ABSTRACT
Nicotinic acetylcholine receptors (nAChRs) exist as a diverse family of physiologically important ligand-gated ion channels active in classic, excitatory neurotransmission and perhaps in more novel forms of neurochemical signaling. Because of their critical functional roles centrally and peripherally, nAChRs are ideal targets for the regulation of nervous system function. nAChRs also are targets of nicotine, which acts acutely like acetylcholine to stimulate nAChR function. Here, we report studies using model cell culture systems testing the general hypothesis that more chronic nicotine exposure has unique effects on nAChRs. Chronic nicotine treatment induces increases in numbers of human muscle-type nAChRs containing \textit{alpha}-1, \textit{beta}-1, \textit{gamma} and \textit{delta} subunits, a human ganglionic nAChR subtype containing \textit{alpha}-3 and \textit{beta}-4 subunits and a human ganglionic nAChR containing \textit{alpha}-7 subunits in intracellular and (except for \textit{alpha}-7 nAChRs) in cell surface pools. However, the half-maximal potency with which nicotine has these effects differs across these nAChR subtypes, as do rates and magnitudes of the “nicotine-induced nAChR up-regulation.” These changes in nAChR numbers are not attributable to either transient or sustained changes in nAChR subunit mRNA levels. Nicotine exposure more potently, more rapidly, and with nAChR-subtype specificity, induces two phases of losses in functional responsiveness of muscle-type nAChRs and \textit{alpha}-3 \textit{beta}-4 nAChRs, including a “persistent inactivation” that is distinct from classically defined “desensitization.” Based on these results, we hypothesize that chronic nicotine treatment induces persistent functional inactivation and numerical up-regulation of all nAChR subtypes via distinct post-transcriptional mechanisms and with potencies, at rates and with magnitudes that are nAChR-subtype specific. We also hypothesize that chronic nicotinic exposure produces long-lasting changes in nervous system function, at least in part, by disabling rather than activating nicotinic cholinergic signaling.

nAChRs are prototypical members of the ligand-gated ion channel superfamily of neurotransmitter receptors. nAChRs have been valuable models in work to establish basic concepts pertaining to mechanisms of drug action, synaptic transmission, and diversity in structure and function of transmembrane signaling molecules (see reviews by Lindstrom, 1996; Lukas, 1995, 1998). nAChRs are found throughout the nervous system (e.g., in muscle, autonomic ganglia and the central nervous system) and exist as multiple, diverse subtypes composed of unique combinations of homologous but genetically distinct subunits. Mammalian muscle-type nAChRs are composed of \textit{alpha}-1, \textit{beta}-1, \textit{delta} and either \textit{gamma} (fetal) or \textit{epsilon} (adult) subunits. One form of vertebrate ganglionic nAChR contains \textit{alpha}-3, \textit{alpha}-5 and \textit{beta}-4 subunits, and another ganglionic nAChR subtype contains \textit{alpha}-7 subunits. \textit{Alpha}-7 subunits are also found in a vertebrate central nervous system nAChR subtype, and nAChRs containing \textit{alpha}-8 or \textit{alpha}-8 plus \textit{alpha}-7 subunits have been identified in chick. A major species of vertebrate central nervous system nAChR contains \textit{alpha}-4 and \textit{beta}-2 subunits. \textit{Alpha}-9 subunits are components of a novel class of nAChR. There may also be additional nAChR subtypes of yet undefined subunit composition, particularly given that \textit{alpha}-2, \textit{alpha}-6 and \textit{beta}3 subunits have not yet been assigned to specific nAChR subtypes and that there still may exist nAChR subunits and genes that have not yet been cloned. A complete understanding is lacking about fundamental properties of different nAChR subtypes and their genes and the physiological significance of nAChR diversity. However, a consequence of nAChR diversity is that each
nAChR subtype has a unique profile for sensitivity to nicotine and other agents. Nicotine is a biologically important substance in tobacco. Nicotine exposure for different times and at different doses is reported to produce a range of physiological effects in laboratory animals or humans ranging from elevated locomotor activity, seizures, and changes in body temperature to real or perceived enhancement of cognition or attention, relief of depression, and anxiolysis (Gray et al., 1994; Henningfield et al., 1995; Warburton, 1995; see Lindstrom, 1996; Lukas et al., 1996 for overviews). Nicotine is not popularly viewed, as are narcotics, as an intoxicating and/or performance/judge-ment-altering drug of abuse, consumption of which acutely endangers the user and/or other members of society. However, habitual users of tobacco products are suggested to experience, as do users of recognized addictive drugs, craving, tolerance, physical and psychological (mild euphoriant) dependence, relapse during abstinence and withdrawal symptoms (op. cit). Moreover, nicotine-dependent tobacco consumption is reported to contribute to health problems in a population much larger than the population of narcotic users and at higher costs (Peto et al., 1992). Hence, regardless of whether nicotine truly represents a model substance for studies of narcotic addiction and abuse, an improved understand- ing of mechanisms underlying effects of nicotine on nervous system function could provide fundamental insight into drug-receptor interactions and a rational basis for public health policy relating to tobacco products.

