An Extracellular Protein Microdomain Controls Up-regulation of Neuronal Nicotinic Acetylcholine Receptors by Nicotine*

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In smoker’s brain, rodent brain, and in cultured cells expressing nicotinic receptors, chronic nicotine treatment induces an increase in the total number of high affinity receptors for acetylcholine and nicotine, a process referred to as up-regulation. Up-regulation induced by 1 mM nicotine reaches 6-fold for α3β2 nicotinic receptors transiently expressed in HEK 293 cells, whereas it is much smaller for α3β4 receptors, offering a rationale to investigate the molecular mechanism underlying up-regulation. In this expression system binding sites are mainly intracellular, as shown by [3H]epibatidine binding experiments and competition with the impermeant ligand carbamylcholine. Systematic analysis of β2/β4 chimeras demonstrates the following. (i) The extracellular domain critically contributes to up-regulation. (ii) Only residues belonging to two β2 segments, 74–89 and 106–115, confer up-regulation to β4, mainly by decreasing the amount of binding sites in the absence of nicotine; on an atomic three-dimensional model of the α3β2 receptor these amino acids form a compact microdomain that mainly contributes to the subunit interface and also faces the acetylcholine binding site. (iii) The β4 microdomain is sufficient to confer to β2 a β4-like up-regulation. (iv) This microdomain makes an equivalent contribution to the up-regulation differences between α4β2 and α4β4. We propose that nicotine, by binding to immature oligomers, elicits a conformational reorganization of the microdomain, strengthening the interaction between adjacent subunits and, thus, facilitating maturation processes toward high affinity receptors. This mechanism may be central to nicotine addiction, since α4β2 is the subtype exhibiting the highest degree of up-regulation in the brain.

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Chronic exposure to nicotine causes a strong addiction, which post-mortem analysis of brain slices from smokers (5, 6) and from rats or mice chronically treated with nicotine (7–10) reveals large increases in the number of high affinity nicotinic binding sites, a phenomenon termed up-regulation. Nicotine up-regulates the α3β2 (11, 12), α4β2 (13–18), α7 (11, 19), and α12β2γ (19) nAChR subtypes reconstituted in cell lines or in Xenopus oocytes.

In addition to the increased number of sites, modulation of the magnitude of the nicotine-elicited electrophysiological response is also observed upon chronic nicotine incubation of cell lines expressing recombinant nAChRs. This functional potentiation of the response is observed with nAChR oligomers α4β2 and α3β2 reconstituted in mammalian cell lines (12, 20). But functional depression almost systematically takes place with the same combination of subunits reconstituted in Xenopus oocytes (13, 21, 22). The functional potentiation of nAChRs by chronic nicotine treatment in vivo (23, 24) is thought to contribute to tobacco addiction (4, 25).

Yet the molecular mechanisms underlying these plasticity phenomena remain to be elucidated. Transcriptional processes (15) have so far been disregarded in favor of post-translational recruitment of preexisting subunits (12) or stabilization of already assembled pentamers and protection against turnover (15) or both. Equilibrium binding and electrophysiological recordings reveal that upon stable expression in HEK 293 cell lines, α3β2 receptors are both strongly up-regulated and potentiated by chronic nicotine exposure. In contrast, α3β4 receptors were unaffected by chronic nicotine treatment (12). This observation offers a rationale to investigate the molecular mechanisms underlying up-regulation by identifying the protein regions controlling this process.

In the present study, we focus our analysis on up-regulation of the number of binding sites for nicotine. By constructing β2/β4 chimeras, we identified key amino acids that form a compact microdomain, contributing to the differences in up-regulation between α3β2 and α3β4. This microdomain also accounts for the up-regulation differences between α4β2 and α4β4 at nicotine concentrations compatible with those found in the blood of tobacco smokers. We propose a molecular mechanism of up-regulation where nicotine, binding to immature receptor precursors, causes a conformational reorganization of this microdomain.

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1 The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ECD, extracellular domain; HEK cells, human embryonic kidney cells; nH, Hill number; PBS, phosphate-buffered saline.
MATERIALS AND METHODS

Construction of Chimeras between β2 and β4 Subunits—cDNAs encoding β2 and the β4 rat subunits were excised as a NsiI/XhoI fragment from the clones kindly supplied by J. Boulter and introduced into the pMT2 vector for expression (26). Synthetic genes coding for the extracellular domain (ECD) of β2 and β4 were constructed by PCR amplification (two-polymerase, Roche Applied Science) using 60-mer oligonucleotides with an overlap of 20 base pairs, containing the minimum set of mutations allowing adequately distributed common restriction sites. We then fused these synthetic genes to the C-terminal SpeI/XhoI-amplified β4 (two-polymerase, Roche Applied Science). Chimeras were then constructed by simple subcloning between the two synthetic constructs. Single amino acid mutations were performed by subcloning hybridized pairs of oligonucleotides containing the appropriate mutation into the β4 synthetic gene. Synthetic genes and restriction sites are shown on Fig. 1.

