Endogenously Expressed Muscarinic Receptors in HEK293 cells Augment Up-regulation of Stably Expressed α4β2 Nicotinic Receptors

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*Running Title: Muscarinic receptors augment nAChR up-regulation

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Background: Nicotinic ligands up-regulate neuronal nicotinic receptors in vitro and in vivo. Results: Carbachol or oxotremorine plus nicotine up-regulate α4β2 nicotinic receptors more than nicotine alone. Conclusion: Muscarinic receptor activity augments nicotinic receptor up-regulation by increasing α4 and β2 subunit mRNA and protein. Significance: This study reveals a novel target for modulating neuronal nicotinic receptor expression.

SUMMARY

Nicotine-induced up-regulation of neuronal nicotinic receptors (nAChRs) has been known and studied for more than 25 years. Other nAChR ligands can also up-regulate nAChRs, but it is not known if these ligands induce up-regulation by mechanisms similar to nicotine's. In this study we compared up-regulation by three different nicotinic agonists and a competitive antagonist of several different nAChR subtypes expressed in HEK293 cells. Nicotine markedly increased α4β2 nAChR binding site density and β2 subunit protein. Carbachol, a known nAChR and muscarinic receptor agonist, up-regulated both α4β2 nAChR binding sites and subunit protein 2-fold more than did nicotine. This increased up-regulation was shown pharmacologically to involve endogenously expressed muscarinic receptors, and stimulation of these muscarinic receptors also correlated with a 2-fold increase in α4 and β2 mRNA. Muscarinic receptor activation in these cells appears to affect CMV promoter activity only minimally (~1.2 fold), suggesting that the increase in α4 and β2 nAChR mRNA may not be dependent on enhanced transcription. Instead, other mechanisms may contribute to the increase in mRNA and a consequent increase in receptor subunits and binding site density. These studies demonstrate the possibility of augmenting nAChR expression in a cell model through mechanisms and targets other than the nAChR receptor itself.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels comprised of α (α2-α10) and β (β2-β4) subunits. These subunits assemble as pentamers to make either homomeric receptors (e.g., α7 nAChRs) or heteromeric receptors (e.g., α4β2 nAChRs) in the CNS, peripheral ganglia, and adrenal gland. Activation of nAChRs by acetylcholine changes the confirmation of the receptor to allow passive ion-flow through the receptor channel. This ion-flow, if sufficient, depolarizes neurons, which in turn contributes to CNS functions such as cognition, affect and reward, and activates important responses in the autonomic and sensory nervous systems. The predominant nAChRs in mammalian brain contain α4 and β2 subunits (1-5), forming the α4β2, α4β2α5 and perhaps other receptor subtypes (2, 4-6).

The density of nAChRs in rodent and human brain increases (up-regulates) during chronic nicotine exposure (7-11); and this in vivo increase appears to be restricted primarily to the α4β2
receptor subtype (1, 4, 12). The nicotine-induced up-regulated nAChRs are functional (13-17) and thus conceivably contribute in substantial ways to nicotine addiction and/or dependence. In addition to their roles in normal physiology and in nicotine addiction, nAChRs are consistently found to be decreased in brains from Alzheimer’s disease patients (18-23); therefore, up-regulation of these receptors could be a potential target for treatment of this and possibly other neurodegenerative diseases.

The exact mechanisms that lead to nicotine-induced nAChR up-regulation are still unclear, but the initial signal appears to originate at the agonist binding site (24). The up-regulation is not accompanied by an increase in steady-state levels of subunit mRNA, indicating that the increase in receptors is independent of transcription or mRNA stabilization (25-28). Studies in cells that heterologously express nAChRs, suggest that nicotine acts as a “pharmacological chaperone”, enhancing the incorporation of β2 subunits into assembling receptors (29, 30) and thereby increasing the number of α4β2 nAChRs and possibly changing the stoichiometry from (α4)2(β2)2 to (α4)3(β2)3 (31, 32). The ability of nicotine to chaperone β2 subunits into the nAChR assembly appears to be due, in part, to an action of nicotine binding at a microdomain located on the extracellular portion of the β2 subunit (33). Moreover, nicotine has also been found to promote nAChR subunit assembly by activating protein kinase C-dependent phosphorylation of immature α4 subunits (34). In addition to its action to promote subunit assembly, there is strong evidence that, in cell models heterologously expressing nAChRs, nicotine also induces up-regulation by decreasing the degradation rate of the receptor (26, 29). Although both increased receptor assembly and decreased receptor degradation are well-supported theories regarding the mechanism for nAChR up-regulation, neither has been confirmed in neurons or in vivo.

Nicotine is not the only nicotinic ligand that up-regulates nAChRs. Cytisine, a plant-derived partial agonist at α4β2 nAChRs up-regulates these receptors after chronic administration in vivo in rat brain (24), as do the algae-derived full agonist (+)-anatoxin (13) and the synthetic partial agonist varenicline (35). Moreover, other nAChR ligands such as the agonists epibatidine and methylcarbamylcholine (36), carbachol (37-39), and choline (40), and even the nAChR competitive antagonist dihydro-β-erythroidine (DHβE) (14, 30, 38, 41), up-regulate α4β2 nAChRs in vitro in cells models. However, the mechanism(s) of nAChR up-regulation by carbachol and other agonists or antagonists have not been examined. Interestingly, a recent study demonstrated that tumor necrosis factor-α (TNF-α) can also up-regulate α4β2 nAChRs heterologously expressed in cells. Furthermore, the up-regulation by TNF-α effect was synergistic with nicotine (38) and appeared to involve p38 MAP kinase (38). In that study, the authors also noted that these receptors are possibly up-regulated more by carbachol than by nicotine (38). To investigate this further, we examined differences in α4β2 nAChR up-regulation induced by nicotine, carbachol, choline and DHβE.

MATERIALS AND METHODS

Materials and Chemicals: Dulbecco’s modified eagle medium, fetal bovine serum, and antibiotics were purchased from Invitrogen (Carlsbad, CA). [3H](±)epibatidine ([3H]EB), [3H]quinuclidinyl benzilate ([3H]QNB), and [125I]α-bungarotoxin ([125I]α-BTX) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Polyclonal antibodies sc-1772 (α4) and sc-1449 (β2) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). DHβE was obtained from National Institute of Drug Abuse. Nicotine hydrogen tartrate, carbamylcholine chloride (carbachol), choline chloride, oxotremorine sesquifumarate, atropine sulfate, and quinuclidinyl benzilate (QNB) were purchased from Sigma Aldrich.

Cell Culturing and Treating: HEK293 cells were maintained and grown as previously described (39, 42, 43). Cell culturing medium contained Dulbecco’s modified eagle medium supplemented with 10% fetal bovine serum, 100 unit/ml penicillin G, 100 µg/ml streptomycin, and 0.7 mg/ml genetin (G418). Cells were grown in 72 cm² Falcon T-flasks (Thermo Fisher Scientific; Pittsburgh, PA) and kept at 37°C under 5% CO₂ in a humidified incubator. After cells reached approximately 90% confluency, drug treatments were performed by replacing old culturing medium with new medium containing the desired concentration of drug(s). Treated cells were again
kept at 37°C under 5% CO2 in a humidified incubator for the desired treatment time.