Acute exposure to nicotine (or to the endogenous neurotransmitter acetylcholine) activates nAChR function, which may account for some of nicotine’s physiological effects. How- ever, more chronic exposure to nicotine, which occurs in habitual users of tobacco products, must have different or additional effects to account for processes such as nicotine dependence, tolerance and the unpleasant effects associated with nicotine withdrawal. Chronic nicotine exposure induces increases in numbers (up-regulation) of central nervous sys- tem radioligand binding sites (which probably represent alpha-4 beta-2 nAChR and central nervous system alpha-7 nAChR subtypes) in animals and in human smokers in vivo (for overviews, see Lukas, 1991; Lukas et al., 1996). Up-regulation of native or transgenic radioligand-binding alpha-4 beta-2 nAChRs in central nervous system neurons or non-neuronal expression systems also occurs on chronic nicotine treatment in vitro (Peng et al., 1994; Zhang et al., 1994; Bencherif et al., 1995a). Chronic nicotine treatment produces a rapid and persistent loss of nicotine-sensitive nAChR func- tional activity in the brain (for overviews, see Lukas, 1991; Lukas et al., 1996). Function of ganglionic alpha-3 beta-4 nAChRs or muscle-type alpha-1 beta-1 gamma delta nAChRs also is reported to be rapidly and persistently lost on chronic nicotine exposure (for overview, see Lukas, 1991). Hence, it is clear that nicotine can induce a puzzling loss of nAChR function while increasing apparent numbers of nAChRs. However, relationships between effects of nicotine exposure on numbers and function of nAChRs are poorly understood. For example, it is not clear whether functional responses affected by chronic nicotine treatment occur far downstream from nAChR activation and whether the same or different populations of nAChRs are being up-regulated/functionally inactivated in whole animals or in preparations composed of heterogeneous cell populations. It is not clear whether these effects are exclusive to central nervous system nAChRs, and mechanisms involved in these effects have not been fully elucidated.

The current study was undertaken to begin a systematic investigation of effects and mechanisms involved in nicotine’s ability to regulate expression of its own receptors. These studies involve use of well-characterized cloned cell lines as models that naturally express different nAChR subtypes. Preliminary accounts of some of these findings have appeared (Lukas et al., 1996). The results suggest that chronic nicotine exposure induces numerical up-regulation and persistent functional inactivation of several nAChR subtypes, contributing to physiological effects of chronic nicotine use and providing molecular bases for nicotine dependence.

**Experimental Procedures**

**Materials.** Unless otherwise noted, all chemicals, including (—)nicotine ditartrate, were of analytical grade and purchased from Sigma Chemical (St. Louis, MO). [25]I-labeled a-bungarotoxin (I-Bgt: Amershalm; Arlington Heights, IL) was diluted with unlabeled a-bungarotoxin (Bgt) to achieve working specific activities of 50–100 dpm/fmol. [3H]ACh (American Radiolabeled Chemicals, St. Louis, MO; 100–250 dpm/fmol) was used without modification. DEME, trypsin, penicillin/streptomycin solution, amphotericin B and horse sera were from GibCO BRL (Gaithersburg, MD), and fetal calf sera were from Hyclone (Logan, UT). The BCA protein determi- nation kit was obtained from Pierce Chemical (Rockford, IL). 86Rb or 32P was from New England Nuclear (Boston, MA), and native Bgt was prepared as described by Lukas (1984).

