Up-regulation of Nicotinic Receptors by Nicotine Varies with Receptor Subtype*

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Recent evidence suggests that in addition to α4β2 and α3-containing nicotinic receptors, α6-containing receptors are present in midbrain dopaminergic neurons and involved in the nicotine reward pathway. Using heterologous expression, we found that α6β2, like α3β2 and α4β2 receptors, formed high affinity epibatidine binding complexes that are pentameric, trafficked to the cell surface, and produced acetylcholine-evoked currents. Chronic nicotine exposure up-regulated α6β2 receptors with differences in up-regulation time course and concentration dependence compared with α4β2 receptors, the predominant high affinity nicotine binding site in brain. The α6β2 receptor up-regulation required higher nicotine concentrations than for α4β2 but lower than for α3β2 receptors. The α6β2 up-regulation occurred 10-fold faster than for α4β2 and slightly faster than for α3β2. Our data suggest that nicotinic receptor up-regulation is subtype-specific such that α6-containing receptors up-regulate in response to transient, high nicotine exposures, whereas sustained, low nicotine exposures up-regulate α4β2 receptors.

The addictive actions of nicotine are initiated by its binding to nicotinic acetylcholine receptors (nAChRs)² (1, 2). nAChRs are ligand-gated ion channels (3). Muscle nAChRs are pentamers containing four different subunits, α, β, γ (or ε), and δ. Neuronal nAChRs are homologous to muscle nAChRs and fall into two different pharmacological classes. One class binds α-bungarotoxin with high affinity and is predominantly pentamers composed of only α7 subunits (4–6). The other class of neuronal nAChRs bind agonists with high affinity and is composed of α (α2-α6) and β (β2-β4) subunits, homologous to the muscle subunits (7, 8).

After chronic nicotine exposure, high-affinity agonist binding to nicotinic receptors is increased in murine (9, 11, 62) and human brains (12). This process, termed nicotine-induced up-regulation, is linked to nicotine addiction. The sites up-regulated appear to be predominantly α4β2 receptors (13). Yet other nAChR subtypes that display high affinity binding in the presence of cytisine (10, 14) are up-regulated with nicotine exposure (15). Whether these nAChRs are α3-, α6-, or even α4-containing receptors is unknown (16, 17). Recent evidence using α-conotoxin MII, an antagonist specific for α6- and α3-containing receptors (18–20) and α6 null mice (16, 21), suggest that these additional nAChRs are α6-containing receptors.

The identification of different neuronal nAChR subtypes has been hampered by a lack of subtype-specific ligands and antibodies, receptor heterogeneity, and low levels of expression (2). Because of these problems heterologous expression of nAChR subunits has been used to help identify nAChR subtypes and to study up-regulation (22). Several features of the up-regulation allow it to be studied in heterologous systems. First, the mechanisms underlying up-regulation are predominantly, if not exclusively, posttranslational (62, 45) and, thus, independent of elements regulating nAChR transcription and translation. Second, the features of the up-regulation appear to be independent of the cells in which the nAChRs are expressed, and up-regulation is “cell autonomous” (65).

Studies of α6 subunits have been difficult because α6 subunits appear to aggregate instead of forming functional pentameric receptors (23–26). In this work we report the successful expression of functional α6β2 receptors in heterologous cells and compare the nicotine-induced up-regulation of α6β2 receptors to that of α3β2 and α4β2 receptors. We find that the time course and concentration dependence of α6β2 and α3β2 receptor up-regulation is similar but ~10-fold faster than α4β2 receptor up-regulation, requires much higher nicotine concentrations to up-regulate, and occurs without the 2–3-h delay observed with α4β2 receptor up-regulation. These differences suggest that up-regulation of these nicotinic receptor subtypes could occur during different phases of nicotine intake that are observed during smoking. Our findings suggest that the transient, high nicotine levels in brain achieved during a single cigarette are capable of up-regulating α6-containing receptors but not α4β2 receptors. In contrast, our findings suggest that α4β2 receptors, but not α6-containing receptors, up-regulated by the nicotine levels that slowly rise and are sustained during and after many hours of smoking.

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EXPERIMENTAL PROCEDURES

cDNA Constructs, Cell Culture, and Gene Transfer—Rat α3, α6, and β2 subunit cDNAs (a gift from Dr. J. Boulet, University of California, Los Angeles, CA) were subcloned into pCB7, which contains a cytomegalovirus promoter and the hygromycin resistance gene used to select for cells stably expressing α3β2 hemagglutinin (HA) or α6(FLAG)/β2(HA) receptors. The HA epitope (27), YPYDVPDYA, and a stop codon were inserted after the last codon of the 3’-translated region of the subunit DNA of the B2 using the extension overlap method (28) as described in Vallejo et al. (29). TheFLAG epitope, DYKD-DDDK (30), and a stop codon were inserted after the last codon of the 3’-translated region of the α3 and α6 subunit DNA using the extension overlap method. The α6FLAG construct was then subcloned into the pCDNA3.1 vector. The human embryonic kidney (HEK) cell line tsA201 was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10,000 units/ml penicillin and streptomycin (Invitrogen) and 10% calf serum (HyClone, Logan, UT) at 37 °C in the presence of 5% CO2. Unless otherwise noted, cells were moved to 30 °C 24 h before experimentation. DNA was expressed by transient transfection into tsA201 cells using the calcium phosphate method (31). Stable cell lines were established from tsA201 cells using calcium phosphate transfection and hygromycin B selection (Calbiochem) at 0.42 μg/ml.