**Radioligand Binding:** Following drug treatment, cells were harvested to make membrane homogenates as previously described (39). The cell medium was first replaced with 25 ml of 50 mM Tris buffer (pH 7.4 at 4°C), the cells were then gently scraped off the flasks and centrifuged at 1,000 g for 10 min at 4°C to wash and collect whole-cells. The resulting cell pellet was resuspended in 25 ml of buffer and homogenized using a polytron homogenizer or by sonication. The cell suspension was centrifuged again at 32,000 g for 10 min at 4°C. Pellet resuspension, homogenization, and high-speed centrifugation were repeated twice before resuspending the final pellet in Tris buffer. Membrane homogenates were then aliquoted into incubation tubes (approximately 25-100 µg of protein/tube, depending on the subtype of receptor being measured) and incubated with [3H]EB for 2-4 hr at room temperature to determine total binding. Nonspecific binding (NSB) was determined by the inclusion of 300 µM nicotine. Bound radioligand was separated from free radioligand by vacuum filtration over Whatman GF/C filters pre-soaked in 0.5% polyethylenimine. Filters were then measured for radioactivity by liquid scintillation counting. Binding to α7 nAChR was measured with 0.5 nM [125I]α-BTX in the presence of 0.5% bovine serum albumin to decrease NSB to the filters. NSB was measured in the presence of 1.5mM nicotine. The total density of muscarinic acetylcholine receptors was measured with 0.5 nM [3H]QNB, and NSB was determined in the presence of 50 µM atropine. NSB was subtracted from the total radioligand bound to determine specific binding. Values were normalized to the total protein as determined by a BCA protein assay.

**Western Blotting:** Crude homogenized membranes from untreated and treated cells were aliquoted into loading buffer containing 50 mM dithiothreitol and 2% sodium dodecyl sulfate. Samples were boiled for 5 min and 10 µg of total protein was loaded and separated by SDS-PAGE. The concentration of protein in each sample was determined before loading by a BCA protein assay. Following SDS-PAGE, protein was transferred from the gels onto polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were then blocked with 1% bovine serum albumin in TBST buffer (20 mM Tris-Cl, 147 mM NaCl, and 0.1% Tween-20; pH 7.6) for 30 min at room temperature, followed by 1 wash in TBST. Primary polyclonal antibodies directed at the α4 subunit (sc-1772) and the β2 subunit (sc-1449) were diluted to 1 µg/ml in TBST containing 1% BSA and 0.01% sodium azide and incubated with PVDF membranes overnight at 4°C while gently rocking. After incubation, membranes were washed with TBST buffer 4 times and incubated with anti-goat IgG conjugated to horseradish peroxidase in TBST with 1% BSA for 30 min at room temperature. Membranes were again washed with TBST 4-times before adding SuperSignal west pico chemiluminescent substrate (Thermo Fisher Scientific, Pittsburg, PA). The probed membranes were then visualized on autoradiography film. Following visualization, membranes were washed with TBST buffer 4-5 times over 2 hr while gently shaking at room temperature. India ink, diluted 1:1,000, was then introduced to the blots for 15-60 min while gently rocking at room temperature, similarly to previous studies (44, 45). Blots sufficiently stained with India ink were air dried. Autoradiography film and ink-stained membranes were recorded using a computer scanner. Cropping and sizing of images was performed with Corel Photo-Paint 11 software (Corel Corporation, Ottawa, Canada). Quantification of band densities and India ink staining was measured using Image J (National Institute of Health). Band densities were normalized to the total protein density in each lane as indicated by the India ink staining. Standard curves of α4 and β2 blots were carried out to ensure that band densities were within the linear range of the film.

**RNA Isolation and Quantitative Real Time RT-PCR:** HEK cells stably expressing rat αβ2 nAChRs were plated on poly-D-lysine treated Nunclon™ Delta Surface 6-well dishes (Thermo fisher Scientific; Pittsburgh, PA) and grown to 90% confluency before treating with nicotine, carbachol, and atropine. After an appropriate treatment time, media was aspirated, cells were lysed with 1 ml of TRizol (Invitrogen; Carlsbad, CA), and total RNA was isolated according to the manufacturer’s protocol. Isolated RNA was then transferred to a spin cartridge from a PureLink™ RNA Mini Kit (Life Technologies Corporation;
Carlsbad, CA), and RNA was purified according to the manufacturer’s protocol. Reverse transcription was used to create cDNA from 1µg of purified RNA using a RT² EZ First Strand cDNA Kit (SABiosciences; Frederick, MD) as described in the protocol provided by the manufacturer. Following reverse transcription, 1µL of cDNA was used for quantitative real-time PCR (qPCR). To quantify α4 mRNA, 300 nM of sense primer (CAGCAAGCCACCAGCTCCC) and 300 nM antisense primer (GCCTCCGCCCTGAGACAG) were used. Quantification of β2 mRNA was performed with 300 nM of sense primer (GCCGACGCAACGAGACCA) and 300 nM antisense primer (CGAGGGAGGTGGGAGCACA). All relative changes in α4 and β2 mRNA expression were normalized to GAPDH mRNA expression, which was determined using 600 nM of sense primer (GAGTCAACGGATTTGGTCGT) and 600 nM antisense primer (GATCTCGCTCCTGGAAGATG). RT² SYBR® Green (SABiosciences; Frederick, MD) was added to visualize amplification of cDNA with a CFX96 Real-Time System (Bio-Rad; Hercules, CA) using the following cycling protocol: 1 cycle of 95°C for 10 min followed by 30 cycles of 95°C for 10 seconds, 64°C for 30 seconds, and 72°C for 45 seconds. Following 30 cycles, a melting curve was produced to ensure a single product was amplified. In addition, samples were run on a 1.2% agarose electrophoresis gel after qPCR to ensure a single product was amplified with the expected base-pair sizes. Efficiency values of each primer pair was determined as previously described (46). These values were then considered during analysis of quantitative data using the Pfaffl equation (46) to determine relative changes in mRNA expression.

Luciferase Reporter Assay: HEK cells stably expressing α4β2 nAChRs were plated on Nunclon™ Delta 60mm dishes (Thermo fisher Scientific; Pittsburgh, PA) and grown to 90% confluency. Cells were then split into poly-D-lysine treated Nunc MicroWell 96-well optical-bottom plates (Thermo fisher Scientific; Pittsburgh, PA), and incubated overnight at 37°C under 5% CO₂. The following morning, cells were treated with nicotine, carbachol, and atropine for 6 or 24 hr. A Steady-Glo® Luciferase Assay system (Promega Corporation; Madison, WI) was used to lyse cells and introduce luciferase substrate following the manufacturer’s protocol. Relative luminescence was measured with an EnVision 2104 Multilabel Reader (PerkinElmer).