**Model cell lines and cell culture.** Cells of the SH-SY5Y human neuroblastoma express ganglionic nAChRs containing alpha-3, alpha-4, beta-2 with or without beta-2 subunits (“alpha-3 beta-4 nAChRs”) as high-affinity, specific binding sites for [3H]ACh and as functional nAChRs detectable by 86Rb influx assays (Lukas et al., 1993; see Lindstrom, 1996). They also express neuronal nAChRs that contain alpha-7 subunits and have high-affinity binding sites for I-Bgt but do not contribute to 86Rb influx responses, probably due to the very fast kinetics of channel closing on agonist exposure (alpha-7 nAChRs; Lukas et al., 1993; Puchaz et al., 1994). TE671/BD human clonal cells express muscle-type nAChRs containing alpha-1, beta-1, gamma and delta subunits (alpha-1 nAChRs) that bind either I-Bgt or [3H]ACh with high affinity and whose function is detectable using 86Rb influx assays (Lukas, 1986a, 1989, 1990; Luther et al., 1988). BC2-H1 cells express mouse muscle-type nAChRs that can be quan- tified using I-Bgt binding assays (Lukas, 1993). SH-SY5Y, TE671/BD or BC2-H1 cells were maintained at low passage (less than passage 25) in DMEM supplemented with antibiotics and serum as described previously (Lukas, 1986a, 1993; Lukas et al., 1993; Bencherif and Lukas, 1993). Control cultures and cultures for nicotinic ligand treat- ment were seeded at the same time in 100-mm diameter plates (for binding assays) or in poly-L-lysine-coated wells of 12-well trays (for 86Rb influx assays). Studies of temporal effects of nicotinic ligand exposure were designed so indicated drug treatments ended at about the same time for all samples and cells had achieved confluence. Stock nicotinic ligands were prepared in sterile DMEM (pH adjusted to 7.4) at 100 times the highest concentration to be used. At the end of drug treatment, ligands were removed by aspiration, and plated cells were rinsed three times with ice-cold (for ligand binding assays) or room-temperature (for functional assays) Ringer’s buffer within 20 sec. For radioligand binding assays using cell membranes, cells were harvested by scraping, and crude membranes were made by centrifugation of cells at 40,000 x g for 10 min, resuspension of cells into hypotonic 5 mM Tris at 0°C for 5 min, Polytron homogenization (setting 90 for 30 sec) and centrifugation at 40,000 x g for 10 min. Membrane pellets were resuspended in Ringer’s buffer, collected
again by centrifugation at 40,000 × g for 10 min and resuspended in desired volumes of Ringer’s buffer again using brief sonication to aid in obtaining of a uniform suspension of material. Processing of cells for measurement of intracellular or cell surface binding sites is described below.

