Molecular Pharmacology Fast Forward. Published on July 11, 2006 as doi: 10.1124/mol.106.027326
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MOLPHARM/2006/027326

β3 Subunits Promote Expression and Nicotine-Induced Upregulation of Human Nicotinic α6* AChRs Expressed in Transfected Cell Lines

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MOLPHARM/2006/027326

Running Title: α6 AChRs

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Number of text pages: 44

Number of tables: 3

Number of figures: 7

Numbers of references: 40

Words in abstract: 247

Words in introduction: 739

Words in discussion: 1495

ABBREVIATIONS: AChR, acetylcholine receptor; αBgt, α bungarotoxin; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; MIR, main immunogenic region; mAb, monoclonal antibody; PBS, phosphate buffered saline; TPBS, 0.5% Triton X-100 in PBS

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 ACh 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 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 upregulation was not due to a β3 induced increase in affinity for nicotine, but probably due to a direct effect on assembly of AChR subunits. HEK cells express only a small amount of mature α6β2 AChRs, but many of these are on the cell surface. This contrasts with *Xenopus* oocytes which express a large amount of incorrectly assembled α6β2 subunits which bind cholinergic ligands but form large amorphous intracellular aggregates. 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 upregulation by nicotine.

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Nicotinic acetylcholine receptors (AChRs) are composed of five homologous subunits (Sine and Engel, 2006; Lindstrom, 2000). 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 et al., 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 upregulates 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; Salette et al., 2005; Kuryatov at al., 2005; Corringer et al., 2006).

Putative assembly intermediates the size of α4β2α4β2 tetramers have been identified which could assemble with accessory subunits in a final step to produce mature AChRs (Kuryatov et al., 2005). Nicotine also contributes to upregulation by increasing the lifetime of surface membrane AChRs (Kuryatov et al., 2005). Nicotine applied to transfected HEK cell lines for five days upregulated 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* oocytes, human α6β4 AChRs were functional and α6β4β3 AChRs were expressed at a higher level (Kuryatov et al., 2000). Although co-expression 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 *Xenopus* oocytes (Wang et al.,

1996; Gerzanich et al., 1998), but in HEK cells they do not assemble efficiently unless upregulation is induced by nicotine or culture at low temperatures (Wang et al., 1998). Co-expression in HEK cells of α3β2 with α5 increases expression, but co-expression of α3β4 with α5 somewhat decreases expression (Wang et al., 1998). In oocytes, α5 subunits alter desensitization, pharmacology, and Ca^{++} 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 upregulation 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 lab as previously described (Anand and Lindstrom, 1990; Gerzanich et al., 1997; Kuryatov et al. 2000). Human β3 was obtained from Christopher Grantham at the Janssen Research Foundation (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 Life Technologies.

Cell Line Culture and Transfection: Human embryonic kidney tsA201 cell lines (Margolskee et al., 1993) were maintained in Dulbecco Modified Eagle Medium (DMEM, high glucose) (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine at 37º C, 5% CO_2 at saturating humidity in a water jacket incubator. HEK cells were transfected using the Fugene 6 DNA Transfection Kit, 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 Blasticidin (5µg/ml) starting 72 hours after transfection. β2 and β4 cell lines were similarly selected with Geneticin (600 µg/ml). α6β2 and α6β4 AChRs were assayed for expression and screened by [^{3}H] epibatidine binding. Stably transfected α6β4 and α6β2 lines were co-transfected with human β3 (pcDNA 3.1/Zeocin(+)) to produce α6β4β3 and α6β2β3 AChRs. Selective pressure for cell lines containing β3 was applied using Zeocin (500 µg/ml) starting 72 hours after transfection. AChRs containing β3 were selected for high expression based on liquid phase radioimmune assays. β3 transfected cell lines were plated in serial dilution on Costar 96 well plates. Surviving colonies were plated individually to Costar 35mm culture plates and then grown to confluency. Cells were detached with ice cold DMEM and extracted as described above into a 1.5 ml microfuge tube. AChRs were incubated with 2.5 nM [^{3}H] epibatidine and 5 µl of β3 antiserum, acquired from Santa Cruz Biotechnologies (Santa Cruz, CA), for 1-2 hours at room temperature. AChRs were precipitated by secondary antibody

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incubation with rabbit anti-goat antiserum for 1-2 hours at room temperature and then pelleted at 13,000x *g* for 10 minutes. Pellets were washed three times with 1 ml of 0.5% Triton X-100 in PBS +10mM 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 ml of Optiphase 'SuperMix' (PerkinElmer Life Sciences) was added to each sample. Samples with scintillation fluid were set on rotation for 1 hour at room temperature before counting in a 1450 Microbeta Trilux Liquid Scintillation & Luminescence Counter (Perkin-Elmer Life 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 confluency on Costar 35 mm tissue culture plates (approximately 1x 10^6 cells/plate) and then detached by cold DMEM and collected by centrifugation at 500x *g*. Cells were washed once with 1 ml buffer A (50 mM $NaPO_4$ pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 15 mM iodoacetamide, 2 mM phenylmethylsulfonylflouride), pelleted by centrifugation at 13,000x *g* and then resuspended in buffer A. Cells in buffer A were incubated with 2 nM [^{3}H] epibatidine for 30 minutes at room temperature with agitation. Cell membrane fractions were washed three times with PBS +10 mM NaN_3 on Whatman glass filters (GF/F) pretreated with 1% polyethylenimine for 1 hour and dried on blotting paper. Filters containing AChRs bound to radioligands were counted in 1.5 ml microfuge tubes using 1ml of Optiphase SuperMix scintillation fluid in a 1450 Microbeta Trilux liquid scintillation counter (Perkin-Elmer Life Sciences). Results were standardized to the wet weight of the cells after the first wash in buffer A.

Previously Used Antisera and mAbs: 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 (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) 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 (Pharmacia) for purifying AChRs for use in immunization or with cell culture supernatants in protein A-coated microtiter wells for solid phase RIA.

Female BALB/c mice, 3-4 weeks of age, were obtained from Charles River Laboratories. All animals were handled in accordance with guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania under an approved protocol on file with that office. IACUC 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 three-week intervals with 40 μg per mouse of bacterially-expressed subunit constructs lacking the transmembrane domains of the subunits in TiterMax (TiterMax USA, Inc.). The titers were monitored by test bleeds against

corresponding subunits in ELISA assay. Five days after a final boost with antigen, the harvested splenic lymphocytes (~ 10^8) from the animal with the highest titers were fused with SP2/0 myeloma cells (1×10^8) using 50% polyethylene glycol (Kodak 1450) and inoculated into 24 96-well plates (Costar 3595).

