The primary target for nicotine in the brain is the neuronal nicotinic acetylcholine receptor (nAChR). It has been well documented that nAChRs respond to chronic nicotine exposure by up-regulation of receptor numbers, which may underlie some aspects of nicotine addiction. In order to investigate the mechanism of nicotine-induced nAChR up-regulation, we have developed a cell culture system to assess membrane trafficking and nicotine-induced up-regulation of surface-expressed $\alpha_4\beta_2$ nAChRs. Previous reports have implicated stabilization of the nAChRs at the plasma membrane as the potential mechanism of up-regulation. We have found that whereas nicotine exposure results in up-regulation of surface receptors in our system, it does not alter surface receptor internalization from the plasma membrane, postendocytic trafficking, or lysosomal degradation. Instead, we find that transport of nAChRs through the secretory pathway to the plasma membrane is required for nicotine-induced up-regulation of surface receptors. Therefore, nicotine appears to regulate surface receptor levels at a step prior to initial insertion in the plasma membrane rather than by altering their endocytic trafficking or degradation rates as had been previously suggested.

Neuronal nicotinic acetylcholine receptors (nAChRs) in mammalian brain compose a family of proteins encoded by 11 genes ($\alpha_2$-$\alpha_7$, $\alpha_9$-$\alpha_{10}$ and $\beta_2$-$\beta_4$) that assemble into pentameric ligand-gated ion channels (1–4). Although all combinations of subunits that form functional nicotinic receptors can bind nicotine, $\alpha_4$- and $\beta_2$-containing receptors form the high affinity nicotine binding site (5–9) and are therefore thought to be the primary nAChR subtype affected by the relatively low nicotine concentrations (100–500 nM) found in the blood of smokers (10).

Chronic exposure of $\alpha_4\beta_2$ receptors to nicotine, as occurs in smokers, results initially in receptor desensitization, followed by subsequent up-regulation of high affinity nicotine binding sites (11). Upon nicotine withdrawal, the increased number of nAChRs recover from desensitization, resulting in excess activity in the nAChR system. It has been proposed that this cycle of nicotine-induced receptor up-regulation may contribute to the negative symptoms associated with nicotine withdrawal, resulting in continued tobacco consumption and, eventually, nicotine dependence (12).

The mechanisms and cellular machinery required for nicotine-induced up-regulation remain unknown. However, a large body of research supports a consensus on several relevant points. First, up-regulation is observed in the brains of human smokers (13) as well as in chronically nicotine-treated animal models (8, 14) and in cultured cells heterologously expressing nAChRs (15–18), suggesting that up-regulation requires basic, conserved cellular processes. It has been convincingly shown that increased nicotine binding reflects an increase in receptor number rather than receptor affinity (15, 19–21), implying that up-regulation involves receptor protein dynamics rather than intrinsic changes in the existing receptors. Finally, chronic nicotine treatment does not increase nAChR subunit mRNA levels (14), making transcriptional regulation unlikely.

The functionally relevant pool of nAChRs in neuronal cells exists at the plasma membrane, where exposure to neurotransmitter regulates membrane excitability. In many cases, cell surface receptor activity is regulated by insertion and removal of receptors at the plasma membrane (22). For example, it is becoming increasingly clear that modulation of neuronal synaptic transmission is regulated at least in part by the dynamic localization and turnover of neurotransmitter receptors (23, 24). Furthermore, ligand-induced opiate receptor endocytosis has been shown to be a determinant of the addictive potential of opiate agonists (25). Therefore, mechanisms dictating the number of surface receptors at the plasma membrane may also be involved in nicotine-induced up-regulation.

To directly test this hypothesis, we analyzed the up-regulation and trafficking of surface $\alpha_4\beta_2$ nAChRs using a cell culture model that expresses functional surface-localized $\alpha_4\beta_2$ receptors. We show that in this system, chronic nicotine treatment induces the up-regulation of surface $\alpha_4\beta_2$ nAChRs in a manner that is similar to nAChR up-regulation observed in the brains of human smokers and in animal models chronically exposed to nicotine. Our analysis of nicotine-dependent nAChR trafficking suggests that up-regulation is not mediated via modulation of endocytic trafficking but at a biosynthetic step prior to insertion of $\alpha_4\beta_2$ nAChRs into the plasma membrane.

**MATERIALS AND METHODS**

**Cell Culture, Transfection, and DNA Constructs—Human embryonic kidney cells (HEK293T) were maintained at 37 °C in 5% CO2 and passaged in HEPES-buffered Eagle’s medium (Specialty Media, Phillipsburg, NJ) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM $\beta$-glutamine (Specialty Media), and 50 units/ml penicillin and streptomycin (Invitrogen). Transient transfection of cDNA constructs into HEK293T cells was accomplished by calcium phosphate precipitation (26). Mouse cDNAs encoding the $\alpha_4$ and $\beta_2$ nAChR subunits were a generous gift from Dr. Jerry Stützel (University of Colorado). HA epitope-tagged mouse SKD1...
and SKD1(E235Q) clones were a gift from Dr. Scott Emr (University of California, San Diego) and Dr. Markus Babst (University of Utah).

Electrophysiology—HEK293T cells were co-transfected by the calcium phosphate method with separate plasmids containing α2β2 and EGFP at a 1:1:0.05 ratio. Whole-cell recordings were performed at room temperature (–21 °C) from EGFP-expressing cells 24–48 h after transfection. Coverslips were transferred from the incubator (37 °C, 8% CO2) to a recording chamber (RC-26G, Warner Instruments) fitted to the stage of an upright microscope (AxioScope, Zeiss). Cells were continuously superfused with a HEPES-buffered extracellular solution composed of 146 mM NaCl, 2.5 mM KCl, 1.2 mM CaCl2, 1 mM MgCl2, 20 mM sucrose, 20 mM glucose, 10 mM Hepes, 1 μM atropine, pH 7.4 (adjusted with NaOH), 310 mosmol. Patch pipettes were pulled on a micropipette puller (P83; Sutter Instruments) from borosilicate glass capillaries (GC150F-10; inner diameter, 0.86 mm; outer diameter, 1.50 mm; Harvard Apparatus) and fire-polished on a microforge (MF-83, Narishige, Japan) to a final tip open resistance of 3–5 megohms. Patch pipettes were filled with a CsCl-based internal solution composed of 140 mM CsCl, 4 mM NaCl, 0.5 mM CaCl2, 5 mM EGTA, 10 mM HEPES, 0.5 mM NaGTP, and 2 mM MgATP, 290 mosmol, pH 7.3 (adjusted with CsOH).