Nicotine-induced Up-regulation and 125I-labeled Epibatidine Binding—Unless otherwise noted 300 or 30 μM nicotine were used for the α3β2 and α6β2 receptors for 24 h at either at 30 or 37 °C. Transiently transfected tsA201 cells were treated 24 h after transfection, and stably expressing cells were used once confluent. In general, cells were washed 4–5 times and collected by gentle agitation with PBS followed by incubation in 2 mM 125I-labeled epibatidine in 500 μl of PBS for 20 min at room temperature. All binding was terminated by vacuum filtration through Whatman filters using a Brandel 24 channel cell harvester. Bound 125I-labeled epibatidine (2200 Ci/mmol) was determined by γ counting (PerkinElmer Life Sciences) with nonspecific binding estimated by 125I-labeled epibatidine binding to untransfected tsA201 cells. There was no significant binding to the parent HEK cell line in the absence and presence of nicotine treatment. This background is subtracted from total binding. Sample sizes are 1/3 of a 6-cm plate that contains on average a total of 1–2 mg of protein for the entire plate.

Western Blots—Cultures of transiently or stably transfected tsA201 cells were rinsed 3 times with PBS, scraped, pelleted at 5000 × g for 3 min, and resuspended in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.02%NaN3, plus 1% Triton X-100) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, and chymostatin, pepstatin, leupeptin, and tosyllysine chloromethyl ketone at 10 μg/ml). In each case parallel samples were used to estimate protein content using the BCA protein assay (Pierce). No difference in total protein concentration was detected between the 30 and 37 °C conditions. Lysates were incubated with Y-11 anti-HA (Santa Cruz Biotechnology) or anti-FLAG agarose (Sigma) overnight at 4 °C. Anti-HA antibody–antigen complexes were precipitated with protein G-Sepharose (Amersham Biosciences). Purified subunits were analyzed along with 1/6 of a sample of whole cell lysates on a 7.5% SDS–PAGE gel that was transferred to nitrocellulose membrane and probed with Y-11 or M2 anti-FLAG antibody (Sigma). Secondary antibodies conjugated to a cy5 fluorophore were detected using the BioRad Phars FX Molecular Imager system. This method allowed for more sensitive detection of protein, which could be quantified using the Quantity One program. Bands were quantified after background subtraction, and saturated samples were rescanned at a lower intensity to ensure quantitative analysis.

Methanethiosulfonate Ethylammonium Biotinylation Assays—To biotinylate surface receptors, intact, confluent cultures of transiently or stably transfected tsA201 cells were treated with 100 μM methanethiosulfonate ethylammonium (MTSEA)-biotin (Toronto Research Chemicals, Toronto, Canada) for 30 min at room temperature. To biotinylate surface plus intracellular receptors, cells were incubated in 10 mM phosphate buffer containing 0.5% saponin, 0.1% bovine serum albumin, and 10 mM EDTA for 5 min, washed twice with PBS, and treated with MTSEA-biotin as before. After MTSEA-biotin treatment, cells were washed with PBS and solubilized at 4 °C in lysis buffer containing 1% Triton X-100 and protease inhibitors. Lysates with 75 μl of streptavidin immobilized on beaded agarose were rotated overnight at 4 °C. Afterward, biotinylated subunits were subjected to 125I-labeled epibatidine binding or analyzed on an SDS–PAGE gel, transferred to nitrocellulose, and blotted with either Y-11 anti-HA or M2 anti-FLAG antibodies.

Sucrose Gradient—B23 cells stably expressing AChRs (32) were bound with 2 nM 125I-labeled bungarotoxin for 1 h at room temperature. B23 cells and stable α6β2 cells were washed 3 times with PBS, harvested, and lysed in lysis buffer with 1% Triton X-100. Triton X-100-soluble fractions were layered on a 5–20% sucrose gradient prepared in lysis buffer with 1% Triton X-100. Gradients were centrifuged at 40,000 rpm for 14.25 h in a Beckman SW 50.1 rotor. A total of 18 fractions of 300 μl each were collected. Fractions of B23 were counted in the γ-counter to determine the amount of 125I-labeled bungarotoxin bound to each fraction. α6β2 fractions were bound with 2 nM 125I-labeled epibatidine for 20 min at room temperature, and binding was halted by vacuum filtration through Whatman filters using a Brandel 24 channel cell harvester. Bound 125I-labeled epibatidine was determined by γ counting.

Electrophysiology—tsA201 cells stably expressing α6β2 were recorded from 35-mm cultures 2–3 days after plating as described previously (22). Cells were impaled and maintained in voltage clamp in the whole-cell configuration technique using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 1 kHz and digitized at 5 kHz by a PCI card (National Instruments, Austin, TX). Values were stored on the hard disk of a Macintosh computer (Apple Computers, Cupertino, CA) and analyzed using MacDatac (personal program). All recordings were done at room temperature in the following extracellular medium: 130 mM NaCl, 5 mM Na2CO3, 2 mM CaCl2, 2 mM MgCl2, and 10 mM HEPES, pH 7.4, with NaOH. Patch electrodes made of borosilicate electrodes (3–8 megohms) were filled with the following: 130 mM potassium
gluconate, 5 mM NaCl, 2 mM MgCl₂, 10 mM HEPES, and 5 mM EGTA, pH 7.4, with KOH.