Cell Culture and Transient Transfection of Human HEK 293 Cells—Human HEK 293 cells, an embryonic kidney cell line, were maintained in minimum essential medium (Invitrogen) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% fetal calf serum (Invitrogen) in a CO₂ (15%) incubator at 37 °C. Cells were grown in 10-cm dishes and transfected using the calcium phosphate method (27). The transfectant was rinsed 24 h after transfection and replaced by fresh medium to allow expression for an additional 24 h.

[3H]Epibatidine Binding, Nicotine Up-regulation—Up-regulation of α3β2 and α3β4 receptors transiently transfected in the HEK 293 cell line was examined by allowing expression of the receptors to reach a near steady-state level for 1 day and then incubating the cells with culture medium supplemented with (up-regulation conditions) or without (control conditions) the concentrations of nicotine given in the corresponding sections of the text (0.01–1000 μM) for 24 h. Nicotine was then eliminated from the culture medium for 30 min 2 times at 37 °C. Cells were then rinsed 3 times with PBS (137 mM NaCl, 2.7 mM KCl, 5.3 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.2) and harvested using 5 mM EDTA in PBS. After 5 min of centrifugation at 1500 rpm, the cells were resuspended in 1 ml of PBS. [3H]Epibatidine binding was carried out for 30 min at 37 °C at a concentration of 5 nM in 250 μl of the cell suspension. This mixture was then filtered 2 times on GF/C filters (Whatman) with ice-cold PBS. The filters were then counted using biodegradable counting scintillant (BCS, Amersham) in a 1209 Rackbeta counter (LKB Wallak). Nonspecific binding was assessed using 5 μM nicotine. For α3β4, a significant up-regulation took place at 1 μM nicotine and reached more than 2-fold at 10 μM nicotine but sometimes resulted in a large decrease in binding sites due to the toxicity of nicotine at this concentration.

Membrane Preparation—The cells were pelleted at 1500 rpm for 5 min, resuspended in 1 ml of homogenization buffer (ice-cold PBS containing 10 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride). Cell disruption was performed through sonication (3 times for 10 s) as previously described (17), and binding was performed in the usual conditions.

Molecular Modeling—Molecular models were constructed with Modeler (28) and Autodock (29) as described elsewhere (30). Handling of models and generation of pictures were performed with Deep-View (31), Rasmol (32), and ViewerLite (Accelrys).

RESULTS

Up-regulation Properties of α3β2 and α3β4 Receptors Transiently Transfected in HEK 293 Cells—The identification of the amino acids responsible for the differences in up-regulation between β2 and β4 subunits by a chimeric approach required the generation and screening of numerous chimeras. To do so, we designed an up-regulation procedure after transient transfection in HEK 293 cells. Cells expressing α3 along with β2, β4, or a β2/β4 chimera were cultured for 24 h without (control condition) or with nicotine at a given concentration. After 1 h of washing to remove the nicotine bound to the receptor, the number of nicotinic binding sites was measured by equilibrium binding with [3H]Epibatidine, a high affinity agonist. In all experiments, subunits from rat, previously designed to dissect the molecular determinant of desensitization (33), were used.

Fig 1A shows the average [3H]Epibatidine binding site expression of the wild-type α3β2 and α3β4 receptors in control condition and upon up-regulation by 1 mM nicotine. Data are taken from a series of eight independent experiments. Each experiment consisted of expressing both receptors in control and up-regulated conditions on the same day on the same batch of
cells. Fig. 1A indicates that the high affinity binding site expression is variable, with S.D. around 50% in the four conditions. However, we found that this variability was mainly dependent on the state of the cells at the time of the transfection. Indeed, within each experiment the relative expression level between both receptors is stable. For instance, the ratio of the expression of up-regulated α3β4 to up-regulated α3β2 is 1.00 ± 0.17.