Brief pulses (300 ms) of acetylcholine (1 mM) were applied onto the cells every 10 min for up to 60 min. Acetylcholine-evoked whole-cell currents were recorded using a patch-clamp amplifier (Axopatch 200A; Axon Instruments). Membrane currents were isolated using whole-cell patch-clamp configuration. Currents were filtered on-line at 2 kHz using an eight-pole Bessel low pass filter (902LPF, Frequency Devices, Haverhill, MA), digitized at 1 kHz using an analog-to-digital converter (Digidata 1322A; Axon Instruments), and stored on the hard drive of a personal computer (Dimension XPS T600, Dell) using pClamp8 (Axon Instruments). Data analysis was carried out using Clampfit8 (Axon Instruments). Drugs and reagents were obtained from the following sources: nicotine was from Research Diagnostics, Inc. (Raritan, NJ) and chlorisondamine was from Sigma Chemical Co. (St. Louis, MO).

[3H]Epibatidine Binding Assays—Cells were transfected with plasmids containing α2 and β2 subunit genes either singly or in combination and grown for 24 h following transfection. Cells were washed once in Ringer’s binding buffer (140 mM NaCl, 1.5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 25 mM HEPES, pH 7.5) resuspended from culture dishes in Ringer’s binding buffer and harvested by centrifugation at 1500g for 10 min at 4 °C. Cells were resuspended in ice-cold TS buffer and homogenized by passing through a 27-gauge needle 15 times. Lysates were cleared by a brief centrifugation step at 1000g for 2 min.

The total membranes were fractionated from the supernatant by centrifugation at 30,000 rpm in a TLA100 ultracentrifuge and washed a total of three times in Ringer’s binding buffer before resuspension in water. Membranes were then directly assayed for binding. The number of nicotine binding sites was determined by equilibrium binding of [3H]epibatidine (Amersham Biosciences) to [3H]epibatidine-expressing cells. Following incubation, the membranes were blocked and permeabilized in IF buffer (PBS containing 1% bovine serum albumin and resuspended in PBS containing 1 μg/ml propidium iodide (Sigma) to differentiate between live and dead cells. The fluorescence intensity of 30,000 single, live cells was collected for each sample using a BD Biosciences FACScan flow cytometer. Each experimental value reported in the figures is the average from at least three independent experiments.

Fluorescence bleed-through between channels was not detected in any double labeling experiments.

Immunofluorescence and Microscopy—Surface staining of live cells was performed on cells grown on glass coverslips and transfected with plasmids containing α2 and β2 subunit genes. At 24 h post-transfection, cells were transfected with 4°C for 10 min to inhibit membrane trafficking. Growth medium was replaced with fresh chilled medium containing primary antibody, mAb 299 or mAb 270 (Covance, Richmond, CA) diluted at 1:500 against the α2- or β2-αNaChBnununits, respectively, and incubated for 1 h at 4°C. Cells were washed twice with chilled phosphate-buffered saline (PBS) to remove unbound antibodies and then fixed first with 2% paraformaldehyde for 10 min and then with 4% paraformaldehyde for an additional 30 min. Fixed cells were washed twice in PBS and then blocked and permeabilized in IF buffer (PBS containing 1% bovine serum albumin and 0.1% saponin) plus 4% fetal bovine serum for 30 min. Following the blocking step, the specimens were incubated with Cy3 goat anti-rat secondary antibodies (Chemicon, Temecula, CA) diluted at 1:1000 in IF buffer containing mAb 299 and goat anti-rabbit Cy3 or goat anti-mouse fluorescein isothiocyanate (Jackson Immunoresearch, West Grove, PA) against HA and Lamp1, respectively. Following secondary antibody incubations, cells were washed and mounted for confocal microscopy as described above. Co-localization experiments with transferrin receptor and SKD1 mutants and calnexin and the β2 receptor subunit were performed as described above, except cells were first fixed and then labeled with primary antibodies at 1:500 each against transferrin receptor (Zymed Laboratories Inc., San Francisco, CA) and HA or calnexin (Stressgen, San Diego, CA) and β2 (Covance, Richmond, CA) concurrently. Confocal images were viewed with a Zeiss confocal microscope. Fluorescence bleed-through between channels was not detected in any double labeling experiments.

Quantitation of Surface Expression and Up-regulation—For experiments with agonists/antagonists and/or other pharmacological agents, the compounds were added to the cells at 24 h post-transfection and then incubated for the indicated times prior to the start of the experiment. Once cells were harvested, they were maintained at 4°C at all times, and all centrifugation steps were done at 1000×g.

Transfected cells expressing α2β2 receptors were grown to confluence and washed twice with room temperature borate buffer (10 mM borate, 100 mM NaCl). Cell surface proteins were biotinylated with 6 mg/ml sulfo-NHS-biotin (Pierce Biochemicals). Cells from 35-mm dishes were blocked and permeabilized in IF buffer (PBS containing 1% bovine serum albumin and resuspended in PBS containing 1 μg/ml propidium iodide (Sigma), pH 7.4, 150 mM NaCl, 1% Triton X-100) containing protease inhibitor mixture tablets (Roche Applied Science) and incubated for 10 min on ice and then clarified by centrifugation at 14,000×g for 10 min at 4°C. Clarified extracts were incubated with streptavidin-agarose for 3 h at 4°C to precipitate biotinylated proteins. Streptavidin-agarose precipitates were washed twice in SWB containing 0.1% Triton X-100 and then resuspended (50 mg/ml HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100) containing protease inhibitor mixture tablets (Roche Applied Science). The specimens were incubated for 10 min in ice and then centrifuged at 14,000×g for 10 min at 4°C. Clarified extracts were incubated with streptavidin-agarose for 3 h at 4°C to precipitate biotinylated proteins. Streptavidin-agarose precipitates were washed twice in SWB containing 0.1% Triton X-100 and then resuspended (50 mg/ml HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100) containing protease inhibitor mixture tablets (Roche Applied Science). The proteins were then resuspended in 125 mM Tris, pH 6.8, 20% (v/v) glycerol, 6% SDS, 0.005% bromphenol blue, 0.002% β-mercaptoethanol, separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by immunoblotting with antibodies against α2 (Covance, Richmond, CA).

