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Nicotine Acts as a Pharmacological Chaperone to Up-Regulate Human $\alpha4\beta2$ Acetylcholine Receptors

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ABSTRACT

Human neuronal nicotinic acetylcholine receptor (AChR) $\alpha4$ subunits and an $\alpha4$ mutant (S247F$\alpha4$) found in autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE) were expressed along with $\beta2$ in permanently transfected tsA201 human embryonic kidney cell lines. Their sensitivity to activation, desensitization, and up-regulation by cholinergic ligands was investigated. Up-regulation after 3 to 24 h resulted primarily from an increase in assembly of AChRs from large pools of unassembled subunits, but up-regulation also resulted from a 5-fold increase in the lifetime of AChRs in the surface membrane. Up-regulation does not require current flow through surface membrane AChRs, because up-regulation occurs in the presence of the channel blocker mecamylamine and with the $\alpha4$ mutant, which prevents nearly all AChR function. Both membrane-permeable ligands like nicotine and much less permeable quaternary amine cholinergic ligands can act as pharmacological chaperones within the endoplasmatic reticulum to promote the assembly of AChRs. Agonists are more potent pharmacological chaperones than antagonists, presumably because activated or desensitized conformations assemble more efficiently. Assembly intermediates are disrupted by solubilization in Triton X-100, but chemical cross-linking stabilizes a putative assembly intermediate approximately the size of an $\alpha4\beta2\alpha4\beta2$ tetramer.

The effects of nicotine are complex. Nicotine is an agonist that can also function as a time-averaged antagonist by desensitizing nicotinic AChRs (Collins and Marks, 1996; Olale et al., 1997; Meyer et al., 2001; Gentry et al., 2003). Nicotine also causes an increase in the number of AChRs (Peng et al., 1994; Wang et al., 1998; Whiteaker et al., 1998; Cooper et al., 1999; Perry et al., 1999; Gentry and Lukas, 2002).

AChRs have five homologous subunits organized around a central cation channel (Fig. 1) (Lindstrom, 2000). Acetylcholine binding sites in heteromeric neuronal AChRs are formed at the interface between an $\alpha2$, $\alpha3$, $\alpha4$, or $\alpha6$ subunit and a $\beta2$ or $\beta4$ subunit. A fifth "accessory" subunit, which does not take part in an acetylcholine binding site, is typically $\beta2$, $\beta4$, $\alpha5$, or $\beta3$ (Lindstrom, 2000), but an $\alpha4$ subunit can also occupy this position (Fig. 1) (Nelson et al., 2003; Zhou et al., 2003).

$\alpha4\beta2$ AChRs are the primary brain subtype with high affinity for nicotine (Flores et al., 1992; Lindstrom, 2000) and are essential for nicotine self-administration (Tapper et al., 2004; Maskos et al., 2005). Human $\alpha4\beta2$ AChRs in our transfected HEK cell line are present in two stoichiometries: $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ (Nelson et al., 2003; Fig. 1). The $(\alpha4)_2(\beta2)_3$ stoichiometry is more sensitive to activation and up-regulation by nicotine and desensitizes more slowly. Properties of each stoichiometry expressed individually in *Xenopus laevis* oocytes have been characterized (Zhou et al., 2003).

When $\alpha4\beta2$ (Peng et al., 1994; Gopalakrishnan et al., 1997; Pacheco et al., 2001; Nelson et al., 2003; Xiao and Kellar, 2004; Sallette et al., 2004, 2005) or $\alpha3\beta2$ AChRs (Wang et al., 1998; Xiao and Kellar, 2004) are expressed in transfected cells, nicotine can cause a large increase in the quantity of AChRs. Up-regulation of $\alpha3\beta2$ AChRs occurs to the level at which $\alpha3\beta4$ AChRs are constitutively expressed (Wang et al., 1998; Sallette et al., 2004). Susceptibility to nicotine-induced up-regulation of $\alpha3\beta2$ and $\alpha4\beta2$ AChRs is regulated by a $\beta2$ microdomain located just above the acetylcholine binding site (Sallette et al., 2004). Nicotine-induced up-regulation of transfected $\alpha3\beta2$ AChRs (Wang et al., 1998) and $\alpha4\beta2$ AChRs (Sallette et al., 2005) results primarily from an increase in the assembly of large pools of pre-existing subunits, but there

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ABBREVIATIONS: AChR, acetylcholine receptor; ACh, acetylcholine; ADNFLE, autosomal-dominant nocturnal frontal lobe epilepsy; DHβE, dihydro-β-erythroidine hydrobromide; DMEM, Dulbecco's modified Eagle's medium; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; DSP, Dithiobis (succinimidyl propionate); ER, endoplasmic reticulum; HEK, human embryonic kidney; mAb, monoclonal antibody; MCC, methylcarbamylcholine; PBS, phosphate-buffered saline; Sulfo-NHS-LC biotin, sulfosuccinimidyl-6-(biotinamido)hexanoate.

is also an increase in the half-life of surface membrane AChRs (Peng et al., 1994; Wang et al., 1998).

Here, we show that nicotine acts as a pharmacological chaperone on an intracellular assembly intermediate to cause up-regulation by promoting the assembly of $\alpha4\beta2$ AChRs, primarily by inducing an active or desensitized conformation, which assembles more efficiently. During the prolonged incubation necessary for up-regulation, quaternary amine agonists enter the lumen of the ER, in which they can also bind to nascent AChR binding sites and trigger increased AChR assembly. Nicotine further contributes to up-regulation by increasing the lifetime of surface AChRs. In the continued presence of the concentrations of nicotine required for up-regulation as found in typical smokers' sera (Benowitz, 1996), virtually all of the AChRs are desensitized. However, after removing nicotine, most, but not all, of the increased numbers of surface AChRs are functional. The S247F$\alpha4$ mutation, which causes ADNFLE (Steinlein, 2004), exhibits use-dependent activation in *X. laevis* oocytes (Kuryatov et al., 1997; Figl et al., 1998) but is virtually without function when expressed in HEK tsA201 cells. The homozygous mutant might similarly lack function in ADNFLE neurons.

Materials and Methods

Cloning and Tissue Culture. The cDNAs for human $\alpha4$, S247F$\alpha4$, and $\beta2$ subunits were cloned in this laboratory and have been described previously (Wang et al., 1996; Kuryatov et al., 1997). The subcloning of cDNA for human $\beta2$ into the expression vector pRc/CMV (Invitrogen, Carlsbad, CA) was described by Wang et al. (1998). The cDNAs for human $\alpha4$ and S247F$\alpha4$ were subcloned into the restriction sites XhoI and BamHI of the selective mammalian expression vector pcDNA3.1/Zeo(−) (Invitrogen), which carries the Zeocin resistance gene. To establish stable cell lines, equal amounts of plasmids encoding $\alpha4$ and $\beta2$ subunits were transfected into HEK tsA201 cells using the FuGene6 transfection agent (Roche Diagnostics, Indianapolis, IN) at a ratio of 5 μg of DNA per 15 μl of FuGene6 per 100-mm dish. Cloning rings were used to isolate individual clones. These were subsequently screened for highest stable expression using [^{3}H]epibatidine (PerkinElmer Life and Analytical Sciences, Boston, MA) binding to live cells. Transfected cells were maintained in DMEM with penicillin (100 U/ml), streptomycin (100 μg/ml) (Invitrogen), and 10% fetal bovine serum (Hyclone, Logan, UT) as described previously (Wang et al., 1998). Zeocin (0.5 mg/ml) was used for selection of $\alpha4$, and G418 (0.6 mg/ml; both from Invitrogen) was used for the selection of $\beta2$ subunit expression.

Monoclonal Antibodies Used and Solid-Phase Radioimmunoassay. The rat IgG monoclonal antibody (mAb) 299 to $\alpha4$ subunits, mAb 210 to the main immunogenic region on human $\alpha1$, $\alpha3$, $\alpha5$, and $\beta3$ subunits, and mAb 295 to $\beta2$ subunits have been described previously (Lindstrom, 2000). These mAbs bind to the extracellular surface. mAb 295 binds strongly to $\beta2$ subunits when they are assembled with $\alpha3$, $\alpha4$, or $\alpha6$ subunits (Wang et al., 1998; Kuryatov et al., 1997, 2000) but not when they are expressed alone in HEK cells (Ren et al., 2005). The mouse IgG mAb 371 was raised to bacterially expressed human AChR $\alpha4$ subunits as described subsequently. It binds to the cytoplasmic surface of the $\alpha4$ subunit.

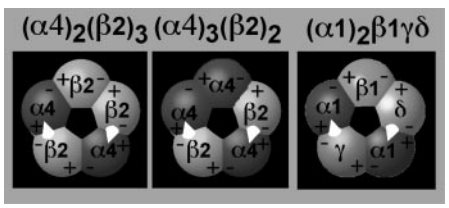

Fig. 1. Comparison of the putative subunit arrangements in alternate $\alpha4\beta2$ AChR stoichiometries with the subunit arrangement in *T. californica* AChR.

Immulon 4 (Dynex Technologies, Chantilly, VA) microtiter wells were coated with mAbs as described previously (Conroy et al., 1990). The cells were detached from plates by ice-cold PBS (100 mM NaCl and 10 mM sodium phosphate, pH 7.4) with 5 mM EDTA and homogenized by repetitive pipetting in buffer A containing 50 mM Na_2HPO_4-NaH_2PO_4, pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 15 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride. The membrane fractions were collected by centrifugation (20 min at 13,000*g*). AChRs were solubilized by incubating the membrane fractions in buffer A containing 2% Triton X-100 at 25°C for 1 h. Insoluble material was removed by centrifugation for 20 min at 13,000*g*. Solubilized AChRs from cells were used directly for all assays. mAb-coated microtiter wells were incubated with Triton-solubilized AChRs and 2 nM [^{3}H]epibatidine at 25°C for 3 h. Then the wells were washed three times with PBS and 0.05% Tween 20 buffer before elution with 0.1 M NaOH and transfer to Eppendorf tubes. The amount of bound radioactivity was determined using a 1450 Trilux Microbeta liquid scintillation counter (PerkinElmer Wallac, Turku, Finland) with OptiPhase "Supermix" (PerkinElmer Wallac). Nonspecific binding determined by incubation of extracts in parallel with 100 μM nicotine was subtracted from the total and was less than 1% of total. Total protein concentration of solubilized AChRs was determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL).