Data Analysis: All data were analyzed and graphed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Most up-regulation data was graphed as fold-increase compared to untreated tissue, which provides easy comparison of up-regulation between different nAChR subtypes, as well as across figures that measure up-regulation of radioligand binding, subunit protein, and mRNA. Statistical analysis was performed with a one-way ANOVA, post-hoc Tukey’s test.

RESULTS

Carbachol up-regulates α4β2 nAChRs with a greater efficacy than nicotine. In addition to nicotine, several other nicotinic ligands up-regulate nAChRs; however, it is not known if they all up-regulate the receptors to the same extent or by the same mechanisms. To compare ligand-induced up-regulation, we examined the concentration-dependence of nicotine, carbachol, choline and DHβE to up-regulate α4β2 nAChRs stably expressed in HEK293 cells. Cells were treated with a range of drug concentrations for 24 hr before measuring [3H]EB binding in membrane homogenates. The concentration-effect curves for up-regulation are shown in figure 1. Of the drugs tested, nicotine was the most potent to up-regulate α4β2 nAChRs; DHβE was the next most potent, followed by carbachol and choline. The maximum up-regulation ($E_{\text{max}}$) of α4β2 nAChRs induced by choline was similar to that induced by nicotine. The fitted $E_{\text{max}}$ of DHβE-induced up-regulation appeared to be less than nicotine, but this difference did not reach statistical significance. Most interesting, however, was that the maximum up-regulation induced by carbachol was ~2-fold more than by nicotine ($p<0.001$). The observation that carbachol induces a greater up-regulation than
Nicotine indicates that carbachol might up-regulate receptors through different and/or multiple mechanisms compared to nicotine.

**Nicotine-, Carbachol-, and Choline-induced Up-regulation of other nAChR Subtypes.** We also tested nicotine-, carbachol-, and choline-induced up-regulation of several other nAChR subtypes α3β4, α7, α4β4, α6β2, and α3β2 nAChRs stably expressed in HEK293 cells (39, 43). Concentrations of nicotine, carbachol, and choline that resulted in near maximal or maximal up-regulation of α4β2 receptors after 24 hr (Fig. 1) were used to treat these other nAChR subtypes. These high concentrations of the three agonist ligands used here allowed the E$_{max}$ for up-regulation of each receptor subtype to be estimated. The relative increase (fold increase) in binding sites after incubation with the three agonists compared to untreated cells for each nAChR subtype is shown in Table 1. The α3β4 nAChR subtype appeared to be modestly up-regulated by a 24 hr exposure to nicotine, carbachol and choline, but the maximum increase observed was ~60% or less. The α7 receptor subtype also demonstrated a modest up-regulation, which was statistically significant only with 100 μM nicotine and 1 mM carbachol treatments. The α4β4 nAChR was modestly up-regulated by nicotine and carbachol, but not by choline. The largest increases in nicotine- and carbachol-induced up-regulation were found in the α6β2 and α3β2 nAChRs, with maximal increases of more than 20-fold and 45-fold, respectively. The large increases in these two nAChR subtypes probably reflect their low density in the untreated control cells. Choline also increased these receptors, but to an extent similar to or less than that seen in the α4β2 receptors. Interestingly, the carbachol-induced increase was statistically greater than the nicotine-induced increase only in the α4β2, α4β4 and α3β2 receptor subtypes.

Nicotine, Carbachol and choline increase the density but not the affinities of α4β2 nAChRs for nicotinic ligands. We next investigated possible differences between nicotine-, carbachol- and choline-induced up-regulation of α4β2 nAChRs. The α4β2 receptor subtype was selected because it is the major subtype in brain and is up-regulated by nicotine in vivo (1, 4, 12). Comparison of [3H]EB saturation binding to α4β2 nAChRs that were up-regulated by a 24 hr treatment with maximal concentrations of nicotine, carbachol or choline revealed that that each curve fit best to a one-site binding model; in addition, these studies demonstrated that all three drugs increased the density but not the affinity of [3H]EB for the binding sites and that the increase by carbachol was ~ twice that by nicotine or choline (figure 2A).

Nicotine binding competition curves at α4β2 nAChRs from untreated, nicotine-, carbachol- and choline-treated cells were then compared. In each case, the competition binding curves were again fit best by a one-site binding model, and, more importantly, the [3H]EB binding was completely displaced by nicotine with similar K$_i$ values for untreated cells or cells up-regulated by nicotine, carbachol, or choline (Fig. 2B).

The time-courses for up-regulation of α4β2 nAChRs by concentrations of nicotine, carbachol, and choline that produce nearly maximal up-regulation are shown in figure 2C. The increase in carbachol-induced up-regulation compared to nicotine-induced up-regulation was first observed at 6 hr of treatment. Carbachol continued to induce receptor up-regulation at an increased rate and with increased efficacy compared to nicotine or choline for up to 12 hr of treatment, after which the rate of increase in receptors decreased and was similar to that of nicotine and choline-treated cells. The observations that carbachol up-regulates α4β2 nAChRs with a faster rate as well as with greater efficacy compared to nicotine or choline again suggests that, at least during the first 12 hr, carbachol up-regulates these receptors through more than one mechanism.

**Muscarinic acetylcholine receptor antagonists attenuate nAChR up-regulation by carbachol.** Carbachol is an agonist at both muscarinic acetylcholine receptors and nAChRs, and HEK293 cells are reported to endogenously express functional M3 muscarinic receptors (47-51). Consistent with those reports, we found the density of muscarinic receptors labeled by a saturating concentration of [3H]QNB, a high affinity muscarinic antagonist, to be 64 ± 3 fmol/mg protein. To determine if the greater increase in nAChRs induced by carbachol compared to nicotine involved signaling through muscarinic receptors, the effects of 10 μM atropine, a muscarinic antagonist, on nicotine- and carbachol-induced up-regulation were examined.
This concentration of atropine was used because the EC$_{50}$ for carbachol to activate muscarinic receptor-mediated PI-hydrolysis is approximately 2.5 μM, and the K$_i$ for atropine to inhibit this response is approximately 1 nM (52). Therefore, in the presence of 1 mM carbachol, the calculated IC$_{50}$ of atropine to inhibit muscarinic receptor function is approximately 400 nM, as calculated from the Cheng-Prusoff equation (53). A 10 μM atropine concentration was therefore selected to ensure nearly complete (96%) muscarinic receptor blockade in the presence of 1 mM carbachol. Up-regulation of nAChRs was expressed as fold-increase in $[^3H]$EB binding compared to untreated cells. Atropine treatment alone did not affect the basal expression of receptors, nor did it affect up-regulation induced by nicotine. Interestingly, however, atropine inhibited the up-regulation induced by 1 mM carbachol by approximately 30%, resulting in an increase of α4β2 nAChRs that was not significantly different from the increase induced by nicotine (fig. 3A).