Quantitation of Surface Expression and Up-regulation—For experiments with agonists/antagonists and/or other pharmacological agents, the compounds were added to the cells at 24 h post-transfection and then incubated for the indicated times prior to the start of the experiment. Once cells were harvested, they were maintained at 4°C at all times, and all centrifugation steps were done at 1000×g.

Transfected cells expressing α2β2 receptors were grown to confluence (48 h post-transfection) and suspended by gently pipetting in ice-cold PBS. The cells were harvested and resuspended in HEPES medium containing an additional 10% fetal bovine serum and incubated at 4°C with rotating for 30 min. Following blocking, cells were harvested and then resuspended and incubated in mAb 299 antibodies against surface α2β2 receptors diluted at 1:1000 in PBS plus 1% bovine serum albumin for 1 h. Cells were then washed three times in cold HEPES medium and resuspended in biotinylated anti-rat secondary antibodies (Chemicon, Temecula, CA (1:1000) and incubated 30 min at 4°C. Following secondary antibody incubations, the cells were again washed three times in HEPES medium and resuspended in PBS/bovine serum albumin containing fluorescein conjugated to streptavidin at 1:500 (Vector, Burlingame, CA) concurrently. Following streptavidin incubation, the cells were washed twice in PBS/bovine serum albumin and resuspended in PBS containing 1 μg/ml propidium iodide (Sigma) to differentiate between live and dead cells. The fluorescence intensity of 30,000 single, live cells was collected for each sample using a BD Biosciences FACScan flow cytometer. Each experimental value reported in the figures is the average from at least three independent experiments.
**RESULTS**

**Functional \( \alpha_4\beta_2 \) nAChRs Are Surface-expressed in HEK293T Cells**—Many previous studies of nicotine-induced nAChR up-regulation have used membrane-permeable ligands to assess the total number of cellular receptor binding sites (16–18). These studies have shown in at least one cell culture model that a large percentage of ligand binding sites are localized intracellularly (17). Since, under physiological conditions, the relevant receptors that contribute to the function and excitability of neuronal cells reside at the cell surface, we wanted to focus specifically on this pool of receptors. Up to this point, it has been problematic to examine the effects of nicotine on the surface-expressed \( \alpha_4\beta_2 \) nAChR protein in living cells, in part due to the difficulties in achieving significant surface expression in cultured cells (28). In addition, it remains technically impracticable to perform studies of specific native receptor subtypes in neuronal cell models due to the complex expression patterns of the nAChR subunits, the lack of antibodies able to discriminate between individual subunits, and the lack of quantitative methods for measuring surface expression in neurons. Therefore, to find an appropriate and tractable model system to study up-regulation and trafficking of surface \( \alpha_4\beta_2 \) nAChRs, we examined surface expression of both rat and mouse \( \alpha_4\beta_2 \) nAChRs in various cultured cell types.

To assess surface expression, we performed immunofluorescence in cultured cells transiently transfected with \( \alpha_4 \) and \( \beta_2 \) subunit cDNAs. At 24 h post-transfection, live cells were labeled with antibodies directed against the extracellular N-terminal domain of the \( \alpha_4 \) subunit to specifically label surface-expressed receptors. Since neither \( \alpha_4 \) nor \( \beta_2 \) is capable of assembling into functional homo-oligomeric receptors (29), no surface labeling was observed when cells were mock-transfected or transfected with either \( \alpha_4 \) or \( \beta_2 \) subunits alone (data not shown). However, when transfected together, mouse \( \alpha_4 \) and \( \beta_2 \) subunits formed receptors that were detected at the surface of HEK293T cells by immunofluorescence with antibodies against the \( \alpha_4 \) (mAb 299) subunit (Fig. 1A).

To determine whether the surface-expressed \( \alpha_4\beta_2 \) nAChRs detected by immunofluorescence corresponded to functional receptors, transfected HEK293T cells were assayed 24–48 h post-transfection using whole-cell patch clamp recordings. Although we could not detect nicotine-evoked currents in untransfected cells or cells expressing \( \alpha_4 \) alone (data not shown), cells co-expressing the \( \alpha_4 \) and \( \beta_2 \) subunits responded in a concentration-dependent manner to pulses of acetylcholine (ACH) in HEK293T cells transfected with \( \alpha_4 \) and \( \beta_2 \) subunit genes. Cells were challenged with 300-ms applications of acetylcholine every 5 min at a holding potential of −60 mV. C, total membranes were prepared from \( \alpha_4 \) and \( \beta_2 \)-transfected HEK293T cells grown in the absence or presence of 500 nM nicotine for 12 h. The number of specific \(^{[3]H}\)epibatidine binding sites was determined. Nicotine induced a significant \((***, \ p \leq 0.001)\) increase in \(^{[3]H}\)epibatidine binding activity as compared with untreated controls.
capable of inducing significant up-regulation of total binding sites in this cultured cell system. Although total [3H]epibatidine binding sites were up-regulated significantly, we were primarily interested in the up-regulation of the surface-expressed receptors. To assess the ability of nicotine to drive up-regulation specifically of the surface pool of nAChRs, we performed quantitative cell surface labeling of a,β2 receptors using a flow cytometry/FACS-based assay. This assay was designed to overcome several problems associated with quantitation of surface protein expression levels in cultured cells. First, the assay was performed in living cells, as defined by propidium iodide staining, to assure that the labeling was representative of surface protein in live cells. Second, cells were selected based on shape and size to measure only fluorescence of single cells and not clusters of cells. Finally, each data point represents the average fluorescence intensity of 30,000 randomly selected cells, which allows normalization of the total population and more accurately represents the expression levels than analysis of individual cells.

Using this assay, we determined both the concentration and time dependence of surface nAChR up-regulation. Transfected HEK293T cells were exposed to nicotine for 12 h at concentrations ranging from 10 nM to 100 μM for 12 h. The cells were then harvested and labeled with antibodies against the α4 subunit (mAb 299) at 4 °C. The cells were extensively washed and then labeled at 4 °C first with biotinylated secondary antibodies and finally with fluorescein isothiocyanate-labeled avidin. The cells were then briefly treated with propidium iodide, and fluorescence intensity of live cells was assessed by FACS analysis. As was previously seen with total receptor binding assays, incubation of cells with nicotine over a 12-h period increased cell surface expression of α,β2 receptors in a concentration-dependent manner (Fig. 2A) with maximal up-regulation of ~2-fold over untreated controls achieved between 10 and 100 μM nicotine.

