human α6β4β3, α3β4α5, or α4β2 AChRs. The mAbs reacted strongly only with α6 subunits and not at all with the other subunits present. C, extent of expression of α6 subunits in the four α6* AChR cell lines was compared using an equal amount of protein from each line and mAb 349. mAbs were used at a 1/1000 dilution and ^{125}I-goat anti-mouse IgG purified antibodies were used at 2 nM. D, reaction of mAb 353 with chimeras (Kuryatov et al., 2000) consisting of the extracellular domains of α3 or α6 in combination with the remainder of the other of these subunits on Western blots. The transmembrane orientation of the epitopes of the α6 mAbs was determined using Western blots of equal amounts of chimeric AChRs from *X. laevis* oocytes injected with 25 ng per subunit of cRNA for the subunit combinations of α3/6β2 or α6/3β2. All six mAbs to α6 reacted strongly with chimeric α3/6 subunits and not at all with α6/3 subunits. Only the Western blot of mAb 353 is shown.

β2-containing α6* AChRs exhibited higher affinities for agonists. Cytisine discriminated by more than 7-fold in affinity between α6β2* and α6β4* AChRs. The affinity for nicotine is not significantly altered by the presence or absence of β3 subunits.

Both equilibrium binding studies of epibatidine (Fig. 6) and competitive binding studies with nicotine and other ligands (Table 2) indicate that the presence of β3 does not alter the ACh binding site or its affinity for nicotine. This is not surprising, because β3 assembles in the accessory position and is not part of an ACh binding site.

***β3* Greatly Increased Sensitivity of *α6** AChRs to Nicotine-Induced Up-Regulation.** Table 3 shows that the presence of β3 increased the sensitivity to nicotine-induced up-regulation of α6β2β3 by 11-fold compared with α6β2 AChRs and of α6β4β3 AChRs by 6.6-fold compared with α6β4 AChRs. The binding data of Fig. 6 and Table 2 show that the presence of β3 does not alter the ACh binding sites and greatly increases affinity for nicotine. So, how might β3 have such a large effect on nicotine-induced up-regulation? Nicotine probably acts on α6* AChRs as a molecular chaperone, as it does on α4β2 AChRs (Kuryatov et al., 2005; Sallette et al., 2005). Binding of nicotine to α6β2α6β2 or α6β4α6β4 assembly intermediates could produce activated or desensitized conformations that would assemble more efficiently with β3 than β2 or β4 subunits in the accessory position in a final assembly step to form mature pentamers. The greater AChR expression observed with β3-containing AChRs in the absence of nicotine (Fig. 1) shows that β3 promotes assembly of mature AChRs. On the other hand, nicotine could act as a molecular chaperone on α6β2β3 or α6β4β3assembly intermediates, and the presence of β3 could promote conformational changes to the active or desensitized conformations, which assemble more efficiently. The accessory subunit α5 influences the sensitivity to activation and desensitization of α3* AChRs (Gerzanich et al., 1998).

