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Chronic Nicotine Treatment Up-Regulates α3 and α7 Acetylcholine Receptor Subtypes Expressed by the Human Neuroblastoma Cell Line SH-SY5Y

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SUMMARY

Chronic exposure to nicotine has been reported to increase the number of nicotinic acetylcholine receptors (AChRs) in brain. The mechanism of up-regulation for the α4β2 AChR subtype, which accounts for the majority of high affinity nicotine binding in mammalian brain, has previously been shown to involve a decrease in the rate of α4β2 AChR turnover. Here, we report an investigation of the extent and mechanism of nicotine-induced up-regulation of α3 AChRs and α7 AChR subtypes expressed in the human neuroblastoma cell line SH-SY5Y. Up-regulation of human α3 AChRs and α7 AChRs, unlike α4β2 AChRs, requires much higher nicotine concentrations than are encountered in smokers; the extent of increase of surface AChRs is much less; and the mechanisms of up-regulation are different than with α4β2 AChRs. The mechanisms of up-regulation may be different for α3 AChRs or α7 AChRs. Chronic treatment with nicotine or carbamylcholine, but not *d*-tubocurarine, mecamylamine, or dihydro-β-erythroidine, induced a 500–600% increase in the number of α3 AChRs but only a 30% increase in α7 AChRs. Chronic nicotine treatment did not increase affinity for nicotine or increase the amount of RNA for α3 or α7 subunits. The effect of nicotine on up-regulation of α7 AChRs was partially blocked by either *d*-tubocurarine or mecamylamine. The effect of nicotine treatment on the number of α3 AChRs was only slightly blocked by the antagonists *d*-tubocurarine, mecamylamine, or dihydro-β-erythroidine at concentrations that efficiently block α3 AChR function. Most of the nicotine-induced increase in α3 AChRs was found to be intracellular. The α3 AChRs, which accumulate intracellularly, were shown to have been previously exposed on the cell surface by their susceptibility to antigenic modulation. The data suggest that chronic exposure to nicotine may induce a conformation of cell surface α3 AChRs that at least in this cell line are consequently internalized but not immediately destroyed.

It is well established that chronic nicotine exposure results in increased binding of [^{3}H]nicotine and ^{125}I-αBgt in brain (1–8). AChRs composed of α4 and β2 subunits have high affinity for nicotine and ACh and account for most of the high affinity nicotine binding in rat brain (9–11). AChRs composed of α3 subunits in combination with β2, β4, and/or α5 subunits have lower affinity for ACh and nicotine than do α4β2 AChRs and account for a small amount of high affinity nicotine binding in brain (12). Flores *et al.* (4) showed that α4β2 AChRs are increased in the cortex of rats chronically treated with nicotine. In addition, Collins *et al.* (5, 6) reported that chronic exposure to nicotine or the antagonist mecamylamine increased mouse brain [^{3}H]nicotine binding in numerous regions to various extents without increasing the levels of α4 or β2 AChR subunit mRNAs. We found that chronic treatment of *Xenopus laevis* oocytes expressing α4β2 AChRs or a mouse fibroblast cell line permanently transfected with chicken α4β2 AChRs with nicotine or mecamylamine caused a ~2-fold increase in α4β2 AChRs (13). The nicotine concentration dependence, time course, and extent of α4β2 AChR up-regulation are similar to those reported for α4β2 AChRs in mammalian brains. The nicotine-induced increase in α4β2 AChRs is due to a decrease in the rate of α4β2 AChR turnover (13). This induction mechanism does not seem to require cation flow through α4β2 AChRs because the channel blocker mecamylamine causes up-regulation (6, 13). Nicotine and mecamylamine are synergistic in causing up-regulation (6, 13) because mecamylamine preferentially blocks open channels and nicotine is an agonist, so together they are more effective at accumulating the inactive conformation of α4β2 AChR,

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ABBREVIATIONS: αBgt, α-bungarotoxin; AChR, acetylcholine receptor; mAb, monoclonal antibody; DHβE, dihydro-β-erythroidine; EGTA, ethylene glycol bis(α-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SSPE, standard saline/phosphate/EDTA; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

which is turned over more slowly (13). Neuronal AChRs that bind αBgt have been found to contain α7, α8, or α9 subunits (14–16). α7 AChRs are the predominant form of αBgt binding protein in brain (14); they have higher affinity for nicotine than for ACh but much lower affinity for nicotine than do α4β2 AChRs (17). Marks *et al.* (7) reported that chronic intravenous infusion of mice with nicotine elicited an increase in brain ^{125}I-αBgt binding. The extent and duration of nicotine-induced up-regulation of ^{125}I-αBgt binding in rat brains were less than the increase in [^{3}H]nicotine binding (7). More recently, Barrantes *et al.* (18) reported that chronic nicotine treatment of hippocampal neurons with nicotine elicits a 40% increase in the number of ^{125}I-αBgt binding sites. These results indicate that the up-regulation of α7-containing AChRs requires a higher dose of nicotine and a longer exposure time than does up-regulation of α4β2 AChRs.

After we and others (13, 19, 20) determined that chronic exposure to nicotine causes up-regulation of the α4β2 AChR subtype by reducing turnover, it seemed important to determine whether other AChR subtypes were similarly regulated. Differences in the effects of chronic nicotine exposure on various AChR subtypes might help to account for variations in the extent of nicotine-induced up-regulation of [^{3}H]nicotine binding in various brain regions (5) and for complexities in functional effects of chronic exposure to nicotine in smokers or chronic exposure to other nicotinic agonists that might be used for therapeutic purposes. The human neuroblastoma cell line SH-SY5Y, like chick ciliary ganglion neurons (21), expresses both α3 AChRs and α7 AChRs (22, 23). These cells resemble human fetal sympathetic neurons grown in primary culture and express mRNAs for α3, α5, α7, β2, and β4 subunits (24). They express α3-containing AChRs of at least two subtypes, half of which contain β2 subunits (23), and which contain some mixture of α3β2, α3β2α5, α3β4, α3β4α5, and α3β2β4α5 subtypes. They also express α7 AChRs that are either homomers of α7 subunits or contain α7 assembled with other, unidentified subunits (22, 24, 25). Experiments were undertaken to investigate whether chronic exposure to nicotine of these cells induces up-regulation of α3 AChRs and α7 AChRs and the mechanisms that may be involved in this up-regulation.