Furthermore, we found that up-regulation was independent of the absolute expression level. Indeed, for each independent experiment the up-regulation ratio (r) was calculated by dividing the number of binding sites in the up-regulated condition by the number of binding sites in the control condition. This ratio remained stable (r = 1.73 ± 0.29 and 6.15 ± 1.19 for α3β4 and α3β2, respectively; Fig. 1A, right panel), independent of the absolute expression level inherent to the state of the cells.

Thus, in our system, α3β2 up-regulates strongly, whereas α3β4 displays relatively weak but significant up-regulation. Because α3β2 expression is much lower than α3β4 in the absence of nicotine up-regulation and since both receptors display nearly identical expression in up-regulated conditions, the difference in up-regulation is the consequence of different levels of expression in control conditions. Further pharmacological analysis in our system shows the following. (i) Performing a saturation analysis of [3H]epibatidine binding for each combination of subunits did not reveal differences in the affinity for [3H]epibatidine between control and up-regulated receptors. Fitting the data points according to the empirical Hill equation yielded Kd values of 0.4 ± 0.1 nM (nH = 1.5 ± 0.2) and 0.35 ± 0.1 nM (nH = 1.6 ± 0.1) for α3β2 and Kd values of 0.25 ± 0.15 nM (nH = 1.7 ± 0.1) and 0.25 ± 0.2 nM (nH = 1.6 ± 0.1) for α3β4, respectively, after exposure to control medium or to nicotine-supplemented medium. A concentration of 5 nM [3H]epibatidine was selected to measure the total amount of ACh binding in our system.

(ii) The up-regulation of α3β2 was concentration-dependent, with a half-maximal effect obtained around 30 μM nicotine and a maximal effect around 1 mM nicotine (Fig. 1B). For α3β4 the EC50 was more difficult to evaluate due to the lower up-regulation level and to the observation that nicotine starts to be toxic for the cells at 10 mM concentration, preventing a reliable evaluation of up-regulation at this concentration. Still, high concentrations of nicotine were required, apparently with an EC50 in the range of 100 μM.

(iii) None of the antagonists tested (mecamylamine (1 μM), d-tubocurarine (1 μM), dihydro-β-erythroidine (0.1 μM)) was found to cause significant up-regulation for both α3β2 and α3β4, and none of them significantly inhibited up-regulation elicited by 1 mM nicotine (data not shown).

Altogether, these data are consistent with those previously reported (12) but with a lower up-regulation ratio for α3β2 (6-fold as compared with 24-fold) and a higher EC50 of up-regulation (30 μM as compared 3 μM). Furthermore, we found that α3β4 exhibited a weak but significant up-regulation. These differences possibly reflect different phenotypes between the rat and human α3-containing nAChRs or, alternatively, differences in the expression systems; that is, transient transfection in HEK 293 cells versus stable expression in tsA210 cells.

**Up-regulation Involves an Increase in Intracellular Binding Sites**—Nicotine and epibatidine are small organic tertiary amines that cross cell membranes in their uncharged form. To distinguish between binding to surface or to intracellular nAChRs, we investigated the ability of carbachol to, an impermeant quaternary amine, to compete with [3H]epibatidine binding. For both the α3β2 and α3β4 receptors, we found that a high concentration (5 mM) of carbachol did not significantly inhibit [3H]epibatidine binding (Fig. 1C). On the other hand, after disruption of the cells by sonication, the same concentration of carbachol was found to displace a large fraction of bound [3H]epibatidine. Thus, in the intact cell, [3H]epibatidine primarily binds to intracellular α3β2 or α3β4 nAChRs, as previously reported for the α4β2 receptor expressed in M10 cells (17). The residual carbachol-insensitive [3H]epibatidine binding observed after sonication (30–40% of total [3H]epibatidine binding) could correspond to sites that are still inaccessible to carbachol after homogenization.

The ECD of the β-Subunit Controls Nicotine Up-regulation—In the subsequent study, mutant receptors were compared both on the basis of their up-regulation ratio (after treatment by 1 mM nicotine) and of their absolute expression level. Although the up-regulation ratios displayed good reproducibility, we have seen that the absolute expression levels were much more variable. To reduce this variability, we systematically included in each experiment the wild-type α3β4 receptor. Absolute expression levels of the various constructs were then normalized to the expression of α3β4 in up-regulated conditions.