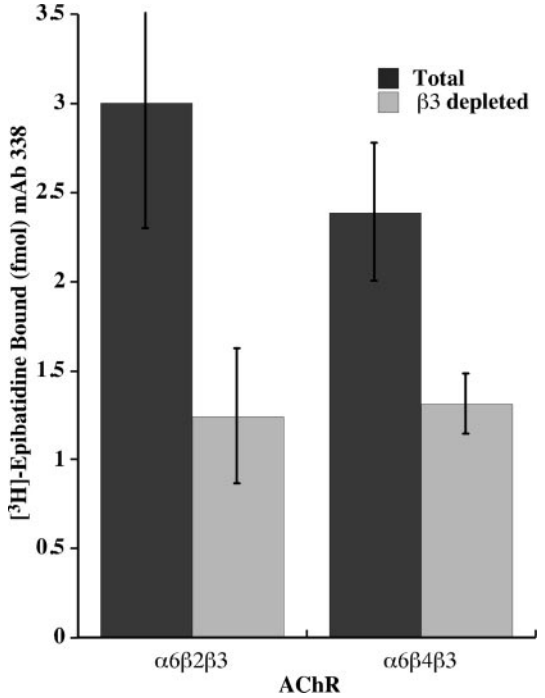

Fig. 4. Incorporation of β3 into α6* AChRs can be detected by immunodepletion using mAb 210 to the MIR. mAb 338 to α6 subunits was used to precipitate AChRs labeled with [^{3}H]epibatidine before and after adsorption with mAb 210 coupled with agarose. The labeled AChRs coupled to mAb 210-agarose were eluted and counted as well. mAb 210 was made to muscle AChR and binds to the MIR on α1 subunits, which includes a sequence similar to that on β3 subunits. mAb 210 agarose does not bind to α6β2 or α6β4 AChRs (data not shown). The values shown are mean ± S.E.M. for triplicate samples.

The EC_{50} for up-regulation by nicotine of α6β2 AChRs (9.8 μM) indicates that at the 0.1 to 0.2 μM concentrations of nicotine sustained in the sera of cigarette smokers (Benowitz, 1996), up-regulation of this subtype would be negligible. By contrast, the EC_{50} for α6β2β3 (0.89 μM) reveals that the presence of β3 confers an order of magnitude more sensitivity to nicotine-induced up-regulation, sufficient to suggest that some up-regulation might occur in a smoker. Still, the sensitivity is much less than that of human α4β2 AChRs expressed in tsA201 HEK cells (EC_{50} = 0.039 μM) (Kuryatov et al., 2005), an AChR subtype that has much higher affinity for nicotine (K_D = 0.0028 μM). β3 also increases the sensitivity to nicotine-induced up-regulation of α6β4β3 AChRs from EC_{50} = 3.55 μM for α6β4 AChRs to EC_{50} = 0.54 μM for α6β4β3 AChRs.

Unlike human α3 AChRs expressed in HEK cell lines, where only β2-containing but not β4 containing AChRs are subject to nicotine-induced up-regulation (Wang et al., 1998), α6* AChRs containing either β2 or β4 subunits are sensitive to nicotine-induced up-regulation. The extent of up-regulation is greater for α6β2β3AChRs than for α6β4β3 AChRs, presumably reflecting the lower baseline assembly efficiency of β2 than β4 subunits (Wang et al., 1998; Sallette et al., 2004).

Culture at 29°C in Combination with Nicotine Dramatically Up-Regulates Expression of *α6β2* AChRs. Cooper et al. (1999) initially observed that culture at 30°C increased expression of α4β2 AChRs and proposed that this resulted from increased assembly and/or slower turnover. The low amount of α6 subunits in the α6β2 cell line by contrast with the large amount after transfection of this line with β3 (Fig. 3C) suggests that unassembled α6 and α6β2 AChRs are unstable and that transfection with β3 promotes assembly and/or stabilizes the resulting AChRs. The large amount of α6 in the other lines shows that, if the AChRs are stabilized by the right subunit combination and ambient conditions, the α6 expression vector can result in substantial amounts of AChRs. Culture at 29°C increased the amount of α6β2 AChRs to that obtained in the presence of β3, and it greatly increased the extent and sensitivity to nicotine-induced up-regulation, somewhat exceeding the effect of β3 on the amount of AChR (Fig. 7). α6β4 AChRs were expressed at a higher level than α6β2 at 37°C, so the effects of culture at 29°C were substantial but less. In the presence of β3, there was little effect on nicotine-induced up-regulation of α6β2β3 AChRs and virtually none on α6β4β3 AChRs. These results are consistent with the idea that low temperature greatly increases the stability of α6β2 assembly intermediates and AChRs, and it increases to a lesser extent the stability of the intrinsically more stable α6β4 assembly intermediates and AChRs, while also promoting their assembly synergistically with the pharmacological chaperone effects of nicotine.