Blue Native Gels—Samples were prepared from tsA201 cells stably expressing αβ2 receptors following basic protocols (33) with modifications. Cells were harvested, washed with cold PBS, resuspended in cold hypotonic buffer (5 mM Tris, pH 7.4, 1 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA) containing 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of chymotrypsin, leupeptin, pepstatin, and tosyl-lysine chloromethyl ketone and Dounce-homogenized (20 strokes) in a 2-ml glass homogenizer. Cell debris and nuclei were removed by centrifugation at 800 × g for 10 min. Samples were transferred to Beckman centrifuge tubes (13 × 51 mm Ultra-clear tubes, Palo Alto, CA), and centrifuged at 100,000 × g for 60 min to isolate membranes. Pellets could be stored at −80 °C at this point or resuspended in 100 mM 6-aminocaproic acid, 50 mM Bis-Tris, pH 7.0. Dodecylmaltoside (10% stock) was added to the sample for a final concentration of 1%. Blue native sample buffer consisted of 500 mM aminocaproic acid, 100 mM Bis-Tris, pH 7.0, and 5% (w/v) Coomassie Blue G was added at a 10:1 ratio (sample to blue native sample buffer). 50-μg samples were loaded into wells of a 4% stack (400 μl of 48% acrylamide, 1.75% bisacrylamide, 1.67 ml of 50 mM Bis-Tris, 500 mM 6-aminocaproic acid, pH 7.0, 2.93 ml of water, 2.5 μl of TEMED, and 28 μl of 10% ammonium persulfate for a 5 ml stack) which overlaid the 4—16% gradient gel. The blue native-PAGE 4—16% gradient gels (48% acrylamide, 1.75% bisacrylamide in 50 mM Bis-Tris, 500 mM 6-aminocaproic acid, pH 7.0) were poured with a gradient maker. Blue native gel was run at 4 °C at a constant voltage of 75 V until the samples entered the resolving gel and then at 200 V until the end of the run. The top reservoir buffer for this separation of protein complexes was 50 mM Tricine, 15 mM Bis-Tris, 0.02% Coomassie Brilliant Blue G250, pH 7.5. The bottom reservoir buffer was 50 mM Bis-Tris, pH 7.0. When the tracking dye reached the second third of the gel height, electrophoresis was stopped, and the reservoir buffer was replaced by colorless cathode buffer. Electrophoresis was stopped when the tracking line of CBB G-250 dye had left the edge. The gels were soaked in transfer buffer containing 0.05% SDS for 30 min and transferred to polyvinylidene difluoride membranes at a constant current of 250 mA overnight. An immunoblot was performed according to standard protocols.

Immunofluorescence Staining—Stable cells expressing α6FL2HA grown on collagen (BD Biosciences)-coated coverslips were stained in one of several ways. (i) For live staining, intact cells were incubated with rabbit anti-HA antibody (1:1000, Covance Research products, Berkley, CA) diluted in media without serum for 1 h at 25 °C. Cells were then washed 2 times with media lacking serum, fixed with 4% paraformaldehyde for 10 min at room temperature, and quenched with 50 mM glycine in PBS for 10 min. Cells were subsequently washed 3 times with PBS and stained with secondary antibody, goat anti-rabbit Alexa Fluor 568 (1:1000, Invitrogen), for 1 h in the dark at room temperature. (ii) Alternatively for anti-FLAG staining, cells were fixed before primary and secondary antibody labeling. All the intermediate washes and antibody dilutions were done with PBS containing 5% serum and 2% bovine serum albumin. Cells were stained with mouse anti-FLAG M2 (1:500, Sigma) for 1 h at room temperature. After 3 washes, cells were incubated with goat anti-mouse conjugated to TRITC for 1 h in the dark at room temperature. (iii) To label the intracellular pool of receptor subunits, cells were permeabilized with 0.1% Triton X-100 post-fixation for 10 min. Primary and secondary antibody incubations and washes were done as mentioned above. Before mounting, the cells were washed 3 times in PBS. Vectashield (Vector Laboratories, Burlingame, CA) was used for mounting the coverslips on glass slides. Slides were examined on either an Olympus DSU spinning disk confocal or an Olympus fluoview scanning laser confocal microscope.

Fluorescence-activated Cell Sorter Analysis—α6FL2HA stable cells were grown on 6-cm plates. Live cell staining was performed with rabbit anti-HA antibody (1:1000) for 1 h at 25 °C in medium without serum. After two washes each with 3 ml of media, cells were incubated with secondary antibody, goat anti-rabbit cyanine5 (cy5) (1:1000) for 1 h in the dark at 250 °C. The cells were washed twice with PBS and allowed to gently lift off the plate with PBS, 10 mM HEPES, 1 mM EDTA for 10 min. The suspended cells were collected in tubes. Cell analyses were performed on a DakoCytomation CyanADP equipped with 405, 488, and 635 nm lasers emission filters. Background subtraction was done using stable cells stained with secondary antibody alone or with parent TSA201 cell lines stained with both primary and secondary antibodies. All analyses and sorts were repeated three times. The percentage of cells stained for the fluorophor was calculated from 50,000 cells. The data were analyzed using FlowJo.