Materials and Methods

Cell cultures. SH-SY5Y cells were initially provided by June Biedler and Barbara Spengler of the Sloan Kettering Institute for Cancer Research (26). Cultures were grown in a 1:1 mixture of Ham's F12 medium and Eagle's minimal essential medium containing 1×10^{-4} M nonessential amino acids, supplemented with 10% fetal bovine serum, in a 95% air/5% CO_2 humidified incubator at 37°. pH of the L-nicotine (Sigma) solutions was adjusted with 10 N NaOH before being added to media. The cell monolayers were washed with PBS saline, scraped, pelleted in a microfuge at 4°, and stored at −80° until use.

mAbs. mAb210 was initially raised to the main immunogenic region on the extracellular surface of mammalian muscle α1 subunits (27) and was shown to cross-react with human α3 and α5 subunits (23). mAb306 was prepared using as antigen a mixture of affinity-purified native and denatured αBgt-binding AChRs from the brains of chickens and rats (14) and was found to cross-react with human α7 subunits in the SH-SY5Y cell line (22).

Labeling reagents. L-[^{3}H]nicotine (72 Ci/mmol) and [^{3}H]epibatidine (56.6 Ci/mmol) were obtained from New England Nuclear Research Products (Boston, MA) (72 Ci/mmol). mAbs 210 and 306 were labeled with ^{125}I to a specific activity of 7×10^{17} cpm/mol. αBgt was labeled with ^{125}I to a specific activity of 1.07×10^{18} cpm/mol.

Northern blot. Total cellular RNA was isolated according to the method of Chomczynski and Sacchi (28) from SH-SY5Y cells that had been treated with or without 1×10^{-3} M nicotine for 4 days. Subsequently, poly(A)$^+$-tailed mRNA was isolated, and 3 μg was electrophoresed for 4 hr at 90 V in a 1% agarose gel containing 1.1 M formaldehyde, 0.02 M MOPS, 0.05 M sodium acetate buffer, pH 8.0, and 0.01 M EDTA. The gels were rinsed in water treated with 0.1% (v/v) diethylpyrocarbonate and then soaked for 45 min in 10× SSPE (1× SSPE contains 180 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA). RNA was vacuum transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) and UV cross-linked. Hybridization was performed by using a random-primed, ^{32}P-dATP-labeled, human α3 or α7 subunit cDNA fragment or ^{32}P-UTP-labeled human β-actin RNA fragment in 40% formamide, 5× Denhardt's solution (1× Denhardt's solution contains 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.5% SDS, 5× SSPE, and 0.15 mg/ml denatured salmon sperm DNA and incubated overnight at 42° for cDNA probes and at 60° for RNA probes. Human β-actin was used as a heterologous probe to determine the amount of human β-actin RNA expressed in the SH-SY5Y cell line by allowing for normalization of α3 and α7 mRNA signals within each lane. Membranes were washed at 50° in 1× SSPE/0.1% SDS and exposed to Kodak XAR-5 film at −70°. Scanned images were quantified using NIH Image 1.54 software.

Cell surface binding. Confluent cells in 60-mm dishes were treated with or without 1×10^{-3} M nicotine for 4 days. To label α3 AChRs, cells were incubated with 1 ml of medium containing 1×10^{-8} M ^{125}I-mAb210 for overnight at 4°. Nonspecific binding was determined in the presence of 1×10^{-6} M unlabeled mAb210. α7 AChRs were similarly labeled using 1×10^{-8} M ^{125}I-αBgt overnight at 4°. Nonspecific binding was determined in the presence of 1×10^{-6} M unlabeled αBgt. In both cases, cells were washed three times with 1 ml of PBS, detached, pelleted, and resuspended before γ-counting.

Membrane fraction binding assays. Cells were grown just as for cell surface binding experiments and then harvested in PBS. Cells were lysed by incubation for 1 hr in 4° hypotonic buffer (5 mM Tris·HCl, pH 7.5) followed by homogenization (34). Centrifugation at 40,000 × *g* for 20 min yielded a crude membrane pellet, which was resuspended in PBS. Labeling with 1×10^{-8} M ^{125}I mAb210 or ^{125}I-αBgt was conducted overnight at 4° with gentle shaking. Unbound labels were removed by pelleting followed by three washes in PBS and repelleting before resuspension and γ-counting. Nonspecific binding was determined in the presence of 1×10^{-6} M unlabeled ligand.

Immunoisolated AChR binding assays. For the [^{3}H]nicotine binding assay, α3 AChRs from SH-SY5Y were solubilized in 5 volumes of lysis buffer (containing 2% Triton X-100, 50 mM NaCl, 50 mM sodium phosphate buffer, pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 5 mM iodoacetamide) through brief vortexing followed by 20 min of gentle rotation at 4° and then a 20-min centrifugation in a microfuge at 4°. The α3 AChRs were immunoisolated by incubating overnight at 4° the solubilized AChR with mAb210-coated Immulon 4 microwells (Dynatech Labs, Chantilly, VA). The microwells were then washed three times, and 100 μl of 2×10^{-8} M [^{3}H]nicotine in 0.5% Triton X-100 PBS buffer was added and incubated 1 hr at 4°. After three rapid washes, bound [^{3}H]nicotine was removed using sample buffer (2.5% SDS, 5% β-mercaptoethanol) and measured using a scintillation counter. For the ^{125}I-αBgt binding assay, solubilized AChRs were incubated with mAb306-coated Immulon 4 microwells overnight at 4°. The microwells were then rinsed and incubated with 100 μl of 5×10^{-9} M ^{125}I-αBgt in 0.5% Triton X-100 PBS buffer, pH 7.5. After three washes, bound ^{125}I-αBgt was measured using a γ-counter. Nonspecific binding was measured using wells lacking mAb.

Electrophysiology. Electrophysiological recordings from *X. laevis* oocytes injected with 5 ng each of cRNAs for human AChR subunits in the combinations $\alpha3\beta2$, $\alpha3\beta4$, or $\alpha3\beta2\beta4\alpha5$ were made as previously described (12, 23). Data were collected 3 days after injection using oocytes voltage-clamped at −50 mV.

Antigenic modulation. Three 60-mm dishes of confluent SH-SY5Y cells for each condition were grown for 3 days with or without 1×10^{-3} M nicotine and/or 1×10^{-7} M mAb210. Cells from each dish were harvested separately and then lysed by incubation for 1 hr in 5 mM Tris·HCl buffer, pH 7.5, followed by homogenization for 10 sec using a Polytron. Membrane fragments were pelleted by centrifugation for 20 min at 40,000 × *g* and then resuspended in 200 μl of PBS. After incubation with 1×10^{-8} M [^{3}H]epibatidine for 2 hr at 4° with gentle agitation, the membranes were washed three times by filtration on Whatman GF/C filters with 3 ml of PBS. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled epibatidine. Bound [^{3}H]epibatidine was measured by scintillation counting.

Statistical analysis. Two-tailed *t* tests were used.