Next, similar experiments were performed to establish a time course for surface receptor up-regulation. Transfected cells were exposed to 500 nM nicotine for varying time periods ranging from 2 to 24 h prior to antibody labeling and FACS analysis. At 500 nM nicotine concentration, maximum up-regulation of surface α,β2 receptors of more than 2-fold over untreated controls was observed following 20–24 h of nicotine exposure (Fig. 2B). Therefore, up-regulation of α,β2 surface nAChRs is both time- and concentration-dependent, and the maximal extent of up-regulation is ~2–2.5-fold over untreated controls.

Since nicotine is membrane-permeable, we wanted to determine whether agonist exposure to only the surface α,β2 nAChRs could also induce surface receptor up-regulation. Just as we observed up-regulation of the receptors with membrane-permeant nicotine, the membrane-impermeant agonist tetramethylammonium produced significant up-regulation (Fig. 2C) of surface α,β2 nAChRs. Therefore, nicotine action on intracellular binding sites is not required for the induction of up-regulation. To address whether ion channel function was required for up-regulation, we tested several antagonists that inhibit α,β2 nAChR activity by distinct mechanisms. First, exposure to dihydro-β-erythroidine, a competitive antagonist, was capable of inducing significant up-regulation in the absence of nicotine (Fig. 2C). However, mecamylamine, an antagonist that acts as an open channel blocker, did not induce up-regulation of surface α,β2 nAChRs (Fig. 2C). Together, these data suggest that occupancy of the binding site, but not ion channel activity, are required for surface receptor up-regulation. Consistent with this observation, the combination of mecamylamine, an open channel blocker, and nicotine together induced up-regulation to the same extent as nicotine alone.

![Fig. 2. Nicotine effects on α,β2 nAChR endocytosis. A, live HEK293T cells expressing α,β2 nAChRs were labeled at 4 °C with antibodies against α4 (mAb 299) to examine surface expression levels by FACS before and after treatment with the indicated increasing concentrations of nicotine for 12 h. B, live HEK293T cells expressing α,β2 nAChRs were labeled at 4 °C with mAb 299 antibodies against α4 to examine surface expression levels by FACS following incubation with 500 nM nicotine for the indicated times. nAChR surface expression increases in a concentration- and time-dependent manner. C, α,β2-expressing cells were incubated for 12 h with indicated nicotinic agonists and antagonists. Surface receptors were labeled with mAb 299, and FACS analysis was performed to examine up-regulation efficiency of the various compounds (TMA, tetramethyl ammonium; DHβE, dihydro-β-erythroidine; MAA, mecamylamine). Significant up-regulation was seen under all treatment conditions (+++, p ≤ 0.001; **, p < 0.005) except with mecamylamine treatment, in which no significant change in surface expression was observed. In A–C, the reported values are normalized to untreated α,β2 controls. Therefore, nicotine is capable of inducing up-regulation of surface α,β2 receptors even in the absence of channel activity (Fig. 2C).

Together, the characteristics of surface nAChR up-regulation that we observed in transfected HEK293T cells are similar to up-regulation of total nAChR populations observed in human smokers, animal models, and other cultured cell systems (11, 13, 15–17, 20, 31). Furthermore, since HEK293T cells are tractable for cell biological studies and allow specific and quantitative analysis of the surface-expressed pool of receptors, they are a useful model system to examine the contribution of receptor trafficking to nicotine-induced up-regulation.

Nicotine Exposure Does Not Influence the Internalization Rates of Surface nAChRs—It has been shown for opiate receptors that interaction with various agonists and antagonists differentially affects their endocytosis (25). In a similar manner, ligand-dependent changes in endocytosis of nAChR could also result in stabilization of receptor protein and up-regulation. We therefore wanted to determine whether endocytosis of the α,β2 nAChRs from the plasma membrane was affected by nicotine. Since the trafficking patterns of α,β2 nAChRs have
nAChR Up-regulation Requires Exocytic Trafficking

Fig. 3. Quantitative internalization rates of α4β2 nAChRs under nicotine treatment conditions. A, surface α4β2 receptors in live transfected HEK293T cells were labeled with mAb 299 against the extracellular domain of the α4 subunit at 4 °C for 1 h. Following labeling, the cells were incubated for the indicated times at 37 °C in medium with or without 100 μM nicotine. Following internalization, cells were fixed, permeabilized, and labeled with Cy3-conjugated secondary antibodies. Time-dependent translocation of the labeled surface receptors from the plasma membrane to internal punctate structures was monitored by immunofluorescence and confocal microscopy. B, FACS-based internalization assays were performed on HEK293T cells expressing α4β2 nAChRs. Surface receptors were labeled with mAb 299 at 4 °C and then allowed to internalize at 37 °C in growth medium for the indicated time points. Cells were treated with (gray triangles) or without 500 nM nicotine (black squares) during the chase period (acute treatment) or for 12 h (gray circles, chronic treatment) prior to surface labeling with mAb 299. The data are expressed as percentage of untreated control to demonstrate up-regulation of nAChRs in the chronically nicotine-treated cells. The rate constants are 0.0364 min⁻¹ for the untreated control, 0.0366 min⁻¹ for acute, and 0.0369 min⁻¹ for chronic nicotine treatment conditions. There is no statistical difference between the rate constants of internalization for either acute (p = 0.884) or chronic (p = 0.609) nicotine treatment as compared with untreated controls. Each data point is the average of three independent experiments.

not been extensively characterized, we first analyzed internalization of surface nAChRs under normal conditions by immunofluorescence. Surface receptors in transfected cells were labeled at 4 °C for 1 h with an antibody (mAb 299) against the extracellular domain of the α4 subunit. The cells were washed and incubated at either 4 °C or at 37 °C in growth medium for 30 min and then fixed, permeabilized, labeled with secondary antibodies, and prepared for immunofluorescence. As expected, when surface-labeled cells were retained at 4 °C (0-min chase), the labeled receptors remained at the surface (Fig. 3A). However, when labeled cells were shifted to 37 °C for 30 min, the surface receptors were translocated in a time- and temperature-dependent manner to intracellular punctate structures, consistent with efficient internalization through the endocytic pathway (Fig. 3A). To assure that the translocation we observed in these assays was due to endocytosis of the surface receptors, we performed internalization experiments in the presence of sucrose, which is known to inhibit endocytosis, and found that the labeled nAChRs were retained at the surface throughout the chase period (data not shown). In addition, we performed internalization experiments with Fab antibody fragments generated from mAb 299. Fab fragments do not cluster receptors and therefore generally do not initiate artificial endocytosis when bound to surface receptors. Using Fab fragments, we observed identical translocation of surface receptors as with the intact antibody. These results indicate that the internalization we observed was unlikely to be the result of antibody interference (data not shown).