RESULTS

Expression of Subunits into Assembled Receptors—To characterize α3β2 and α6β2 receptors, we fused a FLAG epitope tag to the C termini of the α3 and α6 subunits. We had previously shown that the β2 subunit with a HA tag inserted at the C terminus can be used in studies of α4β2 receptors and have demonstrated that the addition of this short tagging sequence does not significantly alter the receptor properties (29). Consequently, we used these epitope-tagged subunits to identify cells expressing the corresponding transcript. Cell lines that stably express α3β2HA and α6FLAGβ2HA were established, and transient transfection of α3FLAGβ2HA receptors was used in experiments to express FLAG-tagged α3 subunits. Both transfection methods produced comparable amounts of ligand binding sites and levels of nicotine-induced up-regulation (data not shown).

We first assayed expression of α6β2 or α3β2 receptors by binding the agonist, 125I-labeled epibatidine (Fig. 1, A and B), to stably transfected α6FLAGβ2HA cells and transiently transfected α3FLAGβ2HA cells. When cells were incubated at 37 °C, α3β2 and α6β2 subtypes showed little epibatidine binding. This is different from 125I-labeled epibatidine binding to α4β2 receptors as reported in Vallejo et al. (29). At 37 °C, 1/5 of a 6-cm culture of HEK cells stably expressing α4β2 receptors typically bound 0.67 pmol of 125I-labeled epibatidine, whereas the same size culture of α6β2- and α3β2-expressing cells bound 0.031 ± 0.009 and 0.005 ± 0.002 pmol, respectively (Fig. 1, A and B). These comparisons of the two cell lines were performed with approximately equal numbers of cells and total protein, on
average 1.5 mg of protein per 6-cm plate for confluent cultures, as well as similar amounts of total β2 subunit expression in the cell lines as assayed by Western blots (data not shown). Thus, the large differences in 125I-labeled epibatidine binding to α6β2, α3β2, and α4β2 receptors in the cell lines does not appear to be caused by differences in cell number or subunit synthesis. Much higher levels of 125I-labeled epibatidine binding to cells expressing α6β2 or α3β2 receptors were observed when the cells were cultured at 30 °C instead of 37 °C for 24 h (Fig. 1, A and B) as previously noted for heterologously expressed α3β2 receptors (34) and α6β2 receptors (35). The shift in temperature from 37 to 30 °C did not alter cell numbers or total protein concentration in any of the cell lines used in this study. The total protein per 6-cm plate for the samples in Fig. 1 was 1.82 ± 0.28 at 37 °C and 1.84 ± 0.25 at 30 °C. When the stably transfected α6FLAGβ2HA cells and transiently transfected α3FLAGβ2HA cells were cultured at 30 °C, 125I-labeled epibatidine binding was increased 6.5-fold for α6β2 receptors (0.2 ± 0.02 pmol) and 12.4-fold for α3β2 receptors (0.066 ± 0.01 pmol) compared with cultures maintained at 37 °C (Fig. 1, A and B). We performed additional experiments to test how the temperature change from 37 to 30 °C affected receptor assembly. Co-immunoprecipitation of the subunits was used to assay subunit assembly. We immunoprecipitated α6FLAG or α3FLAG subunits with anti-FLAG antibodies to determine β2 subunit co-immunoprecipitation and β2HA subunits with anti-HA antibodies for the α6 or α3 subunit co-immunoprecipitation (Fig. 1, C and D). Significant numbers of the α6 and α3 subunits co-precipitated with β2 subunits and visa versa, indicating that α6 and β2 subunits assemble into complexes like α3 and β2 subunits. Using fluorescent secondary antibodies, we were able to quantify the estimated levels of co-precipitated subunits and levels of the subunits available in whole cell lysates and found that 40–60% of the total subunit pool was assembled into receptors from the 5–7 experiments performed under each condition in Figs. 1, C and D. From the same quantification, we determined how the difference in culture temperature, 37 versus 30 °C, affected levels of the assembled subunits. As displayed in Figs. 1, A, C, and E, we observed similar effects of temperature on subunit protein and 125I-labeled epibatidine binding for α6β2 receptors. For α3β2 receptors, the change in subunit protein (Figs. 1, D and F) was somewhat less than the change in 125I-labeled epibatidine binding with the temperature shift (Fig. 1B).

**Assembly into α6β2 Pentamers**—Several studies have suggested that expression of α6 and β2 without other nicotinic receptor subunits results in aggregation and no assembly into α6β2 pentamers (23). To determine whether this phenomenon occurred in our expression system at 30 °C, we performed sucrose gradient sedimentation similar to the experiments of Kuryatov et al. (23). In parallel, sucrose gradient sedimentation was performed using another cell line stably expressing muscle αβδ receptors (32). Solubilized α6β2 receptors from the stable tsA201 cell line were size-fractioned on a sucrose gradient, and 125I-labeled epibatidine binding was performed. These experiments confirmed previous observations made by Kuryatov et al. (23). The epibatidine binding sites progressively increased starting at fraction 7 until the bottom of the gradient at fraction 1 (Fig. 2A), with a small peak at fraction 6. This is in contrast to the sedimentation of the muscle αβδ receptors (Fig. 2B), which migrated predominantly in a single peak centered at fraction 5 (9–10 Svedberg units). This could result from aggregation of α6 and β2 subunits that fail to assemble into pentamers. Another interpretation is that aggregation occurs after solubilization of the cells, a mechanism that would be similar to that observed for the α7 subunits. Aggregates of α7 subunits formed because the highly reductive condition in the cytoplasm of cells is lost during solubilization resulting in the formation of de novo disulfide bonds (36). Aggregates of α3 and β4 subunits were also observed when analyzed on non-denaturing blue native gels (37). The α3 and β4 subunits were aggregated similar to α7 subunits on the gels, but the subunits that assembled into muscle αβγδ receptors and 5HT3 receptors were aggregated to a much lesser extent (see Fig. 2B) (37). In addition to aggregates, α3β4 receptor pentamers were clearly demonstrated by Nicke et al. (37). These pentamers migrated slightly faster than the muscle αβγδ receptors, suggesting that the small peak we observe in Fig. 2A, centered at fraction 6, may contain α6β2 pentamers.