Results

Chronic exposure to nicotine of cultured SH-SY5Y cells caused a 575% increase in the amount of immunoisolated $\alpha3$ AChRs but only a 30% increase in the amount of immunoisolated $\alpha7$ AChRs (Fig. 1). mAb210, which binds to both $\alpha3$ and $\alpha5$ subunits (23), and mAb306, which binds to $\alpha7$ subunits (22), were used to tether AChRs that had been solubilized with Triton X-100. Measurement of total $\alpha3$ AChRs by binding ^{125}I mAb210 or total $\alpha7$ AChRs by binding ^{125}I-αBgt to cell membrane fragments gave results similar to those obtained using immunoisolated solubilized AChR subtypes (data not shown). The half-maximally effective concentrations of nicotine for up-regulation (EC_{50}), assuming that 1×10^{-3} M nicotine gave the maximum response, were 1×10^{-4} M for $\alpha3$ AChRs and 6.5×10^{-5} M for $\alpha7$ AChRs. This contrasts with the much lower EC_{50} value for up-regulation of $\alpha4\beta2$ AChRs of 2×10^{-7} M that we had previously observed (13). At a concentration of 1×10^{-3} M, nicotine did not affect cell proliferation. At 10^{-2} M nicotine, cells started to detach from the dishes, so 1×10^{-3} M was the highest concentration used. Unlike the case with $\alpha4\beta2$ AChRs (13), mecamylamine at a concentration of 1×10^{-3} M did not cause up-regulation of either $\alpha3$ or $\alpha7$ AChRs.

Kinetics of up-regulation of $\alpha3$ AChRs differed from those of $\alpha7$ AChRs (Fig. 2). The increase of $\alpha3$ AChRs was seen as early as 5 hr after nicotine exposure, and the maximum effect was seen after 3 days. Up-regulation of $\alpha7$ AChRs was seen after an 8-hr exposure and was complete within 24 hr.

The up-regulation resulted from an increase in the amount of AChR rather than from an increase in the affinity of $\alpha3$ AChRs for nicotine. This was shown by Scatchard plots of [^{3}H]nicotine binding to control and chronically nicotine-treated SH-SY5Y cells (Fig. 3). There are two populations of $\alpha3$ AChRs in the SH-SY5Y cell line that differ in affinity for nicotine. The affinity of each population for nicotine was not significantly changed after up-regulation.

Northern analysis was used to determine whether the increased AChRs were due to an increased RNA level. Poly(A)$^+$-tailed mRNA was isolated from cells treated with or without 1×10^{-3} M nicotine for 4 days. Nicotine treatment did not up-regulate the steady state amounts of mRNA for $\alpha3$ or $\alpha7$ subunits in SH-SY5Y cells (Fig. 4). The average values from two independent experiments revealed ratios of nicotine-treated to control values of 1.0 for $\alpha3$ mRNA and 0.84 for $\alpha7$ mRNA. These results indicated that nicotine up-regulates both $\alpha3$ and $\alpha7$ AChRs via post-transcriptional mechanisms.

Most of the $\alpha3$ AChRs induced by nicotine were found in an intracellular compartment (Fig. 5). To test whether this might be due to nicotine acting inside the cells to facilitate $\alpha3$ AChR synthesis or assembly, up-regulation by carbamylcholine was also tested. Although nicotine is a tertiary amine that can cross cell membranes, carbamylcholine is a quaternary amine that cannot penetrate the cells to act on $\alpha3$ AChR synthesis. Carbamylcholine also caused an increase in intracellular $\alpha3$ AChRs (Fig. 5); this suggests that either carbamylcholine mediated an increase in internal $\alpha3$ AChRs through mechanisms subsequent to cation flow through $\alpha3$ AChRs that it stimulated on the surface or that the internal $\alpha3$ AChRs had been on the surface to interact with carbamylcholine at some time during the 4-day incubation. Surface $\alpha3$ AChRs were quantified by binding of ^{125}I-mAb210 to intact cells, and total $\alpha3$ AChRs were quantified by binding to membrane fragments. Measurements of total $\alpha3$ AChRs by binding of [^{3}H]nicotine to detergent-solubilized $\alpha3$ AChRs immunoisolated on mAb210-coated microwells gave similar results (data not shown). Chronic treatment with either nicotine or carbamylcholine caused a > 300% increase in the total amount of $\alpha3$ AChRs in SY-SY5Y cells but only about a 30% increase in $\alpha3$ AChRs on the cell surface.

Chronic treatment of SH-SY5Y cells with a high concen-

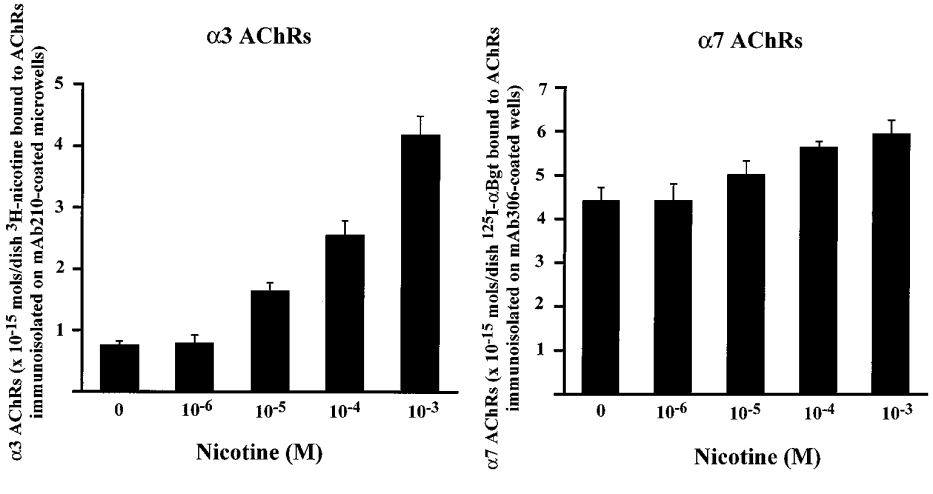

Fig. 1. Dose dependence of nicotine-induced up-regulation of $\alpha3$ and $\alpha7$ AChRs in SH-SY5Y cells. Triplicate cell cultures were treated with the indicated concentrations of nicotine for 4 days. Cells were then harvested, and AChRs were solubilized using Triton X-100. $\alpha3$ AChRs were immunoisolated on microwells coated with mAb210 (which binds to both $\alpha3$ and $\alpha5$ subunits) and quantified by labeling with [^{3}H]nicotine. $\alpha7$ AChRs were immunoisolated on microwells coated with mAb306 (which binds to $\alpha7$ subunits) and quantified by labeling with ^{125}I-αBgt. *Bars,* mean value of three dishes.