The differences in α6β2 expression between HEK cells shown here and *X. laevis* oocytes (Kuryatov et al., 2000) demonstrate the importance of cell-specific factors on AChR assembly, and they suggest the possibility that in some cell

types (e.g., the aminergic neurons in which α6* AChRs are exclusively found in vivo) chaperone proteins might be able to produce effects equivalent to culture at 29°C.

Discussion

Four subtypes of human α6* AChRs (α6β2, α6β2β3, α6β4, and α6β4β3) were stably expressed in human tsA201 HEK cell lines, and six mAbs to human α6 and one mAb to human β3 subunits were made to aid in the characterization of α6* AChRs. These α6mAbs were found to be directed at sequences C-terminal of the extracellular domain. These lines will permit more detailed studies of these AChR subtypes than is possible with brain neurons containing small amounts of complex mixtures of AChR subtypes. Even more complex α6* AChR subtypes such as α6α4β2β3, which have been identified in brain and retina (Zoli et al., 2002; Champtiaux et al., 2003; Gotti et al., 2005a,b), will also need to be studied in transfected cell lines. α6* AChRs are potential drug targets, for example, in Parkinson's disease (Quik and McIntosh, 2006).

Host cell type influences the expression of AChRs, presumably as a reflection of differences the complement of chaperone proteins and enzymes for post-translational modification. For example, the chaperone protein Ric-3 is expressed in the human neuroblastoma cell line SH-SY5Y, which endogenously expresses α7 AChRs, but is not expressed in tsA201 HEK cells, which efficiently express transfected α7 AChRs only after cotransfection with Ric-3 (Landsdell et al., 2005; Williams et al., 2005). α6* AChRs have only been reported in aminergic neurons, which may contain particular chaperones for the assembly and transport of α6* AChRs.

Expression of α6β2 in *X. laevis* oocytes results in the formation of large amounts of epibatidine binding sites but no mature pentameric AChRs on the cell surface (Kuryatov et al., 2000). Instead, α6 and β2 subunits form amorphous intracellular aggregates. In HEK cells only small amounts of α6β2 AChRs are made, but a large proportion are on the cell surface. α6 is closely related in sequence to α3 (Lindstrom, 2000; Le Novere et al., 2002). α3β2 forms functional AChRs in oocytes (Gerzanich et al., 1998). In tsA201 HEK cell lines, α3β2 expresses less well than does α3β4 (Wang et al., 1998), resembling the relationship between α6β2 and α6β4. In HEK cell lines, α3β2 is greatly up-regulated by nicotine but human α3β4 is not (Wang et al., 1998). α6β4 is up-regulated by nicotine. Thus, there are both cell type and subunit-specific factors that influence expression.

Culture at 29°C dramatically increases the expression of α6β2 and its sensitivity to up-regulation by nicotine but has a smaller effect on α6β4 (that is intrinsically expressed at a higher level). The low temperature apparently greatly stabilizes α6β2 AChRs or their assembly intermediates, permitting the accumulation of large amounts under the influence of nicotine. Nicotine probably acts as a pharmacological chaperone (Kuryatov et al., 2005; Corringer et al., 2006). In HEK cells, nicotine dramatically increases the amount of α3β2 AChRs (22-fold) without causing a dramatic increase (perhaps 2-fold) in the amount of α3 subunits on Western blots (Wang et al., 1998), indicating that, as in an α4β2 line