Heteromeric neuronal α3(α4/β2/β4) nAChRs are pentameric transmembrane channel receptors. All subunits possess a similar transmembrane organization; that is, a large extracellular N-terminal domain followed by three transmembrane segments, a cytoplasmic domain, a fourth transmembrane segment, and an extracellular C terminus. To investigate which domain(s) of the β subunit is responsible for the differences in up-regulation between β2- and β4-containing receptors, we constructed chimeras β2-(1–212) consisting of the ECD of β2 and the transmembrane and cytoplasmic domains of β4 (all chimeras are named according to the β2 primary sequence they contain, the balance of the primary sequence belonging to β4) and β2-(212–407), possessing the complementary organization. The up-regulation ratio of β2-(1–212) indicates a β2-like phenotype (r = 7.26), and that of β2-(212–407) indicates a β4-like phenotype (r = 1.43) (Fig. 3), showing that the ECD confers up-regulation when inserted into β2 into β4 and conversely inhibits up-regulation when transferred from β4 into β2. In addition, chimeras β2-(1–212) and β2-(212–407) displayed expression levels nearly 2-fold higher than that of β2 and β4, respectively. This suggests that specific interactions between the ECD of β2 (respectively, β4) and the transmembrane domain of β4 (respectively, β2) increase absolute high affinity protein expression.

**A Compact Microdomain of Two β2 Segments, β2-(74–89) and β2-(106–115), Confers Up-regulation to β4**—Further dissection of the contribution of the ECD to the up-regulation process was achieved by taking α3β4 as a reference and introducing progressively larger portions of the β2 subunit at both the N- and C-terminal ends of the β4 ECD. To achieve this goal we constructed synthetic genes encoding the β2 and β4 subunits, with common restriction sites within their ECD (Fig. 2). We verified that the gene encoding synthetic and wild-type β4 displayed comparable expression and electrophysiological functional properties (data not shown).

This approach showed that segment 38–115 of β2 contains the critical elements conferring up-regulation to β4. Indeed, β2-(1–37) and β2-(116–212) exhibited a β4-like phenotype (r = 1.83 and 1.76, respectively), whereas β2-(1–89) and β2-(90–212) displayed an up-regulation ratio higher than the one of α3β4 (r = 2.86 and 5.26, respectively) (Fig. 3). It is noteworthy that chimeras β2-(1–37), β2-(1–89), and β2-(90–212) displayed absolute expression levels much lower that the wild-type receptors, indicating that these macrochimeras may experience destabilizing interactions within their structure or during their maturation.
conditions, and the lower bar respectively, the expression levels normalized to up-regulated left panel.

...corresponds to both the H9252 and H9251 numbers correspond to both the H9252 and H9251 expression levels similar to that of H9254. Third, β2-(90–115) presented a very weak expression, preventing an accurate measurement of the number of binding sites in control conditions. Still, the evaluated up-regulation ratio (3.48 ± 1.11, data not shown) suggested that this cassette may confer strong up-regulation to H9254. We split this cassette into β2-(90–105) and β2-(106–115) with r = 1.82 and 4.43, respectively. The segment β2-(106–115) was finally separated into 2 microchimeras, β2-(106–110) and β2-(111–115), with respective up-regulation ratios of 3.34 and 2.87 and expression levels similar to that of H9254.

Altogether, the critical residues of β2 conferring up-regulation to H3β4 belong to two separate segments, β2-(74–89) and β2-(106–115). Within β2-(74–89), β2-(74–79) produced weaker effects. Within β2-(106–115), the two half-cassettes, β2-(106–110) and β2-(111–115) nearly equally contributed to the up-regulation. We also performed single amino acid mutants β2-(74), β2-(75), β2-(76), β2-(77), β2-(78), β2-(79), β2-(80), β2-(90), β2-(110), β2-(112), β2-(113), and β2-(114). All of them exhibited up-regulation ratios and absolute expression levels similar to that of H9254 (data not shown), indicating that within the sub-domains β2-(74–79), β2-(106–110), and β2-(111–115) a combination of amino acids generates the phenotypes.

Visualization of the β2 residues conferring up-regulation to H9254 on an atomic model of the H3β2 receptor extracellular domain derived from AChBP (30, 34) (Fig. 5) revealed that these amino acids form a compact microdomain. This “up-regulation domain” is located in the upper part of each subunit and is composed of 1) two β strands (β3 and β5) interacting together from the main β-sheet of the structure (β3: Val-78 and Leu-80; β5: Ser-107, Ala-109, and Val-110) and 2) loops flanking these β strands (loop 1 (Asp-73, Asn-74, Met-75, Lys-76, Lys-77, Val-78, loop 2 (Ser-82, His-84, and Ile-85), and loop 3 (Ser-112, Tyr-113, and Asp-114)). The up-regulation domain, thus, encompasses a large part of the interface between β and α. Moreover, its lower part is adjacent to the agonist binding site.