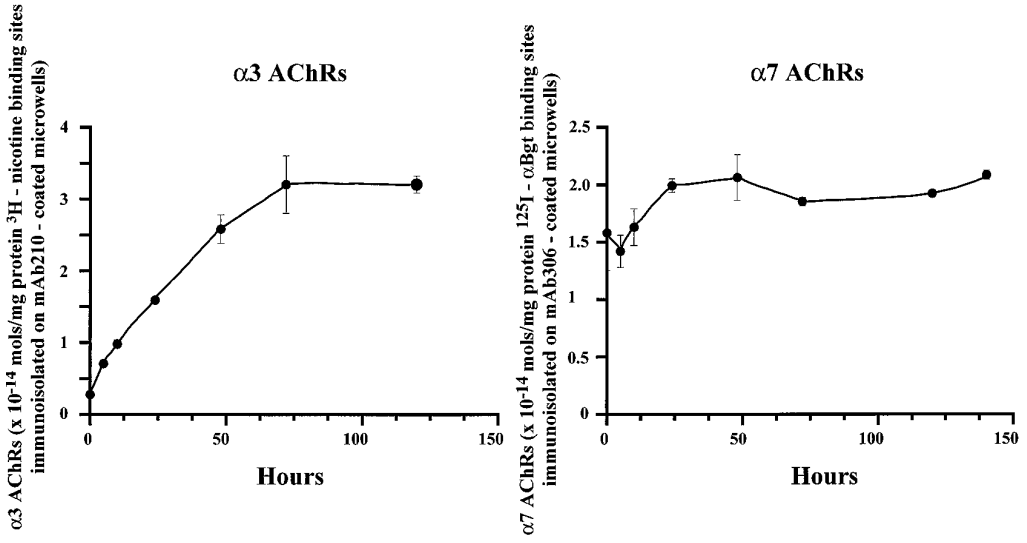

Fig. 2. Time course of nicotine-induced up-regulation. Confluent cells in 60-mm dishes were treated with 1×10^{-3} M nicotine to produce a maximum effect. The cells were washed, harvested at the indicated time points, and subjected to [^{3}H]nicotine or ^{125}I-αBgt binding assays. *Points,* mean value of three dishes.

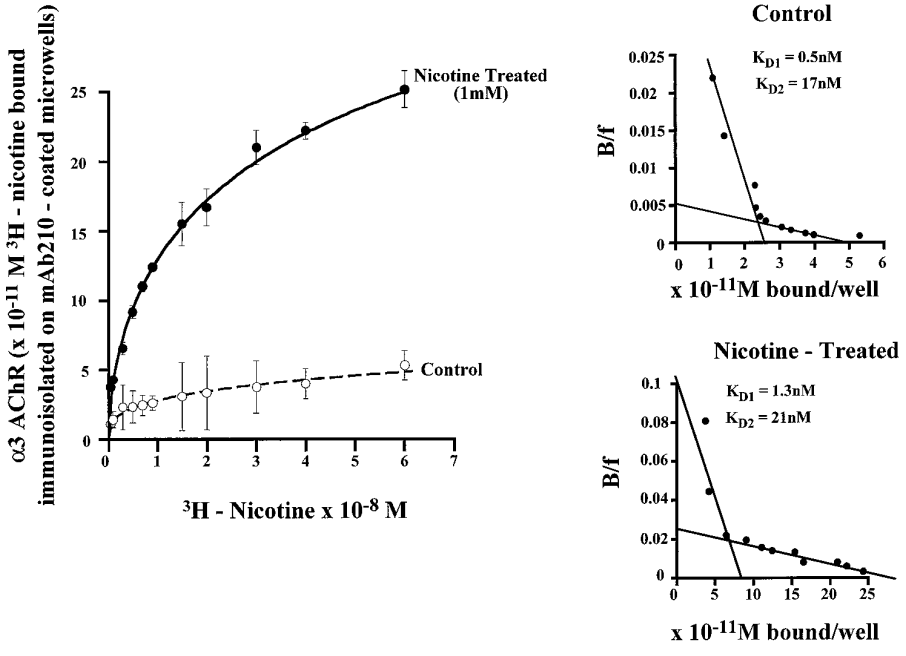

Fig. 3. Nicotine-induced up-regulation does not increase the affinity of α3 AChRs for nicotine. Cells were treated with or without 1×10^{-3} M nicotine for 4 days. AChRs were solubilized with Triton X-100, immunoisolated on microwells coated with mAb210, and then assayed for binding with various concentrations of [^{3}H]nicotine. Scatchard plots revealed two different binding affinities for α3 AChRs, with no significant increase in binding affinities after nicotine treatment.

tration (1×10^{-3} M) of the competitive antagonist *d*-tubocurarine or DHβE or with the noncompetitive open channel blocker mecamylamine did not change the number of α3 or α7 AChRs (data not shown). Up-regulation of α3 AChRs by 5×10^{-4} M nicotine was not inhibited by a 2×10^{-4} M concentration of any of these antagonists (data not shown). Even if we decreased the concentration of nicotine to 1×10^{-5} M and increased the concentration of the antagonists to 1×10^{-3} M to provide a 100-fold molar excess of antagonist, the up-regulation of α3 AChRs was not significantly blocked (Fig. 6). A 100-fold molar excess of the antagonists was very effective at blocking cation flow through α3 AChRs (Fig. 6). This suggests that up-regulation of α3 AChRs induced by agonists does not require cation flow through these AChRs. A 100-fold molar excess of the noncompetitive antagonist, as expected, did not inhibit [^{3}H]nicotine binding. The competitive antagonists at this concentration ratio inhibited [^{3}H]nicotine binding substantially but not completely (Fig. 6). This suggests that during the 4-day incubation with both nicotine and antagonists, a substantial fraction of the ACh binding sites were occupied by nicotine at any given moment (100% for mecamylamine, 24–28% for curare and DHβE). Antagonists bound to one or more of the ACh binding sites or to the cation channel would prevent cation flow through these α3 AChRs. However, nicotine bound to the remaining ACh binding sites might be able to produce some level of functional desensitization and perhaps other consequent effects, such as internalization and up-regulation.