FLEXstation Experiments. For functional assays we used a FLEXstation II (Molecular Devices, Sunnyvale, CA) bench-top scanning fluorometer basically as described by Fitch et al. (2003). The cells were plated at 70,000 cells/well on poly(D-lysine)-coated black-wall/clear-bottom 96-well plates (BD Biosciences, San Jose, CA) the day before the experiment. Membrane potential (excitation wavelength, 530 nm; emission wavelength, 565 nm) and Calcium Plus Assay kits (excitation wavelength, 485 nm; emission wavelength, 525 nm) (Molecular Devices) were used according to manufacturer's protocols. Serial dilutions of drugs were prepared in V-shaped 96-well plates (Fisher Scientific Co., Pittsburgh, PA) and were added in separate wells at a rate of 80 μl/s during recording. Each point on the curves represents the average response of four wells. The Hill equation was fitted to the concentration-response relationship using a nonlinear least-squares error curve-fit method (KaleidaGraph, Synergy Software, Reading, PA): $I(x) = I_{max}[x^n/(x^n + EC_{50n})]$, where $I(x)$ is the current measured at the agonist concentration x, I_{max} is the maximal current response at the saturating agonist concentration, EC_{50} is the agonist concentration required for the half-maximal response, and n is the Hill coefficient.

Binding of [^{3}H]Epibatidine to Living and Fixed Cells. Binding to living cells attached to 35-mm plates was done in DMEM at 4°C for only 15 min with 0.5 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, 10 mM concentration of the quaternary amine carbamylcholine was added together with 0.5 nM [^{3}H]epibatidine to inhibit binding to cell-surface AChRs. Nonspecific labeling (approximately 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.

Before fixation, cells were grown to confluence in 100 μl of the media described above in 96-well microtiter plates with clear bottoms (Corning Glassworks, Corning, NY). Cells were then fixed with 100 μl of 4% phosphate buffered formaldehyde (Fisher Scientific) per well added for 1 h at room temperature. After fixation, the cells were washed three times with 200 μl of PBS. Then 2.0 nM [^{3}H]epibatidine

was added in 100 μl of PBS for 2 h at room temperature. Assays were done in quadruplicate. Nonspecific binding was determined with the addition of 1 mM nicotine. Inhibition experiments were performed under the same conditions with added antagonists. To completely permeabilize fixed cells, they were treated for 1 h with 100 μl of 0.1% Triton X-100 in PBS, then washed three times with 200 μl of PBS before labeling with epibatidine or iodinated mAbs. After labeling with [^{3}H]epibatidine, the wells were washed three times with 200 μl of PBS, and then 25 μl of 0.1 M NaOH was added to elute [^{3}H]epibatidine. Bound radioactivity was determined in the same wells using 200 μl of scintillation fluid per well. Fixation in 2% formaldehyde under these conditions results in the same amount of [^{3}H]epibatidine binding observed when labeling live cells for 30 min with 0.5 nM [^{3}H]epibatidine (91 ± 17%) and a similar K_D for epibatidine binding to membrane fractions (34 ± 2 pM) and fixed cells (17 ± 2 pM), but it avoids the confounding problems of up-regulation induced during labeling and prevents loss of cells during washing steps.

Up-Regulation in the Presence of Mecamylamine. The cells were plated on 24-well plates and grown until they reach 70 to 90% confluence. At this point, 50 μM mecamylamine and 1 μM nicotine, 100 μM MCC, or 100 μM DMPP were added for 3 h. After this incubation, the cells were fixed with equal volume of 4% phosphate buffered formaldehyde (Fisher Scientific) and then washed three times with 1 ml of PBS before being incubated with 2 nM [^{3}H]epibatidine for 30 min. After this incubation, the cells were washed three times with 1 ml of PBS before radioactive material was eluted by 200 μl of 0.1 M NaOH. Bound radioactivity was determined in Eppendorf tubes using 1 ml of scintillation fluid per tube. Protein concentrations were determined in a parallel experiment.

Up-Regulation of Epibatidine Binding Sites in Transfected Cells. Cells were plated at a density of 50,000 cells per well in 96-well plates. The next day, when the cells were nearly confluent, ligands were added. The cells were incubated for another day and were fixed as above by adding 100 μl of 4% phosphate buffered formaldehyde per well for 1 h. All experiments were repeated at least twice. The data represent the average of all experiments.

DSP Cross-Linking. Cells were detached from a 10-cm dish using 10 ml of ice-cold PBS, centrifuged, and then resuspended in 1 ml of PBS. Cross-linking used a 1 mM concentration of DSP (Pierce) in 1 ml of PBS for 2 h on ice. The reaction was stopped by adding 10 μl of 1 M Tris, pH 7.5, for 15 min and then washing three times with 1 ml of ice-cold PBS.

Sucrose Gradients. Linear 5 to 20% sucrose gradients in 11.4 ml of 0.5% Triton X-100, PBS, and 10 mM NaN_3 were layered with 200-μl samples and sedimented for 16 h at 40,000 rpm in a Beckman SW41 rotor. Samples contained extracts from two 10-cm dishes plus 1-μl aliquots of 2 mg/ml purified *Torpedo californica* electric organ AChR as an internal sedimentation standard. After centrifugation, 10-drop fractions were collected from the bottom. From each fraction, 20 μl was removed for assay of *T. californica* AChR on microwells coated with mAb 210 (to α1 subunits). After labeling for 3 h at 4°C with 2 nM ^{125}I-α-bungarotoxin and then washing three times with 200 μl of PBS plus 0.5% Triton X-100, these microwells were assayed in a γ counter. From each fraction, another 20 μl was placed in microwells coated with mAb 295 (to β2 subunits) along with 2 nM [^{3}H]epibatidine. After incubation at room temperature for 3 h, the wells were washed and placed in scintillation fluid before measurement in a scintillation counter.

Biotinylation. Cells from a confluent 10-cm dish were detached by 10 ml of ice-cold PBS containing 1.8 mM $CaCl_2$ and 7.2 mM KCl and then washed in 10 ml of this buffer. The resuspended cells were labeled by EZ-link Sulfo-NHS-LC biotin (Pierce) at 1 mg/ml according to the manufacturer's protocol. After labeling, the cells were plated on 35-mm dishes and incubated again, with or without nicotine. On the other hand, AChRs were immediately solubilized in buffer A with 2% Triton X-100 as above. Biotin-labeled solubilized AChRs were detected after extraction by binding to streptavidin agarose beads (Invitrogen) or streptavidin (Sigma-Aldrich, St. Louis, MO)-coated microtiter wells along with 2 nM [^{3}H]epibatidine.

Polyacrylamide Gel Electrophoresis. AChRs were extracted with 200 μl of 2% Triton X-100 in PBS per 10-cm dish. Samples were separated on 8% polyacrylamide tris-glycine gels or precast Novex 7% polyacrylamide tris-acetate gels (Invitrogen) for α4 subunits and 10% polyacrylamide tris-glycine for β2 subunits.

Preparation of mAb 371. Female BALB/c mice, 3 to 4 weeks of age, were obtained from Charles River Laboratories Inc. (Wilmington, MA). Human α4 subunits lacking the transmembrane domains were constructed in the pET-26b(+) vector (Novagen, Madison, WI) and were expressed in bacteria. They were solubilized in 3% SDS and 100 mM dithiothreitol and then purified by gel exclusion chromatography using Ultrogel AcA 34 (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with 0.1% SDS in PBS buffer. Most of the SDS was removed by dialysis before the purified α4 was used to immunize mice.

Mice were immunized at 4 intradermal sites in the lower back and then boosted at 3-week intervals with 40 μg/50 μl per mouse of α4 subunit construct emulsified in an equal volume of TiterMax adjuvant. One final boost consisted of a mixture of 1 μg per mouse of α4β2 AChRs expressed in HEK cells isolated on mAb 295-coated beads and 10 μg per mouse of the bacterially expressed subunit construct in incomplete Freund's adjuvant. The titers were monitored by test bleeds against α4 subunit in an enzyme-linked immunosorbent assay. Five days after a final boost with antigen, splenic lymphocytes (~10^8) were harvested from the mouse with the highest titer and were fused with SP2/0 myeloma cells (1×10^8) using 50% polyethylene glycol (Kodak 1450) before culture in 24 96-well plates (Corning Life Sciences, Acton, MA). Initial screening was done by enzyme-linked immunosorbent assay as follows: Culture supernatant from each well was diluted 50-fold in PBS, and 100 μl was added to corresponding wells of 24 96-well Immulon 4HBX flat-bottomed microtiter plates (Thermo Electron Corporation, Waltham, MA) coated with 5 μg/ml the α4 subunit and incubated overnight at 4°C. After three washes, bound antibodies were detected by incubation for 1 h with a 1:2000 dilution of biotin-labeled $F(ab)_2$ fragment to mouse IgG(H+L), 1-h incubation with peroxidase-labeled streptavidin, and a final incubation with 100 μl/well of SureBlue TMB microwell peroxidase substrate (all from Kirkegaard and Perry Laboratories, Gaithersburg, MD). Every incubation was followed by three washes, except for the final one. A 100-μl sample of 1 M phosphoric acid was added to each well to stop the reaction. The absorbance at a wavelength of 450 nm was monitored with a Titertek Multiskan MCC/340. To eliminate those mAbs that cross-reacted with closely related subunits, cross-reaction with α3 in a similar solid-phase assay was tested. Then mAbs that could detect both denatured α4 subunits and native α4β2 AChRs were selected using [^{3}H]epibatidine-labeled native α4β2 AChRs in radioimmunoassays. Positive hybridomas were cloned by a limiting dilution method.

Results

Differential Activation and Up-Regulation of Two AChR Stoichiometries. Our α4β2 AChR cell line expresses both an $(\alpha4)_2(\beta2)_3$ and an $(\alpha4)_3(\beta2)_2$ stoichiometry. The arrangements of subunits in these two stoichiometries are shown in Fig. 1. At first, we demonstrated these using electrophysiological methods in combination with quantification of methionine-labeled subunits (Nelson et al., 2003), and we further characterized properties of the two AChR stoichiometries expressed individually in *X. laevis* oocytes (Zhou et al., 2003). Similar responses characteristic of the two stoichiometries can be obtained using fluorescent indicators sensitive to membrane potential or Ca^{2+} concentration using cells grown in 96-well plates assayed in a Molecular Devices

FLEXstation, as shown in Fig. 2. These two-component response curves have been observed in another HEK cell line transfected with human $\alpha4\beta2$ AChRs (Vallejo et al., 2005) but not in SH-EP1 cells transfected with human $\alpha4\beta2$ (Pacheco et al., 2001) or in HEK cells expressing rat or mouse $\alpha4\beta2$ AChRs (Fitch et al., 2003; Karadsheh et al., 2004). The EC_{50} value for activation of the $(\alpha4)_3(\beta2)_2$ stoichiometry by nicotine (2.7 μM) is close to values reported for another human cell line in HEK cells (1.6 μM) (Buisson et al., 1996; and 4.0 μM, Gopalakrishnan et al., 1997) and for human $\alpha4\beta2$ expressed in SH-EP1 cells (2.4 μM) (Pacheco et al., 2001).