To further test for α6β2 pentamers, we assayed α6β2 complexes in the α6β2 stable cell line using blue native gels similar to the analysis of Nicke et al. (37). In Fig. 2C, under non-denaturing conditions (lanes 1–3), α6β2 complexes migrate as both
aggregates and pentamers on the gels. Aggregates and pentamers on the immunoblots were stained similarly with anti-\(\alpha_6\) (lane 1) or anti-\(\beta_2\) (lanes 2 and 3) antibodies, and therefore, both contain \(\alpha_6\) and \(\beta_2\) subunits. Under "mild" denaturing conditions (0.1% SDS and 0.1 M dithiothreitol (DTT), lane 4) pentamers and aggregates begin to break up and resolve into a ladder of smaller components in which monomers, dimers, trimers, tetramers, and pentamers of the subunits are observed. Under stronger denaturing conditions (2% SDS, lane 5) pentamers and aggregates have virtually disappeared, and subunit monomers, dimers, trimers, and tetramers predominate. We quantified protein migration along each lane using densitometry (Fig. 2D). Under non-denaturing conditions (lanes 1 and 2) the profiles along the lanes are similar to the profile obtained for \(^{125}\)I-labeled epibatidine binding sites on sucrose gradients (Fig. 2A). These results provide additional evidence that \(\alpha_6\beta_2\) receptors exist as both pentamers and larger subunit aggregates after solubilization.

Cell-surface Expression of \(\alpha_6\beta_2\) and \(\alpha_3\beta_2\) Receptors—Studies have also suggested that \(\alpha_6\) and \(\beta_2\) subunits alone do not form functional \(\alpha_6\beta_2\) receptors at the cell surface (23, 25). We employed several different assays to examine this issue. As a test of cell-surface expression of \(\alpha_6\beta_2\) receptors, we used immunostaining to visualize both the surface and intracellular receptor subunits in the stable \(\alpha_6\)FLAG/\(\beta_2\)HA cells incubated at 30 °C. The HA tag at the C terminus of the \(\beta_2\) subunit places the HA epitope in an accessible position in the extracellular domain of the \(\alpha_4\beta_2\) receptor (29). Similarly, the FLAG tag at the C terminus of the \(\alpha_6\) subunit places this epitope in the extracellular domain. Using this feature of the subunits, we stained \(\alpha_6\beta_2\) receptors with anti-FLAG and anti-HA Abs on the surface of intact and permeabilized cells. Anti-FLAG and anti-HA Abs both stained the cells stably expressing \(\alpha_6\beta_2\) receptors (Fig. 3, A and B) but did not significantly stain the parent HEK cells or cells stably expressing only \(\beta_2\) subunits (data not shown). Most of the permeabilized cells were stained with either Ab, and the staining showed a perinuclear pattern. Only the "rim" of the intact cells was stained by Abs whether the cells were first fixed before staining with anti-FLAG Ab (Fig. 3A, left panel) or whether living cells were first stained with anti-HA Ab and then fixed (Fig. 3B, left panel). However, staining of living cells with

![Figure 2](image-url)

FIGURE 2. Solubilized \(\alpha_6\beta_2\) receptors are both pentamers and aggregated complexes. Solubilized \(\alpha_6\beta_2\) complexes from the \(\alpha_6\)FLAG/\(\beta_2\)HA stable cell line were sedimented on continuous 5–20% sucrose gradients and

![Figure 3](image-url)

FIGURE 3. Solubilized \(\alpha_6\beta_2\) receptors are both pentamers and aggregated complexes. Solubilized \(\alpha_6\beta_2\) complexes from the \(\alpha_6\)FLAG/\(\beta_2\)HA stable cell line were sedimented on continuous 5–20% sucrose gradients and
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Ab before fixation resulted in clusters of the stained receptors (Fig. 3B, left panel) not observed when cells were fixed before Ab staining (Fig. 3A, left panel), indicating that the Abs were cross-linking receptors if the cell were not fixed. Comparing Ab staining of intact cells to that of permeabilized cells, it was clear that a significant fraction of permeabilized cells that express α6β2 receptors did not have significant Ab staining on the cell surface. We performed flow cytometry measurements on living cells stained with anti-HA Abs as assayed by flow cytometry. In conclusion, our data demonstrate that a fraction of the α6β2-expressing cells transport both subunits to the cell surface.