Evidence that the internalized α3 AChRs induced by agonists had been on the cell surface at some point during the 4 days of incubation with agonist was provided by showing that nicotine-induced α3 AChRs on SH-SY5Y cells were susceptible to antigenic modulation by mAb210 (Fig. 7). It is well known that both antibodies to the main immunogenic region on the extracellular surface of muscle AChR α1 subunits (e.g., mAb210) and autoantibodies to muscle AChRs that are from patients with myasthenia gravis cause down-regulation of AChRs via the process of antigenic modulation (35). This

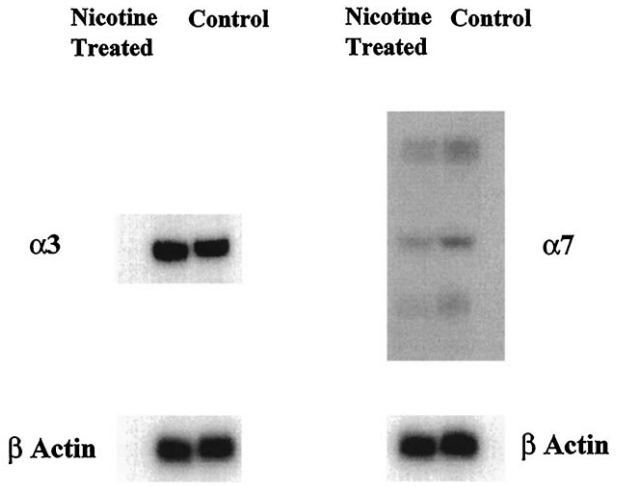

Fig. 4. Nicotine treatment does not change the level of AChR mRNAs. Amounts of α3 and α7 subunit mRNAs were measured using a Northern blot. A series of exposures ensured that the measurements were made in the linear range. Each set of blots was successively used to quantify an AChR subunit, and then β-actin was used as an internal control. Quantification by scanning of the autoradiographs and normalization to the β-actin control revealed average ratios of nicotine treated to control values of 1.0 for α3 and 0.84 for α7 for the two experiments.

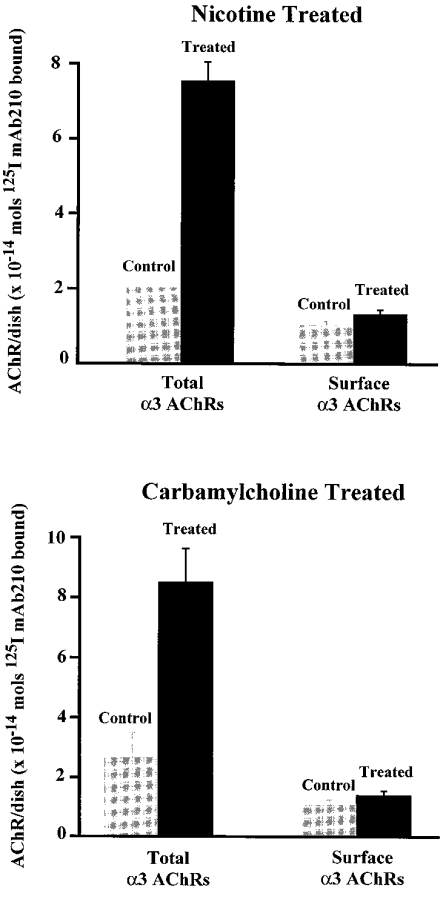

Fig. 5. Nicotine and carbamylcholine preferentially increase internal α3 AChRs. Confluent cells were cultured in 60-mm dishes treated with or without 1×10^{-3} M agonist for 4 days. Surface α3 AChRs were determined by labeling intact cells with ^{125}I-mAb210, and total α3 AChRs were determined by labeling membrane fragments with ^{125}I-mAb210. Each value represents the mean of three dishes.

involves cross-linking of AChRs by antibodies, which facilitates their endocytosis and lysosomal destruction. Because mAbs cannot cross cell membranes, demonstration that mAb210 can prevent most of the nicotine-induced increase in α3 AChRs shows that those α3 AChRs must have been exposed on the surface membrane, where they were accessible to binding by the mAb. The observation that mAb210 did not significantly reduce the amount of α3 AChRs in cells that were not exposed to nicotine may result from the normally very low density of α3 AChRs in these cells, which may make it difficult to cross-link these AChRs into aggregates sufficiently large to speed endocytosis.

The rate of degradation of α3 AChRs in SH-SY5Y cells was assayed using the same approach that we had used to detect a nicotine-induced decrease in the rate of degradation in $\alpha4\beta2$ AChRs permanently transfected into mouse fibroblasts (13) (Fig. 8). This method involves the use of cycloheximide to prevent the synthesis of new AChRs, followed by measurement of the rate of loss of existing AChRs in the presence or absence of nicotine. Unlike what we had observed with $\alpha4\beta2$ AChRs (13), the presence of nicotine did not slow the rate of loss of α3 AChRs (Fig. 8). This was unexpected because it seemed reasonable to suppose that the nicotine-induced increase in internalized α3 AChRs that we had observed would be reflected in a decrease in turnover rate. However, it may be that prevention of lysosomal destruction of the internalized α3 AChRs that accumulate in the presence of nicotine depends on the continued synthesis of a protein, with the result that when protein synthesis is blocked with cycloheximide, not only the synthesis of new α3 AChRs but inhibition of the destruction of the internalized α3 AChRs is prevented.

The slight extent of up-regulation of α7 AChRs induced by nicotine was substantially blocked by both curare and mecamylamine (Fig. 9). The differences in extent and antagonist sensitivity of up-regulation of α7 AChRs compared with those of α3 AChRs suggest that different mechanisms may be involved in nicotine-induced up-regulation of these two AChR subtypes.

Discussion

We found that human α3 and α7 AChRs are up-regulated by chronic exposure to nicotine but only at concentrations of nicotine much higher than those required for up-regulation of $\alpha4\beta2$ AChRs (13). The maximum extent of up-regulation was least for α7 AChRs, intermediate for $\alpha4\beta2$ AChRs, and highest for α3 AChRs. However, the large amounts of nicotine-induced α3 AChRs are found intracellularly, where they would not be functional; this is summarized in Table 1. In normal rat brains, there seems to be approximately equal amounts of α7 and $\alpha4\beta2$ AChRs but much fewer α3 AChRs. $\alpha4\beta2$ AChRs are up-regulated with an EC_{50} value of 2×10^{-7} M nicotine, which also is a serum concentration that is typical of tobacco users (29), whereas up-regulation of α3 and α7 AChRs requires nicotine concentrations of ≥400-fold higher. Both α3 and α7 AChRs require much higher nicotine concentrations for activation than do $\alpha4\beta2$ AChRs, and the equilibrium binding affinity for nicotine of their presumably desensitized states is much lower than that of $\alpha4\beta2$ AChRs. There is no precise correlation between K_D values for binding or EC_{50} values for activation and EC_{50} values for up-regulation