The Up-regulation Microdomain of H9254 Abolishes β2-Like Up-regulation—After demonstrating that the insertion of the β2 up-regulation microdomain into β4 confers to β4 a β2-like up-regulation, we tested the converse process by inserting the H9254 into the β2-(1–212) chimera. Insertion of each segment independently resulted in a large decrease in the absolute expression level but no significant changes in the up-regulation ratios (Fig. 6). Insertion of both segments strongly decreased the up-regulation of the β2-(1–212) chimera to the β4 level, still with a large decrease in...
absolute expression level. Thus, the up-regulation microdomain confers up-regulation when inserted from \( \alpha^2\beta2 \) into \( \alpha^2\beta4 \) and, conversely, inhibits up-regulation when inserted from \( \alpha^2\beta4 \) into \( \alpha^2\beta2 \).

**The Microdomain Contributes to the Different Up-regulations of \( \alpha^2\beta2 \) and \( \alpha^2\beta4 \)** — In the rat brain, up-regulation of nicotine binding sites is primarily attributed to \( \alpha^2\beta2 \) receptors (35). To investigate the possible contribution of the microdomain to the up-regulation of \( \alpha^4 \) containing receptors, we investigated the up-regulation pattern of \( \alpha^4 \) in combination with \( \beta2 \), \( \beta4 \), and with selected critical \( \beta2/\beta4 \) chimeras. Fig. 7 shows that similar results are obtained with \( \alpha^4\beta2 \) and \( \alpha^4\beta4 \) as compared with \( \alpha^3\beta2 \) and \( \alpha^3\beta4 \) but with much lower concentrations of nicotine required for up-regulation. (i) On \( \alpha^4\beta2 \) receptors, up-regulation reaches 4.02- and 6.85-fold at 1 \( \mu \)M and 100 \( \mu \)M nicotine concentration, respectively, indicating an EC\(_{50}\) in the micromolar range; in contrast, \( \alpha^4\beta4 \) receptors up-regulated to a much lower degree, with a maximal up-regulation of 1.14- and 2.09-fold at 1 and 100 \( \mu \)M nicotine concentration. (ii) Analysis of \( \beta2-(1–212) \) and \( \beta2-(212–407) \) chimeras demonstrate that the extracellular domain controls the different up-regulations of \( \alpha^4\beta2 \) and \( \alpha^4\beta4 \). (iii) Introduction of either the \( \beta2 \) segments 1–37 or 90–115 within \( \beta4 \) did not alter the up-regulation significantly, pointing to a critical role of the 38–114 segment. (iv)
and playing higher and lower expression levels as compared with primary sequence. Lowering of up-regulation phenotypes are associated with large decreases in affinity binding site expression in control conditions without significantly changing the expression in up-regulated conditions. This suggests that α3β4 receptors are constitutively up-regulated, whereas α3β2 receptors are weakly expressed, a feature surmounted by nicotine action.

A microdomain within the Extracellular Domain Controls the Different Up-regulation of β2- and β4-containing Receptors—Chimeras in which the extracellular domains were exchanged between β2 and β4 demonstrate that the ECD of the β subunit critically contributes to up-regulation. This indicates that the transmembrane and intracellular domains of the protein are not responsible for the difference in phenotypes between α3(α4)β2 and α3(α4)β4. In particular, specific sequences for phosphorylation or interaction with intracellular factors can be discarded.

The critical residues we identified as conferring an α3β2-like up-regulation to α3β4 and, conversely, as conferring an α3β4-like up-regulation to α3β2 form a compact microdomain when visualized in the α3β4 ECD model derived from AChBP. This microdomain comprises a significant amount of the interface with the adjacent subunit and faces the agonist binding site (Fig. 5).