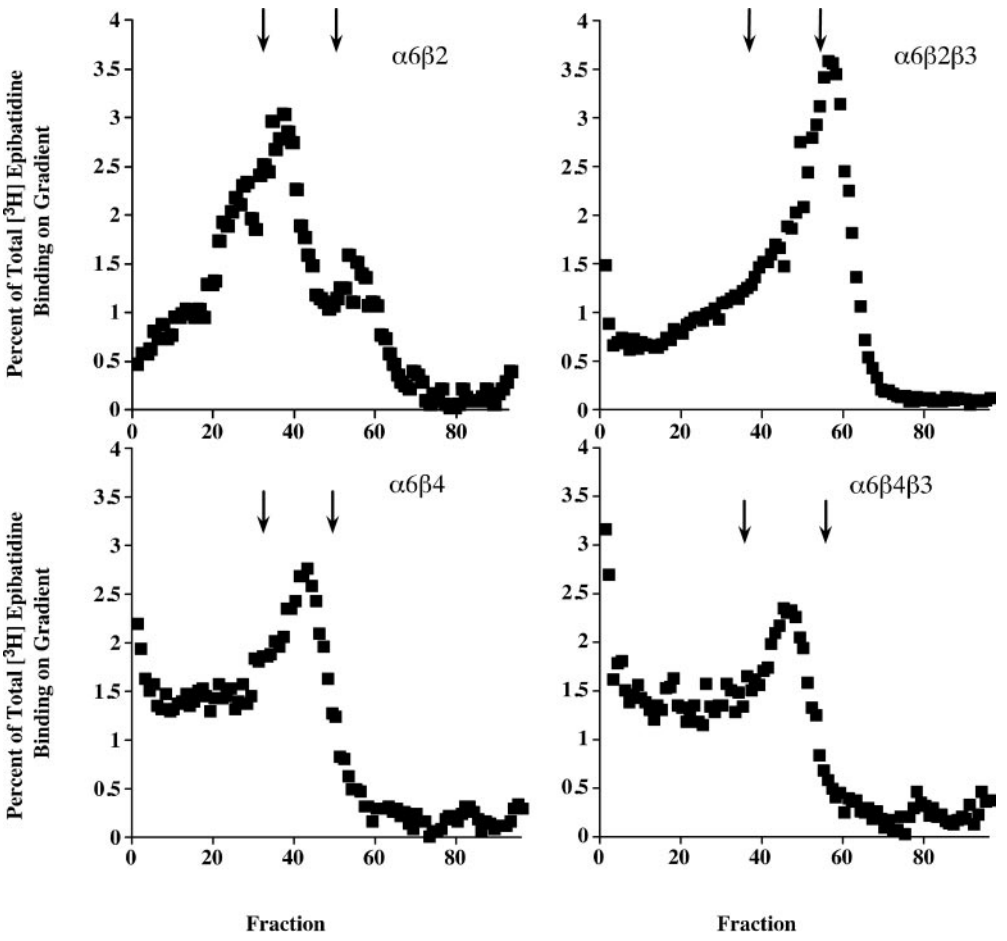

Fig. 5. Sucrose gradient sedimentation of α6* AChRs. Sedimentation on SW41 5 to 20% sucrose gradients used *T. californica* AChRs as internal standards. *T. californica* AChRs were immunoisolated from gradient fractions on wells coated with mAb 210 and then labeled with ^{125}I-α-bungarotoxin. Arrows indicate the positions of the peaks corresponding to the 9.5S monomers and 13S dimers of *T. californica* AChRs. α6β2 and α6β2β3 AChRs were isolated on microwells coated with mAb 295 to β2 subunits. α6β4 and α6β4β3 AChRs were isolated on microwells coated with mAb 338 to α6 subunits. Isolated α6* AChRs were labeled with [^{3}H]epibatidine. mAb 338 to α6 and mAb 337 to β4 subunits (Nelson et al., 2001) were equally effective when coated on microwells at adsorbing α6β4 and α6β4β3 AChRs. However, mAb 338 was much less effective at adsorbing α6β2 and α6β2β3 AChRs. Thus, mAb 295-coated wells were used. The epitope for mAb 338 is probably near the subunit interface and influenced by the β subunit present. The sucrose gradients shown were representative of several similar gradients analyzed.