Radioligand binding assays. ³H]ACh or I-Bgt binding assays were conducted using cellular membrane fractions prepared as described above or intact cells handled as previously described (Lukas, 1990; Bencherif and Lukas, 1993). To determine specific [³H]ACh binding to membrane fractions, levels of nonspecific [³H]ACh binding defined using assay samples containing 10 nM [³H]ACh plus 100 μM Carb were subtracted from levels of [³H]ACh binding defined using assay sample containing 10 nM [³H]ACh but no other nicotinic ligands. To determine “total” I-Bgt binding to membrane fractions, levels of nonspecific I-Bgt binding were defined using samples that contained 10 nM I-Bgt plus 1 μM α-bungarotoxin and subtracted from binding obtained in samples containing 10 nM I-Bgt without competitor. Importantly, we and others (Lukas, 1986a, 1986b; Walker et al., 1988; Conroy et al., 1990; Bencherif et al., 1995b) have noted that only a fraction of specific I-Bgt binding to TE671/RD cells is blocked by small nicotinic ligands such as Carb or d-tubocurarine or can be immunoprecipitated with antibodies that recognize electric tissue nAChRs. By contrast, only a single class of I-Bgt binding sites fully sensitive to blockade by small nicotinic ligands are found in preparations from Torpedo electricus or mouse BC₁-H₁ cells (muscle-type nAChRs) or from cells of the PC12 rat pheochromocytoma or SH-SY5Y/IMR-32 human neuroblastomas (alpha-7 nAChRs; see below and Lukas, 1986b, 1990; 1993; Lukas et al., 1993). Other investigators concluded that small nicotinic drug-insensitive I-Bgt binding sites in TE671/RD cells (or in rat embryonic muscle cells; Carlin et al., 1986) represent incompletely assembled alpha-1 subunits (Conroy et al., 1990). However, our studies (see below) indicate that small nicotinic ligand-insensitive I-Bgt binding sites are expressed on the cell surface, where immature nAChR precursors would not be expected. nAChR variants have been found in TE671/RD cells that contain an elongated alpha-1 subunit encoded by an alternatively spliced alpha-1 subunit message containing an additional exon 3A (Beeson et al., 1990). However, variant nAChRs containing alphai-1(3A+) subunits do not bind I-Bgt with high affinity, do not form functional receptors responsive to agonists and are not reactive with antibodies against the “main immunogenic region” (Newland et al., 1995) and therefore cannot account for small ligand-insensitive I-Bgt binding sites in TE671/RD cells. Further studies are warranted to determine the identity of I-Bgt binding site subsets in TE671/RD cells. However, for the purposes of this study, the “Carb-sensitive” subset of I-Bgt binding in TE671/RD cell preparations was operationally defined by subtracting binding occurring in samples containing 10 nM I-Bgt plus 1 mM Carb from binding occurring in samples lacking that drug. The “Carb-insensitive” subset of I-Bgt binding was operationally defined as the difference between “total” and “Carb-sensitive” I-Bgt binding (i.e., binding occurring in samples containing 10 nM I-Bgt plus 1 mM Carb minus that occurring in sample containing 10 nM I-Bgt plus 1 μM Bgt). The same definitions of total, Carb-sensitive and Carb-insensitive I-Bgt binding sites from TE671/RD cells were applied when assays were run using whole cells in suspension (using centrifugations at 2000 × g for 30 sec to gently collect harvested cells and to separate free I-Bgt from bound I-Bgt and intact cells) or intact cells maintained in situ on culture dishes (done by simply adding reagents to medium used to bathe cells to initiate assays and gentle cell rinses to resolve free from cell-bound I-Bgt) to define “cell surface” I-Bgt binding sites. Experiments conducted in parallel indicated that numbers of cell surface I-Bgt binding sites determined using these two approaches were the same. In some cases, differences between numbers of specific I-Bgt binding sites in membrane fractions and numbers of specific I-Bgt binding sites on the cell surface were calculated (after full normalization of data to numbers of binding sites per unit of total cell protein in samples used for cell surface assays or to generate membrane preparation) to determine numbers of specific I-Bgt binding sites in intracellular pools. Numbers of intracellular I-Bgt binding sites were also determined directly in some experiments by incubating cells for 1 hr in the presence of 10 nM native Bgt, rinsing cells free of excess toxin and processing cells into membrane fractions for I-Bgt binding assays. Material balance determinations demonstrated that calculated and experimentally determined levels of I-Bgt binding to intracellular pools were the same and that the sum of cell surface and intracellular I-Bgt binding equaled that occurring in total membrane fractions. Proportions of total I-Bgt binding sites that were bound on the cell surface or in intracellular pools and that were Carb- or Carb-insensitive are provided where relevant in the text and/or figure legends.

¹⁸⁶Rb⁺ influx assays. A modification of the ¹⁸⁶Rb⁺ influx assay described by Robinson and McGee (1985) was used to quantify effects of nicotinic ligand treatment on nAChR function at 20°C and intact TE671/RD or SH-SY5Y cells cultured on 12-well plates according to Bencherif et al. (1995b). Levels of nonspecific ion flux were equivalent whether defined using samples containing agonist plus 100 μM d-tubocurarine or using blank samples that contained no agonist. Specific nAChR function was defined as total, experimentally determined ion flux in the presence of agonist minus nonspecific ion flux. As shown below, we are able to detect two phases of loss of nAChR function using ¹⁸⁶Rb⁺ influx assays, and we apply operational definitions to characterize losses of function due to both or just one of these processes. To quantify losses in nAChR function due to both “desensitization” (which describes a rapid in onset and quickly reversible loss of function induced on brief exposure to nicotinic agonists and probably represents the process classically described by Katz and Thesleff, 1957) and “persistent inactivation” (defined below), cells were pretreated with nicotinic ligand for a specified period. Over the last minute of this pretreatment period, ouabain was added to the medium to a final concentration of 1 mM. At the end of this period, medium was removed, and a sodium-free, iso-osmotic influx assay buffer containing 1 mM ouabain, ¹⁸⁶Rb⁺ (∼3 μCi/ml) and 1 mM Carb with or without 100 μM d-tubocurarine (to define nonspecific/total influx) was applied to initiate the 20-sec influx period. Assays were terminated by three rapid washes of cells using a laminar flow technique with fresh influx assay buffer to remove extracellular ¹⁸⁶Rb⁺, and ¹⁸⁶Rb⁺ uptake was quantified by Cerenkov counting of cells harvested in 0.01% sodium dodecyl sulfate and 0.1 N NaOH. To quantify losses in nAChR function due to persistent inactivation alone, drug-treated cells were rinsed three times with fresh DMEM over a 4-min period and treated for an additional minute with sodium-free influx assay buffer supplemented with 1 mM ouabain. Fresh buffer containing ¹⁸⁶Rb⁺, ouabain, and Carb with or without d-tubocurarine was then applied to initiate the influx assay as described above. Hence, “persistent inactivation” is operationally defined as the loss of nAChR function that is not reversed during a 5-min period of recovery from agonist exposure.