We also used cell-surface biotinylation to test whether α6β2 receptors are transported to the plasma membrane. Previously, we had developed a biotinylation method using MTSEA-biotin that biotinylated the cell-surface pool of α4β2 receptors when the MTSEA-biotin was applied to intact HEK cells and biotinylated the total pool of α4β2 receptors when the MTSEA-biotin was applied to HEK cells permeabilized with saponin (29). The same methods were applied to the tsA201 cells stably expressing α6β2 receptors or the tsA201 cells transiently transfected with α3FLAGβ2HA receptors (Fig. 3, C–F). We purified receptor subunits using streptavidin-agarose after biotinylation and performed 125I-labeled epibatidine binding to the different pools of receptors. Consistent with the previous studies of α6β2 receptors, we failed to measure a significant amount 125I-labeled epibatidine binding to the surface pools of α6β2 receptors cultured at 37 °C (Fig. 3C). Similarly, cells expressing α3β2 receptors incubated at 37 °C showed no significant amount of binding (Fig. 3C). In contrast, surface receptors as well as the total cellular pool bound 125I-labeled epibatidine when the cells were maintained at 30 °C. As shown in Fig. 3, C and D, incubation of the cells at 30 °C increased 125I-labeled epibatidine binding, and the percentage of binding sites on the cell surface were estimated to be 13% for α6β2 and 43% for α3β2 receptors. For cells expressing α4β2 receptors, 20% of the binding sites were on the cell surface when incubated at 37 °C (29).

We analyzed the MTSEA-biotinylated α6β2 and α3β2 receptors using SDS-PAGE. When immunoblots were stained to visualize FLAG-tagged α6 and α3 subunits or HA-tagged β2 subunits, significant amounts of all subunits were observed on

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de the cell surface at 37 and 30 °C (Fig. 3, E and F). As with the co-immunoprecipitation experiments, the increase in protein levels is similar to the increase in epibatidine binding. These data indicate heterologous expression results in the assembly of subunits into α6β2 and α3β2 receptors with epibatidine binding sites. When cells were maintained at 37 °C, there were few to no 125I-labeled epibatidine binding sites on the cell surface and in intracellular pools, although receptor assembly occurred (Fig. 1, C and D), and subunits were observed on the cell surface. However, when the cells were cultured at 30 °C for 24 h, we observed a significant increase in 125I-labeled epibatidine binding both at the cell surface and for the intracellular pools.

Expression of Functional α6β2 Receptors—To assess α6β2 receptor functionality, electrophysiological experiments were carried out on cells stably expressing the α6FLAG and β2HA subunits. Whole cell currents were recorded from cells maintained either at 30 °C and 37 °C. Brief exposure to acetylcholine (ACh) test pulses evoked currents in both conditions. Typical currents evoked by a series of ACh concentrations are shown in Fig. 4A. Concentration dose-response curves were obtained by plotting the amplitude of the ACh-evoked current as a function of the logarithm of the agonist concentration and were well fitted with a Hill equation (continuous line in Fig. 4B). The estimated EC50 value obtained from the fits to the Hill equation was 150 μM with a Hill slope of 1. ACh-evoked currents were observed only in 15% of cells (n = 254) that were maintained overnight at 30 °C. Although a comparable fraction of responsive cells was observed in culture maintained at 37 °C, the amplitude of ACh-evoked currents was significantly smaller (166 ± 19 pA; n = 8 versus 27 ± 7 pA; n = 5 tested at 300 μM ACh). The small fraction of responsive cells suggests that few cells express functional α6β2 receptors in their membrane, which corroborate the immunocytochemistry and flow cytometry results (Fig. 3, A and B). These data are also consistent with the lower percentage of MTSEA-biotin-labeled α6β2 receptors on the cell surface compared with α3β2 (Fig. 3, C and D) and α4β2 receptors (29).

A Comparison of Nicotine-induced Up-regulation of α6β2, α3β2, and α4β2 Receptors—Because there are conflicting reports as to whether α6-containing receptors are up-regulated in vivo by nicotine exposure (40–42), we assayed nicotine-induced up-regulation of α6β2 receptors. After nicotine treatment for 24 h at 30 μM nicotine, 125I-labeled epibatidine binding to α6β2 receptors increased significantly at 37 and 30 °C. With the up-regulation varying from 3- to 15-fold at both 37 and 30 °C. This range is broader but similar to what was observed for α4β2 where the -fold increase after nicotine treatment varied 3–6-fold (29).

To further characterize the up-regulation, cells stably expressing α6FLAGβ2HA receptors were exposed to different concentrations of nicotine for 24 h (Fig. 5, A and C) and to different exposure times at 5, 10, and 30 μM nicotine (Fig. 6, A, C, and E). The same sets of experiments were also carried out on tSA201 cells stably expressing α3β2HA receptors (Fig. 5, B and D, and Fig. 6B, D and F) both at 37 and 30 °C. 125I-Labeled epibatidine binding as a function of nicotine concentration was well fit by a single Hill equation with a Hill slope of 1 that would correspond to a bimolecular reaction (continuous curves, Fig. 5). In agreement with these observations, the relationship between 125I-labeled epibatidine binding and exposure time to nicotine can be fitted with a single exponential process (Fig. 6, A, B, E, and F).

A striking difference between the nicotine-induced up-regulation of α4β2 receptors and that of α6β2 and α3β2 receptors was the time course of the up-regulation for α6β2 and α3β2 receptors, which occurred on a much faster time scale than for α4β2 receptors. At 37 °C, the time course of the α6β2 and α3β2
up-regulation is essentially complete after 2 h, and no significant changes were observed with longer nicotine incubations. In contrast, the α4β2 up-regulation takes 2–3 h to initiate and 20 h to complete (29). The early time course of the up-regulation was characterized in more detail for α6β2 receptors (Fig. 6C) and for α3β2 receptors (Fig. 6D). Within 5 min of nicotine treatment, we observe significant increases in 125I-labeled epibatidine binding to the receptors and a 3–4-fold up-regulation after 1 h of treatment.