Initial screening was done by ELISA assay as previously described (Kuryatov, et al., 2005). To eliminate mAbs which crossreacted with closely related subunits, the crossreaction of mAbs with a subunit which has the highest homology with the immunogen was tested (α3 in the case of α6 and α5 in the case of β3). mAbs which could bind both denatured subunits and native AChRs, were selected using [^{3}H] epibatidine-labelled native AChRs in RIAs, 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 (Zymed Laboratories) were 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 4,000x g, the supernatant was removed by aspiration. The pellet was washed with 1 ml of TPBS and then suspended in 100 µl of 0.1N NaOH. The suspension was mixed with 900 µl of Optiphase "SuperMix" scintillation fluid (Fisher Scientific). 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 were gravity-blotted onto

prewetted nitrocellulose membranes in 100 µl of TPBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) using a Bio-Dot apparatus (Bio-Rad). 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 hr with a 1µg/ml of 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 hr with a 1:2000 dilution of biotinylated goat anti-mouse IgG (Kirkegaard & Perry Laboratories), followed by a 1 hour incubation with peroxidase-labeled streptavidin (Kirkegaard & Perry Laboratories), and a final incubation in peroxidase substrate to visualize antibody binding. A 1:2000 dilution of biotinylated goat anti-rat IgG (Kirkegaard & 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 cm or 15 cm plates and then upregulated 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 500x *g*. DMEM was aspirated from the pellet. Cells were suspended with 1 ml of buffer A, then transferred to a 1.5 ml Eppendorf microfuge tube and collected via centrifugation at 13,000x *g* for 15 minutes at 4˚ C. Buffer A was aspirated from the pellets, which were then weighed. AChRs were extracted with three volumes of the pellet weight using buffer C (buffer A with 2% Triton X-100). The suspension was gently rotated for 1 hour at room temperature. Cell debris was pelleted by centrifugation at 13,000x *g* for 15 minutes

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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 *Xenopus* oocytes expressing either α6β2, chimeric α6/α3 β2, or chimeric α3/α6 β2 AChRs. Samples were electrophoresed in pre-cast Novex 10% polyacrylamide tris-glycine gels (San Diego, CA) under reducing conditions. Western transfer was done using a semi-dry electroblotting chamber (Semi-Phor, Hoefer Scientific Instruments, San Francisco, CA) to Trans-Blot ® Medium PVDF membrane (Bio-Rad, Hercules, CA). Blots were quenched with 5% dried milk (Carnation) 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 $2.5x10^{18}$ 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 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% confluency. Nicotine was added to induce upregulation of AChRs. Cells were incubated overnight at 37º C. Cells grown to confluency were exposed to one volume of 10% formalin added directly to the growth medium for 1 hour at room temperature in order to fix the cells to the wells. Cells were washed free of agonist and formalin with 200µl of PBS + 10 mM NaN_3 three times and stored at 4ºC with one 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 hours with gentle agitation. Plates were again washed to remove unbound ligands as above. [^{3}H] epibatidine was eluted from the AChRs by 50 µl of 0.1 N NaOH. 200 µl of Optiphase 'SuperMix' (PerkinElmer Life Sciences) scintillation fluid were added to each well. Plates were then shaken at room temperature for 1-2 hours before scintillation counting. Comparison of the maximum number of epibatidine binding sites in all four cell lines after upregulation 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 due to 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 5-20% sucrose gradient. Gradients were built in Beckman Quick-Seal centrifuge tubes (13x51 mm). 400 µl of cell extract were combined with 2 µl of extract of the electric organ from *Torpedo californica* (~1 µM αBgt binding sites) for an internal size marker and loaded onto the top of each gradient. Gradients were centrifuged at 40,000 rpm for 16 hours at 4º C in a Beckman XL-90 ultracentrifuge using a Beckman SW-41 rotor. After ultracentrifugation, the tubes were punctured and fractions were collected from the bottom into mAb coated Immulon flatbottom 4HBX wells using a fraction collector set to collect 10 drops per well (approx 130 µl/well). 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 4º C with gentle agitation. After incubation, 30 µl from each fraction were transferred onto an Immulon plate coated with mAb 210 to bind *Torpedo* AChRs, and 70 µl of 2% Triton X-100 in Buffer A were

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added. Solid phase radioimmuno assays were probed for 2 hours at room temperature with agitation with 2 nM [^{125}I] αBgt to detect *Torpedo californica* AChR binding on mAb 210 plates, or 2 nM [^{3}H] epibatidine to detect α6* AChR binding on mAb 295 or mAb 338 plates. Then plates were washed 3 times with TPBS. Radioligand was dissociated by denaturing the samples with 50 µl 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 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 5,000x g centrifugation for 5 minutes and then AChRs were precipitated with mAb 338. All samples were denatured with 50 µl 0.1 N NaOH for 5 minutes and then shaken with 1 ml Optiphase SuperMix for 2 hours. 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 above on 35mm dishes. One day prior to assay, nicotine was added into the growth media at a final concentration of 10 µM for β3-containing cells and 100 µM for others. Binding to living cells attached to 35 mm plates was done in DMEM at 4^{o}C for only 15 min with 1 nM [^{3}H] epibatidine to minimize ongoing upregulation and penetration of quaternary amines inside the

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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 1ml ice cold PBS with 5 mM EDTA and washed three times with 1ml ice cold PBS by centrifugation (5 min at 500x g) in Eppendorf tubes. The washed pellets were dissociated with 100 μl of 0.1M 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 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 to α3β2 (200 fmoles/mg, Wang et al., 1998) or α4β2 AChR HEK cell lines (900 fmol/mg, Kuryatov et al., 2005). Co-expression with β3 doubled the amount of AChR, much as co-expression with α5 doubled the amount of α3β2 AChRs (Wang et al., 1998). α6β4 expression was twice that of α6β2. Similarly, α3β4 expression in a HEK line exceeded α3β2 expression by four fold (Wang et al., 1998). The increased expression of β4-containing compared to β2-containing AChRs reflects less efficient assembly of β2 subunits due to a microdomain near ACh

binding site (Sallette et al., 2004), and results in increased sensitivity of β2–containing AChRs to the molecular chaperone effects of nicotine which selectivity promote increased assembly of β2-containing AChRs (Wang et al., 1998; Kuryatov et al., 2005; Fig 1). Co-expression with β3 increased α6β4 expression a further 6 fold. By contrast, co-expression 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, upregulation significantly increased surface expression in β2-containing cells (Fig. 1B). After upregulation a greater fraction of the AChRs was found inside the cells (Fig. 1C). This was also observed after nicotine-induced upregulation 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 upregulation by 100 μM nicotine was similar for all of the cell lines (Fig. 2). Upregulation was half complete within 3 hours and close to maximal by 24 hours. This is similar to the kinetics of upregulation 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 which cross reacted between subunits by dot blots of bacterially-expressed subunits were eliminated, leaving 6 mAbs to α6 and

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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 crossreaction 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.