Northern analysis. Poly(A)⁺ RNA was extracted from cells using a modification of the Invitrogen Fast-Track method and resolved on 1% agarose gels. blotting and hybridization with nAChR cDNA probes were performed as described in Bencherif et al. (1995a) using probes described in Lukas et al. (1993). Depending on the probe used, a stringent final wash (0.2 × SSPE at 65°C for 30 min) was performed.

Data analysis. Time dependencies for up-regulation of radioligand binding were fit by

\[ y = c + f(1 - e^{-kt}) + s(1 - e^{-kt}) + d[e^{-kt}] \]

(1)

as appropriate, where \( y \) equals the observed level of radioligand binding, \( e \) indicates the number \( e \) raised to the power of the subsequence term, \( t \) is the time of nicotine exposure (usually in hours), \( c \) (or \( c + d \)) equals the level of radioligand binding in control samples (set at 100%), \( f \) is the increase in radioligand binding due to a fast process characterized by rate constant \( k_f \) (∼0.693/\( t_f \), where \( t_f \) is the time constant for that process), \( s \) is the increase in radioligand binding...
due to a slow process characterized by rate constant $k_f$ and time constant $\tau_f$ and $d$ is the fraction of original radioligand binding sites subject to a decrease via a process characterized by rate constant $k_d$ and time constant $\tau_d$.

Time dependencies for losses of nAChR function were fit by

$$y = c + f(e^{-(k_f t)} + s[e^{-(k_d t)}])$$

as appropriate, where $y$ is the observed specific $^{86}$Rb$^+$ influx, $e$ indicates the number $e$ raised to the power of the subsequent term, $t$ is the time of nicotine exposure (usually in min), $c$ is the amount of $^{86}$Rb$^+$ influx resistant to loss, $f$ is the amount of $^{86}$Rb$^+$ influx subject to fast inactivation described by rate constant $k_f$ (where $\tau_f$ is the time constant for that process) and $s$ is the amount of $^{86}$Rb$^+$ influx subject to slower inactivation described by rate constant $k_d$ and time constant $\tau_d$.

The general formula used to fit radioligand binding saturation curves was

$$y = B_{\text{max}}/[1 + (10^{y/KD})^n]$$

where $y$ is the observed level of radioligand binding, $B_{\text{max}}$ is the maximal level of radioligand binding at saturation, $x$ is the log radioligand concentration, $c$ is the log $K_D$ value and $n$ is the Hill coefficient for radioligand binding.

The general equation to describe dose-dependent up-regulation of nAChR numbers on chronic nicotine exposure was

$$y = a + [[b/[1 + (10^y)^g]] + [[g/[1 + (10^y)^g]]/[1 + (10^y)^g]]]$$

where, as appropriate, $y$ equals the observed level of radioligand binding, $x$ is the log nicotine concentration, $a$ equals the level of radioligand binding in control samples (set at 100%), $b$ is the increase in radioligand binding due to the process half-maximally evident at the log nicotine concentration $c$ exhibiting a Hill coefficient $n$, $g$ is the increase in radioligand binding due to the process (when evident) that is half-maximal at log nicotine concentration $h$ and exhibits a Hill coefficient of $p$ and $d$ is the log nicotine concentration at which there is half-maximal decrease in radioligand binding due to a process (when evident) exhibiting a Hill coefficient of $q$.

The general equation describing dose dependence of nAChR functional loss was

$$y = a + [(100 - a)/[1 + (10^y)^g]]$$

where $y$ equals the observed level of function, $a$ is the percentage of control function resistant to loss, $x$ is the log nicotine concentration, $a$ is the log nicotine concentration that gives a half-maximal loss in function (which may or may not equal the IC$_{50}$ value for nicotine at which there is a 50% loss of function) and $n$ is the Hill coefficient.