Table 1 summarizes the EC50 and time constant values for α6β2, α3β2, and α4β2 for the different experimental conditions. These data illustrate the contribution of the α subunit and experimental conditions. Only α4β2 receptors are significantly up-regulated at 37 °C by nicotine concentrations comparable with those reported for serum levels in smokers (10−7 – 10−6 M). However, because nicotine is lipophilic, it can accumulate and achieve levels 10-fold or more in brain than in serum (43, 44). Thus, nicotine levels in brain may reach levels up to 10−5 M (10 μM), which is almost the midpoint of the concentration dependence curve at 37 °C (Fig. 5A), high enough to up-regulate α6β2 receptors. The time course of α6β2 up-regulation at 37 °C treated with submaximum nicotine concentrations of 5 and 10 μM are displayed in Fig. 5A. These time courses are similar to that of the higher nicotine concentration, and at 5 and 10 μM nicotine we observed significant up-regulation, 1.8- and 2.6-fold, respectively.

Importantly, α6β2 receptors incubated at 30 °C are up-regulated by nicotine concentrations similar to those effective for α4β2 receptors (Table 1). The EC50 values of both α6β2 and α3β2 receptors are shifted by about 2 orders of magnitude when cells are kept at 30 °C instead of 37 °C. This finding that the concentration dependence of nicotine-induced up-regulation is highly temperature-sensitive is potentially significant because of what is observed in vivo. Cigarette smoking lowers skin temperature by 1.5 °C in humans (38) and causes a similar hypothermic change in rats (39). Thus, during smoking the EC50 values for the nicotine dependence of the up-regulation are likely to be significantly shifted to lower values, further suggesting that nicotine levels in brain may reach levels high enough to significantly up-regulate α6β2.

**DISCUSSION**

In this study we have examined α6β2 nAChRs using heterogeneous expression. Contrary to the results of others (23), we find that α6 subunits assemble with β2 subunits (Fig. 1, C and D) into pentamers (Fig. 2), are transported to the cell surface (Fig. 3), and produce ACh-evoked current at the cell surface (Fig. 4). In addition to the fully formed α6β2 receptors, we also observed assembly of these subunits into the large aggregates (Fig. 2) reported by Kuryatov et al. (23). It is possible that some aggregation occurs in the cells before solubilization as a consequence of subunit misfolding. However, it is unlikely that the
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FIGURE 6. The time course of nicotine-induced up-regulation for α6β2 and α3β2 receptors. tsa201 cells stably expressing α6FLAGβ2HA (A, C, and E) and α3β2HA (B, D, and F) receptors were exposed to nicotine (30 or 300 μM, respectively) for varying lengths of time. Nicotine exposure was again assayed by 125I-labeled epibatidine (EB) binding at 2 nM. The full time course of nicotine exposure at 37 °C is displayed in A and B and at 30 °C in C and D. Panel A also shows the time course of up-regulation at 37 °C with 5 and 10 μM nicotine. The nicotine-induced up-regulation for much briefer nicotine exposures is displayed in C and D. Up-regulation of 125I-labeled epibatidine binding to α6β2 receptors with 30 μM nicotine at 37 and 30 °C occurred with a T value of 1.2 h (A) (n = 3) and 2.3 h (E) (n = 3), respectively. Up-regulation of binding to α6β2 with 5 and 10 μM nicotine occurred with a T value of 0.76 h (A) (n = 3) and 1.09 h (E) (n = 3), respectively. Up-regulation of 125I-labeled epibatidine binding to α3β2 with 300 μM nicotine occurred at 37 and 30 °C with a T value of 1.5 h (B) (n = 5) and 3.2 h (F) (n = 4), respectively. Data are represented as the averages ± S.E.

| Subtype | Temperature °C | EC50 (nM) | T (h) |
|---------|----------------|-----------|-------|
| α6β2    | 37             | 4.6 × 10^-5 | 1.2   |
| α3β2    | 37             | 1.3 × 10^-4 | 1.5   |
| α6β2*   | 37             | 2.7 × 10^-7 | 14.5  |
| α3β2    | 30             | 3.6 × 10^-10| 2.3   |
| α6β2    | 30             | 3.8 × 10^-6 | 3.2   |

* Taken from Vallejo et al. (29).

TABLE 1

A comparison of the dose and time dependence of nicotine-induced up-regulation for different receptor subtypes

Instead, the subunit aggregation appears to occur in large part during the extraction procedure as observed for other neuronal nicotinic receptor subtypes (36, 37).

The levels of α6β2 and α3β2 receptor expression we and others (23, 34, 45) observe based on epibatidine binding and functional expression were significantly less than what was observed for α4β2 receptors (29, 45, 46). A plausible explanation is that unlike α4β2 receptors, neither α6β2 nor α3β2 complexes contain the subunit composition that occurs in vivo. Muscle nAChR subunit assembly is less efficient when these nAChRs lack the full complement of subunits (31, 47), suggesting that if additional subunits were available to assemble with α6β2 and α3β2 (e.g. α4, α5, or β3) transport to the surface would increase. However, other studies suggest that the addition of α5 (34) or β3 (35) subunits to α3β2 or α6β2 receptors, respectively, increases 125I-labeled epibatidine binding 2-fold or less and, thus, does not account for the ~100-fold higher binding to α4β2 receptors.