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 6 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 (25μg) amounts of mAbs in a 200 μl reaction mix containing 0.2 nM

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[^{3}H] epibatidine labeled rat brain AChRs precipitable by mAb 295 to β2 subunits. Of these AChRs, 4% were bound by mAb 350, and about 0.25% were bound by mAbs 338 and 351.

Immune adsorption 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 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 immune precipitation 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 percent of these AChRs which contain β3 due to the low affinity of mAb 210 for β3 in these α6 AChRs. Since 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. Previously we observed that, when expressed in *Xenopus* oocytes, α3β2 AChRs sedimented on sucrose gradients

Molecular Pharmacology Fast Forward. Published on July 11, 2006 as DOI: 10.1124/mol.106.027326
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as a uniform 11 S component intermediate in size between monopentamers and dipentamers of *Torpedo* 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 11 S (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 endoplasmatic 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 which is nearly the size of 9.5 S *Torpedo* 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 a component corresponding to monopentamers and larger aggregates (Fig. 5). The larger proportion of aggregates may bias the monopentamer peak to appear to sediment more rapidly than in the case of α6β2β3 AChRs. The formation of aggregates on sucrose gradients which is prominent with α6* AChRs is not observed with α1*, α3β2, or α4β2 AChRs (Kuryatov et al., 2000; 2005).

Molecular Pharmacology Fast Forward. Published on July 11, 2006 as DOI: 10.1124/mol.106.027326
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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 of 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 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 Upregulation. Table 3 shows that the presence of β3 increased the sensitivity to nicotine-induced upregulation of α6β2β3 by 11 fold compared to α6β2 AChRs and of α6β4β3 AChRs by 6.6 fold compared to α6β4 AChRs. The binding data of Fig. 6 and the Table 2 show that the presence of β3 does not alter the ACh binding sites and greatly increase

affinity for nicotine. So how might β3 have such a large effect on nicotine-induced upregulation? 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 which 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. Alternatively, nicotine could act as a molecular chaperone on α6β2β3 or α6β4β3 assembly intermediates and the presence of β3 could promote conformation 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).

The EC_{50} for upregulation by nicotine of α6β2 AChRs (9.8 μM) indicates that at the 0.1-0.2 μM concentrations of nicotine sustained in the sera of cigarette smokers (Benowitz, 1996) upregulation 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 upregulation, sufficient to suggest that some upregulation 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 which has much higher affinity for nicotine (K_D = 0.0028 μM). β3 also increases the sensitivity to nicotine-induced upregulation of α6β4β3 AChRs from EC_{50} = 3.55 μM for α6β4 AChRs to EC_{50} = 0.54 μM for α6β4β3 AChRs.

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Unlike human α3 AChRs expressed in HEK cell lines, where only β2-containing but not β4 containing AChRs are subject to nicotine-induced upregulation (Wang et al., 1998), α6* AChRs containing either β2 or β4 subunits are sensitive to nicotine-induced upregulation. The extent of upregulation is greater for α6β2β3 AChRs than for α6β4β3 AChRs, presumably reflecting the lower base line assembly efficiency of β2 than β4 subunits (Wang et al., 1998; Sallette et al., 2004).

Culture at 29 °C in Combination with Nicotine Dramatically Upregulates 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 condition, 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 greatly increased the extent and sensitivity to nicotine-induced upregulation, 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 upregulation 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 increases to a lesser extent the stability of

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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 *Xenopus* oocytes (Kuryatov et al., 2000) demonstrate the importance of cell-specific factors on AChR assembly, and 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 α6 mAbs 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

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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 co-transfection with Ric-3 (Williams et al., 2005; Landsdell 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 *Xenopus* 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; LeNovere 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 upregulated by nicotine but human α3β4 is not (Wang et al., 1998). α6β4 is upregulated by nicotine. Thus, there are both cell type and subunit-specific factors which influence expression.

Culture at 29 °C dramatically increases the expression of α6β2 and its sensitivity to upregulation by nicotine, while having 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 (Kuryatov et al., 2005), there are large pools of unassembled α3 subunits present. By contrast, western blots of α6β2 reveal that only small amounts of α6 are present, even though transfection of this line with β3 results in much larger amounts of α6, and parallel α6β4 and α6β4β3 lines contain much more α6. Thus unassembled α6 as well as α6β2 AChRs must be relatively unstable. Using chimeras between α6 and α3 or α4 subunits, the part of the α6 subunit which limits expression of mature AChRs with β2 in *Xenopus* oocytes was mapped to sequences C-terminal of the extracellular domain of α6 (Kuryatov et al., 2000). In α6* AChR-expressing neurons there may be chaperones which have effects similar to culture at 29 °C in promoting the assembly and stability of α6β2 AChRs.

The presence of β3 in the accessory position greatly increased expression of α6β2 and α6β4, increased sensitivity to upregulation by nicotine, and negated any additional effect of culture at 29 °C. In these respects, the effects of β3 were more dramatic than the effects of α5 on α3 AChRs (Gerzanich et al., 1998; Wang et al., 1998). The effects of temperature, nicotine, and subunit composition suggest that in these cell lines regulation of AChR expression occurs primarily at the post translational level. Studies of the amount of AChR α4 and β2 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., submitted). In both *Xenopus*

oocytes (Peng et al., 1994), HEK cells (Kuryatov et al., 2005) and rodent brain (Flores et al., 1992) nicotine-induced upregulation of α4β2 AChRs occurs by post-translational mechanisms.

The complete aggregation of α6β2 AChRs expressed in *Xenopus* oocytes (Kuryatov et al., 2000) and extensive aggregation of α6* AChR subtypes expressed in HEK cells suggests that this reflects a particular property of α6 subunits. The detergent Triton X-100 used for solubilization may partially dissociate some α6 AChRs subtypes and subsequent re-aggregation might account for the sedimentation properties observed. The presence of β3 greatly increased the proportion of monopentamers in α6β2β3 AChRs compared to α6β2 AChRs, perhaps by stabilizing them against dissociation. It will be interesting to determine if 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 upregulation by nicotine of α6* AChRs to the range where the 0.1-0.2 μM concentration of nicotine sustained in the sera of cigarette smokers (Benowitz, 1996) would be expected to cause some upregulation of these α6* AChR subtypes in brain. However, these α6* AChRs are much less sensitive to upregulation than are α4β2 AChRs (Kuryatov et al., 2005).