These equations were fit to normalized and pooled data by an iterative process to derive nonlinear regression least-squares curves and the parameters mentioned in the text and/or figure legends.

Unless otherwise indicated, data points on graphs or data values presented in the text are mean ± S.D. values, whereas parameters derived from curve fitting to data points are calculated mean ± S.E.M. values.

**Protein determination.** Protein contents for harvested or asayed cells or for membrane preparations were determined using the method of Lowry et al. (1951) or the BCA assay normalized to bovine serum albumin.

**Results**

**Time dependence of nicotine-induced up-regulation of muscle-type nAChRs.** Numbers of muscle-type nAChR radioligand binding sites in membrane preparations containing both cell surface and intracellular pools of sites from TE671/RD cells increase as a function of duration of nicotine exposure whether measured using I-Bgt or $[^3H]$ACh as probes (fig. 1). However, numbers of specific $[^3H]$ACh binding sites increase ~5-fold over 3 days of nicotine exposure, whereas numbers of total, specific I-Bgt binding sites increase only ~2.5-fold. The ratio between total I-Bgt binding sites and $[^3H]$ACh binding sites decreases during exposure to nicotine, but the ratio between Carb-sensitive I-Bgt binding sites and $[^3H]$ACh binding sites remains constant throughout the course of nicotine treatment (2.68 ± 0.36). There is dissociation of $[^3H]$ACh from ~25% of specific $[^3H]$ACh binding sites during sample processing, whereas there is negligible dissociation of I-Bgt from its specific binding sites during sample processing (Lukas, 1990). Furthermore, under conditions of the assays used, nearly all specific I-Bgt binding sites are occupied by I-Bgt, whereas there is occupancy of only about one half of specific $[^3H]$ACh binding sites ($K_f$ for $[^3H]$ACh binding $≈ 10$ nM; see Lukas, 1990). Hence, when these correction factors (×0.75 and ×0.5) are applied, the
ratio of Carb-sensitive I-Bgt binding sites in TE671/RD cells to specific [3H]ACh binding sites is 1.01 ± 0.14. Collectively, these findings suggest that the same species of nAChR is detected using specific [3H]ACh binding and Carb-sensitive I-Bgt binding assays.

Results of more extensive kinetic studies (fig. 2A) show that levels of I-Bgt binding become stable after 2 to 5 days of nicotine exposure. The increase in numbers of Carb-sensitive I-Bgt binding sites was ~4-fold (i.e., ~500% of control levels) over 2 to 5 days of nicotine exposure and was well fit \( (r^2 = .96) \) to an equation for a biphasic exponential process. A more modest increase in numbers of Carb-insensitive I-Bgt binding sites at time of exposure to nicotine increased was fit \( (r^2 = .71) \) to a single exponential process with a rate constant equal to that for the slower process affecting Carb-sensitive I-Bgt binding levels. Results for time-dependent increases in numbers of total I-Bgt binding sites during nicotine exposure were also well fit \( (r^2 = .94) \) to a curve representing a weighted admixture of contributions from Carb- and Carb-insensitive binding sites. Studies using specific [3H]ACh binding (taken only to 72 hr of nicotine exposure; fig. 2B) showed a 4-fold increase in numbers of sites, just as was the case for studies of Carb-sensitive I-Bgt binding, and the data were well fit \( (r^2 = .98) \) to a two-phase process using the rate constants derived from analysis of effects on Carb-sensitive I-Bgt binding. Nevertheless, reasoning that some form of heterogeneity in I-Bgt binding sites in these preparations was confounding data analysis, further studies were done using intact cells and membrane fractions in parallel to distinguish effects of nicotine exposure on I-Bgt binding site subsets.

Assays were conducted to measure numbers of I-Bgt binding sites in intracellular pools of TE671/RD cells. The results (fig. 3A) indicate that numbers of intracellular, Carb-sensitive sites increase ~4-fold over 2 to 3 days of nicotine exposure according to a two-phase process for a 341 ± 37% increase in numbers of sites with a rate constant, \( k_f \), of 0.027/hr \( (\tau_s = 25.7 \text{ hr}) \) and an 85 ± 18% increase in numbers with a rate constant, \( k_s \), of 1.0/hr \( (\tau_f = .69 \text{ hr}; r^2 = .96) \). The more modest increase in Carb-insensitive sites is well fit by a two-phase process with the same rate constants (a 46 ± 14% increase for the process with \( \tau_s = 25.7 \text{ hr} \) and an 11 ± 7% increase for the process with \( \tau_f = .69 \text{ hr}; r^2 = .79 \)). The increase in total numbers of I-Bgt binding sites (137% due to