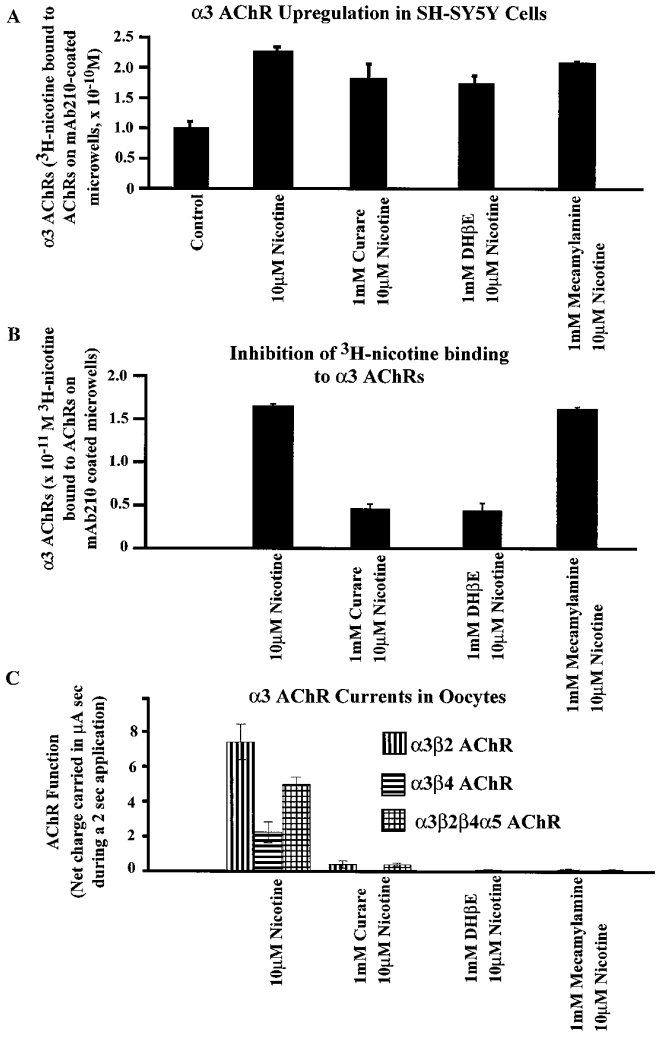

Fig. 6. Antagonists at 100-fold molar excess do not block nicotine-induced up-regulation of α3 AChRs, even though nicotine binding is substantially reduced and current flow is virtually completely blocked. A, α3 AChR up-regulation. SH-SY5Y cells were treated with nicotine with or without antagonists at the indicated concentrations for 4 days. Cells were then harvested, and AChRs were solubilized before solid-phase radioimmunoassay. Each value represents the mean of three dishes. Up-regulation was not significantly blocked ($p > 0.05$). B, Inhibition of [^{3}H]nicotine binding. α3 AChRs solubilized from SH-SY5Y cells were immunoisolated on mAb210-coated microwells and then incubated with 2×10^{-8} M [^{3}H]nicotine with or without a 100-fold molar excess of antagonists for 1 hr at 4°. After three rapid washes, bound [^{3}H]nicotine was measured by scintillation counting. Each value is the mean of duplicate experiments (range is shown). Binding of [^{3}H]nicotine was substantially, but not completely, blocked by the competitive antagonists curare and DHβE but not affected by the channel blocker mecamylamine. C, α3 AChR currents. Currents carried by cloned human $\alpha3\beta2$ AChRs, $\alpha3\beta4$ AChRs, or the mixture of subtypes typical of SH-SY5Y cells obtained through expression of equal amounts of α3, β2, β4, and α5 subunits in *X. laevis* oocytes revealed blockages by the antagonists of a 1×10^{-5} M nicotine response of 95–100%. Each value represents the mean value of four oocytes.

of any of these AChR subtypes. In all cases, the EC_{50} value for up-regulation is closer to the EC_{50} value for activation than it is to the K_D value for equilibrium binding. However, as we discussed previously regarding $\alpha4\beta2$ AChRs (13) and as shown in the current study for α3 AChRs (Fig. 6), the inability of channel blockers to prevent nicotine-induced up-

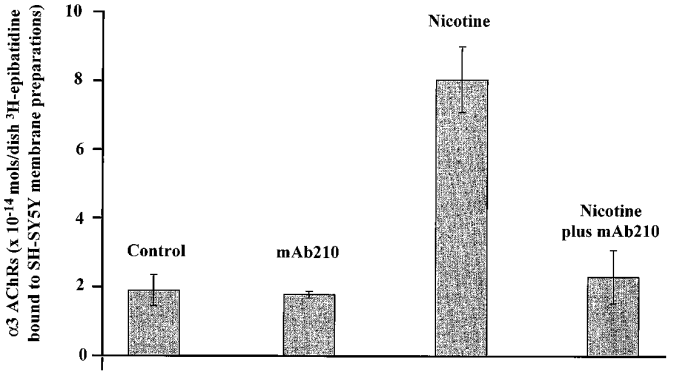

Fig. 7. Nicotine-induced α3 AChRs are susceptible to antigenic modulation by mAb210. SH-SY5Y cells were cultured for 3 days with or without 1×10^{-3} M nicotine and 1×10^{-7} M mAb210. Then, their total content of α3 AChRs was determined by measurement of binding of [^{3}H]epibatidine to membrane fragments. *Bars,* mean of determinations on triplicate 60-mm culture dishes. The loss of most of the nicotine-induced increase in α3 AChRs as a result of the presence of mAb210 suggests that these α3 AChRs appeared on the cell surface and were then cross-linked by the mAbs into aggregates that were endocytosed and destroyed in lysosomes.

regulation argues strongly that for those subtypes, AChR activation is not required for up-regulation. In the case of α7 AChRs, both curare and mecamylamine seemed to block the small amount of nicotine-induced up-regulation, so in the case of this subtype, up-regulation may depend on AChR activation.

Total α3 AChRs in SH-SY5Y cells could be up-regulated to a larger extent (575%) (Fig. 1) than the 100% that is typical of chick $\alpha4\beta2$ AChRs (13) or mammalian brain $\alpha4\beta2$ AChRs (4), but the extent of surface up-regulation of α3 AChRs was only 30–40% (Fig. 5). Thus, if the effects in SH-SY5Y cells reflect the effects of chronic nicotine exposure on human brain and ganglia, one might expect little nicotine-induced increase in the number of surface α3 AChRs or α7 AChRs in contrast with a doubling of surface $\alpha4\beta2$ AChRs. However, because nicotine can also induce functional desensitization of AChRs and the extent and reversibility of this desensitization may vary with AChR subtype, the net relative sensitivity of various AChR subtypes after chronic exposure to nicotine may not be reflected even in the relative amounts of various AChR subtypes in cell surfaces.