Once we were satisfied that our assay was monitoring endocytic events, we examined the effect of acute nicotine treatment on internalization. Cells were treated in the same manner as in the constitutive internalization assays, except that 100 μM nicotine was added to the cells during the chase period. Just as in the untreated controls, nAChRs were translocated into internal structures within the 30-min chase period in the presence of nicotine (Fig. 3A), suggesting that acute exposure to nicotine does not dramatically change the initial internalization of these receptors from the plasma membrane. Relatively high concentrations of nicotine were used in these assays to ensure that any effects would be as dramatic and apparent as possible. However, at both high (100 μM) and low (500 nM; data not shown) concentrations, no differences in internalization could be discerned.

To confirm the immunofluorescence results by an independent method, we utilized a quantitative FACS-based assay to examine the kinetics of internalization following both acute and chronic nicotine exposure. The surface α4β2 nAChRs were first labeled with primary antibodies against the α4 subunit and then returned to standard growth medium at 37 °C for the indicated time points. The cells were then cooled to 4 °C, and remaining labeled surface nAChRs were labeled with secondary and tertiary antibodies. Under these conditions, only those receptors that were labeled by the primary antibody and remained at the cell surface during the chase period were accessible to secondary antibodies at any particular time point. Thus, the loss of a population of pulse-labeled receptors from the surface over time could be determined. To examine the efficacy of this assay, we performed internalization experiments using mAb 299 to label surface receptors. The labeled surface receptors were cleared from the plasma membrane efficiently during the chase period, with only ~40% of labeled receptors maintained at the surface following the 30-min chase period (Fig. 3B). Furthermore, we obtained identical internalization rates using either full-length mAb 299 or Fab fragments to label the surface receptors and observed that maintaining the cells either at 4 °C or under glucose-deprivation conditions dramatically inhibited internalization over the course of the chase period (data not shown). Therefore, this assay monitors a temperature- and glucose-dependent internalization process.

We then examined the effect of nicotine treatment on internalization of surface α4β2 nAChRs using this assay. Again, in agreement with the immunofluorescence experiments, when nicotine was added to labeled cells during the 30-min internalization period, only ~35% of surface receptors remained following the 30-min chase, and the rate of clearance from the plasma membrane (0.0366 min⁻¹) was not statistically different from that of untreated cells (0.0364 min⁻¹) (Fig. 3B).

Although acute nicotine treatment did not appear to influence the internalization of the nAChRs, it was possible that chronic incubation with nicotine sufficient to induce up-regu-
Immunoblotting with mAb 299. were precipitated from cell lysates with streptavidin-agarose, and the cytosis from the cell surface.

Exposure to nicotine changes the rate of nAChR protein endocytosis from the plasma membrane is similar to that of untreated cells. Therefore, neither acute nor chronic protein is internalized from the plasma membrane is similar to that of untreated cells. Therefore, neither acute nor chronic nicotine treatment with mAb 299 directed against the αβ2 nAChR protein endocytosis was affected by nicotine. First, to identify the intracellular compartment that the surface receptors are directed to from the plasma membrane, we performed co-localization experiments with internalized αβ2 receptors and Lamp1, a protein marker of lysosomal membranes. At 4 °C, labeled αβ2 nAChRs were seen at the plasma membrane, distinctly localized from the internal punctate Lamp1 compartments (Fig. 4A, 0-min chase). However, following 30 min of chase, the internalized αβ2 receptors exhibited significant co-localization with Lamp1-positive compartments (Fig. 4A). Interestingly, at this same time point, we saw no significant co-localization of internalized nAChRs with either transferrin receptor or EEA1 (data not shown), two markers of early endosomal compartments from which proteins are recycled to the plasma membrane. Therefore, it appears that under normal growth conditions, surface αβ2 receptors are internalized and rapidly directed to lysosomal compartments.

If nicotine changed the rate of transport to the lysosome, we would expect to observe dramatic changes in the degradation rate of the surface receptors. Therefore, we examined the stability of the surface-expressed pool of receptors using a surface biotinylation degradation assay in either the presence or absence of nicotine. Intact transfected cells were reacted with NHS-sulfo-biotin, a membrane-impermeant biotinylation reagent that exclusively labels surface proteins. The fate of the biotinylated proteins was monitored over time by streptavidin immunoprecipitation and subsequent detection with mAb 299 directed against the α4β2 subunit of nAChRs. In the absence of nicotine, the surface nAChR signal was dramatically reduced following 30 min at 37 °C (Fig. 4B). This experiment suggests that internalized receptor is not recycled to the plasma membrane but is directly routed to the lysosome and degraded. These results are consistent with our immunochemistry and FACS data and provide additional evidence that the internalization that we observe using antibodies to label surface receptors is not likely to be due to antibody-induced internalization.

Interestingly, the addition of nicotine during the chase period did not dramatically stabilize the surface nAChRs (Fig. 4B) as compared with the untreated control cells. Together, these data suggest that exposure to nicotine does not affect the degradation of surface expressed αβ2 nAChRs in the lysosome.