To confirm that this effect of atropine was most likely due to muscarinic receptor blockade, the effects of quinuclidinyl benzilate (QNB), another high affinity muscarinic receptor antagonist, on nicotine- and carbachol-induced up-regulation was examined. QNB is more potent than atropine, so a lower concentration of QNB (1 μM) was used to inhibit muscarinic receptor function. This QNB treatment did not affect the basal level of nAChRs or the up-regulation induced by nicotine, but it inhibited ~34% of the up-regulation induced by carbachol (fig. 3B); thus, similar to the results with carbachol in the presence of atropine, the increase in nAChR induced by carbachol in the presence of QNB was not significantly greater than the increase induced by nicotine.

**Up-regulation of nAChR binding densities correlates to increases in the β2 subunit protein.** Western blot analyses were carried out to determine if increased $[^3H]$EB binding densities reflected increases in α4 and/or β2 subunit protein. Before carrying out westerns blots on cells treated with the agonists and/or atropine, the specificity of these commercially available antibodies for probing α4 and β2 subunits was verified. Figure 4A shows western blots of proteins corresponding to the α4 and β2 subunits in membranes from untreated cells expressing α4β2 nAChRs (nAChR density ~500 fmol/mg protein), cells treated for 24 hr with carbachol (~8000 fmol/mg protein), cells expressing α3β4 nAChRs (~8,000 fmol/mg protein) and untransfected HEK 293 cells. To gauge total protein loading, an India ink stain was performed for each blot. The α4 subunit band was clear and measurable in membranes from untreated α4β2-expressing cells, but it was more prominent in membranes from cells in which the nAChRs were up-regulated with carbachol. In contrast, no band corresponding to the α4 subunit was seen in the membranes from cells that express α3β4 nAChRs or from untransfected cells. Similar results were found for the protein band probed with the β2 subunit antibody, except the band in the membranes from untreated α4β2-expressing cells was much less prominent than the α4 band (Fig. 4A). These studies thus demonstrate that the antibodies used here are specific and sensitive enough to detect the α4 and β2 subunit proteins in these transfected cells, which express a high density of nAChRs.

Western blots were then carried out on membranes from untreated cells and cells treated for 24 hr with nicotine or carbachol in the presence or absence of atropine. Figure 4B shows representative western blots probed for α4 and β2 nAChR subunit proteins, along with the loading control India ink stain. Figures 4C and 4D show the summarized densitometric analyses from western blots of the two subunit proteins, expressed relative to measurements in untreated cells. The α4 subunit protein appeared to be slightly increased by carbachol, and atropine blocked this increase (Fig. 4C). In contrast to the α4 subunit protein, both nicotine and carbachol treatments markedly increased the density of the β2 subunit protein (Fig. 4D); moreover, this increase was in proportion to the increase in nAChR binding sites (compare to Fig. 3). Thus, the β2 subunit protein was consistently increased more from cells treated with carbachol than with nicotine (Fig. 4D), and treatment with atropine, which alone did not affect the density of β2 subunit protein nor block the increase in β2 protein induced by nicotine, decreased the effect of carbachol treatment on β2 protein, so that in cells treated with carbachol plus atropine, the density of the β2 subunit protein was similar to that from nicotine-treated cells (Fig. 4D).
Muscarinic receptor agonists augment nicotine-induced up-regulation of nAChRs.

Because muscarinic receptor antagonists attenuated carbachol-induced up-regulation of α4β2 nAChRs, the muscarinic agonist oxotremorine was tested to determine if it augmented nicotine-induced up-regulation or otherwise affected nAChRs. As shown in figure 5A, 200 µM oxotremorine alone significantly up-regulated α4β2 nAChRs; moreover, up-regulation by this concentration of oxotremorine was only partially blocked by atropine. Treatment with oxotremorine plus nicotine produced significantly greater up-regulation than nicotine alone (Fig. 5A), similar to the effect observed with carbachol. Furthermore, blocking muscarinic receptors with atropine nearly fully prevented the oxotremorine augmentation of nicotine-induced up-regulation, resulting in binding values similar to those of nicotine alone (Fig. 5A).

To determine if the increased [3H]EB binding sites induced by oxotremorine retained their fundamental nAChR characteristic (i.e., high affinity for nicotine), we assessed the competition by nicotine for [3H]EB binding sites that were up-regulated with 200 µM oxotremorine alone. As shown in figure 5B, nicotine competed for all of the [3H]EB binding to the α4β2 nAChRs receptors that were up-regulated by oxotremorine; furthermore, the Hill coefficient for these binding curves was close to 1, indicating that only a single affinity class of binding site was present, and the calculated Kᵢ of nicotine for these sites, 5.5 nM, was similar to the values determined for untreated and nicotine agonist up-regulated, α4β2 nAChRs, as shown in figure 2B.

Because oxotremorine alone and even in the presence of atropine significantly increased α4β2 nAChRs (Fig. 5A), the affinity of oxotremorine for α4β2 nAChRs labeled by [3H]EB was examined and compared to nicotine, carbachol, choline, atropine, and QNB. As shown in figure 5C, nicotine had the highest affinity for α4β2 nAChRs, and carbachol demonstrated the next highest affinity for binding. Interestingly, choline and the muscarinic receptor ligands oxotremorine and atropine also competed for α4β2 receptors; however, the affinity of these ligands was 3,000 to 8,000 times lower than that of nicotine. QNB at concentrations up to 300 µM did not compete for α4β2 nAChR binding sites.

Considering that the calculated Kᵢ value for oxotremorine at α4β2 receptors was 32 µM, it is likely that the up-regulated nAChRs following treatment with 200 µM oxotremorine shown in figure 5A resulted from oxotremorine binding to the nAChR agonist recognition site, which would explain why atropine only partially inhibited oxotremorine-induced up-regulation. In contrast, the augmentation by oxotremorine of nicotine-induced up-regulation was nearly completely blocked by atropine. The probable explanation for this is that in the presence of 1 µM nicotine (~200-times its Kᵢ at α4β2 nAChRs), 200 µM oxotremorine (6-times its Kᵢ at α4β2 nAChRs) will theoretically bind few, if any, nAChRs. Therefore, under these conditions oxotremorine can augment receptor up-regulation by binding muscarinic receptors, but would not be expected to induce up-regulation directly via the nAChR binding site already occupied by nicotine. That would explain why atropine nearly fully blocked the augmentation by oxotremorine of nicotine-induced receptor up-regulation but not the effects of oxotremorine alone directly on the receptor. To test this explanation, the effects of a much lower concentration of oxotremorine were measured. Oxotremorine at a concentration of 3 µM, which is approximately 10-times lower than the calculated Kᵢ for α4β2 nAChRs (Fig. 5C) but 3 times higher than the EC₅₀ for muscarinic receptor activation (52), did not significantly up-regulate α4β2 nAChRs; however, the same concentration of oxotremorine did significantly augment up-regulation in the presence of nicotine. Moreover, the augmentation of up-regulation by this low concentration of oxotremorine was completely blocked by a low concentration (300 nM) of atropine (fig. 5D).