Nicotine selectively up-regulates the sensitive $(\alpha4)_2(\beta2)_3$ stoichiometry, confirming the results previously detected electrophysiologically (Nelson et al., 2003) (Fig. 3). This also explains the observation of Vallejo et al. (2005), that up-regulation "alters the functional state", resulting in increased sensitivity to activation and decreased desensitization. At first, there is a two-component dose-response curve for nicotine (EC_{50} = 116 and 2700 nM for the two stoichiometries). After nicotine treatment, only a single component of the nicotine response curve is resolved (EC_{50} = 345 nM) because of a greater increase in the amount of the sensitive $(\alpha4)_2(\beta2)_3$ stoichiometry. Differences in sensitivity to activation by acetylcholine of the two AChR stoichiometries are sufficient to resolve separate EC_{50} values both before and after up-regulation (Table 1). However, the two stoichiometries seem to exhibit similar sensitivities to activation by both DMPP and cytisine so that two components cannot be resolved in the response curves (Table 1).

Equilibrium binding of [^{3}H]nicotine reveals only a single component, presumably corresponding to a single desensitized state common to both stoichiometries (K_D = 2.7 ± 0.2 nM for wild-type $\alpha4\beta2$ and 2.4 ± 0.7 nM for S247F$\alpha4\beta2$). Likewise, only a single binding component is resolved for [^{3}H]epibatidine (K_D = 0.0172 ± 0.0018 nM for wild-type and 0.0396 ± 0.0041 for S247F$\alpha4\beta2$). Our wild-type $\alpha4\beta2$ AChR cell line expresses 0.9 pmol epibatidine binding sites per mg of Triton X-100-extracted protein before up-regulation and 7.1 pmol/mg after maximum up-regulation. For the S247F$\alpha4\beta2$ cell line, these values are 2.1 and 6.3 pmol/mg.

Blocking AChR Function. The function of $\alpha4\beta2$ AChRs can be blocked in several ways (Fig. 4). Mecamylamine noncompetitively blocks the cation channel (IC_{50} = 770 nM). The competitive antagonist dihydro-β-erythroidine (DHβE) blocks acetylcholine binding (IC_{50} = 88 nM). Nicotine, when added for a prolonged period, acts as a time-averaged antagonist by inducing a desensitized conformation. Nicotine is far more potent (IC_{50} = 6 nM) than the other antagonists shown and is more potent as an antagonist than as an agonist (EC_{50} = 116 and 2700 nM on the two stoichiometries) (Table 1). After 6 h at the 200 nM nicotine concentration characteristic of serum from cigarette smokers (Benowitz, 1996), 90% of function is desensitized (Fig. 4).

Because nicotine-induced up-regulation occurs over a matter of hours, the time-averaged antagonist effect of nicotine in accumulating desensitized AChRs is probably more important for up-regulation than its short-term agonist effect. Comparison of the data in Figs. 3 and 4 and Tables 1 and 2 makes it clear that nicotine at its 35 nM EC_{50} value for up-regulation exceeds its 6 nm IC_{50} value for antagonism, so that under the conditions used for up-regulation, virtually all of the mature surface-membrane AChRs are desensitized. Assembly intermediates that could bind nicotine, such as $\alpha4\beta2$ dimers or $\alpha4\beta2\alpha4\beta2$ tetramers, are probably also similarly desensitized. The sensitivity to desensitization of such assembly intermediates might be less than that of mature AChRs in the cell surface. This could account for the difference between the EC_{50} value for up-regulation and the IC_{50} value for desensitization, if desensitization were a critical step in up-regulation. The 35 nM EC_{50} value for up-regulation is lower than the 116 nM EC_{50} value for activation of $(\alpha4)_2(\beta2)_3$ AChRs in the cell surface and far below 2700 nM EC_{50} value for activation of $(\alpha4)_3(\beta2)_2$ AChRs. Over the 6-h exposure to nicotine in Fig. 4, at concentrations that would produce no apparent short-term activation of $(\alpha4)_3(\beta2)_2$ AChRs, all of the AChRs end up in a desensitized state. The long periods of incubation used in binding experiments with [^{3}H]nicotine or [^{3}H]epibatidine similarly convert all of the AChRs to the same desensitized state and reveal monotonic binding curves rather than the two-component activation curves seen in Figs. 2 and 3.

Up-Regulation Does Not Require Activation of Surface AChRs, but Membrane-Permeable Ligands Act Intracellularly as Molecular Chaperones. An HEK cell line transfected with the ADNFLE S247F$\alpha4$ mutation and $\beta2$ exhibits virtually no function by patch-clamp recording (data not shown). No function could be detected with the mutant cell line by assay using membrane potential-sensitive fluorescent indicators under control conditions. After up-regulation by nicotine, a very small response was detectable, but the maximum was approximately 3% that of similarly up-

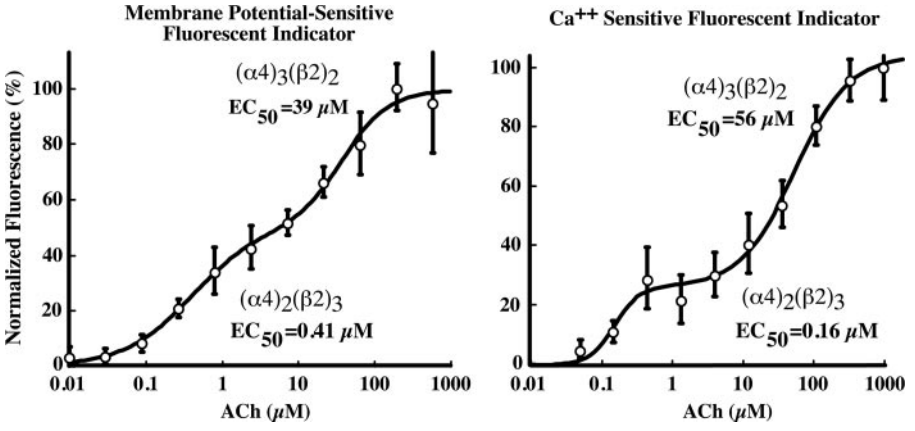

Fig. 2. Activation by acetylcholine of $\alpha4\beta2$ AChRs expressed in the HEK cell line assayed in microwell plates using the FLEXstation and its proprietary fluorescent indicators reveals activity of both the sensitive $(\alpha4)_2(\beta2)_3$ stoichiometry and the insensitive $(\alpha4)_3(\beta2)_2$ stoichiometry. The higher proportion of response detected in the Ca^{2+}-sensitive indicator reflects the higher Ca^{2+} permeability of the $(\alpha4)_3(\beta2)_2$ stoichiometry (data not shown). Responses were normalized to the maximum response.

regulated wild-type $\alpha4\beta2$ AChRs (Fig. 5). Nonetheless, cholinergic ligands potently induce up-regulation in the mutant cell (Fig. 5 and Table 2).

Nicotine as well as the quaternary amines MCC and DMPP can induce up-regulation of both wild-type and S247F$\alpha4\beta2$ AChRs in the presence of 50 μM mecamylamine (Fig. 6), a concentration that blocks all current flow (Fig. 4). This further indicates that current flow is not required for up-regulation. Up-regulation of the amount of AChR is 3.3-fold more sensitive to the tertiary amine agonist nicotine and 7.6-fold to the tertiary agonist cytisine than is activation, but it is 3.4-fold less sensitive to the quaternary amine agonist DMPP (Tables 1 and 2). That may correlate with the delay in penetration of quaternary amines inside the cells (see Fig. 9).

Up-Regulating AChR Function. The activity of $\alpha4\beta2$ AChRs desensitized by nicotine can be recovered after removing the nicotine, resulting in a net gain in function caused by up-regulation during the period of agonist exposure (Fig. 7). The extent of up-regulation in function is directly proportional to the increase in amount of AChRs on the cell surface (Fig. 8), thereby clearly revealing the mechanism by which increased assembly of AChRs results in increased function of AChRs.

This contrasts with the interpretation that nicotine causes an allosteric conformation change in pre-existing surface AChRs to account for the increased function observed (Vallejo et al., 2005). The slowing in desensitization and enhanced sensitivity observed after up-regulation, which Vallejo et al. (2005) attribute to a conformation change of AChRs in the surface membrane, in fact reflects the increase in proportion of the $(\alpha4)_2(\beta2)_3$ stoichiometry after up-regulation (Fig. 3; Nelson et al., 2003). The $(\alpha4)_2(\beta2)_3$ stoichiometry is more sensitive to nicotine and desensitizes less rapidly than the $(\alpha4)_3(\beta2)_2$ stoichiometry (Nelson et al., 2003; Zhou et al., 2003).

The extent of up-regulation of function is less than the extent of up-regulation of surface AChRs (i.e., the slope of the line in Fig. 8 is less than 1). This probably reflects irreversible desensitization of some of the AChRs (Gentry and Lukas, 2002; Gentry et al., 2003). An alternate explanation would be that nicotine, which had accumulated within the cells, partitioned out slowly, sustaining the AChRs in a reversibly desensitized state (Jia et al., 2003).

Acetylcholine Can Induce Up-Regulation. Incubation of cells with 300 μM acetylcholine does not induce up-regulation (Fig. 9A), nor does a 10 μM concentration of the acetylcholinesterase inhibitor neostigmine. Added together so that acetylcholine remains intact, they cause up-regulation equivalent to 1 μM nicotine (Fig. 9A). Acetylcholine up-regulates both native $\alpha4\beta2$ AChRs (Fig. 9A) and nonfunctional S247F $\alpha4\beta2$ AChRs (data not shown). This indicates that the endogenous quaternary amine acetylcholine can increase the amount of AChRs by a mechanism that does not require function of the cation channel in AChRs on the cell surface.

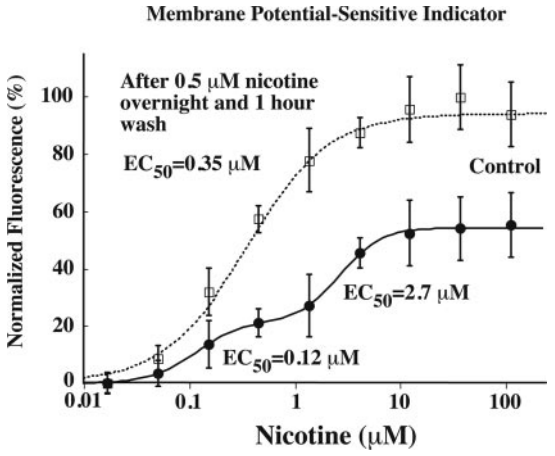

Fig. 3. Nicotine selectively up-regulates the sensitive $(\alpha4)_2(\beta2)_3$ stoichiometry in the $\alpha4\beta2$ AChR cell line. AChR function was assayed using the FLEXstation. Both an $(\alpha4)_2(\beta2)_3$ and an $(\alpha4)_3(\beta2)_2$ stoichiometry are clearly present in control cells. The more sensitive $(\alpha4)_2(\beta2)_3$ stoichiometry predominates after nicotine treatment. Responses were normalized to the maximum $\alpha4\beta2$ AChR response after treatment with nicotine. A membrane potential-sensitive indicator was used.