(Kuryatov et al., 2005), there are large pools of unassembled $\alpha 3$ subunits present. By contrast, Western blots of $\alpha 6\beta 2$ reveal that only small amounts of $\alpha 6$ are present, even though transfection of this line with $\beta 3$ results in much larger amounts of $\alpha 6$, and parallel $\alpha 6\beta 4$ and $\alpha 6\beta 4\beta 3$ lines contain much more $\alpha 6$. Thus, unassembled $\alpha 6$ as well as $\alpha 6\beta 2$ AChRs must be relatively unstable. Using chimeras between $\alpha 6$ and $\alpha 3$ or $\alpha 4$ subunits, the part of the $\alpha 6$ subunit that limits expression of mature AChRs with $\beta 2$ in *X. laevis* oocytes was mapped to sequences C-terminal of the extracellular domain of $\alpha 6$ (Kuryatov et al., 2000). In $\alpha 6$* AChR-expressing neurons, there may be chaperones that have effects similar to culture at 29°C in promoting the assembly and stability of $\alpha 6\beta 2$ AChRs.

The presence of $\beta 3$ in the accessory position greatly increased expression of $\alpha 6\beta 2$ and $\alpha 6\beta 4$, increased sensitivity to up-regulation by nicotine, and negated any additional effect of culture at 29°C. In these respects, the effects of $\beta 3$ were more dramatic than the effects of $\alpha 5$ on $\alpha 3$ AChRs (Gerzanich et al., 1998; Wang et al., 1998). The effects of temperature, nicotine, and subunit composition suggest that regulation of AChR expression in these cell lines occurs primarily at the post-translational level. Studies of the amount of AChR $\alpha 4$ and $\beta 2$ subunit protein in the brains of AChR subunit knockout mice similarly demonstrate that in brain, the regulation of AChR expression also occurs primarily at the post-translational level (Whiteaker et al., 2006). In *X. laevis* oocytes (Peng et al., 1994), HEK cells (Kuryatov et al., 2005), and rodent brain (Flores et al., 1992), nicotine-induced up-regulation of $\alpha 4\beta 2$ AChRs occurs by post-translational mechanisms.

The complete aggregation of $\alpha 6\beta 2$ AChRs expressed in *X. laevis* oocytes (Kuryatov et al., 2000) and extensive aggregation of $\alpha 6$* AChR subtypes expressed in HEK cells suggest that this reflects a particular property of $\alpha 6$ subunits. The detergent Triton X-100 used for solubilization may partially