Parker et al. (2004) reported selective upregulation of α6β2 AChRs compared to 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 upregulate 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 while expressing 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 upregulation of human α4β2 AChRs expressed in HEK cells (EC_{50}=35 nM, Kuryatov et al. (2005)), but 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 upregulate AChRs in nerve endings as well as on the cell body. Upregulation by this mechanism might also selectively affect high affinity α4β2* AChRs. Most nicotine-induced brain AChR upregulation is of highly sensitive α4β2 AChRs (Flores et al.,

1992). Autonomic ganglia express a mixture of α3, β2, β4, and α5 subunits, but preferentially assemble α3β4 AChRs (Davila-Garcia et al., 2003; Nguyen et al., 2003). The nicotine concentrations obtained in vivo result in negligible upregulation of the less sensitive α3* AChRs.

The large increase in amount of α6* AChRs as well as their sensitivity to nicotine-induced upregulation which results from the β3 subunit are consistent with the hypothesis that assembly of the β3 subunit in the accessory position is the final step in assembly of mature AChRs. β3 subunit appears to preferentially assemble in the accessory position over β2 or β4 to result in larger amounts of AChRs. β3-containing α6 AChRs did not show increased affinity for nicotine. The increased sensitivity to upregulation by nicotine in the presence of β3 could result from assembly intermediates such as α6β2 or α6β2α6β2 assuming a nicotine-induced desensitized conformation which more efficiently assembles with β3. Alternatively, assembly intermediates incorporating β3 such as β3α6β2 could more efficiently assume a nicotine-induced conformation which assembles more efficiently.

Molecular Pharmacology Fast Forward. Published on July 11, 2006 as DOI: 10.1124/mol.106.027326
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FOOTNOTES

This work was supported by grants to Jon Lindstrom from the National Institute of Health (NS11323) and the Phillip Morris External Research Program.

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Molecular Pharmacology Fast Forward. Published on July 11, 2006 as DOI: 10.1124/mol.106.027326
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LEGENDS FOR FIGURES

Fig. 1. Extent of α6* AChR expression in transfected cell lines. A. Total AChRs were determined by 2nM [^{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 upregulate 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 hour 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 upregulation overnight by 100 μM nicotine. Bars show measured values±S.E.M. for quadruplicate samples.

Fig. 2. Time course of nicotine-induced upregulation 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 prior to 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 percent of the maximum obtained for each line. Figure 1A shows the different base lines and maximum extents of upregulation

for each line. Expressing the data for all lines as a percent of the maximum upregulation allows the kinetics of upregulation in all lines to be compared. Although the base line and extent of upregulation differed with each line, the time courses of upregulation for all were the same. The values shown are mean ± S.E.M. for triplicate samples.

Fig. 3. Reaction of mAbs to α6 and β3 with denatured subunits on blots. A. The subunit specificity of the mAbs was demonstrated by dot blot assays using bacterially-expressed human AChR subunit constructs. Reaction is compared to 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. The 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. The 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 *Xenopus* 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.

Molecular Pharmacology Fast Forward. Published on July 11, 2006 as DOI: 10.1124/mol.106.027326
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Fig. 4. Incorporation of β3 into α6* AChRs can be detected by immune depletion 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.

Fig. 5. Sucrose gradient sedimentation of α6* AChRs. Sedimentation on SW41 5-20% sucrose gradients used *Torpedo* AChRs as internal standards. *Torpedo* AChRs were immunoisolated from gradient fractions on wells coated with mAb 210, then labeled with [^{125}I] α bungarotoxin. The positions of the peaks corresponding to the 9.5 S monomers and 13 S dimers of *Torpedo* AChRs are indicated by arrows. 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.

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Fig. 6. Concentration dependence of binding of [^{3}H] epibatidine by cell lines. α6β2* AChR cells were fixed after upregulation overnight with nicotine (100 μM in case of α6β2 or 10 μM in case of α6β2β3) to provide convenient amounts of AChRs for assay. α6β4 and α6β4β3 cells did not require upregulation. Binding was assayed using the indicated concentrations of [^{3}H] epibatidine. Background binding, assayed in the presence of 1mM nicotine, was subtracted. The values shown are mean ± S.E.M. for quadruplicate assays.

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

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TABLES

Table 1. Immune Precipitation 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 and Kuryatov et al., 2005). A 0.5 nM concentration of AChRs and 1.5 nM of [^{3}H]-epibatidine were used in these assays using triplicate samples.

AChR	Titer of mAb (μmol ^{3}H epibatidine binding sites/l mAb)					
	mAb 338	mAb 339	mAb 349	mAb 350	mAb 351	mAb 353
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

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Table 2. Relative Affinities of α6* 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 software) to Hill equation: $I(x) = I_{max}x^n/(x^n+IC_{50}{}^n)$. Values are shown ±S.E.M.

AChR	IC_{50} (μM)			
	ACh	Nicotine	DMPP	Cytisine
α6β2	2.82±1.04	0.72±0.16	0.51±0.01	0.25±0.10
α6β2β3	1.52±0.01	0.40±0.05	0.93±0.06	0.25±0.01
α6β4	6.30±0.64	1.79±0.17	1.02±0.22	1.92±0.28
α6β4β3	6.88±0.24	1.39±0.03	2.08±0.12	4.48±0.27

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Table 3. Sensitivity to Nicotine-Induced Upregulation

Serial dilutions of nicotine were added to cells close to confluency 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 Hill equation: $I(x) = I_{max}x^n/(x^n+EC_{50}{}^n)$. Values are shown ±S.E.M.