The microdomain largely contributes to the interface with the adjacent subunit, suggesting a critical role in subunit-subunit interaction and/or assembly. In support of this notion, several residues of the microdomain were previously found to play a major role in both the efficacy and specificity of subunit-subunit interaction for other ligand-gated ion channels. (i) β4-(Thr-106) and β4-(Ser-115) correspond to amino acids responsible for the differences in assembly efficacy between rat/mouse and α subunits to form heterodimers (36); (ii) β4-(Gly-74) corresponds to an asparagine on the mouse δ subunit, which provides a crucial glycosylation site for the formation of αδ heterodimers (37); (iii) β4-(Ile-77) corresponds to an amino acid of the α1 subunit of the glycine receptor shown to play an important role in subunit homooligomerization (38); and (iv) β-(473–485) is included in a 20 amino acids zone of the α1 subunit of the γ-aminobutyric acid, type A receptor identified as crucial for the assembly specificity with the γ2 subunit (39). Furthermore, the β4-(106–109) stretch interacts directly with α3S148 within our model, a residue homologous to T150 known to critically contribute to ααγγ tetramer formation (40).

It is noteworthy that the contribution of some of those amino acids (36, 37, 40) occurs at early assembly steps (typically from monomers to dimers formation), indicating a putative maturational role of the microdomain in early assembly of subunits during nAChRs formation.

**Discussion**

Different Contributions of the α and β Subunits to the Up-regulation of Neuronal Heteromeric Receptors—The present study supports the notion that up-regulation is a general property of heteromeric receptors since we found that even β4 produces weak but significant up-regulation when co-expressed with either α3 and α4. Comparison of the up-regulation patterns of α3β2, α3β4, α4β2, and α4β4 documents the contribution of the “principal” (α3, α4) and “complementary” (β2, β4) subunits to the up-regulation phenotypes.

First, the principal subunits play a major role in the sensitivity of the receptor to up-regulation. Indeed, α3β2 and α4β2 display similar amplitude of up-regulation at supra-maximal nicotine concentrations, but the EC50 of up-regulation is 2 orders of magnitude lower for α4β2 than for α3β2.

Second, comparison of α3β2 and α3β4 reveals that the complementary subunits primarily modulate the amplitude of up-regulation. However, the relative sensitivity of these nAChR subtypes to nicotine up-regulation cannot be measured since it is not possible to evaluate the EC50 of α3β4 for technical reasons. Identification of the minimal protein segments from β2 conferring up-regulation to the α3β4 receptor also provides clues on the up-regulation mechanism. All mutations within the microdomain produced up-regulation by lowering high affinity binding site expression in control conditions without significantly changing the expression in up-regulated conditions. This suggests that α3β4 receptors are constitutively up-regulated, whereas α3β2 receptors are weakly expressed, a feature surmounted by nicotine action.

**Fig. 6. The microdomain of β4 inhibits up-regulation when inserted into β2.** Up-regulation ratio (left panel) and expression levels normalized to up-regulated α3β4 (right panel, for each construct, the upper bar corresponds to control conditions, and the lower bar corresponds to 1 mM nicotine-up-regulated conditions) of N-terminal β2/β4 chimeras co-expressed with α3. Up-regulation ratios for wild-type α3β2 and α3β4 are shown in black and gray, respectively. For the subunit schematics, black lines represent the β2 sequence, and gray lines represent β4. Data are ± S.D. from three different experiments.

Within this segment introduction of the critical β2 cassettes 74–79 and 106–115 produced strong phenotypes, characterized by a large decrease in expression level as compared with β4, no significant change in the up-regulation at 1 μM nicotine, but a large increase in up-regulation by 100 μM nicotine. Therefore, we show here that the critical segments of the microdomain confer up-regulation to α4β4 but with an apparent lower sensitivity as compared with α4β2.

**Correlation between Up-regulation Ratios and Absolute Expression Levels**—The data presented in Fig. 1A concerning wild-type nAChRs show that the different up-regulation ratios between α3β2 and α3β4 apparently correspond to a difference in binding site expression in the absence of nicotine, with no significant change in the number of binding sites in up-regulation conditions. This observation might have important implications regarding the actual mechanism of up-regulation but is difficult to interpret since β4 and β2 may intrinsically produce different expression levels due to their large difference in primary sequence.