**Up-regulation of nAChRs Is Independent of Recycling from the Endosome**—Although we did not observe significant changes in internalization or degradation of surface αβ2 nAChRs in the presence of nicotine, the possibility remained that small changes in postendocytic trafficking over extended periods of time may contribute to up-regulation. As another means to assess the contribution of endocytic trafficking to up-regulation, we utilized a dominant negative mutant of SKD1, an endosomal associated AAA-ATPase previously shown to be required for efficient recycling of plasma membrane proteins from endosomal compartments (32). Under normal conditions, the majority of SKD1 is soluble and distributed throughout the cytoplasm. However, a dominant negative mutation in the ATPase domain (E235Q) of SKD1 (SKD1(EQ)) results in the accumulation of endocytic cargoes, as well as the SKD1(EQ) protein itself, in large endosomal structures. Endocytic cargoes are unable to traffic out of these aberrant compartments, resulting in an intracellular accumulation of internalized surface proteins (33). If changes

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**Fig. 4. Lysosomal trafficking and degradation of surface αβ2 nAChRs.** A, live HEK293T cells expressing αβ2 receptors were labeled with mAb 299 and then incubated at either 4 °C (0-min chase) or 37 °C for 30 min. Cells were then fixed, permeabilized, and co-labeled with primary antibodies against Lamp1. Co-localization of internalized receptors following 30 min of internalization at 37 °C with Lamp1-positive late endosomal/lysosomal compartments was monitored by confocal microscopy. B, HEK293T cells transfected with αβ2 nAChR subunits were surface-biotinylated and returned to growth medium with or without 100 μM nicotine for the indicated times. Biotinylated αβ2 receptors were precipitated from cell lysates with streptavidin-agarose, and the stability of the labeled receptors was examined by SDS-PAGE and immunoblotting with mAb 299.

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**Nicotine Does Not Change Postendocytic Degradation Rates of Surface Receptors**—Previous studies of up-regulation suggested that nicotine may act by changing the rate of nAChR turnover, stabilizing αβ2 nAChR protein, and subsequently resulting in up-regulation. To directly test this hypothesis, we wanted to determine whether trafficking of the αβ2 nAChRs from the plasma membrane to the lysosome was affected by nicotine. We performed co-localization experiments with internalized αβ2 receptors and Lamp1, a protein marker of lysosomal membranes. At 4 °C, labeled αβ2 nAChRs were seen at the plasma membrane, distinctly localized from the internal punctate Lamp1 compartments (Fig. 4A, 0-min chase). However, following 30 min of chase, the internalized αβ2 receptors exhibited significant co-localization with Lamp1-positive compartments (Fig. 4A). Interestingly, at this same time point, we saw no significant co-localization of internalized nAChRs with either transferrin receptor or EEA1 (data not shown), two markers of early endosomal compartments from which proteins are recycled to the plasma membrane. Therefore, it appears that under normal growth conditions, surface αβ2 receptors are internalized and rapidly directed to lysosomal compartments.

If nicotine changed the rate of transport to the lysosome, we would expect to observe dramatic changes in the degradation rate of the surface receptors. Therefore, we examined the stability of the surface-expressed pool of receptors using a surface biotinylation degradation assay in either the presence or absence of nicotine. Intact transfected cells were reacted with NHS-sulfo-biotin, a membrane-impermeant biotinylation reagent that exclusively labels surface proteins. The fate of the biotinylated proteins was monitored over time by streptavidin immunoprecipitation and subsequent detection with mAb 299 directed against the α4β2 subunit of nAChRs. In the absence of nicotine, the surface nAChR signal was dramatically reduced following 30 min at 37 °C (Fig. 4B). This experiment suggests that internalized receptor is not recycled to the plasma membrane but is directly routed to the lysosome and degraded. These results are consistent with our immunochemistry and FACS data and provide additional evidence that the internalization that we observe using antibodies to label surface receptors is not likely to be due to antibody-induced internalization.

Interestingly, the addition of nicotine during the chase period did not dramatically stabilize the surface nAChRs (Fig. 4B) as compared with the untreated control cells. Together, these data suggest that exposure to nicotine does not affect the degradation of surface expressed αβ2 nAChRs in the lysosome.

**Up-regulation of nAChRs Is Independent of Recycling from the Endosome**—Although we did not observe significant changes in internalization or degradation of surface αβ2 nAChRs in the presence of nicotine, the possibility remained that small changes in postendocytic trafficking over extended periods of time may contribute to up-regulation. As another means to assess the contribution of endocytic trafficking to up-regulation, we utilized a dominant negative mutant of SKD1, an endosomal associated AAA-ATPase previously shown to be required for efficient recycling of plasma membrane proteins from endosomal compartments (32). Under normal conditions, the majority of SKD1 is soluble and distributed throughout the cytoplasm. However, a dominant negative mutation in the ATPase domain (E235Q) of SKD1 (SKD1(EQ)) results in the accumulation of endocytic cargoes, as well as the SKD1(EQ) protein itself, in large endosomal structures. Endocytic cargoes are unable to traffic out of these aberrant compartments, resulting in an intracellular accumulation of internalized surface proteins (33). If changes
in the postendocytic trafficking of nAChR proteins were required for up-regulation, disruption of this pathway with SKD1(EQ) should prevent up-regulation.

To establish the SKD1 phenotype in the HEK293T cell line, we first examined the distribution of transferrin receptor, a normally recycled protein, in cells transfected with HA epitope-tagged versions of either wild-type SKD1 (SKD1-HA) or dominant negative SKD1 (SKD1(EQ)-HA). In cells expressing wild-type SKD1-HA, transferrin receptor was distributed throughout the cell in small punctate structures as expected (Fig. 5A). However, in cells expressing the mutant SKD1(EQ)-HA, transferrin receptor accumulated with SKD1(EQ)-HA protein in large aberrant endosomal compartments (Fig. 5A), confirming that the SKD1(EQ) mutation disrupts endosomal recycling in the HEK293T cell line, as has been previously shown in other cultured cell lines (33).

When co-transfection experiments were performed with both αβ2 and SKD1(EQ)-HA, internalized αβ2 receptors were detected in SKD1(EQ)-HA-positive compartments (Fig. 5B), suggesting that αβ2 nAChR trafficking to the lysosome normally proceeds via the SKD1 compartment. Importantly, the accumulation of αβ2 was only observed in cells co-expressing αβ2 and SKD1(EQ)-HA. Therefore, the SKD1(EQ) mutant is defective in postendocytic trafficking of αβ2 nAChRs to the lysosome.