AChR	EC_{50}	Bmax
	(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

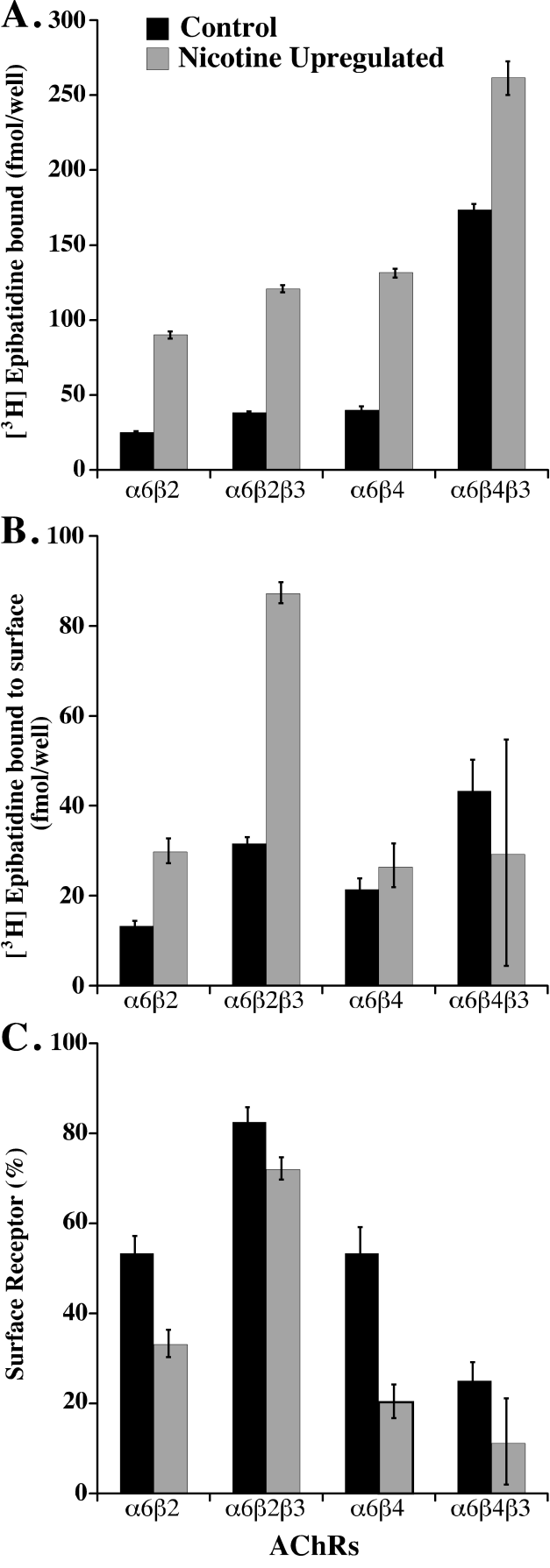
A.
Control
Nicotine Upregulated
[3H] Epibatidine bound (fmol/well)
300
250
200
150
100
50
0
α6β2
α6β2β3
α6β4
α6β4β3
B.
[3H] Epibatidine bound to surface (fmol/well)
100
80
60
40
20
0
α6β2
α6β2β3
α6β4
α6β4β3
C.
Surface Receptor (%)
100
80
60
40
20
0
α6β2
α6β2β3
α6β4
α6β4β3
AChRs

Figure 1

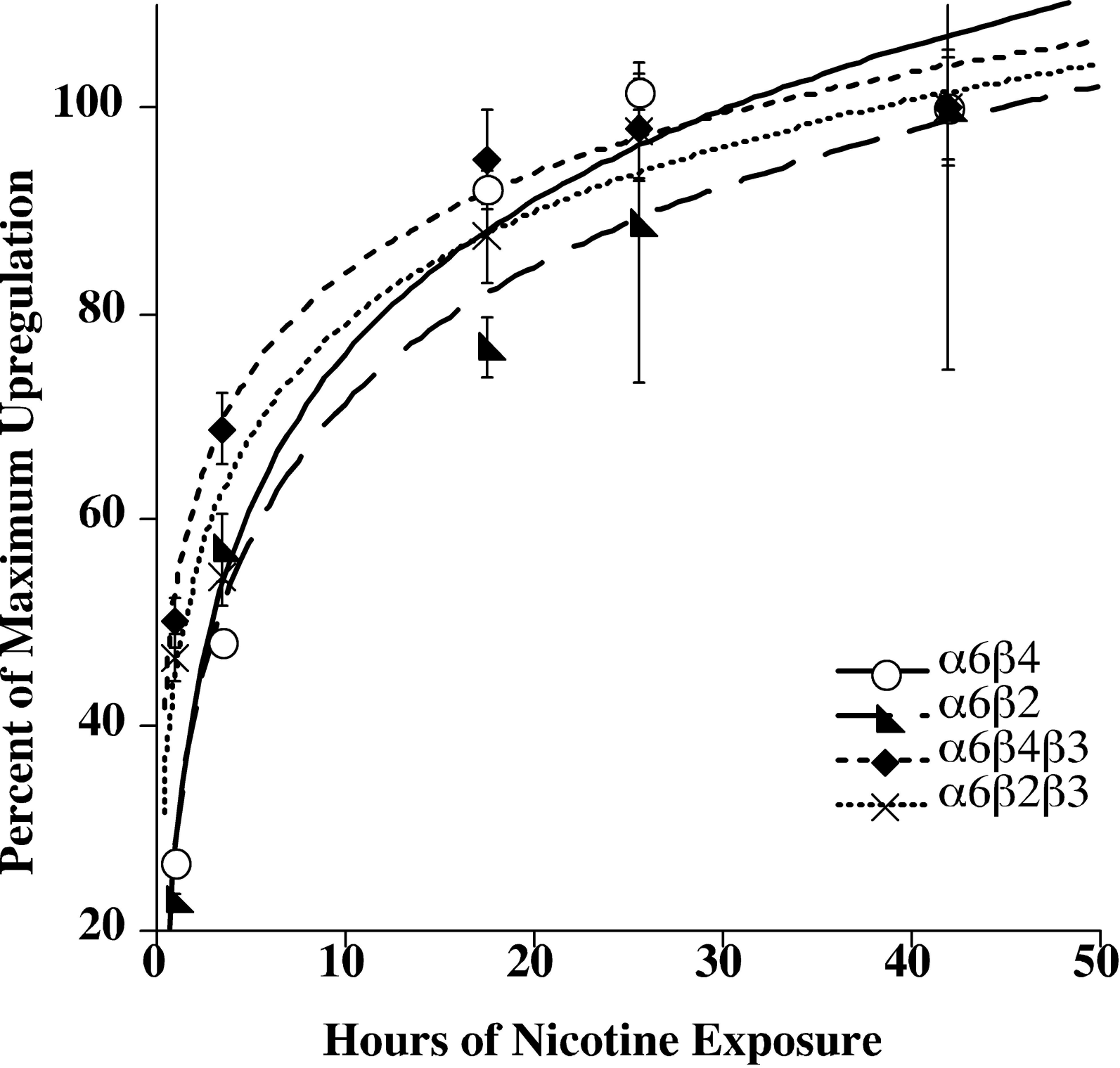
Percent of Maximum Upregulation
100
80
60
40
20
0
10
20
30
40
50
Hours of Nicotine Exposure
α6β4
α6β2
α6β4β3
α6β2β3