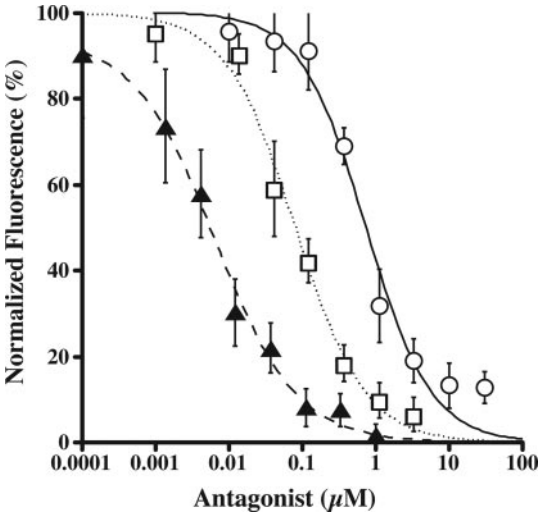

Fig. 4. $\alpha4\beta2$ AChR function can be blocked by competitive and noncompetitive antagonists and even more potently by nicotine, which desensitizes the AChRs. Antagonists were added 30 min before assay. Nicotine was added for 6 h before assay. Responses to 3 μM acetylcholine were determined. ○, antagonism by the noncompetitive channel blocker mecamylamine, IC_{50} = 770 nM; ■, antagonism by the competitive acetylcholine binding site blocker DHβE, IC_{50} = 88 nM; ▲, the time-averaged antagonist effect of desensitization by nicotine, IC_{50} = 6.1 nM. Responses were normalized to control responses without antagonist. A membrane-potential-sensitive indicator was used.

TABLE 1
Activation and Inhibition of $\alpha4\beta2$ AChRs in an HEK cell line

	Receptors	Nicotine	Acetylcholine	DMPP	Cytisine	MCC	DHβE	Mecamylamine
Control					*nM*			
Inhibition IC_{50}	$\alpha4\beta2$	6.1 ± 3.6					88 ± 24	770 ± 220
Activation EC_{50}	$(\alpha4)_2(\beta2)_3$	116 ± 15	230 ± 40	1110 ± 550	57 ± 20	3200 ± 1000	>1,000,000	
	$(\alpha4)_3(\beta2)_2$	2700 ± 200	57,000 ± 20,000					
Nicotine-treated								
Activation EC_{50}	$(\alpha4)_2(\beta2)_3$	345 ± 40	320 ± 10	1320 ± 190			>1,000,000	
	$(\alpha4)_3(\beta2)_2$		71,000 ± 37,000					

Quaternary Amines Penetrate Inside Cells. Nicotine and epibatidine are tertiary amines that can readily cross cell membranes in their unprotonated form. In a 20-min incubation, both were very effective in inhibiting all specific binding of 5 nM [^{3}H]nicotine to AChRs in the $\alpha4\beta2$ cell line (Fig. 9B). Quaternary amines would not be expected to cross cell membranes. After 20 min, a variety of quaternary amine agonists showed limited ability to inhibit the binding of [^{3}H]nicotine to cells, as would be expected if they were binding only to surface AChRs (Fig. 9B). However, after 1 or 2 h, some of these ligands were able to inhibit virtually all specific binding. This surprising phenomenon has also been noted by others (Sallette et al., 2005; Vallejo et al., 2005). Thus, on prolonged incubation, quaternary amine agonists like ACh and tetramethylammonium gain access to the interior of the ER and Golgi apparatus, in which they can bind to the acetylcholine binding sites of nascent AChRs. Darsow et al. (2005) attribute the up-regulation produced by tetramethylammonium to an effect on surface AChRs, but Fig. 9B shows that it can act on AChRs within the cell.

Agonists Are More Potent at Up-Regulation than Are Antagonists. A variety of nicotinic ligands can induce up-regulation in these $\alpha4\beta2$ AChR cell lines (Table 2). For example, the acetylcholine esterase antagonist and allosteric AChR affector galantamine (Samochocki et al., 2003), which is used to treat Alzheimer's disease, can also cause up-regulation. The partial agonist cytisine equally potently up-regulates $\alpha4\beta2$ AChRs and the nonfunctional mutant. Even the competitive antagonist DHβE can cause up-regulation. It is much less potent at up-regulating (EC_{50} = 32 μM) (Table 2) than at antagonizing function (IC_{50} = 0.088 μM) (Fig. 4 and Table 1). The observation that agonists (nicotine, cytisine) are 3.3- to 7.6-fold more potent at up-regulation than at activation of AChRs, whereas the antagonist DHβE is 364-fold less potent at up-regulation than at antagonizing activation of AChRs, suggests that the ability of agonists to induce the conformation changes associated with either activation or desensitization is important for potent up-regulation by these ligands acting intracellularly as molecular chaperones. It has been suggested that up-regulation is initiated by desensitization (Fenster et al., 1999). The maximum extent of activation that can be induced on wild-type $\alpha4\beta2$ AChRs by all agonists averages 5.3-fold, which is significantly greater than the maximum extent of up-regulation by the antagonist DHβE (3.3-fold) or the allosteric affector galantamine (2.2-fold). This further suggests the importance of the ability to induce conformation changes associated with activation or desensitization in potency of up-regulation. Likewise, 17-β-estradiol has been found to potentiate the activation of $\alpha4\beta2$ AChRs by binding to the C terminus of $\alpha4$ subunits (Paradiso et al., 2001), and we found that estrogen at 30 μM increases the extent of up-regulation by nicotine ~25% during a 3-h incubation (data not shown).

Nicotine induces up-regulation much more potently (EC_{50} = 35 nM) than it activates function (EC_{50} = 120 and 2700 nM for the two stoichiometries) but less potently than it binds at equilibrium to desensitized $\alpha4\beta2$ AChRs (K_D = 2.8 nM). Likewise, epibatidine, a very high-affinity agonist, induces up-regulation less potently (EC_{50} = 3.6 nM) than it binds to desensitized $\alpha4\beta2$ AChRs (K_D = 0.017 nM). These results suggest that agonists may induce up-regulation by binding to AChR assembly intermediates with lower affinity than ma-

TABLE 2

Up-regulation of $\alpha4\beta2$ AChRs in HEK cell lines

Values are presented as mean ± S.E.M. Values reported in this table reflect binding of [^{3}H]epibatidine to fixed cells after 16 h of up-regulation. $C_{>95}$ is the concentration of drug required to achieve more than 95% of maximum up-regulation.

Up-Regulation & Line	Ligand Used to Induce Up-Regulation						
	Nicotine	Epibatidine	DMPP	Cytisine	MCC	DHβE	Galantamine
Maximum extent (up-regulated/control)							
WT $\alpha4\beta2$	4.9 ± 0.9	5.4 ± 1.4	5.5 ± 1.1	5.2 ± 1.3	5.8 ± 1.9	3.3 ± 0.6	2.2 ± 0.2
S247F$\alpha4\beta2$	2.9 ± 0.5		2.5 ± 0.9	2.5 ± 0.5	2.4 ± 0.7	1.3 ± 0.2	2.2 ± 0.2
Sensitivity							
WT $\alpha4\beta2$							
EC_{50} (nM)	35 ± 8	3.6 ± 0.2	3800 ± 1300	7.5 ± 2.7	1500 ± 500	3200 ± 1600	3200 ± 900
$C_{>95}$ (nM)	500	50	50,000	100	50,000	500,000	100,000
S247F$\alpha4\beta2$							
EC_{50} (nM)	27 ± 4		1700 ± 700	5.8 ± 2.1	7900 ± 1300	5700 ± 900	8400 ± 2900
$C_{>95}$ (nM)	500		20,000	100	100,000	200,000	500,000

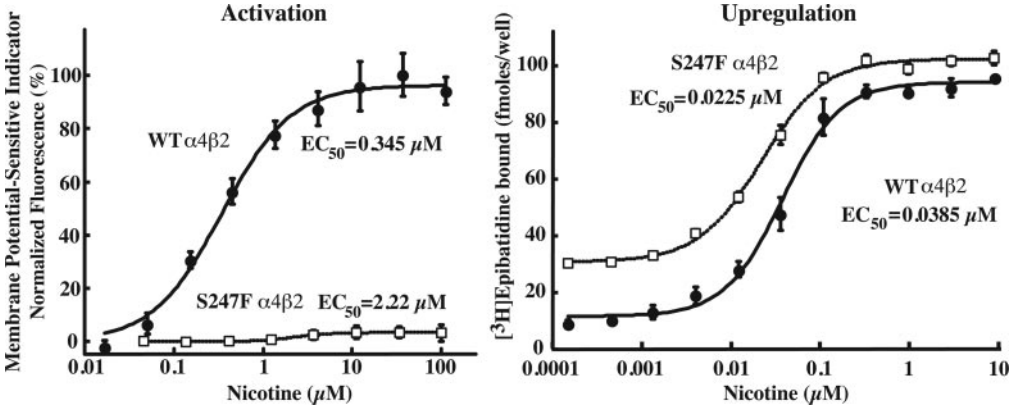

Fig. 5. The S247F$\alpha4\beta2$ line exhibits virtually no AChR function, but nicotine up-regulates the amount of AChRs to the same extent as wild-type $\alpha4\beta2$ AChRs. Function was assayed after up-regulation overnight with 0.5 μM nicotine followed by a wash for 1 h to permit detection of the tiny response of the S247F$\alpha4\beta2$ AChRs. A membrane-potential-sensitive indicator was used to assay function. Responses were normalized to the maximum wild-type $\alpha4\beta2$ AChR response.

ture AChRs. Accurate comparisons are difficult, partly because the concentration of nicotine within the cells is not known. Nicotine crosses cell membranes very quickly (Fig. 9), and in some cells, it can accumulate to much higher than ambient concentrations (e.g., in negatively charged compartments within *X. laevis* oocytes) (Jia et al., 2003; A. Kuryatov, V. Gerzanich, and J. Lindstrom, unpublished data).

Increased Assembly of AChR Subunits Contributes to Up-Regulation. The time course of nicotine-induced up-regulation revealed a rapid increase in mature $\alpha4\beta2$ AChRs within the cells followed by an increase in surface AChRs after a lag of approximately 3 h (Fig. 10A). The initial increase reflects rapid assembly of subunits from pre-existing pools in the ER. The pulse/chase labeling studies of Sallette et al. (2005) led to this conclusion as well. The lag phase presumably reflects the time required for transport through the Golgi apparatus for modification of glycosylation before transport to the surface membrane. The accumulation of up-regulated AChRs within the cells may indicate that processing of AChRs in the Golgi is a rate-limiting step.