Muscarinic receptor activation increases α4 and β2 nAChR mRNA. The mechanism of nicotine-induced up-regulation of α4β2 nAChRs in brain is not dependent on increased transcription of α4 and β2 mRNA (25). To investigate whether transcription plays a role in muscarinic receptor-mediated augmentation of nAChR up-regulation, reverse transcription followed by qPCR was used to measure changes in α4 and β2 mRNA levels after treatment of cells with carbachol. HEK cells stably expressing α4β2 nAChRs were treated with nicotine, carbachol, or carbachol plus atropine for 6 hr before the cells were lysed. A 6 hr treatment
time was selected because the rate of carbachol-induced up-regulation was most rapid between 3-12 hr incubation times, while after 12 hr the rate of up-regulation slowed and was similar to nicotine-induced up-regulation (figure 2C). We hypothesized that if increased mRNA is involved in carbachol-induced up-regulation, the increase would likely be observed during this early and rapid up-regulation rate. Figure 6A shows an agarose gel following qPCR using α4, β2, and GAPDH primers in untreated HEK cells expressing α4β2 nAChRs, demonstrating that each primer pair amplified a single product with the expected band sizes. The results in figure 6B indicate that a 6 hr nicotine treatment did not significantly affect the expression of α4 and β2 transcripts (although it appears that nicotine slightly decreased the expression of β2 mRNA, this decrease was not statistically significant). Surprisingly, carbachol treatments induced a significant 2-fold increase in both α4 and β2 mRNA. Atropine completely blocked this carbachol-induced increase in mRNA, similar to its effect on carbachol-induced increases in nAChR binding sites and subunit protein.

Testing CMV Promoter Enhancement by Carbachol. The expression of α4 and β2 nAChR subunits in stably transfected HEK cells are controlled by a CMV promoter. Although CMV promoters are considered to be constitutively active, our observation that carbachol increases mRNA of α4 and β2 nAChRs suggests that this increase in subunits could be a result of increased transcription. Therefore, we examined the possibility that muscarinic receptor activity increases α4 and β2 mRNA through enhancement of the CMV promoter with a consequent increased transcription of mRNA. The HEK cells stably expressing α4β2 nAChRs were transiently transfected with pEGFPLuc plasmid, which contains the firefly luciferase gene that is expressed from a CMV promoter. After transfection, cells were split into a 96-well optical plate and 24 hr later treated with nicotine, carbachol, or carbachol plus atropine. Cells treated with sodium butyrate, which is known to enhance CMV promoter activity (54), served as a positive control. Enhancement of the CMV promoter was then measured by luciferase activity either 6 or 24 hr after treatment. As shown in figure 7A, nicotine did not affect the expression of luciferase. Carbachol treatments resulted in a small 13% increase in luciferase expression that was not statistically significant, but which nevertheless appeared to be fully blocked by atropine. However, sodium butyrate, the positive control, also did not increase luciferase expression 6 hr after treatment. Therefore, to elicit a clearer and perhaps larger response, luciferase expression was measured 24 hr following treatments. At that time point, sodium butyrate treatment resulted in a statistically significant 2.7 fold increase in luciferase expression (Fig. 7B). Nicotine did not affect the expression of luciferase protein, but again carbachol appeared to induce a small (23%) not statistically significant increase in luciferase expression, which again appeared to be blocked by atropine (Fig. 7B).

DISCUSSION

This study demonstrates that the nAChR agonists nicotine, carbachol, and choline and the competitive antagonist DHβE up-regulate α4β2 nAChRs with varying potencies, which correlate with the rank order of their affinities at the nAChR agonist binding site. Thus, nicotine, which has the highest affinity for α4β2 nAChRs of the drugs tested here, was most potent in up-regulating these receptors, while choline was least potent. Our results with choline confirm a previous report, also using this cell line, that this fairly ubiquitous endogenous acetylcholine precursor can up-regulate α4β2 nAChRs (40).

Previous studies demonstrated that nicotine-induced up-regulation of nAChRs is most likely triggered from the agonist binding site (24, 29, 41); in fact, this up-regulation apparently does not even require ion channel function per se since the nAChR channel blocker mecamylamine does not prevent nicotine-induced up-regulation in rat brain in vivo (24) or in cells in culture (29). Furthermore, as shown here, as well as previously (14, 30, 38, 41), the competitive antagonist DHβE also up-regulates these receptors. Despite the apparent importance of the agonist binding site, the EC\textsubscript{50} concentrations that we found for up-regulation by nicotine, carbachol, choline and DHβE are much higher than their binding affinities to the desensitized receptor, an observation consistent with a previous study (41). In fact, the concentrations of the ligands examined here are closer to their EC\textsubscript{50} values to activate (or,
in the case of DHβE, the IC50 value to block) function (see for example, 14, 16, 55, 56, 57). It is possible that the higher concentrations of nicotinic ligands required to up-regulate these receptors compared to their binding affinity to mature receptors reflect, in part, an action at nascent receptors or even individual subunits, such as the microdomain on the β2 subunit that appears to be a target for a chaperone effect of nicotine that leads to incorporation of the subunit into the receptor and subsequent receptor maturation and up-regulation (29, 30, 33, 58).

Nicotine administered in vivo increases predominantly the α4β2 nAChR subtype in brain (1, 4); moreover, it does not increase the nAChRs in autonomic ganglia or adrenal gland (59, 60), which appear to express predominantly α3β4* nAChR subtypes and a smaller number of α3β2* subtypes (61-64). But in mammalian cells transfected to heterologously express pairs of nAChR subunits that form potential receptor subtypes, exposure to nicotine for 3 days or longer increases [3H]EB binding to several different subunit combinations, including α2β2, α3β2, α4β2, α2β4, α3β4 and α4β4 (27, 37, 64, 65). Similarly, in the current studies, even a 24 hr exposure of cells to either nicotine or carbachol increased almost all of the subtypes to some extent, with the α3β4 subtype being the least affected (Table 1). Exposure to choline had less effect on the different receptor subtypes, in general (Table 1), but the α4β2 subtype was increased even at a choline concentration that might be within reach of its physiological intracellular concentration range. Thus, the EC50 of choline to up-regulate these nAChRs (660 μM) is within the range of values found for its Km for the enzyme choline acetyltransferase (200 μM to 800 μM) in the synthesis of acetylcholine (67-69). This raises the interesting possibility that endogenous choline could play a physiologic role in maintaining an adequate number of α4β2 nAChRs under some circumstances.

Most interesting, however, was the observation that the α4β2 nAChRs in these cells were up-regulated more by carbachol than by nicotine. In both cases the increased receptors retained similar high affinity for [3H]EB and demonstrated the characteristics of a single class of high affinity binding sites for nicotine. The added effect of carbachol compared to nicotine on nAChR binding was seen during the first 12 hr of exposure, after which the rate of increase of the receptors appeared to be the same out to at least 72 hr. This two-phased effect of carbachol implies that it initially acts through a mechanism that nicotine does not activate; moreover, the transient nature of this initial mechanism induced by carbachol could reflect an independently regulated signaling system, such as a second messenger system.