The agonist-induced increase in [^{3}H]nicotine binding to immunoisolated AChRs results from an increase in the amount of AChRs rather than from an increase in the affinity of the AChRs for nicotine (Fig. 3). There are two classes of binding sites for [^{3}H]nicotine in SH-SY5Y cells ($K_{D1} = 0.5$ nM, $K_{D2} = 17$ nM) (Fig. 3). These results are similar to the results reported by Lukas *et al.* (24). These two binding affinities may reflect the relative amounts of α3 AChR subtypes present among the possible $\alpha3\beta2$, $\alpha3\beta2\alpha5$, $\alpha3\beta4$, $\alpha3\beta4\alpha5$, and $\alpha3\beta2\beta4\alpha5$ subunit combinations (23). For example, we also found that half of the α3 AChRs in SH-SY5Y cells contain β2 subunits and that this half of the AChRs is associated with much higher affinity for epibatidine (23). The effect of nicotine-induced regulation of α3 AChRs in SH-SY5Y cells differs from that in chick ciliary ganglion neurons (21). The α3 AChRs expressed in chick ciliary ganglion neurons are reduced 30% by chronic exposure of cultures to carbamylcholine; this might be accounted for by differences in neuronal

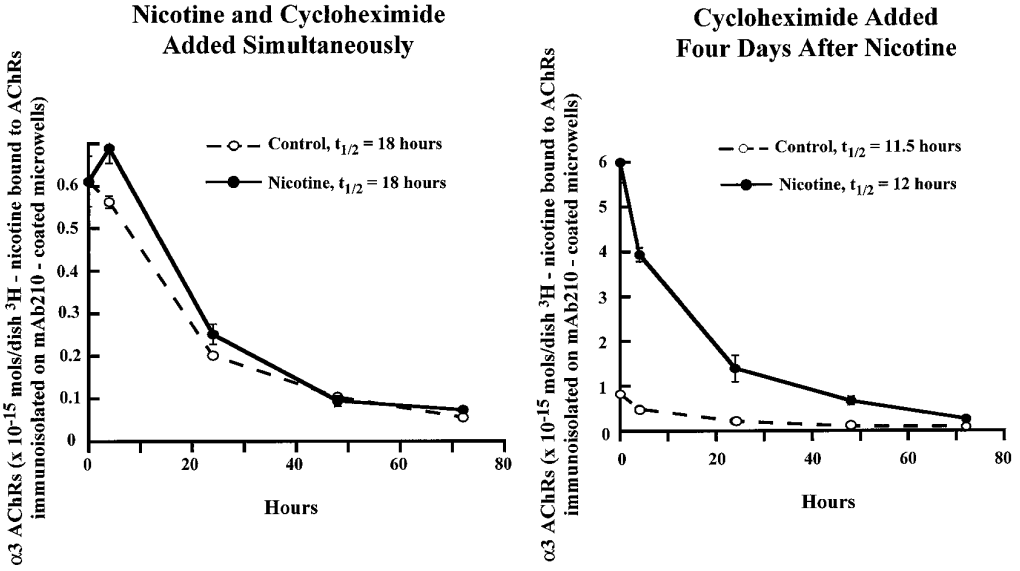

Fig. 8. Nicotine treatment does not seem to change the turnover rate of α3 AChRs. Confluent cells were treated with or without 1×10^{-3} M nicotine for 4 days before the addition of 3.5×10^{-5} M cycloheximide to block protein synthesis, or cells were simultaneously exposed to nicotine and cycloheximide. The total amount of α3 AChRs remaining in cells was then measured by [^{3}H]nicotine binding to immunoisolated solubilized α3 AChRs. Neither of those treatments changed the turnover rate of α3 AChRs. *Points,* the mean of duplicate determinations.

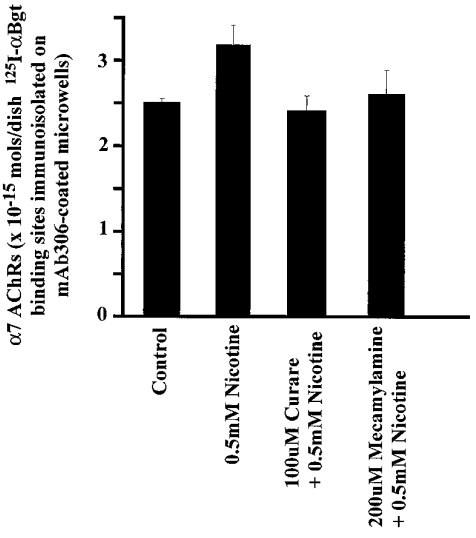

Fig. 9. Antagonists block nicotine-induced up-regulation of α7 AChRs in SH-SY5Y cells. SH-SY5Y cells were treated with nicotine with or without antagonists at the indicated concentrations for 4 days. Cells were then harvested ,and AChRs were solubilized before solid-phase radioimmunoassays. Each value represents the mean of three dishes.

TABLE 1
Effects of Nicotine on AChR Subtypes

Subtype	Activation EC_{50}	Binding K_D	Up-regulation	
			EC_{50}	Maximum extent
	μM	μM	μM	%
$\alpha4\beta2$ AChRs	0.35[a]	0.004[a]	0.21[a]	~100[a]
α3 AChRs	30[b]	0.02	100	~600 (only ~30 on surface)
α7 AChRs	40[c]	1.3[c]	65	~30

[a] ref. 13
[b] ref. 24
[c] ref. 22

cell types, species, or α3 AChR subtypes. In chicken ciliary ganglion neurons, 80% of α3 AChRs have the subunit composition $\alpha3\alpha5\beta4$ (30), but in SH-SY5Y cells, ≥56% of the α3 AChRs contain β2 subunits (23).

α7 AChRs were also up-regulated by nicotine (Fig. 1) but only by very high concentrations of nicotine and to a lesser degree than were $\alpha4\beta2$ AChRs (13). Up-regulation of α7 AChRs reached its maximum within 24 hr (Fig. 2), mimicking the up-regulation observed *in vivo* (7). In mice, higher nicotine doses are required to elicit increases in brain ^{125}I-αBgt sites than are necessary to increase [^{3}H]nicotine binding sites, and the amounts of ^{125} I-αBgt sites change more rapidly (7, 31). The relatively small magnitude of up-regulation for α7 is reminiscent of the small changes in ^{125}I-αBgt binding observed after *in vivo* administration of nicotine (7, 30). Brain ^{125}I-αBgt binding sites are up-regulated only by higher doses of nicotine (32, 33). This also reflected the lower level of up-regulation that resulted from chronic nicotine exposure of αBgt binding sites compared with [^{3}H]nicotine binding sites that was observed in rat brain by Marks *et al.* (7). Up-regulation of muscle-type AChRs expressed by TE671 cells required 1×10^{-3} M nicotine for significant up-regulation to occur (34, 36). Thus, the effective nicotine concentration reflects the sensitivity of the particular nicotinic AChR subtype to this agonist.

The failure of nicotine to up-regulate transcription of α3 and α7 subunit mRNA (Fig. 4) while up-regulating the amount of α3 and α7 AChRs in SH-SY5Y cells is consistent with similar results observed in brain with $\alpha4\beta2$ AChRs (5) and suggests that nicotine also up-regulates α3 and α7 AChRs via post-transcriptional mechanisms. This is also reminiscent of results with $\alpha4\beta2$ AChRs in transfected cells (13, 37).