Figure 2

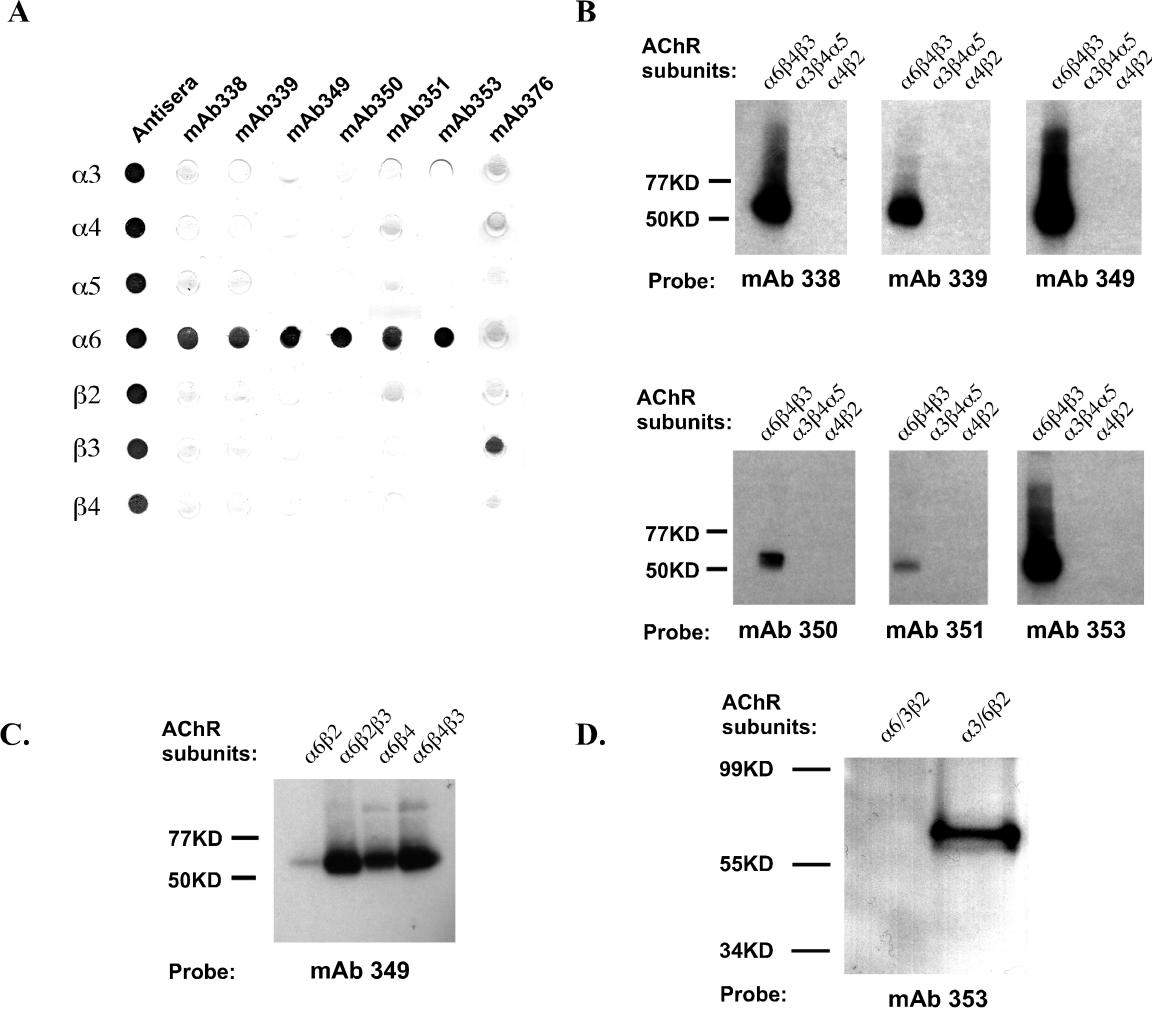
A
Antisera
mAb338
mAb339
mAb349
mAb350
mAb351
mAb353
mAb376
α3
α4
α5
α6
β2
β3
β4
B
AChR subunits:
α6β4β3
α3β4α5
α4β2
77KD
50KD
Probe:
mAb 338
mAb 339
mAb 349
mAb 350
mAb 351
mAb 353
C.
AChR subunits:
α6β2
α6β2β3
α6β4
α6β4β3
77KD
50KD
Probe:
mAb 349
D.
AChR subunits:
α6/3β2
α3/6β2
99KD
55KD
34KD
Probe:
mAb 353

Figure 3

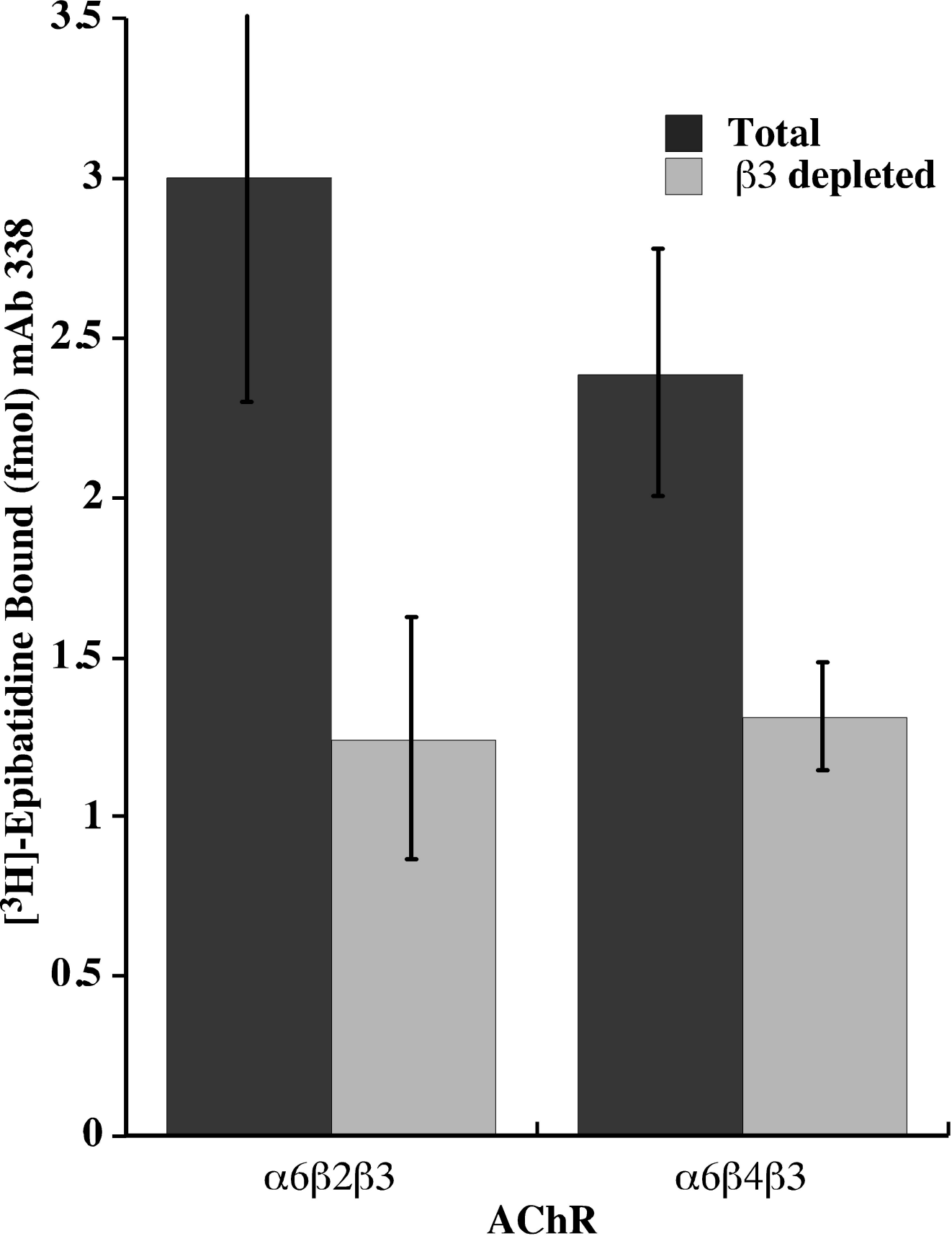
Total
β3 depleted
[3H]-Epibatidine Bound (fmol) mAb 338
3.5
3
2.5
2
1.5
1
0.5
0
α6β2β3
α6β4β3
AChR