The greater efficacy of carbachol to up-regulate α4β2 nAChRs in these cells clearly involves a mechanism mediated by muscarinic receptors, as demonstrated by the pharmacology of the effect. Thus, the added effect of carbachol on the up-regulation was blocked by two different muscarinic antagonists, atropine and QNB, and mimicked by combining nicotine with the muscarinic agonist oxotremorine. These cells express the M3 muscarinic receptor subtype (47-51), which can function via three independent signaling cascades that activate second messengers: 1) Gq coupling to phospholipase C activation to hydrolyze phosphoinositide to diacylglycerol and inositol triphosphate (50, 51, 70, 71); 2) G12 coupling to phospholipase D activation to hydrolyze phosphatidylcholine into phosphatidic acid and choline (71); 3) β-arrestin mediated internalization of the receptor after continued activation, leading to generation of further signaling cascades (50, 51). It is not known which of these pathways is critically involved at this time.

Western blots in these cells show that nicotine markedly increases nAChR β2 subunit protein and to a much less extent the α4 subunit protein. In fact, the increase in β2 subunit protein correlates closely with the increase in binding site density after treatment with nicotine and the further increase in binding sites after treatment with carbachol. The two-fold increase in the α4 subunit protein after carbachol treatment, however, is consistent with the two-fold greater increase in receptor binding sites after treatment with carbachol compared to nicotine. Because these western blots were performed with crude membrane homogenates, the subunit signals measured likely reflect fully assembled nAChRs, as well as unassembled monomers and dimers. We speculate that, at least in these cells, there are more unassembled α4 subunits than unassembled
β2 subunits, which might be more rapidly degraded under basal conditions. Therefore, when these receptors are up-regulated, changes in β2 subunits are more readily apparent because they avoid degradation by being incorporated into assembling receptors. In contrast, increased incorporation of α4 subunits is less apparent and harder to measure because they are assembled from a larger pool of subunits. Similar changes in subunit expression of α4 and β2 subunits in these cells have been observed previously (40), and the authors of that study also speculate that differences in unassembled subunit pools can explain why the change in β2 subunits, but not α4 subunits, is closely related to changes in [3H]EB binding during up-regulation. Interestingly, however, in our study, carbachol was the only drug that induced a statistically significant increase in α4 subunits; and although this increase was only ~2 fold, it too closely tracks the greater increase in the α4β2 nAChRs induced by carbachol compared to nicotine. Nevertheless, taken together these data suggest that the availability of the β2 subunit is limiting in the synthesis of α4β2 nAChRs in these cells.

The muscarinic acetylcholine receptor agonist oxotremorine also augments α4β2 nAChR up-regulation. Our expectation was that the effect of oxotremorine was mediated solely by muscarinic receptors, but surprisingly the response to the high concentration of oxotremorine (200 µM) is only partially blocked by a high concentration of atropine. We subsequently found that, in addition to being a muscarinic agonist, oxotremorine binds to α4β2 nAChRs with a K_d (affinity) of 32 µM. Therefore, we conclude that at high concentrations, oxotremorine up-regulates α4β2 nAChRs by actions at the nicotinic receptor binding site, as well as by activating muscarinic receptors, similar to carbachol-induced up-regulation. That explains why atropine only partially blocked nAChR up-regulation induced by a high concentration of oxotremorine. In addition, however, this concentration of oxotremorine augments nicotine-induced up-regulation, and this augmentation is fully blocked by atropine (see Fig. 5A). In this case, the presence of nicotine at a concentration ~200-times its K_d fully occupies the nAChRs and thus prevents oxotremorine at a concentration ~6-times its K_d from binding to nAChRs; therefore, the augmentation of up-regulation induced by oxotremorine is due virtually entirely to the activation of muscarinic receptors, while nicotine up-regulates via its binding to nAChRs.

A low concentration (3 µM) of oxotremorine alone, which, based on its K_d at α4β2 nAChRs, would be expected to occupy fewer than 9 percent of the nAChRs, did not significantly up-regulate these receptors. It did, however, augment nicotine-induced nAChR up-regulation, and this augmentation was completely blocked by a low concentration of atropine, indicating that it was mediated by muscarinic receptors (see Fig. 5D). It appears, therefore, that activating muscarinic receptors alone does not significantly up-regulate nAChRs, but rather requires a nicotinic agonist (e.g., nicotine), probably acting via a chaperone effect (29, 33) to observe the muscarinic receptor augmentation of nAChR up-regulation. Thus, from these oxotremorine experiments, we speculate that the activation of muscarinic receptor stimulation increases nAChR subunits so that more are available for incorporation into the nAChR pentamer. However, under basal conditions, with the nAChR unoccupied, increasing subunits alone may not result in increased nAChRs because the receptor assembly process in these cells may already be saturated with subunits, thus the availability of subunits is not limiting. Nicotine, which acts as a subunit chaperone, may enhance the rate of receptor assembly enough to make the availability of β2 subunits the limiting factor. Thus, in the presence of nicotine to increase assembly, an increase in the availability of α4 and β2 subunits via muscarinic receptor stimulation results in the augmentation effect observed here.

Muscarinic receptor activation may augment α4β2 nAChR up-regulation by increasing mRNA for both α4 and β2 subunits. The observed 2-fold increase in mRNA in carbachol-treated cells compared to nicotine-treated cells parallels the approximate 2-fold augmentation of nicotine’s effect on subunit protein measured by western blotting (Fig. 5) as well as on binding site densities (e.g., Figs. 1 and 3). It is likely that the increase in nAChR subunit mRNA by muscarinic receptor activation results in an up-regulation of subunits, which under the influence of nicotine’s chaperone effect, are then assembled into more nAChRs.
The relationship between transcription and translation of nAChR subunits was not directly measured here, but the origin of the increased α4 and β2 mRNA was investigated (i.e. increased transcription). Luciferase reporter assays indicated that carbachol induces a very small enhancement of CMV promoter activity (~1.2 fold) measured 6 and 24 hr after treatments. Although this small increase in activity was not statistically significant, it was fully inhibited by atropine at both time points and therefore is consistent with an effect mediated by muscarinic receptors. Moreover, because the precise quantitative relationship between promoter activity and subunit mRNA is not known, a role for promoter activity in the effect of muscarinic receptor augmentation of nicotine-induced up-regulation of nAChRs cannot be ruled out. However, evidence against increased CMV promoter activity playing a major role in the augmentation of α4β2 nAChR up-regulation is found when comparing nicotine- to carbachol-induced up-regulation of other nAChR subtypes stably expressed in the same parent HEK cells and under control of the same CMV promoter. A high concentration of carbachol did not up-regulate α7 or α3β4 nAChRs beyond that seen with nicotine (Table 1). Instead, the up-regulation by carbachol was consistently greater than by nicotine for receptors containing α4 and/or β2 subunits, an effect that was greatest for the receptor that contained both α4 and β2 subunits.