Fig. 2. Time-dependent effects of nicotine exposure on numbers of TE671/RD cell nAChRs measured (A) using I-Bgt binding assays or (B) using [3H]ACh binding assays. Cells of the TE671/RD human clonal line were treated with 1 mM nicotine for the indicated periods of time (abscissa; hours) before being processed to membrane preparations and subjected to I-Bgt or [3H]ACh binding assays to quantify nAChRs (ordinate; specific radioligand binding as a percentage of control values) as described in Experimental Procedures. Results are the mean ± S.D. for data from three (B) or five (A) experiments. A, The solid line drawn through data points for specific I-Bgt binding sensitive to blockade by 100 μM carbamylcholine [Carb-sensitive; •] is fit \( (r^2 = .96) \) to equation 1 where \( k_f = 1.9 \pm 7.6/\text{hr} \) (errors in derived parameters are ±S.E.M.), \( k_s = 0.027 ± 0.009/\text{hr} \) and \( f \) and \( s \) are 40 ± 23% and 340 ± 34% of control values (c = 100%), respectively. Fits of the data for \( s = 0 \) yielded a theoretical curve \( (r^2 = .95; k_f = 0.632 ± 0.008/\text{hr}, f = 309 ± 287\% \) of control values) that clearly fit data for nicotine treatments of 6 hr or less very poorly. The solid line drawn through data points for specific I-Bgt binding sensitive to blockade by 100 μM carbamylcholine [Carb-insensitive; ○] is fit \( (r^2 = .71) \) to equation 1 for fixed \( k_f = 1.9 \) and \( k_s = 0.027 \) and yielded \( f = 0 ± 5.9\% \) and \( s = 38 ± 10\% \) of control values (c = 100%), respectively, but individual data points are not statistically different from controls (t test P > .05). The solid line drawn through data points for specific I-Bgt binding sensitive to blockade by 1 mM native Bgt (total; ◦) is fit \( (r^2 = .94) \) to a weighted admixture of the effects on Carb-sensitive (initially 33.8% of the total) and Carb-insensitive (initially 66.2% of the total) I-Bgt binding contributing to a 14% increase in sites with \( k_f = 1.9/\text{hr} \) and a 140% increase in sites with \( k_s = 0.027/\text{hr} \). Variability across experiments in levels of I-Bgt binding sites after 2 to 5 days of nicotine exposure does not reflect the much lower intraexperimental error for replicate samples (typically <10% of the average value). However, the final magnitude of binding site up-regulation seemed to be attenuated (and numbers of ligand binding sites per unit of cell protein are also lower) if cultures had been initially seeded at lower densities and/or were maintained for longer periods of time before initiation of nicotine treatments. Perhaps some medium components were depleted or cell health was compromised during those longer-term cultures, or perhaps cells need to achieve a threshold density before nAChRs can be expressed at maximum capacity and can be maximally affected by chronic nicotine treatment. Typical specific binding levels (fmol/mg of membrane protein) were 35 to 50 for Carb-sensitive, 100 to 150 for total and 70 to 100 for Carb-insensitive I-Bgt binding. B, The solid line drawn through data points is the best fit \( (r^2 = .99) \) to equation 2 for rate constants for Carb-sensitive I-Bgt binding from fig. 2 (i.e., \( k_f = 1.9/\text{hr}, k_s = 0.027/\text{hr} \)) and yield values for \( f \) and \( s \) of 60 ± 93% and 363 ± 20% of control values (c = 100%), respectively. Typical specific [3H]ACh binding was 12 to 17 fmol/mg of membrane protein.
Fig. 3. Time-dependent effects of nicotine exposure on numbers of I-Bgt binding sites (A) in membrane-bound, intracellular pools from TE671/RD cells or (B) on the surface of the cell surface (see fig. 3B). The solid line drawn through data points for specific I-Bgt binding sensitive to blockade by 100 μM carbamylcholine (Carb-sensitive; □) is for $k_s = 0.027 \pm 0.019/hr$, $s = 341 \pm 37\%$, $k_r = 1.04 \pm 1.2/hr$, $f = 85.4 \pm 18.4\%$ and $c = 100\% (r^2 = 0.96)$; errors are ±S.E.M.). The solid line for Carb-insensitive I-Bgt binding (Carb-insensitive; ○; $r^2 = 0.79$) is for $k_r = 0.027/hr$, $s = 46.3 \pm 14.4\%$, $k_r = 1.0/hr$ and $f = 11.1 \pm 7.1\%$. The solid line drawn through data ($r^2 = .95$) for I-Bgt binding sensitive to blockade by 1 μM native Bgt (total; ●) is the weighted admixture of theoretical curves for Carb-sensitive and Carb-insensitive binding sites (initially 30.7% and 69.3%, respectively, of the total).