Figure 4

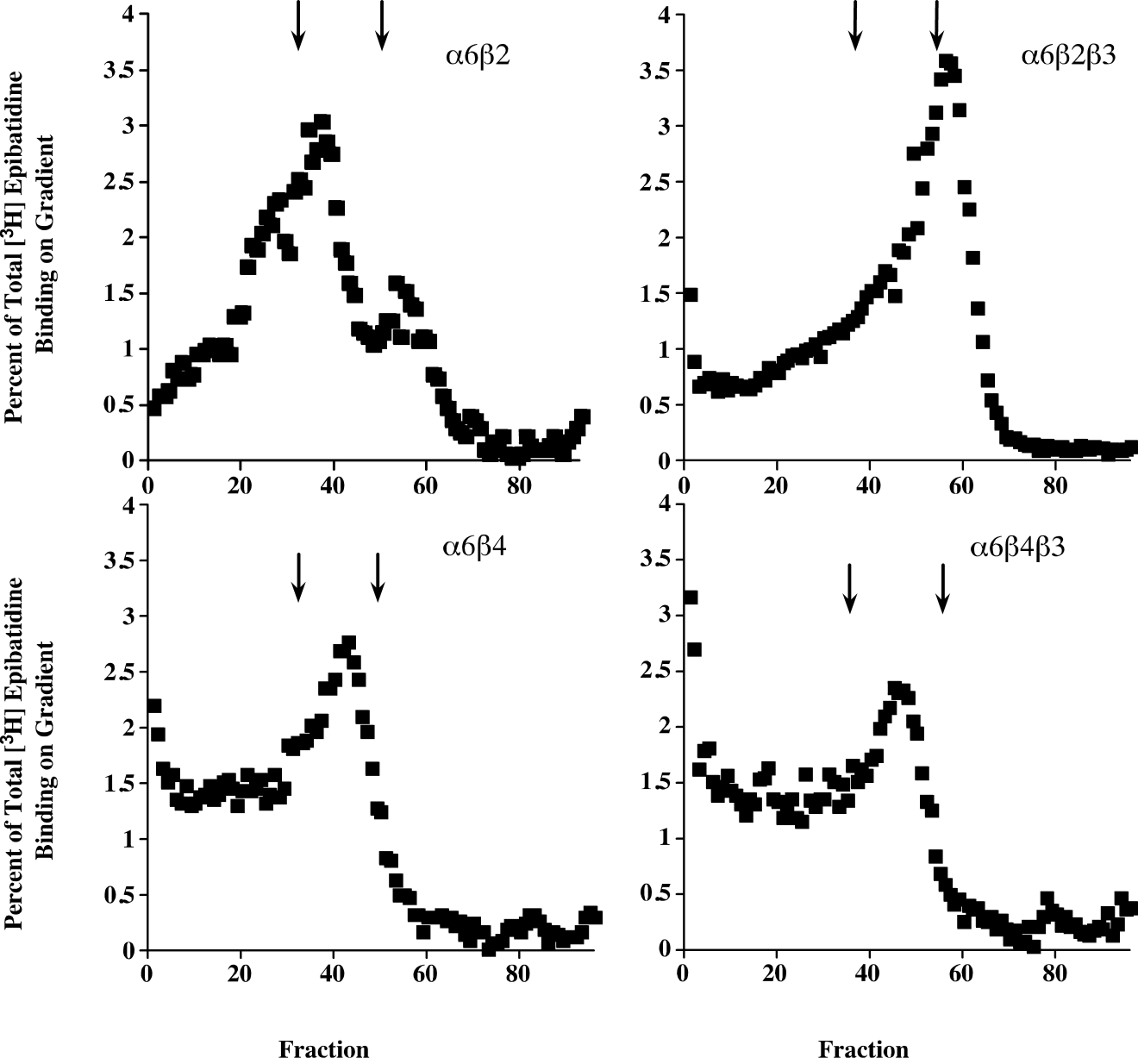
α6β2
α6β2β3
α6β4
α6β4β3
Percent of Total [3H] Epibatidine Binding on Gradient
Percent of Total [3H] Epibatidine Binding on Gradient
Fraction
Fraction
0
0.5
1
1.5
2
2.5
3
3.5
4
0
20
40
60
80