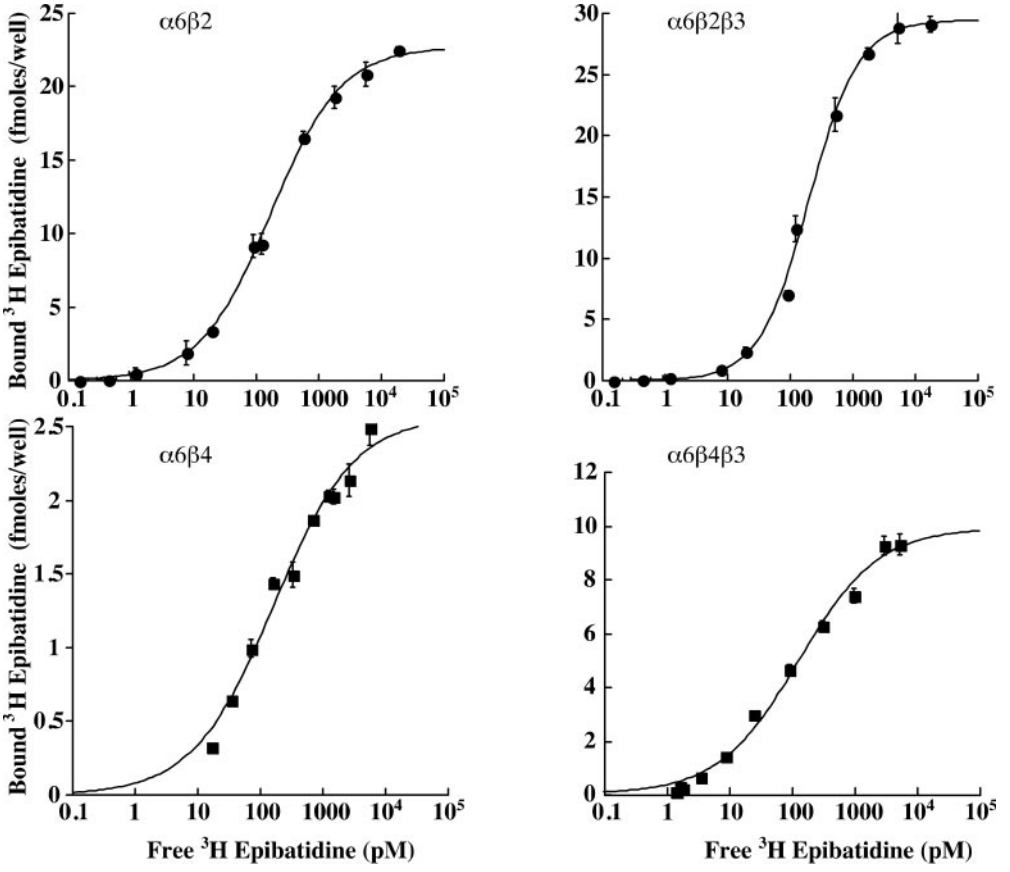

Fig. 6. Concentration dependence of binding of [^{3}H]epibatidine by cell lines. $\alpha 6\beta 2$* AChR cells were fixed after up-regulation overnight with nicotine (100 μM in the case of $\alpha 6\beta 2$ or 10 μM in the case of $\alpha 6\beta 2\beta 3$) to provide convenient amounts of AChRs for assay. $\alpha 6\beta 4$ and $\alpha 6\beta 4\beta 3$ cells did not require up-regulation. Binding was assayed using the indicated concentrations of [^{3}H]epibatidine. Background binding, assayed in the presence of 1 mM nicotine, was subtracted. The values shown are mean ± S.E.M. for quadruplicate assays.

TABLE 2

Relative affinities of $\alpha 6$* AChR subtypes for agonists

Serial dilutions of agonists were applied with [^{3}H]epibatidine at 2 nM to cells fixed with formalin on Costar 96-well white with clear-bottom plates. The concentration-response curves were fitted using a nonlinear least-squares error curve fit method (KaleidaGraph; Abelbeck/Synergy Software, Reading, PA) to the Hill equation $I(x) = I_{max}x^{nH}/(x^{nH} + IC_{50}{}^{nH})$. Values are shown ± S.E.M.

AChR	IC_{50}			
	ACh	Nicotine	DMPP	Cytisine
		μM		
$\alpha 6\beta 2$	2.82 ± 1.04	0.72 ± 0.16	0.51 ± 0.01	0.25 ± 0.10
$\alpha 6\beta 2\beta 3$	1.52 ± 0.01	0.40 ± 0.05	0.93 ± 0.06	0.25 ± 0.01
$\alpha 6\beta 4$	6.30 ± 0.64	1.79 ± 0.17	1.02 ± 0.22	1.92 ± 0.28
$\alpha 6\beta 4\beta 3$	6.88 ± 0.24	1.39 ± 0.03	2.08 ± 0.12	4.48 ± 0.27

DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide.

dissociate some α6 AChR subtypes and subsequent reaggregation might account for the sedimentation properties observed. The presence of β3 greatly increased the proportion of monopentamers in α6β2β3 AChRs compared with α6β2 AChRs, perhaps by stabilizing them against dissociation. It will be interesting to determine whether substitution of one of the two α6 subunits in an AChR pentamer by an α4 or α3 subunit to produce the most abundant and complex subtypes of brain α6* AChRs might reduce or eliminate aggregation of the resulting AChRs, reflecting a stabilizing influence on pentamers.