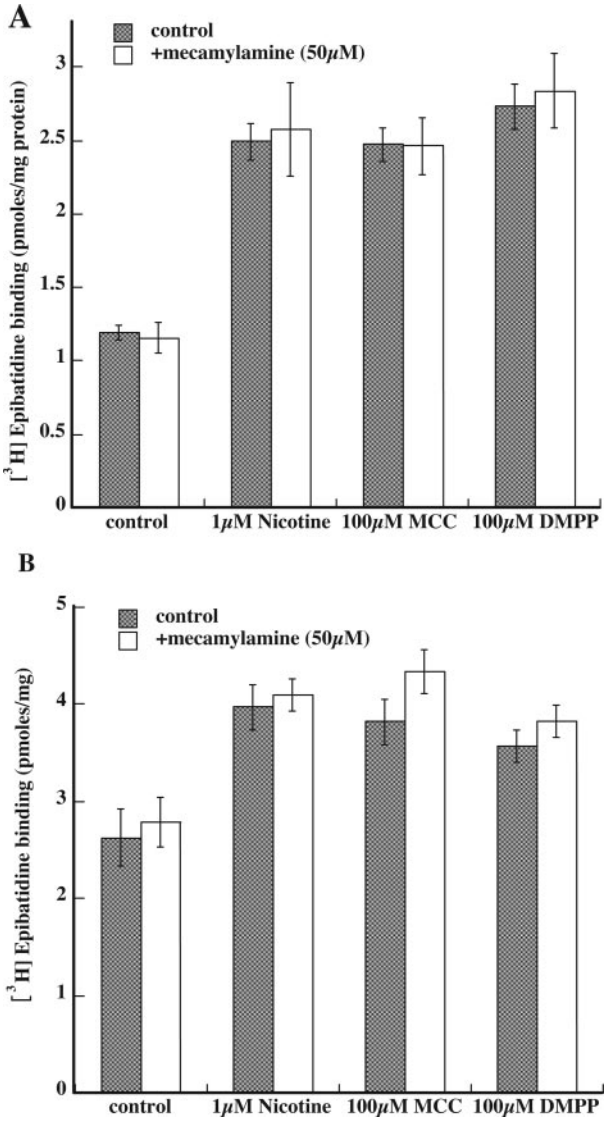

Fig. 6. The channel blocker mecamylamine does not block up-regulation. Mecamylamine was used at 65-fold of its IC_{50} value for noncompetitively blocking function. The amount of AChR after 3 h of up-regulation was measured by [^{3}H]epibatidine binding to fixed cells. Both the tertiary amine nicotine and the quaternary amines MCC and DMPP were used in both wild-type $\alpha4\beta2$ AChRs expressing cells (A) and nonfunctional S247F$\alpha4\beta2$ AChRs cells (B). Failure of channel block to prevent up-regulation and the ability of nonfunctional AChRs to efficiently up-regulate proves that up-regulation is not triggered by current flow through AChRs in the surface membrane.

Figure 10, B through D, uses three different methods to demonstrate that up-regulation by nicotine increases the proportion of total AChRs within the cell. Figure 10B uses surface biotinylation with sulfo-NHS-LC biotin to label surface AChRs, which are solubilized and isolated with avidin before labeling with [^{3}H]epibatidine. This method may underestimate the absolute amount of AChRs on the cell surface if the biotinylation reagent does not label all surface AChRs, but it should accurately reflect changes in the amount of surface AChRs. Surface AChRs isolated on streptavidin-coated wells were 35% of the total AChRs isolated on mAb 295-coated wells before up-regulation. After up-regulation, 25% were on the surface. Figure 10C uses brief incubation with carbamylcholine to inhibit binding only to surface AChRs on live cells. By this assay, 80% of the AChRs were on the surface before up-regulation, and 55% were afterward. These are probably reasonably accurate estimates. The problematic aspect of this assay is that carbamylcholine and epibatidine both induce up-regulation, requiring that the total assay period is brief to minimize this effect. Figure 10D uses binding of ^{125}I-mAb 295 plus or minus permeabilization on fixed cells. The subtleties of labeling with this mAb to $\beta2$ are discussed in the following paragraph. Before up-regulation, mAb 295 labels a small amount of mature AChRs on the cell surface and a much larger amount of assembly intermediates within the cells. After up-regulation, mAb 295 detects an increased proportion of mature AChRs on the cell surface and a consequently decreased proportion of assembly intermediates and fully assembled AChRs within the cells. All of these results are consistent with each other, Figs. 10A and 11 (to be subsequently discussed), and the pulse/chase labeling studies of Sallette et al. (2005) in proving that at short times, up-regulation results primarily from an increase in assembly of AChRs within the ER followed at later times by transport of the mature AChRs to the cell surface.

This contrasts with the hypothesis of Vallejo et al. (2005), who propose that nicotine induces a conformation change in pre-existing nonfunctional surface-membrane AChRs, which permits them to acquire high-affinity binding of epibatidine and the ability to function in response to agonist binding. They applied the unusual approach for assaying surface-membrane AChRs of using the membrane-permeable biotinylation reagent methanethiosulphonate ethylammonium biotin to label the cytoplasmic surface of surface-membrane AChRs. They observed a fixed ratio of surface AChRs before and after up-regulation, and on this basis, they argued that no new synthesis of AChRs was involved in up-regulation. Their actual cholinergic ligand binding data for nicotine-induced up-regulation is consistent with ours and that of Sallette et al. (2005), which is shown by several methods to result from increased assembly of mature AChRs within the cells.

The total amount of ^{125}I-mAb 295 binding to permeabilized formalin-fixed cells increases only 25% (from 5.6 to 7.3 fmol/well) after up-regulation with nicotine. This mAb to $\beta2$ sub-

units has the property that it does not bind with high affinity to $\beta 2$ subunits expressed alone (Ren et al., 2005) but only to assembled $\alpha 3\beta 2$, $\alpha 4\beta 2$, or $\alpha 6\beta 2$ AChRs (but not to $\alpha 3\beta 4$, $\alpha 4\beta 4$, or $\alpha 6\beta 4$ AChRs) (data not shown). Thus, mAb 295 binds to an epitope on $\beta 2$, whose conformation changes upon assembly with α subunits. The rat mAb 290 to $\beta 2$ subunits has very similar properties (Sallette et al., 2005). The substantial amount of ^{125}I-mAb 295 binding to intact membranes before up-regulation suggests that there is a large pool of assembly intermediates consisting of at least $\alpha 4\beta 2$ pairs before up-regulation. The association between $\alpha 4$ and $\beta 2$ within the assembly intermediates in intact membranes is easily disrupted by the detergent Triton X-100, as shown in Figs. 11 and 12. In Triton X-100, the subunits of mature AChR pentamers remain associated, and the AChRs are efficiently solubilized. The assembly intermediates seem to have lower affinity for epibatidine than do mature AChRs. This would account for the higher affinity of [^{3}H]nicotine binding to mature immunoisolated $\alpha 4\beta 2$ AChRs (K_D = 2.7 nM) compared with the EC_{50} value for up-regulation of 35 nM and the K_D = 0.017 nM for binding of [^{3}H]epibatidine compared with its EC_{50} value for up-regulation of 3.6 nM. This would also account for the ~5-fold increase in binding of [^{3}H]epibatidine after up-regulation measured using 2 nM [^{3}H]epibatidine. The 25% increase in binding of ^{125}I-mAb 295 on up-regulation would then reflect that before up-regulation, most $\alpha 4$ and $\beta 2$ subunits are part of assembly precursors, which exhibit the mature conformation of $\beta 2$, moderate ligand binding affinity, and susceptibility to disruption by Triton X-100.

The total amount of binding of ^{125}I-mAb 371 to $\alpha 4$ subunits in the cells is the same before and after up-regulation with 0.5 μM nicotine for 20 h. This indicates that the total pool of $\alpha 4$ subunits remains constant during up-regulation. Unlike mAb 295, mAb 371 binds both to assembled native $\alpha 4$ subunits and to dissociated denatured subunits.

Large pools of dissociated $\alpha 4$ and $\beta 2$ subunits are present in Triton X-100 extracts of this cell line (Fig. 11). Nicotine added overnight causes assembly of these subunits into mature AChRs. Nicotine causes no substantial increase in the total amount of subunits (i.e., it causes assembly of AChRs not synthesis of subunits).

Sucrose gradient sedimentation of Triton X-100 extracts showed that most epibatidine binding sites before and after up-regulation by nicotine are concentrated at 10 S, as expected for pentameric $\alpha 4\beta 2$ AChRs (Fig. 12A). The shoulder at 8.5 S on the peak of epibatidine binding may represent an assembly intermediate smaller than pentamers. The total amount of epibatidine binding sites in Triton X-100 extracts increased 13- to 20-fold (Fig. 12A) compared with a 3- to 5-fold increase of surface binding sites (Figs. 6 and 10) or a 5-fold increase in total epibatidine binding to fixed cells (Table 2). The greater extent of up-regulation of epibatidine binding to mature AChRs in Triton X-100 extracts probably results because much of the epibatidine binding in cells before up-regulation is to assembly intermediates of $\alpha 4$ and $\beta 2$ subunits, which are easily disrupted by Triton X-100.

Western blots of sucrose gradient fractions revealed $\alpha 4$ subunits (Fig. 12B) and $\beta 2$ subunits (data not shown) concentrated in the 10 S peak of mature AChR. There was no peak of unassembled subunits around 4 to 5 S, where monomeric subunits would be expected. Instead, many unassembled subunits were observed at high apparent molecular weight (sedimenting faster than 10 S pentamers), suggesting that they were in association with large protein chaperones or were aggregated. mAb 295 to $\beta 2$ subunits could not immunoprecipitate $\alpha 4$ subunits from fractions along gradients except in the case of 10 S mature AChRs, showing that in the presence of Triton X-100, any assembly intermediates between $\alpha 4$ and $\beta 2$ were either dissociated or disrupted so that they were no longer recognizable by mAb 295 (data not shown).

Chemical Cross-Linking May Stabilize the Labile Assembly Intermediate on Which Nicotine Acts as a Pharmacological Chaperone to Cause Up-Regulation. To try to stabilize an assembly intermediate of $\alpha 4\beta 2$ AChRs, which is disrupted by Triton X-100, and through which binding of nicotine in the ER might promote assembly of mature AChRs, $\alpha 4\beta 2$ AChRs were treated with the cross-linking reagent DSP (Fig. 13). This revealed an epibatidine-binding 8.5 S component that could correspond to an assembly intermediate. This component would have to contain at least one $\alpha 4\beta 2$ subunit pair to bind epibatidine (molecular weight ≈ 119,000). This putative assembly intermediate is larger than 7 S IgG (molecular weight ≈ 150,000) and smaller than 9.5 S *T. californica* AChR monomer (molecular weight ≈ 268,000) and thus could correspond to an $\alpha 4\beta 2\alpha 4\beta 2$ tetramer (molecular weight ≈ 239,000). Cross-linking inhibited some epibatidine binding and increased the sedimentation of mature AChRs somewhat, perhaps caused by linkage to adjacent proteins or by changes in AChR conformation.