Figure 5

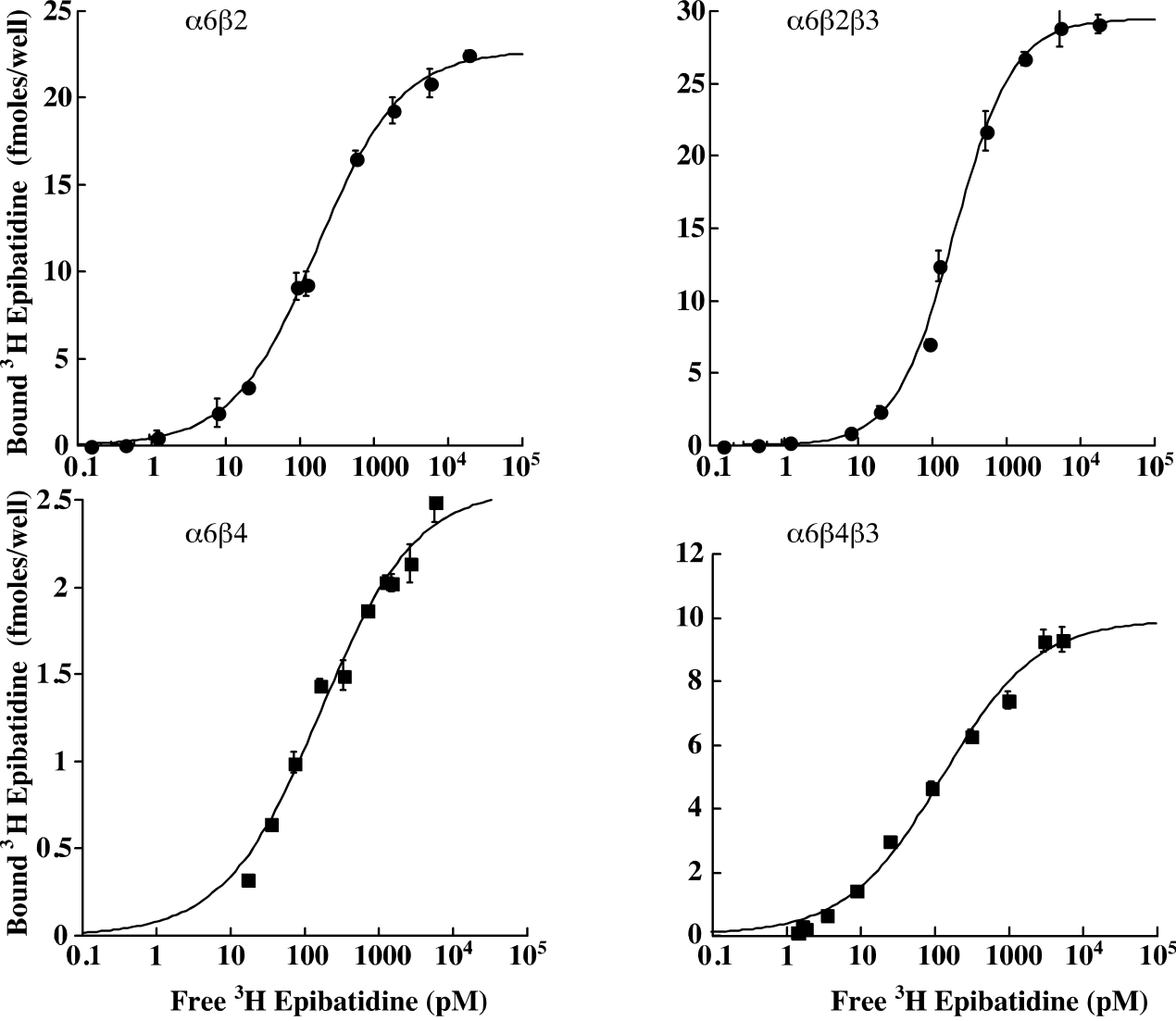
α6β2
α6β2β3
α6β4
α6β4β3
Bound ^{3}H Epibatidine (fmoles/well)
Free ^{3}H Epibatidine (pM)
0.1
1
10
100
1000
10^4
10^5

Figure 6

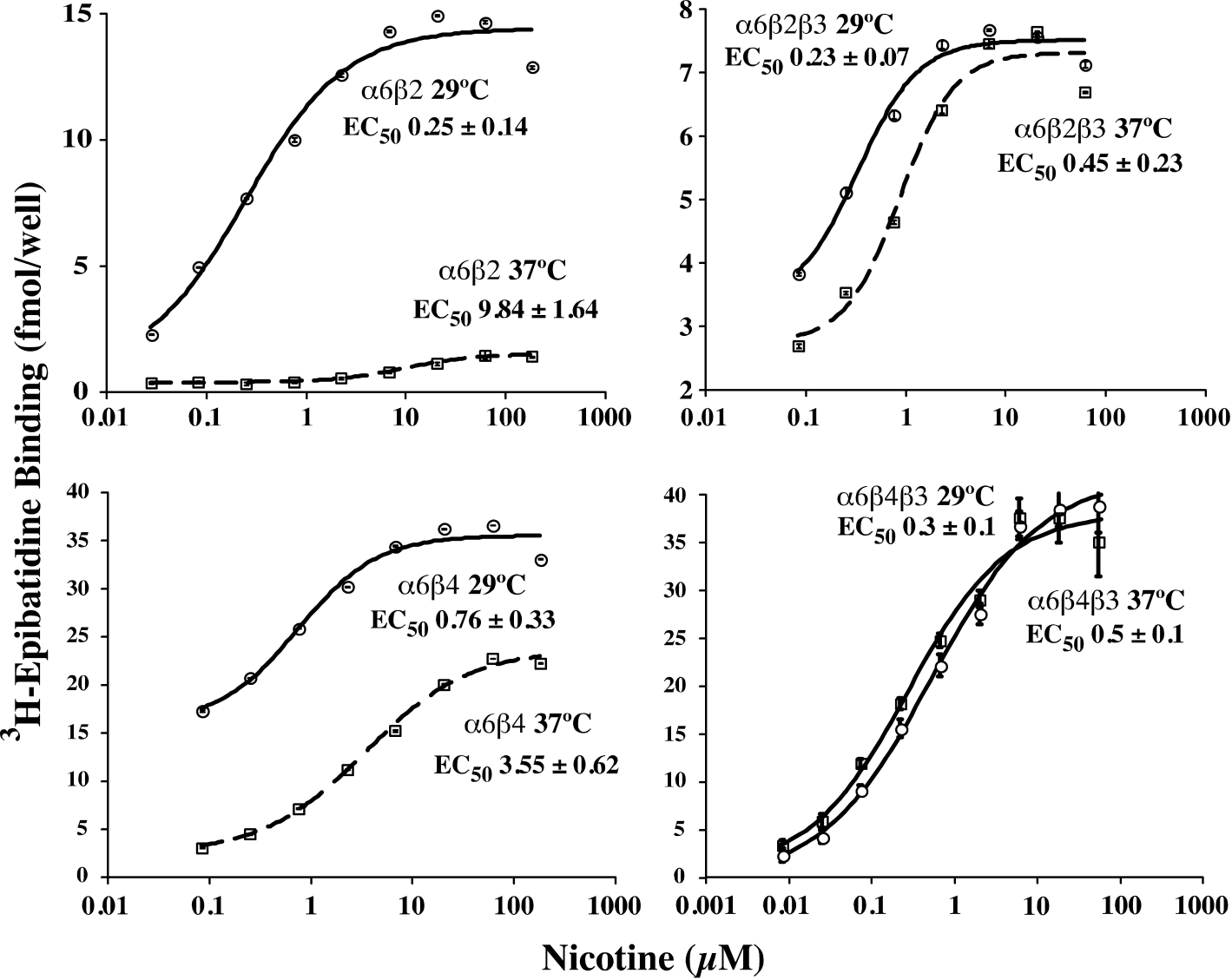
α6β2 29°C
EC50 0.25 ± 0.14
α6β2 37°C
EC50 9.84 ± 1.64
α6β2β3 29°C
EC50 0.23 ± 0.07
α6β2β3 37°C
EC50 0.45 ± 0.23
α6β4 29°C
EC50 0.76 ± 0.33
α6β4 37°C
EC50 3.55 ± 0.62
α6β4β3 29°C
EC50 0.3 ± 0.1
α6β4β3 37°C
EC50 0.5 ± 0.1
3H-Epibatidine Binding (fmol/well)
Nicotine (μM)

Figure 7