The presence of β3 subunits increased sensitivity to up-regulation by nicotine of α6* AChRs to the range where the 0.1 to 0.2 μM concentration of nicotine sustained in the sera of cigarette smokers (Benowitz, 1996) would be expected to cause some up-regulation of these α6* AChR subtypes in brain. However, these α6* AChRs are much less sensitive to up-regulation than are α4β2 AChRs (Kuryatov et al., 2005).

Parker et al. (2004) reported selective up-regulation of α6β2 AChRs compared with α4β2 AChRs in rat brain as a result of long-term self-administration of nicotine. On the contrary, reduction in the amount of α6* AChRs after nicotine treatment in various other ways has been reported in rats, mice, and monkeys (Lai et al., 2005; McCallum et al., 2006; Mugnaini et al., 2006). How can these apparently contradictory results on the ability of nicotine to up-regulate α6* AChRs be explained? Dopaminergic neurons in the ventral tegmental area of rodents express a mixture of α4, α6, β2, and β3 subunits and preferentially transport α4α6β2β3 to their nerve endings in the striatum but express α4β2 AChRs on the cell bodies (Zoli et al., 2002; Champtiaux et al., 2003; Gotti et al., 2005b; Quik et al., 2005). In primates, α3 subunits are also involved, and there is a lower proportion of α4β2 in the endings. In monkey striatum, 70% of nicotine-evoked dopamine release is mediated by α6β2* and/or α3β2* AChRs, whereas in rodents α6β2* AChRs mediate only 30% (Quik and McIntosh, 2006). This complex mixture of AChRs subunits competes for assembly in the endoplasmic reticulum of these neurons. Nicotine acts as a molecular chaperone to promote assembly of AChRs by binding to assembly intermediates (Kuryatov et al., 2005; Sallette et al., 2005; Corringer et al., 2006). The mean plasma nicotine concentration in monkeys given nicotine in their drinking water was 51 nM (McCallum et al., 2006). This would efficiently promote up-regulation of human α4β2 AChRs expressed in HEK cells (EC_{50} = 35 nM; Kuryatov et al., 2005), but it would have little effect on α6β2β3 AChRs (EC_{50} = 890 nM). If the amount of β2 subunits were limiting, at low nicotine concentrations, increased assembly of α4β2 AChRs could decrease assembly of α6β2β3 AChRs by depleting the pool of β2 subunits. Nicotine-induced slowing of mature AChR destruction (Kuryatov et al., 2005) could also up-regulate AChRs in nerve endings as well as on the cell body. Up-regulation by this

TABLE 3

Sensitivity to nicotine-induced up-regulation

Serial dilutions of nicotine were added to cells close to confluence, and the cells were incubated overnight on Costar 96-well white with clear-bottom plates. The cells were fixed with formalin and incubated with 2 nM [^{3}H]epibatidine. The concentration-response curves were fitted using a nonlinear least-squares error curve fit method (KaleidaGraph; Abelbeck software) to the Hill equation: $I(x) = I_{max}x^{nH}/(x^{nH} + EC_{50}{}^{nH})$. Values are shown ± S.E.M.

AChR	EC_{50}	B_{max}
	μM	*fmol/well*
α6β2	9.84 ± 0.03	1.13 ± 0.08
α6β2β3	0.89 ± 0.29	4.56 ± 0.75
α6β4	3.55 ± 0.62	21.3 ± 1.7
α6β4β3	0.54 ± 0.13	42.0 ± 4.1