Further studies will be needed to explore the mechanism of this muscarinic receptor influence on nAChR up-regulation; more specifically, to determine whether nAChR up-regulation is augmented by muscarinic receptor signaling through Gq-, G12- or another G-protein coupling mechanism, or possibly by β-arrestin after internalization. It will also be important to determine if this muscarinic receptor contribution to nAChR up-regulation occurs in native tissues, including brain, which could indicate the potential physiological importance of these findings. In any case, these findings, along with other recent studies (38, 40, 72), reveal the possibility of modulating α4β2 nAChR expression in brain by drugs and mechanisms other than nicotine.

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FOOTNOTES

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†Abbreviations used are: nAChR, neuronal nicotinic acetylcholine receptor; DHβE, Dihydro-β-erythroidine; [3H]EB, [3H]Epibatidine; [3H]QNB, [3H]Quinuclidinyl Benzilate; [125I]α-BTX, [125I]α-Bungarotoxin; NSB, Non Specific Binding; quantitative real-time PCR, qPCR

FIGURE LEGENDS

TABLE 1. Cells were treated with drugs for 24 hr before preparing membrane homogenates. The α7 nAChR subtype was measured in the presence of 0.5nM [125I]α-BTX binding, whereas all other nAChR subtypes were measured using 1.5-2nM [3H]EB binding. Binding densities were normalized to the total protein (mg) in the binding assay. Values are represented as the fold-increase of receptor binding density (mean ± S.E.M) compared to untreated cells. Mean values were obtained from three to six individual experiments. Significance of fold increase from the untreated group is indicated by asterisks (*p < 0.05; **p<0.01; ***p<0.001). Significance of up-regulation induced by 10 mM carbachol and 10 mM choline compared to 100 µM nicotine is indicated by daggers (†p < 0.01; ††p < 0.001).

FIGURE 1. Cells were treated with increasing concentrations of nicotine (Nic), carbachol (Carb), choline (Ch), or DHβE for 24 hr. The density of nAChR binding in membrane homogenates was measured with 1.5-2 nM [3H]EB and was normalized to total protein. The plotted data (mean ± S.E.M.) was obtained from three to six individual experiments, and was fit with a nonlinear regression, dose-response fit (variable slope). The baseline value for binding was 631 ± 58 fmol/mg protein. The determined EC_{50} and E_{max} values (mean ± SEM) were calculated from these curves. EC_{50} values are shown in the figure. The E_{max} values derived from these curves are (fmol/mg protein): nicotine = 4152 ± 178; carbachol = 8733 ± 434; DHβE = 3406 ± 42; choline = 4358 ± 408. Statistical significance of E_{max} was compared for all treatments (***p < 0.001, carbachol vs nicotine). Hill coefficients from the fitted curves were as follows: nicotine = 1.1 ± 0.2; carbachol = 0.76 ± 0.1; DHβE = 1.0 ± 0.3; choline = 0.80 ± 0.2.

FIGURE 2. (A and B) Cells were untreated or up-regulated with 100 µM nicotine (Nic), 10 mM carbachol (Carb), or 10 mM choline (Ch) for 24 hr. Membrane homogenates were then prepared and washed following drug treatment. (A) Aliquots of homogenates were added to 1-1,200 pM [3H]EB. [3H]EB specific binding was normalized to total protein and then plotted against the concentration of free (unbound) [3H]EB in the binding assay. The data shown was obtained from three individual experiments, and is plotted as the mean ± S.E.M. Fitted curves were generated using a nonlinear regression, saturation binding fit. The K_{d} values for [3H]EB binding from these curves are shown in the figure as mean ± S.E.M. (B) Competition binding was performed in membrane homogenates with 0.5nM [3H]EB in the
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Results are presented as the percent of \([{}^{3}H]EB\) specific binding, and are plotted as the mean ± S.E.M. from three individual experiments. A fitted curve was generated with a nonlinear regression, competition curve fit (one-site versus two-site binding). The \(K_i\) values for nicotine from these curves are represented as the mean ± S.E.M. Hill coefficients from the curves are as follows: untreated = 1.06 ± 0.06; nicotine = 0.90 ± 0.06; carbachol = 0.91 ± 0.05; choline 0.96 ± 0.04.

FIGURE 3. Cells expressing \(\alpha_4\beta_2\) nAChRs were treated with 1 \(\mu\)M nicotine (Nic) or 1 mM carbachol (Carb) in the presence or absence of (A) 10 \(\mu\)M atropine (Atr) or (B) 1 \(\mu\)M QNB for 24 hr. Receptor density was measured with \([{}^{3}H]EB\) (1.5-2 nM) in membrane homogenates and binding was normalized to total protein. The results shown are the mean ± S.E.M. from four to six different experiments. Asterisks indicate statistical significance from control (**p<0.01). Carbachol treatments were statistically different from all other treatment groups (**p<0.01).

FIGURE 4. Western blots were probed with either \(\alpha_4\) or \(\beta_2\) polyclonal antibodies, visualized by chemoluminescence, and then washed and stained with India ink to visualize total protein loading. (A) Western blot from untransfected HEK293 cells or cells that stably express \(\alpha_4\beta_2\) or \(\alpha_3\beta_4\) nAChRs. A 24 hr, 1 mM carbachol treatment was used to obtain up-regulated \(\alpha_4\beta_2\) nAChRs. The blot shown is representative of three individual experiments. (B) Western blot from cells stably expressing \(\alpha_4\beta_2\) nAChRs that were treated with 1 \(\mu\)M nicotine (Nic), 1 mM carbachol (Carb), and/or 10 \(\mu\)M atropine (Atr) for 24 hr before. (C and D) Band densities were measured and normalized to the total protein density. The results are graphed as the mean ± S.E.M. fold-increase in band density compared to untreated tissue from four to six individual experiments, each run in triplicate. Statistical significance from untreated samples was calculated (***p<0.001). Carbachol’s effect on \(\beta_2\) subunits was statistically different from all other treatment groups (†††p<0.001).