We then examined surface expression of αβ2 receptors in cells co-expressing αβ2 with either vector alone, SKD1-HA, or the dominant negative SKD1(EQ)-HA mutant. As is usually observed in co-transfection experiments, virtually all cells expressing αβ2 also expressed either SKD1-HA or SKD1(EQ)-HA (Fig. 5B), assuring that phenotypic differences that we observed were from cells expressing both constructs together. As expected, in cells expressing αβ2 alone or αβ2 together with wild type SKD1-HA, we observed nicotine-induced up-regulation following a 12-h incubation with nicotine (Fig. 5C). Interestingly, however, in cells expressing both αβ2 and SKD1(EQ)-HA, although trafficking of nAChRs is disrupted (Fig. 5B), we still observed up-regulation of αβ2 nAChRs (Fig. 5C). These results are consistent with the lack of nicotine effects on internalization and degradation rates of surface receptors and further suggest that up-regulation is independent of post-endocytic protein sorting.

Transport through the Secretory Pathway from the Endoplasmic Reticulum Is Required for nAChR Up-regulation—Together, our data indicate that neither internalization of the αβ2 nAChRs from the plasma membrane nor trafficking in the postendocytic pathway is involved in nicotine-induced up-regulation of surface αβ2 nAChRs. We therefore wanted to examine the contribution of receptor biosynthesis and/or insertion of receptors into the plasma membrane to up-regulation. It has been well documented that nicotine-induced up-regulation of high affinity nicotine binding sites does not result from an increase in mRNA levels, which suggests that up-regulation is not transcriptionally regulated. Furthermore, it has been reported that up-regulation of nicotine binding sites can proceed in the presence of cycloheximide (15), suggesting that de novo protein synthesis is also not required for up-regulation. In our system as well, we have found that the translational inhibitors cycloheximide and emetine do not block up-regulation of surface nAChRs (data not shown).

Following translation, membrane proteins are transported through the secretory pathway to the plasma membrane. If trafficking of protein through the secretory pathway were required for up-regulation, disruption of secretory pathway function should disrupt nicotine-induced up-regulation. Brefeldin A (BFA) is a fungal metabolite that disrupts transport of secreted proteins from the endoplasmic reticulum (ER) to Golgi (34, 35). To assess the contribution of secretory traffic to the plasma membrane in up-regulation, we performed surface labeling experiments in the presence of BFA. BFA was either added individually to transfected cells or co-administered with nicotine for 10 h prior to labeling for surface expression analysis. Following exposure to nicotine alone, transfected cells displayed the typical ~40% up-regulation of surface nAChRs when compared with untreated controls (Fig. 6A). Cells exposed to BFA alone showed a marked decrease in basal surface expression, which is indicative of the effectiveness of BFA in disrupting overall secretory pathway function (data not shown). However, when BFA treatment was combined with nicotine, we could not detect nicotine-dependent up-regulation of surface expression. Instead, nAChR surface expression was slightly, but not sig-
cells but that the transport of up-regulated receptors to the plasma membrane is disrupted. This further suggests that the site of up-regulation is at or prior to the ER, the site of BFA action in the cell.

If up-regulation is regulated at the ER, an internal pool of preexisting nAChR subunits required for up-regulation most likely exists in the ER. To determine in which compartment internal α2β2 protein is localized, we performed co-localization experiments with calnexin, a membrane-bound ER-resident protein. HEK293T cells were transfected with α4 and β2 subunits and grown for 24 h prior to fixation and permeabilization. Permeabilized cells were labeled with antibodies against β2 and calnexin to examine the distribution of the total pools of both proteins. As expected, calnexin was detected in a disperse perinuclear compartment, consistent with ER localization (Fig. 6C). In addition, the β2 protein labeling was predominantly coincident with calnexin (Fig. 6C), suggesting that the majority of the internal β2 protein pool is in the ER, providing an available source of nAChR subunits that can be converted to ligand binding-competent nAChRs by nicotine exposure.

**DISCUSSION**

The phenomenon of nicotine-induced nAChR up-regulation is contrary to conventional models of ligand-receptor interaction. Typically, exposure to ligand results in surface receptor down-regulation to attenuate signaling. However, in the case of nAChRs, chronic exposure to agonist results in up-regulation, which has been proposed to be a compensatory mechanism to replace desensitized receptors at the cell surface (12). Therefore, nicotine-induced up-regulation seems to be novel means to control the activity of cell surface receptors.

We have utilized a culture cell system and a quantitative FACS-based surface expression assay to characterize the up-regulation and trafficking patterns of surface-expressed α4β2 nAChRs in living cells. We have chosen to concentrate specifically on the surface-expressed proteins, which *in vivo* are the functionally relevant receptors required for membrane excitability. In our cultured cell system, we achieve robust and reproducible surface expression of functional mouse α4β2 nAChRs (Fig. 1) and observe nicotine-induced up-regulation of both total and surface α4β2 nAChRs (Figs. 1 and 2). The up-regulation that we observe is similar to previously reported high affinity nicotine binding site up-regulation in both cultured cells and animal models (15–17, 31). This indicates that up-regulation can be achieved using basic, fundamental cellular machinery that is intact in nonneuronal cells. Therefore, understanding the processes responsible for up-regulation of nAChRs may be important not only with respect to nicotine response but potentially for the regulation of other cell surface receptors as well. In addition, mechanisms and components that can be identified using this reduced system may also be involved in regulation of these receptors in neuronal cells and, once identified, can be directly tested in the more complicated context of neuronal cells or whole animals.