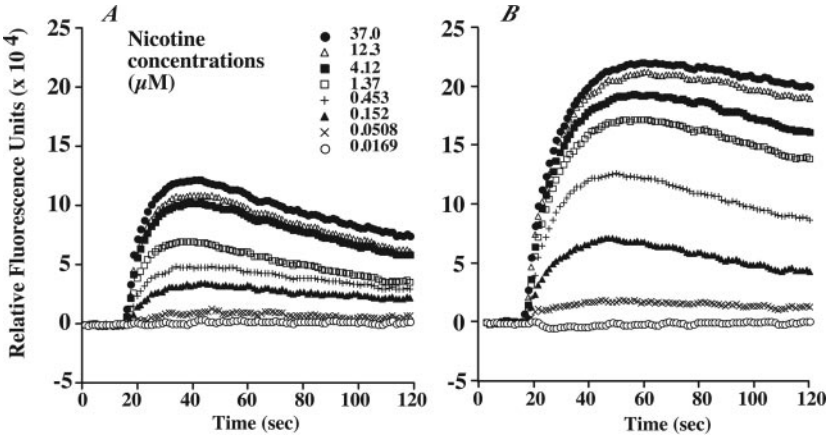

Fig. 7. Nicotine treatment increases the number of functional AChRs detectable after a 1-h wash to reverse the desensitizing effects of nicotine. The responses to various concentrations of nicotine are shown in the absence (A) and in the presence (B) of treatment overnight with 0.5 μM nicotine. A membrane-potential-sensitive indicator was used.

Increased AChR Half-Life Contributes to Up-Regulation. As in our human $\alpha3\beta2$ AChR cell line (Wang et al., 1998) and chicken $\alpha4\beta2$ AChR cell line (Peng et al., 1994), up-regulation of $\alpha4\beta2$ AChR depends not only on increased assembly of new AChRs but also on an increase in the half-life of AChRs in the surface membrane (Fig. 14).

The increased lifetime of AChRs in nicotine apparently requires the continuous presence of nicotine. Gopalakrishnan et al. (1997) reported that if nicotine were removed after up-regulation, the number AChRs decreased with a $t_{1/2}$ = 11.7 h. This is close to the $t_{1/2}$ = 12.6 h, which we measured for the loss of AChRs in cells that were never exposed to nicotine.

Discussion

We propose that nicotine and other nicotinic ligands can act on an assembly precursor as pharmacological chaperones to cause up-regulation by promoting the assembly of mature AChR pentamers. In transfected cells, there are large pools of subunits in the ER (Figs. 11 and 12) (Wang et al., 1998; Sallette et al., 2005). Many of these subunits are in assembly intermediates (Fig. 10D), approximately the size of $\alpha4\beta2\alpha4\beta2$ tetramers (Fig. 13) (Sallette et al., 2004, 2005). The affinity of these assembly intermediates for nicotinic ligands is high, reflecting the potency of these ligands for up-regulating AChRs by this mechanism (Table 2), but it is probably lower than that for mature AChRs. For example, the EC_{50} value for up-regulation by nicotine is 35 nM, whereas the K_D value for binding to mature AChRs is 2.7 nM. The association of the subunits in assembly intermediates is easily disrupted by solubilization in Triton X-100 (Figs. 10–

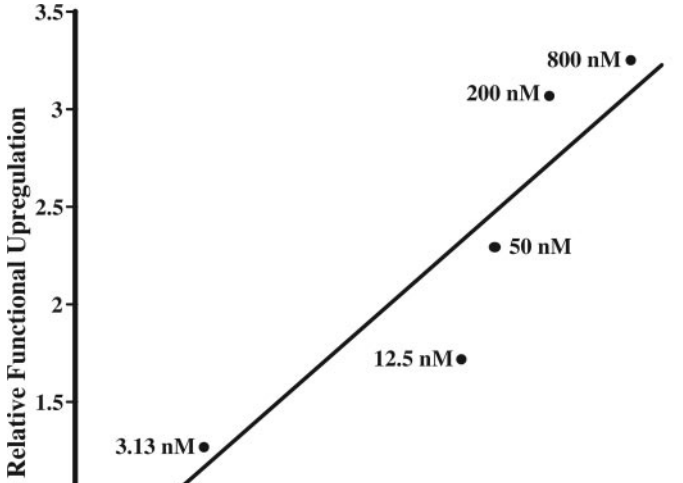

Fig. 8. Overnight exposure to nicotine increases surface $\alpha4\beta2$ AChRs in direct proportion to the increase in AChR function (assayed after a 1-h wash). The slope of the line is less than 1. The decrement reflects the fraction of AChRs, which are irreversibly desensitized under these conditions. The number over each point in the graph is the nicotine concentration used for overnight incubation. Surface up-regulation was determined as the difference between total binding of 0.5 nM [^{3}H]epibatidine for 15 min and its binding in the presence of 10 mM carbamylcholine to block epibatidine binding to surface AChRs. Thus, at 50 nM nicotine, the amount of AChRs on the surface increases 3.3-fold, but the response to acetylcholine increases only 2.3-fold.

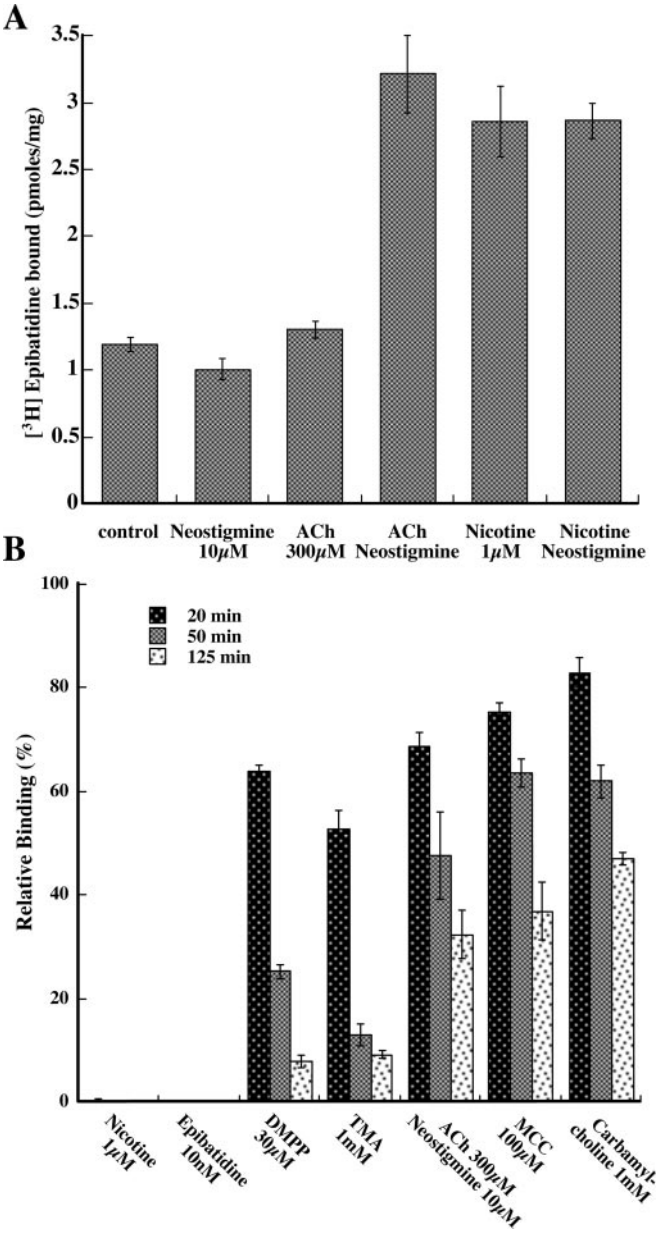

Fig. 9. Quaternary amines can penetrate inside cells and induce up-regulation. A, acetylcholine protected from esterase destruction by neostigmine can induce as much up-regulation in 3 h as can nicotine. Cells on 24-well plates were treated for 3 h with 300 μM acetylcholine, 1 μM nicotine, and 10 μM neostigmine. After incubation, the cells were washed three times with DMEM and fixed with 2% formaldehyde for 1 h before labeling with [^{3}H]epibatidine. Bound radioactivity was eluted by 200 μl of 0.1 M NaOH. Protein concentration was calculated on wells in parallel experiment. B, quaternary amines slowly enter the ER and Golgi apparatus, in which they can compete for binding of [^{3}H]nicotine. The tertiary amines nicotine and epibatidine cross cell membranes very quickly to compete for binding within the cell. Cells on 96-well plates were up-regulated overnight with 0.5 μM nicotine to neutralize the effect of ongoing up-regulation during incubation with quaternary amines. Then, cells were washed three times with 200 μl of DMEM. The drugs were added either 105 or 30 min before [^{3}H]nicotine or together with 5 nM [^{3}H]nicotine and then incubated for an additional 20 min at 37°C and 5% CO_2 before washing the cells with DMEM. The cells were washed three times with 200 μl of DMEM, and then bound radioactivity was eluted by 0.1 M NaOH.

12) but can be stabilized to solubilization in Triton X-100 by cross-linking with DSP (Fig. 13). When nicotine binds to assembly intermediates, it acts as a pharmacological chaperone. Binding of ligands to the ACh binding site at the interface between $\alpha 4$ and $\beta 2$ subunits may stabilize assembly intermediates, resulting in increased amounts of intermediates and consequently of mature AChRs. Binding of agonists may also promote conformation changes in the assembly intermediate corresponding to those the agonist would produce during activation or desensitization, and these conformation changes may further promote the assembly of mature AChRs. This would account for the far greater potency of agonists than antagonists as pharmacological chaperones. If the assembly intermediates were $\alpha 4\beta 2\alpha 4\beta 2$ tetramers with two ACh binding sites, final assembly would consist only of the addition of a $\beta 2$ subunit in the accessory position. The precise pathway of $\alpha 4\beta 2$ AChR assembly remains to be determined.