In the case of $\alpha4\beta2$ AChRs, the competitive antagonist curare blocks nicotine-induced up-regulation of cell surface $\alpha4\beta2$ AChRs, the channel blocker mecamylamine causes up-regulation and is synergistic with nicotine in causing up-regulation (13). This was interpreted to mean that agonists and mecamylamine induced a conformation, probably a desensitized conformation, of the $\alpha4\beta2$ AChRs that is turned over more slowly and that up-regulation does not require cation flow through the AChR.

The mechanism of α3 AChR up-regulation, although similarly post-transcriptional and also apparently not requiring cation flow through the AChRs (Fig. 6), is different from that of $\alpha4\beta2$ AChRs, especially in accumulating a large excess of

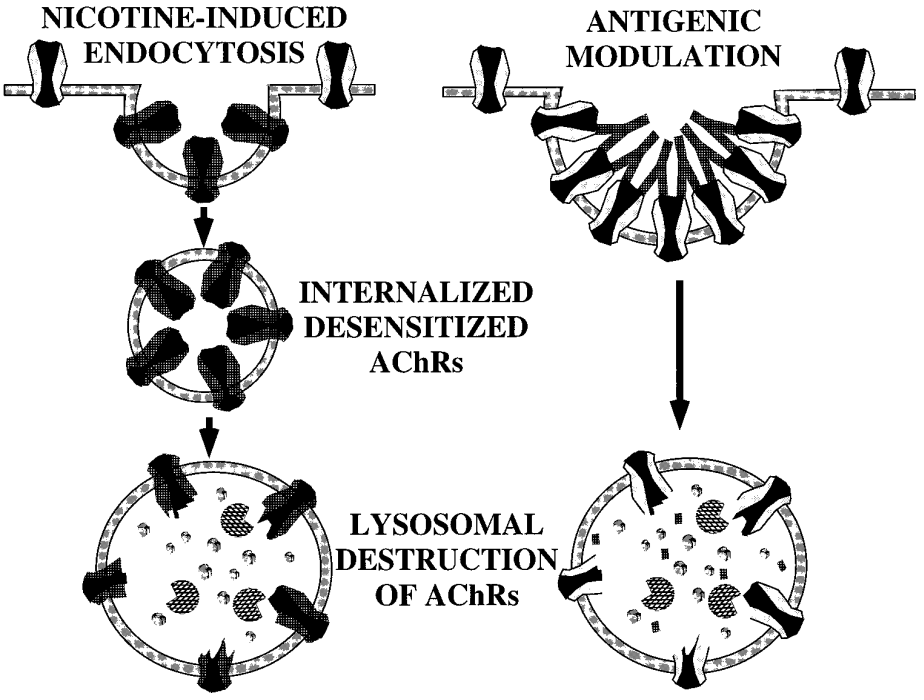

Fig. 10. Comparison of proposed mechanisms of agonist-induced internalization of α3 AChRs in SH-SY5Y cells with antigenic modulation of α3 AChRs. Nicotine is depicted as inducing a desensitized conformation of α3 AChRs that results in their internalization to a compartment in which as a result of a process that depends on protein synthesis, they are not immediately destroyed; ultimately they are proteolytically degraded. Antigenic modulation is depicted as a process in which cross-linking of α3 AChRs by mAbs to the extracellular surfaces of their α3 and α5 subunits causes aggregation, which speeds endocytosis and lysosomal destruction.

internal α3 AChRs in response to chronic exposure to high concentrations of nicotine. Unlike the effect on $\alpha4\beta2$ AChRs (13), the channel blocker mecamylamine itself does not cause up-regulation of α3 AChRs, and it had no synergistic effect with nicotine in causing up-regulation (Fig. 6). This suggests that mecamylamine does not induce the conformation of α3 AChRs required for up-regulation, but neither does it seem to prevent nicotine from inducing this conformation. The observation that after 3 days in the presence of nicotine most of the α3 AChRs induced by agonists were intracellular (Fig. 5) was unexpected; it presented the conundrum of reconciling the observation that a membrane-impermeable agonist could cause accumulation of intracellular α3 AChRs (Fig. 5) with the observation that antagonists could not block up-regulation (Fig. 6), which indicated that ion flow through α3 AChRs could not be used to signal the inside of the cell to more rapidly synthesize α3 AChRs. Demonstration that the nicotine-induced increase in α3 AChRs could be prevented by antigenic modulation (Fig. 7) showed that all of the α3 AChRs affected by nicotine had been on the surface, where they could bind membrane-impermeable quaternary amine ligands. Fig. 10 depicts the mechanism of antigenic modulation. We hypothesize that chronic exposure to agonists causes α3 AChRs to assume a conformation, perhaps a desensitized conformation, that at least in SH-SY5Y cells results in being internalized into a compartment in which they linger for a while before being degraded in lysosomes as they would be normally. This is also depicted diagrammatically in Fig. 10. There is precedent for the idea that agonists can induce internalization of receptors into a compartment in which they are not immediately destroyed, as in the case of β-adrenergic receptors (38).

Nicotine-induced up-regulation of α3 and α7 AChRs may differ not only quantitatively (with α3 AChRs showing more extensive up-regulation; Fig. 1) but also qualitatively in the mechanisms of up-regulation. Ion flow through α3 AChRs does not seem to be required for nicotine-induced up-regulation because the channel blocker mecamylamine does not block up-regulation (Fig. 6). In the case of α7 AChRs, ion flow through the AChR may be involved in nicotine-induced up-regulation because with α7 AChRs, both curare and mecamylamine are effective at blocking nicotine-induced up-regulation (Fig. 9). The EC_{50} value for nicotine-induced up-regulation of α7 AChRs is also close to their EC_{50} value for activation (Table 1).

Although in smokers the effects of nicotine concentrations on the up-regulation of $\alpha4\beta2$ AChRs seem to be large and the effects on surface α3 and α7 AChRs seem to be small, the amount of AChR is not the only important parameter in determination of behavioral responses to nicotine. Nicotine-induced reversible desensitization and permanent functional inactivation are also important. $\alpha4\beta2$ AChRs are subject to desensitization and inactivation by low concentrations of nicotine (13, 39), whereas $\alpha3\beta2$ AChRs are reported to be resistant to such inactivation (39), and α7 AChRs are much more subject to rapid and extensive desensitization than are $\alpha4\beta2$ AChRs (40). Thus, perhaps in chronic smokers, most $\alpha4\beta2$ AChRs are inactivated at average serum nicotine concentrations and some α3 AChR subtypes or other AChR subtypes are left by default to respond to the transient high bolus doses of nicotine in smokers.

The differential effects of nicotinic ligands on the activation, up-regulation, and desensitization of various subtypes of AChRs are likely to be important as new subtype-selective AChR ligands are developed for their cognitive enhancing and neuroprotective effects as possible therapeutic agents for conditions such as Alzheimer's disease, Parkinson's disease, Tourette's syndrome, and schizophrenia (41).

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