**Ligand-dependent Trafficking and Up-regulation—** Our examination of α4β2 nAChR endocytic trafficking has shown that surface-expressed nAChRs are constitutively and rapidly endocytosed from the plasma membrane and directed into a lysosomal degradation pathway (Figs. 3 and 4). Therefore, under normal conditions, surface nAChRs are constantly replaced by new receptors from the secretory pathway. Chronic exposure of surface nAChRs to nicotinic agonists is sufficient for induction of surface receptor up-regulation but does not alter the endocytic trafficking of these receptors (Fig. 3). One mechanism that has been proposed for up-regulation is that nicotine modulates stability of the nAChRs, redirecting internalized receptors from a degradative pathway to increase their surface residence and

**Fig. 6.** Exocytic trafficking of α2β2 nAChRs from the ER is required for up-regulation. A, cells expressing α2β2 receptor subunits were treated with nicotine, BFA, or nicotine in combination with BFA for 10 h. Surface receptors were labeled with mAb 299, and surface expression in the presence of these compounds was quantitated by FACS. The values shown in the figure are the average of three independent experiments. Nicotine alone induced significant up-regulation (***, p < 0.005), but BFA prevented nicotine-induced up-regulation. B, total membranes were prepared from HEK293T cells expressing α2β2 receptor subunits, which were exposed for 8 h prior to the experiment with or without nicotine, BFA, or a combination of nicotine and BFA. [3H]Epibatidine binding was measured in the membrane preparations to determine up-regulation of total binding sites. Significant up-regulation was observed in cells treated with nicotine alone as compared with untreated controls and with the combination of BFA and nicotine as compared with controls treated only with BFA (***, p < 0.001). The reported values are expressed as percentage of untreated controls (without nicotine) and represent the average of three independent experiments. C, cells expressing α2β1 receptor subunits were co-labeled with antibodies against both calnexin and the β1 nAChR subunit. Co-localization between internal β2 protein (left) and calnexin (middle) is observed in merged images (right).
stability (15). However, our data clearly demonstrate that neither acute nor chronic exposure to nicotine has any effect on the clearance of the surface nAChRs from the plasma membrane or their degradation in the lysosome (Figs. 3 and 4). Furthermore, disrupting postendocytic traffic with a dominant negative SKD1 mutant does not prevent up-regulation of nAChRs (Fig. 5). Together, these data strongly suggest that nicotine-dependent regulation of nAChR surface expression is not via changes in endocytic trafficking or the lysosomal degradation rate of the surface nAChRs, a model that has been previously invoked as a post-translational mechanism for up-regulation (15).

In contrast to these previous studies, our degradation and internalization experiments were conducted by pulse-chase analysis of nAChRs in situ, on much shorter time courses and without additional drug treatments. In addition, our experiments specifically analyzed the fate of surface receptors and therefore did not directly address the possibility of changes in the degradation of internal nAChRs through a nonlysosomal pathway. It is possible that the more long term changes that were observed by Peng et al. (15) could be explained by an inhibition of proteasomal degradation of internal receptor protein as a result of extended drug exposure. However, our results clearly demonstrate a lack of a direct effect of nicotine on the lysosomal degradation pathway.

Although postendocytic trafficking does not contribute significantly to up-regulation, the insertion of nAChRs into the plasma membrane through the secretory pathway appears to be required for nicotine-induced up-regulation of surface receptors. Inhibition of secretory pathway function with BFA results in complete loss of nicotine-induced surface receptor up-regulation (Fig. 6). Interestingly, however, de novo protein synthesis does not appear to be required for up-regulation, since translational inhibitors do not inhibit up-regulation (15). There have been contradictory interpretations of the effects of translational inhibitors on up-regulation (15, 16). However, the data in each of these studies show an increase of binding sites in the presence of cycloheximide and nicotine when compared with cycloheximide treatment alone, suggesting that secondary effects following prolonged exposure translational inhibitors may result in changes in protein expression but that nicotine-induced up-regulation still occurs. Furthermore, we have repeated translational inhibitor experiments in our cultured cell system and found no effect of cycloheximide or emetine, two inhibitors that act at distinct points in the protein translation pathway, on up-regulation. Together, these data indicate that a preexisting intracellular pool of protein is sufficient for nicotine-induced up-regulation. In agreement with this, binding experiments done in the presence of BFA indicate that although surface receptor up-regulation is inhibited by disruption of secretory pathway traffic to the plasma membrane, the total pool of receptors is still up-regulated by nicotine exposure. This suggests that the up-regulation event is at or prior to the formation of ligand binding-competent receptors and requires their subsequent transport through the secretory pathway.

Model for Nicotine-induced Up-regulation—Taken together, our data suggest that an internal pool of nAChRs relevant to up-regulation may reside in the endoplasmic reticulum. At the ER, there are several potential steps in receptor biosynthesis that could be regulated by nicotine to affect expression levels. For multisubunit receptors, including nAChRs, folding and assembly into functional receptors is a prerequisite for packaging into secretory vesicles and exit from the ER (38). In fact, recent studies using FRET as a means to monitor assembly of nAChRs have shown that nicotine increases FRET between $\alpha_4$ and $\beta_2$ subunits, which may be indicative of increased assembly into functional receptors (39). Prior to assembly, in order to accomplish appropriate folding, ER-localized chaperone proteins act to prevent proteasomal degradation and promote folding of nascent polypeptide chains (40). Muscle nAChR expression appears to be effected by alterations in the ubiquitin-proteasome system (41), and this quality control step could be influenced by nicotine to affect up-regulation. Finally, once properly folded, many receptor subunits contain exposed ER retention signals that act to keep unassembled components in the ER until assembly is complete, at which time the retention signals are masked, and packaging into forward transport vesicles can proceed. Both muscle and neuronal nicotinic receptor family members have been shown to interact with calcineurin, an ER chaperone protein, and they also contain putative ER retention signals (37, 42, 43).

Finally, although nicotine is membrane-permeant and binds to intracellular sites, we and others have shown that ligand interaction with surface receptors alone is sufficient to induce up-regulation, since exposure to membrane-impermeant ligands also induces up-regulation (Fig. 2) (17). Therefore, it is necessary to invoke a signal initiated by the surface receptors in response to nicotine, to activate a second messenger that acts at a distinct internal site to drive up-regulation. This pathway is unlikely to be dependent upon nAChR channel activity, since chronic treatment of cells with competitive antagonists or the combination of nicotine and channel blocking antagonist, both of which inhibit the ion channel activity of surface receptors, is sufficient to drive up-regulation independent of function (Fig. 2) (15, 16). Together, these considerations suggest a revised model for nicotine-induced up-regulation, where the interaction of nAChR with ligand at the cell surface induces a second messenger signaling system. This results in an increased number of nAChRs transiting from the ER to the surface, mediating the observed up-regulation of surface nicotinic receptors. The challenge now will be to define the nicotine-regulated molecules involved in receptor dynamics at the ER and, once these molecules are in hand, to move into neuronal systems and whole animal models and directly test their involvement in nicotinic receptor trafficking and nicotine-induced behaviors.

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Tamara Darsow, T. K. Booker, Juan Carlos Piña-Crespo and Stephen F. Heinemann

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