The agonist-mediated conformation changes in the acetylcholine binding protein described by Gao et al. (2005) reflect the sorts of interactions that may be involved in molecular chaperone effects. As acetylcholine enters the binding site, it brings together conserved tryptophan residues from adjacent subunits. This might be a "molecular glue" effect shared by agonists and antagonists, which would help to stabilize the association of $\alpha 4$ and $\beta 2$ in assembly intermediates. Acetylcholine was found to cause a large movement of the C loop to close access to the binding site and mediate new contacts

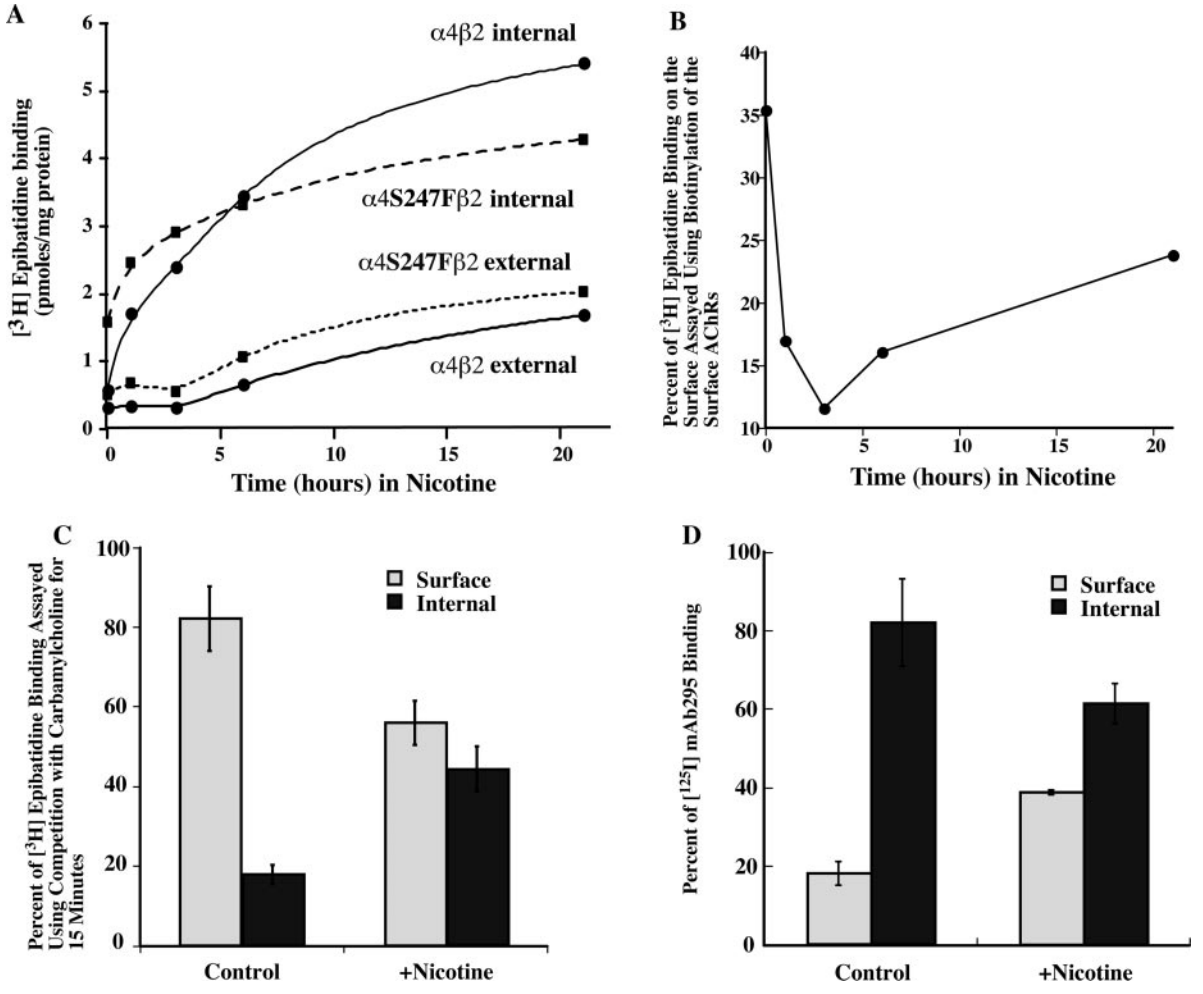

Fig. 10. Nicotine-induced up-regulation of intracellular and surface AChRs. A, the time course of nicotine-induced up-regulation is plotted. There is a rapid increase in intracellular $\alpha 4\beta 2$ AChRs, followed after a lag of 3 h by an increase in AChRs on the surface membrane. The mutant line starts with a somewhat higher level of total expression (2.1 pmol/mg of protein) than wild type (0.9 pmol/mg), which could reflect either results of independent transfection and selection for the two lines or some intrinsic difference in expression of the two AChRs. After incubation in 0.5 μM nicotine for the indicated times, all 35-mm dishes of cells were simultaneously labeled with the membrane-impermeable reagent sulfo-NHS-LC biotin. Triton X-100 extracts from each plate were incubated with [^{3}H]epibatidine in wells coated with mAb 295 to $\beta 2$ subunits to bind all AChRs or coated with streptavidin to bind surface-labeled AChRs. B, the same data as in A for the wild-type cell line are presented as the ratio of surface to total AChRs. C, competitive inhibition of [^{3}H]epibatidine binding to surface AChRs by carbamylcholine was used to determine the proportion of surface and intracellular AChRs before and after up-regulation. Binding to living cells attached to 35-mm plates was done in DMEM at 4°C for only 15 min with 0.5 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, 10 mM concentration of the quaternary amine carbamylcholine was added together with 0.5 nM [^{3}H]epibatidine to inhibit binding to cell-surface AChRs. D, the proportion of the cell surface and intracellular AChRs before and after up-regulation was measured by binding of ^{125}I-mAb 295 plus or minus permeabilization. Cells expressing $\alpha 4\beta 2$ AChRs were labeled with 2 nM ^{125}I-mAb 295 to $\beta 2$ subunits after fixation for 1 h using 2% phosphate-buffered formaldehyde plus or minus permeabilization with 0.1% Triton X-100 for 1 h at room temperature. Incubation with 0.5 μM nicotine overnight increased binding of ^{125}I-mAb 295 to permeabilized cells only by 25%. Binding to the surface of cells that were not permeabilized with Triton X-100 was increased by 300%. Because this mAb binds only to $\beta 2$ in association with $\alpha 4$ (Ren et al., 2005), these results suggest much of the $\beta 2$ in the cells is associated with $\alpha 4$.

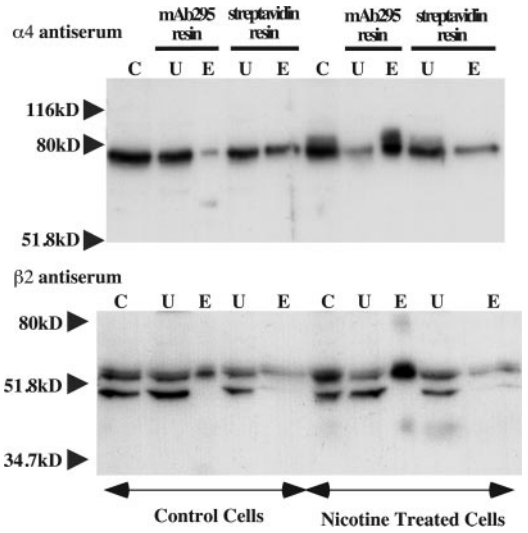

Fig. 11. Large pools of α4 and β2 subunits in the cell line are demonstrated by Western blots of Triton X-100 extracts of control and biotinylated cells. These are seen in crude extracts (C) at similar levels before and after up-regulation overnight with 0.5 μM nicotine. Before up-regulation, most subunits are in the unbound (U) fraction after adsorption with mAb 295 or streptavidin bound to agarose, indicating that there are few mature pentameric AChRs and that any partially assembled α4 and β2 subunits are disrupted by Triton X-100 so that they can no longer bind to mAb 295. In fractions eluted with SDS from these adsorbents (E), initially only a small amount of assembled AChRs are present, but the amount increases greatly after up-regulation. α4 and β2 subunits can be found at more than one molecular weight. This reflects differences in glycosylation. Removal of glycosylation using PNGase F resulted in single bands of lower molecular weights (data not shown). The effects of endoglycosidases that we observed were very similar to those of Sallette et al. (2005). The surface AChRs, which have been biotinylated and adsorbed by streptavidin, indicate the glycosylation state of the mature surface AChRs.

between subunits across the binding site, including hydrogen bonds between side chains on the loop and the opposing β8–β9 linker. This might reflect the agonist-induced conformation change through which nicotine and other agonists gain great potency in promoting the assembly of AChR subunits.

The surprising observation that quaternary amine cholinergic ligands gain access to the interior of the ER after prolonged incubation is also supported by the observations of others (Sallette et al., 2005; Vallejo et al., 2005). Thus, both rapidly membrane-permeable ligands like nicotine and very slowly membrane-permeable ligands like MCC can act as pharmacological chaperones.

The up-regulated AChRs can function in the surface membrane (Figs. 3, 7, and 8) after recovering from desensitization or antagonism by the drug (Fig. 4). Human α4β2 AChRs in the presence of the 0.2 μM nicotine concentrations sustained in the sera of cigarette smokers (Benowitz, 1996) are substantially increased in amount over 6 h (Figs. 8 and 10), but 90% are desensitized (Fig. 4) in the continued presence of this concentration of nicotine. By 1 h after the washout of this concentration of nicotine, 82% of the AChRs present can be activated by nicotine (Fig. 8).

Studies of our human α4β2 AChR cell line have revealed a fundamental new insight into nicotine-induced up-regulation: nicotine can act as a pharmacological chaperone to promote the assembly of AChR subunits. This concept is also supported by the studies of Sallette et al. (2004, 2005). Pharmacological chaperones have been found previously to promote the conformational maturation of single subunit metabotropic receptors for opioids and vasopressin (Morello et al., 2000; Petaja-Repo et al., 2002).