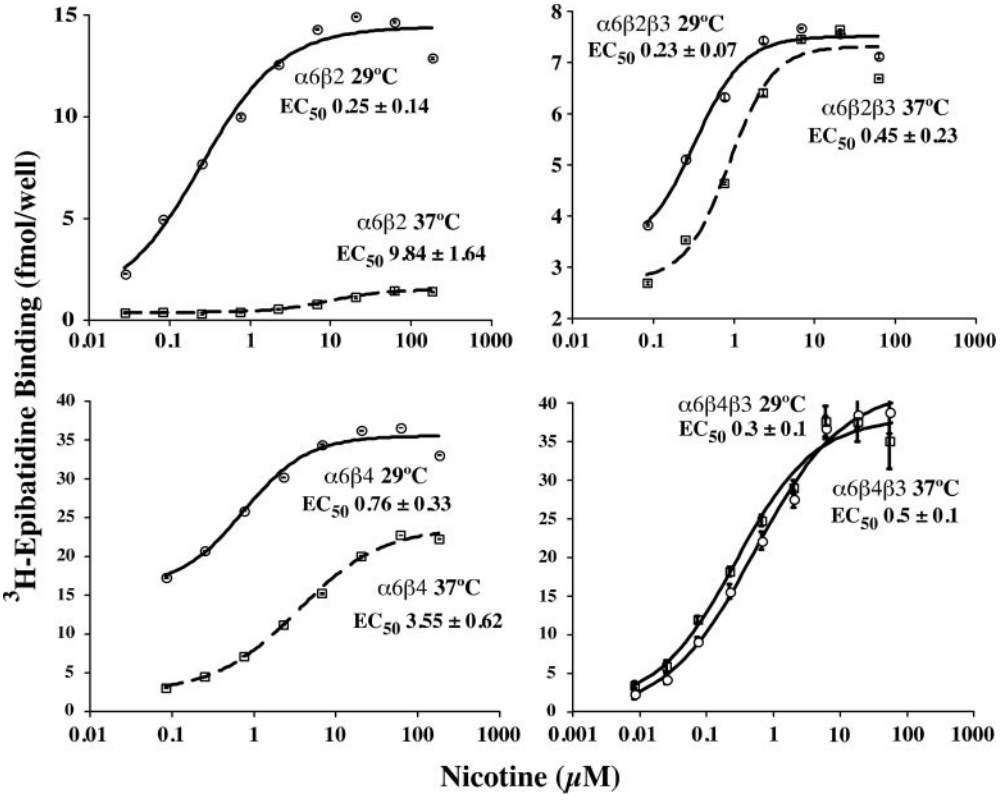

Fig. 7. Culture at 29°C greatly increased expression and sensitivity to nicotine-induced up-regulation of α6β2 and α6β4 AChRs. The indicated cultures were shifted to 29°C for the 12- to 15-h period during which nicotine was applied. Then, cells were fixed before measuring binding of [^{3}H]epibatidine applied at 2 nM. The values shown are mean ± S.E.M. for quadruplicate assays.

mechanism might also selectively affect high affinity $\alpha4\beta2^*$ AChRs. Most nicotine-induced brain AChR up-regulation is of highly sensitive $\alpha4\beta2$ AChRs (Flores et al., 1992). Autonomic ganglia express a mixture of $\alpha3$, $\beta2$, $\beta4$, and $\alpha5$ subunits but preferentially assemble $\alpha3\beta4$ AChRs (Davila-Garcia et al., 2003; Nguyen et al., 2003). The nicotine concentrations obtained in vivo result in negligible up-regulation of the less sensitive $\alpha3^*$ AChRs.

The large increase in amount of $\alpha6^*$ AChRs as well as their sensitivity to nicotine-induced up-regulation that results from the $\beta3$ subunit is consistent with the hypothesis that assembly of the $\beta3$ subunit in the accessory position is the final step in assembly of mature AChRs. The $\beta3$ subunit seems to preferentially assemble in the accessory position over $\beta2$ or $\beta4$ to result in larger amounts of AChRs. $\beta3$-containing $\alpha6$ AChRs did not show increased affinity for nicotine. The increased sensitivity to up-regulation by nicotine in the presence of $\beta3$ could result from assembly intermediates such as $\alpha6\beta2$ or $\alpha6\beta2\alpha6\beta2$, assuming a nicotine-induced desensitized conformation that more efficiently assembles with $\beta3$. On the other hand, assembly intermediates incorporating $\beta3$, such as $\beta3\alpha6\beta2$, could more efficiently assume a nicotine-induced conformation that assembles more efficiently.

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