Our study produced numerous chimeras, some of them displaying higher and lower expression levels as compared with β4, probably as a consequence of structural interactions between the β2 and β4 portions. Yet in the α3β4 context we found that the minimal mutations conferring up-regulation to β4 (namely β2-(74–79), β2-(81), β2-(82–89), β2-(106–110), and β2-(111–115)) caused in all cases absolute expression levels in up-regulated conditions close to those of β4 (Fig. 4). Thus, in these chimeras, up-regulation looks to be associated with the lowering of β4 expression in control conditions, nicotine in the culture medium thus rescuing the expression of the high affinity binding sites to β4 levels. However, when the microdomain was transferred from β2 to β4 in the α4β4 context or when transferred from β4 to β2 in the α3β2 context more complex phenotypes were observed. In these cases the transfer of the up-regulation phenotypes are associated with large decreases in absolute expression levels, suggesting an additional role of the microdomain in protein maturation and/or metabolic stability.
In addition, in its lower part, the microdomain faces the binding site for agonists and competitive antagonists. Indeed, affinity labeling experiments performed on the Torpedo nAChR demonstrated that the ACh binding sites were located at the interface between the /H9251 and /H9253 or /H9254 subunits, particularly through 4 loops of binding homologous to /H9251 3-(Tyr-91), /H9251 3-(Trp-147), /H9251 3-(Tyr-188), and /H9252 4-(Trp-55). An additional binding loop, although not labeled by small ligands of the size of nicotine and epibatidine, was found to contribute directly to d-tubocurarine binding, a macrocylic competitive antagonist of ACh (41, 42). This loop carries /H9254 (Ser-110) that is homologous to /H9252 4-(Arg-111) belonging to the up-regulation microdomain. Thus, the microdomain is close but may not contribute directly to the agonist binding site.

Increased Maturation by Nicotine Contributes to Up-regulation—It is well established that nicotine does not modify levels of mRNA coding for nAChRs and, thus, acts on post-transcriptional processes (9, 11, 15, 19). The more straightforward molecular mechanism of up-regulation would be an action of nicotine on the functional receptors promoting up-regulation either through receptor activation or desensitization. The first hypothesis can be discarded since we showed that competitive antagonists do not inhibit up-regulation, in agreement with previous work (11, 17). The second hypothesis has been nicely demonstrated in the case of the /H9251 4 /H9252 2 receptors expressed in the Xenopus oocytes, where the nicotine concentrations producing up-regulation are identical to those required to occupy the high affinity binding sites and to desensitize the receptors. In this case, up-regulation would be associated with a total inactivation of the receptors, caused by an irreversible desensitization (13). This mechanism also does not apply to our case since the concentrations of nicotine eliciting up-regulation are several orders of magnitude higher than those necessary to occupy the high affinity binding site of the functional receptor (17), i.e. for...
α3β2 and α3β4, we find a nicotine EC50 around 30 and 100 μM, respectively, for up-regulation compared with intrinsic K_i of 16 and 300 nM (43). In addition up-regulation in cell lines is in all cases associated with a strong functional potentiation of the responses (12, 14, 20). It is noteworthy that the EC50 of up-regulation in our case on α4β2 receptors (1 μM) as compared with the EC50 in Xenopus oocyte (10 nM) (15) matches well those observed in the rat brain (around 1 μM) (10), suggesting that the cell lines constitute an expression system much closer to physiological conditions than Xenopus oocytes.

Among the numerous studies dedicated to the up-regulation of heteromeric neuronal receptors expressed in cell lines, the α3β2 receptors was investigated in great detail upon stable expression in tsA201 cells (12). The 24-fold up-regulation of these receptors mainly results from an increase in receptor maturation, since a 12-fold up-regulation was observed in the absence of protein synthesis at a time scale (2–3 h) where the receptors do not undergo any significant degradation due to turnover. This demonstrates that nicotine converted pre-existing pools of unmatured subunits/oligomers toward high affinity receptors. In addition, nicotine was found to decrease the turnover rate of the high affinity receptors in the presence of cycloheximide, but its quantitative contribution to the up-regulation process in standard conditions remains to be established. Furthermore, such maturational mechanism is fully consistent with the pharmacology of up-regulation, which is elicited by much higher concentrations than those required to occupy the high affinity binding sites (this study; see also Refs. 11 and 19).

For α4β2 receptors, more fragmentary data are available in the literature. Nicotine was found to decrease the turnover rate of α4β2 receptors expressed in M10 cells (15), but maturational processes were not investigated in this system (16, 17). Upon expression in HEK 293 cells, no accumulation of subunit protein is associated with the increase in high affinity binding sites (44), suggesting that up-regulation corresponds to a conversion of precursors from low to high affinity entities rather than to the stabilization and accumulation of high affinity receptors. Therefore, it appears premature to propose a complete up-regulation mechanism from the available literature. Although maturational processes, at least for α3β2, were shown to critically contribute to up-regulation, the cellular location of the pools of precursors is still a matter of controversy, since immunmeant ligands such as carbamylcholine are found to promote up-regulation, raising the possibility that the immature precursors might actually be located at the membrane surface (17).