These studies conflict with the mechanism of nicotine-

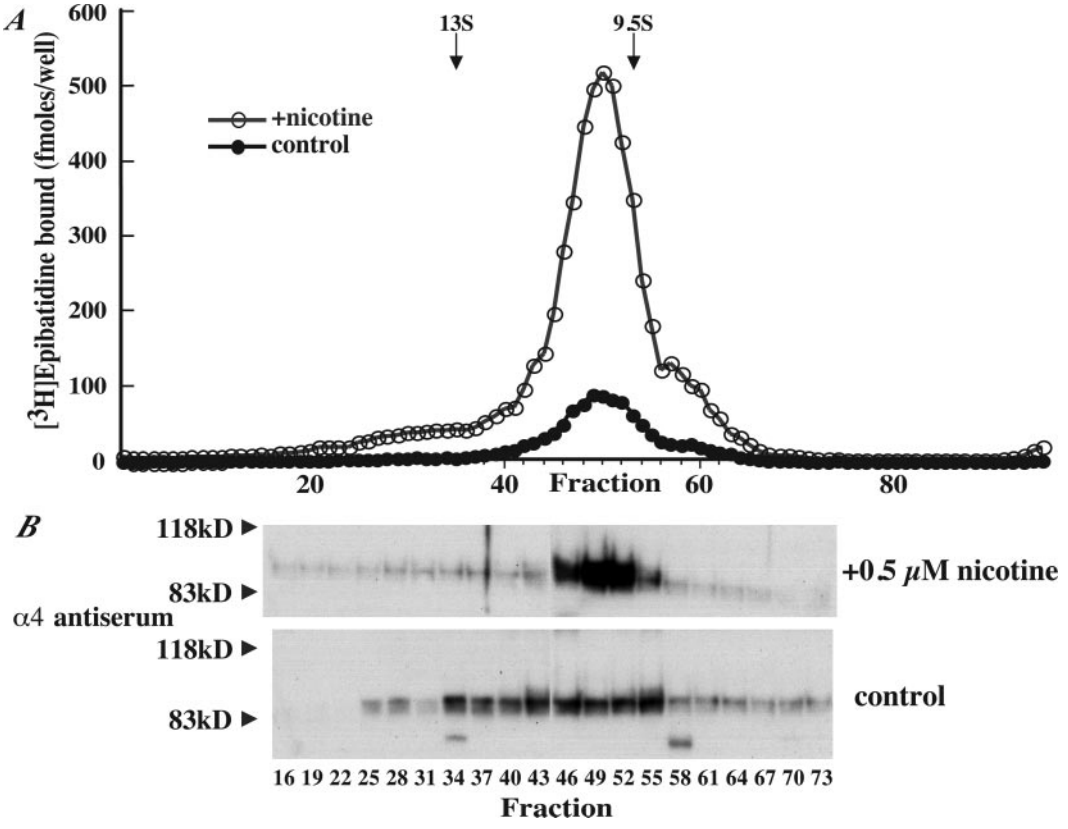

Fig. 12. Sucrose gradient velocity sedimentation of Triton X-100 extracts from the α4β2 cell line shows a large increase in 10 S mature α4β2 AChRs after up-regulation overnight with 0.5 μM nicotine. A, binding of [^{3}H]epibatidine to AChRs immunoisolated from fractions of the gradient. Arrows indicate the position of *T. californica* AChR 9.5 S monomers and 13 S dimers run as internal standards on the gradients. B, Western blots of α4 subunits in fractions from the gradient. β2 subunits were similarly distributed (data not shown). Samples were sedimented 16 h at 40,000 rpm in an SW41 rotor on 5 to 20% sucrose gradients containing 0.5% Triton X-100. α4β2 AChRs were isolated for labeling with [^{3}H]epibatidine on wells coated with mAb 295. The *T. californica* AChRs were isolated for labeling with ^{125}I-α-bungarotoxin on microwells coated with mAb 210.

induced up-regulation proposed by Vallejo et al. (2005). They propose that nicotine converts AChRs already in the surface membrane to a state which binds more epibatidine, is more sensitive to activation, and desensitizes more slowly. We show that the increase in epibatidine binding and increase in response to agonists at 3 to 24 h is primarily caused by the assembly of new, mature 10 S AChRs. The increased sensitivity to activation and reduced rate of desensitization results from the selective up-regulation of the $(\alpha4)_2(\beta2)_3$ stoichiometry, which has these properties.

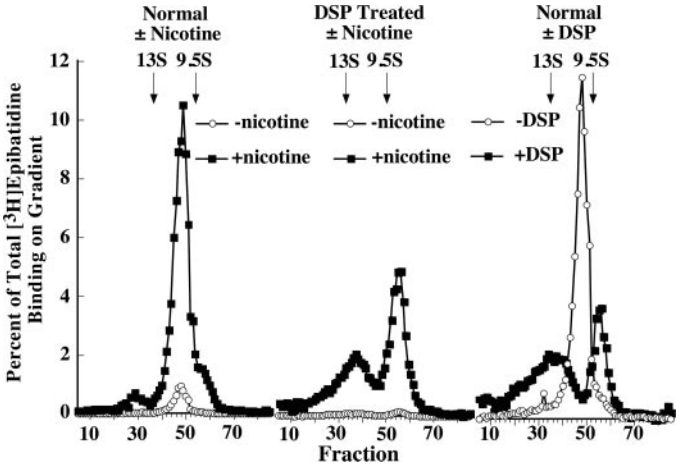

Fig. 13. DSP cross-linking of the $\alpha4\beta2$ cell line reveals an 8.5 S component that can bind epibatidine and may be an assembly intermediate, easily dissociated by Triton X-100, to which nicotine binds to promote assembly of AChR pentamers. Cells were cross-linked for 2 h on ice with 1 mM DSP in PBS before the reaction was stopped by adding 10 mM Tris, pH 7.5, for 15 min, then rinsing three times with PBS. Sucrose gradient sedimentation was as in Fig. 11. Overnight up-regulation with 0.5 μM nicotine increased the number of epibatidine labeled AChRs 13-fold in Triton extract fractions isolated on mAb 295-coated microwells, as shown on the left. Middle, DSP cross-linking before solubilization in Triton X-100 revealed the existence of an 8.5 S peak of partially assembled AChRs, which is usually dissociated by the detergent. Right, the control samples shown in the middle and left. On the right, 100% is defined for each of the gradients as the integrated number of binding sites on that gradient. This allows observing the small control peaks, which are otherwise obscured by the large increase in the amount of AChR in the nicotine-treated samples in the left and middle.

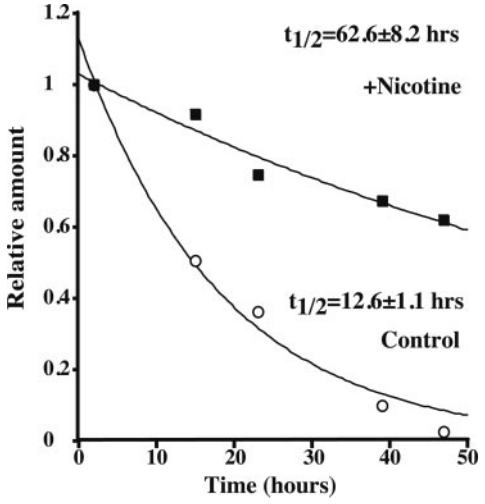

Fig. 14. Nicotine treatment increases the half-life of surface membrane $\alpha4\beta2$ AChRs. Nicotine-treated cells were incubated for 16 h with 0.5 μM nicotine. Before biotinylation, the cells were detached with ice-cold PBS and 5 mM EDTA and then washed twice by centrifugation with PBS only. The cells were surface-labeled with Sulfo-NHS-LC biotin as in Fig. 10 and then replated and grown with 0.5 μM nicotine for nicotine-treated cells and without it for control cells. At the indicated times, Triton X-100 extracts of cells were applied to streptavidin beads to isolate AChRs biotinylated on the cell surface. AChRs were quantified by labeling with [^{3}H]epibatidine. The data represent the average of three experiments.

In addition to promoting the assembly of the new AChRs, nicotine also contributes to up-regulation by increasing the half-life of AChRs in the surface membrane (Fig. 14) (Peng et al., 1994; Wang et al., 1998). Increased half-life of surface AChR might be caused by a post-translational modification subsequent to desensitization. In the presence of nicotine, virtually all AChRs are desensitized (Fig. 4). The 5-fold increase in half-life of human $\alpha4\beta2$ AChRs caused by nicotine (Fig. 14) could contribute substantially to up-regulation over several days. In transfected cells with large pools of unassembled subunits, large increases of AChRs within the cells can accumulate within 3 h as a result of increased assembly, whereas smaller increases in surface AChRs become apparent after 5 h (Fig. 10A). In neurons without large pools of unassembled subunits, up-regulation caused by reduced degradation of surface AChRs alone would be more than sufficient to explain the extent of nicotine-induced up-regulation observed in brain (Perry et al., 1999).

The nicotine-induced increase in lifetime of surface membrane human AChRs, which we describe here, and the nicotine-induced increase in lifetime of chicken $\alpha4\beta2$ AChRs (Peng et al., 1994) and human $\alpha3\beta2$ (Wang et al., 1998) has not been observed in other studies (Darsow et al., 2005; Sallette et al., 2005; Vallejo et al., 2005). All of our studies that detected a nicotine-induced increase in the lifetime of AChRs used permanently transfected lines. Sallette et al. (2005) transiently transfected HEK cells with human $\alpha4\beta2$ AChRs, which may have inhibited the detection of this effect measured in experiments longer than 50 h in our permanently transfected cell line. Vallejo et al. (2005) permanently transfected an HEK cell line with rat $\alpha4\beta2$ AChRs. Species-specific effects may be important in this case. In addition, instead of biotinylating the extracellular surface of AChRs as we did to identify surface AChRs in turnover experiments, they used a reagent which biotinylated the cytoplasmic surface of the AChRs, and this might influence their results. Their biotinylation method also did not detect the initial nicotine-induced increase in newly assembled AChRs in the ER demonstrated by pulse-chase labeling by Sallette et al. (2005) or by three other methods shown in Fig. 10. Darsow et al. (2005), using HEK cells transiently transfected with mouse $\alpha4\beta2$, did not detect a nicotine-induced increase in lifetime. They agree that nicotine increased assembly but detected only 1.8% of the amount of AChRs that we detect in our permanently transfected cell line. The very rapid rate of internalization of surface AChRs that they observe (60% in 30 min) may be a species-specific effect, which accounts for their low level of expression and lack of detection of an effect of nicotine on AChR lifetime.

The lack of function of S247F$\alpha4$ $\beta2$ AChRs (Fig. 5) does not prevent up-regulation of AChRs by ligands acting as pharmacological chaperones. The ADNFLE mutation S247F in the channel lining M2 region of $\alpha4$ subunits alters the function of $\alpha4\beta2$ AChRs expressed in *X. laevis* oocytes (Kuryatov et al., 1997). This mutation virtually completely prevents function when these AChRs are expressed in HEK cells (Fig. 5). Loss of function by this mutant in HEK cells may be in

part because 80% of the AChRs in this cell line have an $(\alpha4)_3(\beta2)_2$ stoichiometry, whereas, at appropriate ratios of subunit mRNAs, virtually all of the AChRs expressed in oocytes can have an $(\alpha4)_2(\beta2)_3$ stoichiometry (Zhou et al., 2003). With the $(\alpha4)_3(\beta2)_2$ stoichiometry of the mutant, three phenyl groups in the M2 channel lining transmembrane domain are in the channel rather than two in the $(\alpha4)_2(\beta2)_3$ stoichiometry. The replacement of three serines in the channel by three phenyl groups may almost completely prevent current flow in the $(\alpha4)_3(\beta2)_2$ stoichiometry of the mutant. This idea is supported by the observation (data not shown) that transient transfection of the mutant line with either $\beta3$ or $\alpha5$ subunits produces AChRs with abundant function. The addition of these accessory subunits would result in $(\alpha4)_2(\beta2)_2\alpha5$ or $(\alpha4)_2(\beta2)_2\beta3$ stoichiometries with only two phenyl groups in the channel. In *X. laevis* oocytes, S247F$\alpha4\beta2$ AChRs are only weakly activated by the first application of agonist, but subsequently they are efficiently activated (Kuryatov et al., 1997; Figl et al., 1998). These mutant AChRs desensitize faster than wild type, have reduced inward rectification, and virtually no Ca^{2+} permeability. If S247F $\alpha4\beta2$ AChRs were as inactive in ADNFLE neurons as in HEK cells, the effects of this mutation in the homozygous state would be greater than experiments in *X. laevis* oocytes have suggested.

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