Another possible explanation for the differences we observe with the expression of the different nAChR subtypes is that 125I-labeled epibatidine binding and function of α6β2 and α3β2 receptors do not directly reflect the number of nAChRs expressed. Instead, both the number of epibatidine binding sites and receptor function could be state-dependent characteristics of the receptors that are regulated without changing the number of assembled receptors. Regulation of the number of functional nAChRs without changes in receptor numbers as monitored by antibody binding has been reported on chick ciliary ganglion neurons (48), although radiolabeled agonist binding was not performed in these studies. Additional evidence for this kind of mechanism is our studies of nicotine-induced up-regulation of α4β2 receptors in which we present evidence for nicotine-induced changes in the number of epibatidine binding sites and receptor functional state without changes in the number of assembled α4β2 receptors (29).

A change in the number of surface receptors with a similar change in temperature was first observed for muscle AChRs (49). The change was caused by the higher temperature dependence of surface turnover relative to surface incorporation. Increases in 125I-labeled epibatidine binding to α4β2 receptors were also observed by reducing cell incubation temperature from 37 to 30 °C (50). In this study by Cooper et al.
(50), they observed no increase in α4 subunit protein that corresponded with the temperature change. This finding suggested that the intracellular pool of receptors decreased as surface incorporation of receptors increased with the temperature change. Others have found that α3β2 receptor expression is increased by reducing the temperature from 37 to 30 °C (51). We found that assembled subunit protein and epibatidine binding to the complexes was increased by the temperature change for both surface and intracellular receptor pools (Figs. 1 and 3) and are contrary to the findings of Cooper et al. (50) with respect to how the intracellular pool changes.

Nicotine-induced up-regulation, and its role in the formation of nicotine addiction has focused principally on α4β2 receptors. This is because the majority of high affinity binding sites in the brain contain α4 and β2 subunits (13, 52), and α4β2 receptors mimic the changes observed in brains chronically treated with nicotine when heterologously expressed (45). Other nAChR subtypes, in particular α6-containing nAChRs, which are concentrated in midbrain dopaminergic neurons in the reward pathway (17, 53, 54), could play a role in mediating chronic exposure. Here we found that α6β2 nAChRs undergo nicotine-induced up-regulation and compared the up-regulation of α6β2 receptors with that of α3β2 and α4β2 receptors. The up-regulation of α6β2 receptors occurs more than an order of magnitude faster than the up-regulation of α4β2 receptors at 37 °C (Table 1) without a 2–3 h delay (29) but requires higher nicotine levels (Table 1). Because the subunit composition of the three receptor subtypes only differs with respect to their α subunits, differences in up-regulation must be attributed to the α subunits. If nicotine up-regulation depends upon binding of nicotine to the ligand binding site as previously suggested (55), then the binding site residues contributed by α subunits have a role in shaping the time course and nicotine concentration dependence of up-regulation. This conclusion is supported by a recent study in which α4β2 receptor up-regulation was altered by mutations within the α4 ligand binding site (56).

Our findings of subtype differences in the time course and nicotine dependence of their up-regulation suggest nAChR subtypes other than α4-containing receptors can contribute to up-regulation in ways previously not considered. The subtype differences in up-regulation time course and nicotine dependence provide new insights into how the receptors might operate in vivo. Specifically, α6β2 and α4β2 receptors could be up-regulated by different phases in the rise of nicotine levels during cigarette smoking. Cigarette smoking causes mean nicotine levels in serum to slowly rise on average to 200 ng during the course of a day (57). This slow rise in nicotine levels over time would clearly up-regulate α4β2 receptors but is too low a nicotine concentration to have a significant effect on α6β2 receptors. Consistent with this is positron emission tomography, showing that cigarette smoking in amounts used by typical daily smokers leads to nearly complete occupancy of α4β2 nAChRs (58). However, each cigarette smoked causes fastest fluctuations in serum nicotine on top of existing levels (59, 64). These rapid rises are amplified in brain where nicotine levels rise more rapidly than serum levels and can achieve 10-fold higher levels than in serum (43, 44) potentially rising to 10 μM given nicotine concentrations reported for serum levels in smokers (10−7–10−6 M). Additionally, cigarette smoking causes a drop in skin temperature of 1.5 °C (38). Based on the steep temperature dependence of the nicotine dependence of up-regulation (Table 1), the EC50 of α6β2 up-regulation would be 15 μM at 35.5 °C. Therefore, α6β2, but not α4β2 receptors, would be expected to be up-regulated during the shorter-lived increases in nicotine during smoking.

Our findings of subtype differences in nicotine up-regulation may also help explain a controversy in the field about whether α6-containing receptors actually are up-regulated in vivo. Some studies support up-regulation of α6-containing receptors by nicotine (35, 40), whereas other studies indicate that α6-containing receptors are not up-regulated by nicotine (41, 60, 61). With respect to this question, differences in either the time course or nicotine dependence of receptor up-regulation have not been considered when designing experiments or interpreting in vivo data. Because the methods for administrating nicotine vary widely in studies evaluating receptor up-regulation, the concentration of nicotine at the receptors sites are likely to vary, and it is possible that doses that up-regulate α4β2 receptors are not sufficient to up-regulate α6-containing receptors. If the up-regulation of α6-containing receptors occurs more rapidly than for α4β2 receptors as our studies indicate, the length of nicotine exposure also needs to be considered. Similarly, if the up-regulation of α6-containing receptors reverses more rapidly than for α4β2 receptors, then time allowed after nicotine exposure and before binding measurements will be important.

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