A Plausible Molecular Mechanism for Up-regulation—Our observation that the same protein region contributes to up-regulation of α3β2 and α4β2 receptors suggests that similar mechanisms occur in both cases. From the above discussion, we will limit our analysis to the maturational mechanism that was convincingly shown to contribute to up-regulation of α3β2 receptors, but a modulation of the receptor turnover by the mentioned microdomain may also contribute to this up-regulation process.

We have seen that the microdomain largely contributes to the subunit interface and carries amino acids previously shown to contribute to subunit assembly. It is noteworthy that subunit assembly has been shown to be rate-limiting in the formation of muscle-type receptors (45). Furthermore, up-regulation of α3β2 was found to be associated with a dramatic increase in co-immunoprecipitation between β2 and α3 (12), indicating that nicotine increased the interaction between subunits. An increase in subunit interaction was also observed for α4β2 receptors, since α4 subunits from the endoplasmic reticulum were shown to co-assemble tightly and consequently retain intracellularly a β2 chimera in the presence of nicotine (44). These data are consistent with a scheme where the microdomain contributes to an αβ assembly-limiting step for the maturation toward high affinity receptors. The β2 microdomain would impair some key molecular interaction between adjacent subunits, causing incomplete maturation, whereas for β4, efficient αβ and/or ββ oligomerization would allow subsequent conformational reorganizations leading to high affinity receptors.

The high concentrations necessary to produce up-regulation suggest that the immature precursors display low affinity for nicotine. A wide range of agonists specific for the binding site, such as nicotine, epibatidine, carbamylcholine, cytisine, di-methyl phenyl piperazinium (12, 15, 16, 46, 47), and even in some cases antagonists such as d-tubocurarine and dihydro-β-erythroidine (14, 46) have been shown to promote up-regulation of heteromeric receptors expressed in cell lines. Thus, a common pharmacological spectrum for up-regulation and high affinity binding is observed. Moreover, a correlation between the ligand binding concentrations and those required to up-regulate has been observed (16, 17, 46, 48). In our opinion, this suggests that the binding site for up-regulation corresponds to a low affinity conformation of the classical binding site that bridges the interface between the α and β subunits. A plausible molecular mechanism providing the simplest interpretation of our data is that nicotine, by binding to the αβ interface within pools of precursors, would allosterically stabilize a conformation of the β2 microdomain similar to the one of β4. This would result in an efficient αβ interaction, allowing further maturation. The up-regulation microdomain would act as a transduction element, converting the binding of nicotine into an efficient assembly signal between neighboring subunits.

This scheme postulates that up-regulation is primarily associated with an alteration of subunit-subunit interactions. It is thus plausible that, in addition to simply rescuing immature precursors, nicotine might favor specific inter-subunit interactions and modulate the final receptor stoichiometry. Such an idea would provide a rational explanation of previous electrophysiological experiments, associating up-regulation of α4β2 receptors with significant changes in receptor activation and desensitization (20). Moreover, recent overexpression and electrophysiological experiments suggest that up-regulation of α4β2 receptors is associated with an enrichment of β2 subunits within pentamers (49).

Conclusion—In the central nervous system, β2-containing nAChRs are critical for nicotine-evoked enhancement of striatal dopamine release and nicotine autoadministration (50). Because chronic nicotine has been shown both to up-regulate β2-containing heteromeric receptors and potentiate striatal dopamine release (24), it is likely that up-regulation contributes to the long term effect of chronic nicotine exposure, including its addictive properties.

It is noteworthy that the pharmacology of up-regulation observed in our expression system, namely that α4β2 receptors are readily up-regulated by micromolar concentrations of nicotine, whereas α3- or β4-containing receptor requires much higher concentrations, matches previous studies performed in the rat brain. Indeed, increases in nAChR binding density in various rat brain areas was found to start at 0.1 μM nicotine, with a stronger effect at 1 μM (10). A majority of the up-regulated receptors were of the α4β2 subtype, with relatively little effects on α3β2-like and α3β4-like receptors (8, 35). Therefore, the up-regulation microdomain is likely to contribute to nicotine up-regulation in the brain. In addition, the β2 and β4 microdomains are highly conserved among rat, mouse, and human species. Our molecular dissection, thus, paves the way to an analysis of the functional consequences of up-regulation through in vivo expression in the mice of β subunits endowed with different up-regulation properties.
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Protein Structure and Folding: An Extracellular Protein Microdomain Controls Up-regulation of Neuronal Nicotinic Acetylcholine Receptors by Nicotine

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