FIGURE 5. Cells expressing \(\alpha_4\beta_2\) nAChRs were treated with: (A) 200 \(\mu\)M oxotremorine (High Oxo) in the presence or absence of 1 \(\mu\)M nicotine (Nic) and/or 10 \(\mu\)M atropine (High Atr) for 24 hr; (D) 3 \(\mu\)M oxotremorine (Low Oxo) in the presence or absence of 1 \(\mu\)M nicotine (Nic) and/or 300 nM atropine (Low Atr) for 24 hr. Receptor densities were measured with \([{}^{3}H]EB\) (1.5-2 nM) in membrane homogenates. Specific binding was normalized to total protein, and the data are displayed as fold-increase in receptor density after treatment. Results were obtained from four to nine individual experiments (mean ± S.E.M). Statistical significance from untreated receptors is indicated by the asterisks (**p<0.01). Statistical significance between treatment groups are indicated by the brackets and daggers (†††p<0.001). (B) Competition binding with membrane homogenates that were up-regulated with 200 \(\mu\)M oxotremorine for 24 hr. Aliquots of washed homogenates were added to 0.5 nM \([{}^{3}H]EB\) and 3–9,000 nM nicotine. Data are plotted as the percent of \([{}^{3}H]EB\) specific binding (mean ± S.E.M from three individual experiments), and the curves were fit using a nonlinear regression competition curve fit (one-site versus two-site). The calculated \(K_i\) of nicotine (mean ± S.E.M) from these curves is displayed as mean ± S.E.M. A Hill coefficient of -0.87 ± 0.05 was also calculated from the fitted curve. (C) \([{}^{3}H]EB\) (0.5 nM) competition binding against increasing concentrations of nicotine (Nic), carbachol (Carb), choline (Ch), atropine (Atr), oxotremorine (Oxo), or QNB using untreated membrane homogenates. The \(K_i\) values shown (mean ± S.E.M) were calculated from three individual experiments. The nicotine binding competition curve was adapted from figure 2B and is shown here for comparison. Hill coefficients calculated from the fitted curves are: nicotine = 1.06 ± 0.06; carbachol = 1.01 ± 0.06; choline = 0.97 ± 0.06; oxotremorine = 1.02 ± 0.10; atropine = 0.91 ± 0.12.
**FIGURE 6.** Cells expressing α4β2 nAChRs were treated with 1 µM nicotine (Nic), 1 mM carbachol (Carb), or 1 mM carbachol plus 10 µM atropine (Carb + Atr) for 6 hr. Isolated RNA from these cells was subjected to reverse transcription and qPCR with α4, β2, and GAPDH specific primers. (A) Following qPCR, samples were separated in a 1.2% agarose gel to ensure a single product was amplified. NRT (No Reverse Transcriptase) indicates RNA that did not undergo reverse transcription, but was still subjected to qPCR with a mixture of α4, β2, and GAPDH primers (negative control). Isolated RNA that did undergo reverse transcription was quantified by qPCR with α4, β2, or GAPDH specific primers separately as labeled in the figure. Primers were expected to amplify the following base pair (bp) sizes: α4 = 139 bp; β2 = 249 bp; and GAPDH = 225 bp. (B) Quantitative data were analyzed using the Pfaffl method (46) to determine relative fold-increase in mRNA expression compared to untreated cells. Data are represented as the mean ± S.E.M. from three separate experiments. Carbachol was the only drug to induce statistically significant increases in mRNA (**p < 0.01; ***p<0.001).

**FIGURE 7.** HEK cells expressing α4β2 nAChRs were transiently transfected with plasmid DNA containing the firefly luciferase gene that is expressed from a CMV promoter. Cells were treated with 1 µM nicotine (Nic), 1 mM carbachol (Carb), 1 mM carbachol plus 10 µM atropine (Carb + Atr), or 2 mM sodium butyrate (Na Butyrate) for (A) 6 hr or (B) 24 hr. Cells were lysed, pre-incubated for 10 min with luciferase substrate, and the luminescence was then measured. The data shown were compiled from four to six separate experiments, each run in triplicate, and are displayed as the mean ± S.E.M of the fold increase in relative luminescent units (RLU) compared to untreated cells. A 24 hr treatment with sodium butyrate was the only treatment that was statistically significant compared to untreated (**p < 0.01).
# TABLE 1:

**Fold Increase of nAChRs Compared to Untreated**

| Subtype | Untreated | Nicotine (1µM) | Nicotine (100µM) | Carbachol (1mM) | Carbachol (10mM) | Choline (1mM) | Choline (10mM) |
|---------|-----------|----------------|-----------------|-----------------|-----------------|--------------|--------------|
| α4β2    | 1.0±0.03  | 6.0±0.7***     | 6.8±0.3***      | 10.9±0.6***     | 13.2±1.4††***   | 4.0±0.5***   | 7.2±0.9***   |
| α3β4    | 1.0±0.05  | 1.3±0.1       | 1.5±0.2         | 1.6±0.3**       | 1.6±0.2*        | 1.1±0.02     | 1.4±0.2      |
| α7      | 1.0±0.07  | 2.0±0.3       | 2.8±0.3**       | 2.7±0.4**       | 2.1±0.3         | 1.8±0.2      | 1.8±0.5      |
| α4β4    | 1.0±0.02  | 1.5±0.1*      | 2.3±0.1***      | 2.5±0.2***      | 3.8±0.3***††    | 1.2±0.1      | 1.4±0.2†     |
| α6β2    | 1.0±0.06  | 2.8±0.6       | 19.7±2***       | 11.8±2***       | 26.0±3***       | 2.2±0.3      | 3.2±0.3††    |
| α3β2    | 1.0±0.1   | 7.5±0.4***    | 45.2±1***       | 38.8±2***       | 62.3±1***††     | 3.4±0.3      | 6.0±0.3††    |

Asterisk: Significance from Untreated (*p<0.05, **p<0.01, ***p<0.001)

Dagger: Significance of 10 mM Carbachol and Choline from 100 µM Nicotine (†p<0.01, †† p<0.001)
FIGURE 1:

- **Nic:** $EC_{50}=0.22 \pm 0.03 \mu M$
- **Carb:** $EC_{50}=63 \pm 12 \mu M$
- **DHβE:** $EC_{50}=24 \pm 5 \mu M$
- **Ch:** $EC_{50}=660 \pm 200 \mu M$

**[H]EB Specific Binding (fmol/mg protein)** vs **Log[Drug] (M)**
FIGURE 2:

A

Up-regulated with:

- Untreated
- Nic
- Carb
- Ch

K_d = 22 ± 8 pM
K_d = 28 ± 1 pM
K_d = 27 ± 4 pM
K_d = 29 ± 6 pM

B

Up-regulated with:

- Untreated
- Nic
- Carb
- Ch

K_i = 6.7 ± 0.6 nM
K_i = 5.9 ± 0.9 nM
K_i = 5.6 ± 0.3 nM
K_i = 6.0 ± 0.4 nM

C

Fold Increase in [3H]EB Binding

- Nic
- Carb
- Ch

Treatment Time (hr)

0 12 24 36 48 60 72

0 5 10 15 20 25

* * * **
FIGURE 3:

A

Fold Increase in [3H]EB Binding

B

Fold Increase in [3H]EB Binding

- Untreated
- Atr
- Nic
- Nic + Atr
- Carb
- Carb + Atr
- Untreated
- QNB
- Nic
- Nic + QNB
- Carb
- Carb + QNB
FIGURE 4:

A

B

C

D

Muscarinic receptors augment nAChR up-regulation
FIGURE 5:

A

Fold Increase in $[^3]$HEB Binding

B

% Specific $[^3]$HEB Bound

C

% Specific $[^3]$HEB Bound

D

Fold Increase in $[^3]$HEB Binding

Muscarinic receptors augment nAChR up-regulation
FIGURE 6:

A

B

Muscarinic receptors augment nAChR up-regulation
FIGURE 7:

Muscarinic receptors augment nAChR up-regulation
Endogenously expressed muscarinic receptors in HEK293 cells augment up-regulation of stably expressed α4β2 nicotinic receptors
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