Fits to equation 1 for $s = 0$ yielded curves that coincided poorly with data between 0 and 6 hr of nicotine exposure and were characterized by $r^2$ values of .88 for Carb-sensitive I-Bgt binding, .74 for Carb-insensitive I-Bgt binding and .87 for total I-Bgt binding. In these studies, before nicotine exposure, intracellular I-Bgt binding sites represented 76.3 ± 5.8% of the total number of sites (see fig. 3B) and absolute numbers of intracellular I-Bgt binding sites ranged between 132 and 200 fmol/mg of cell protein. B, Cells were gently harvested and subjected to I-Bgt binding assays in suspension to quantify cell surface binding sites (ordinate; specific I-Bgt binding; percent of control samples not exposed to nicotine) as described in Experimental Procedures. Results are the means from three experiments (see fig. 3A). Solid lines drawn through data points are derived from equation 1 for the indicated parameters: $y = 50 + 50[1 - e^{(-0.1t)}] + 140[1 - e^{(-0.05t)}] + 50[1 - e^{(-20t)}]$ for I-Bgt binding sensitive to blockade by 100 μM carbamylcholine (Carb-sensitive; □); $r^2 = .95$, or $y = 40 + 60[1 - e^{(-20t)}] + 50[1 - e^{(-0.3t)}] + 60[1 - e^{(-20t)}]$ for I-Bgt binding that is insensitive to blockade by carbamylcholine (Carb-insensitive; ○; $r^2 = .87$); actual data points were not significantly different from controls ($P > .05$). The solid line drawn through the data points for specific I-Bgt binding sensitive to blockade by 1 μM native Bgt (total; ●) is the weighted admixture of the theoretical curves for Carb- and Carb-insensitive binding sites ($r^2 = .93$). In these studies, before nicotine exposure, 23.7 ± 1.8% of the total number of I-Bgt binding sites were on the cell surface (see fig. 3A), absolute numbers of cell surface sites ranged between 41 and 70 fmol/mg of cell protein and 47.3 ± 5.7% of the cell surface sites were sensitive to blockade by Carb.

The slow process and 34% due to the fast process; $r^2 = .95$) is in excellent agreement with the predicted admixture of the large increase in numbers of Carb-sensitive I-Bgt binding sites (initially 30.7 ± 1.8% of the total) and the more modest increase in numbers of Carb-insensitive I-Bgt binding sites (initially 69.3% of the total).

Effects of nicotine exposure on numbers of cell surface I-Bgt binding sites were more complex (fig. 3B). There was a ~50% loss of the original number of Carb- or Carb-insensitive I-Bgt binding sites on the cell surface over the initial 30 to 60 min of nicotine exposure. However, numbers of Carb-sensitive (or total) binding sites were significantly above control levels by 12 hr of nicotine exposure, reflecting in part effects of a fast phase of up-regulation. Numbers of Carb-sensitive I-Bgt binding sites were driven to higher levels due to a slower up-regulatory process and equilibrated after 2 to 3 days of nicotine exposure (triphasic fit to the data yields $r^2 = .95$) at levels ~4-fold higher than those observed at the trough of the transient down-regulation. A two-phase process describing more modest (~50%) increases in numbers of Carb-insensitive I-Bgt binding sites could also be fit to the data ($r^2 = .87$). Numbers of total I-Bgt binding sites changed reflecting the weighted sum of the effects on Carb- and Carb-insensitive I-Bgt binding sites ($r^2 = .93$).

Collectively, these findings suggested that numbers of Carb-sensitive I-Bgt binding sites (or [3H]ACH binding sites when they could be measured) in TE671/RD cells increased ~4-fold over a 2- to 5-day course of nicotine treatment, whether sites were expressed on the cell surface or in the substantial intracellular pool of binding sites. The Carb-insensitive subset of I-Bgt binding sites was more modestly (~50%) up-regulated. Numbers of cell surface I-Bgt binding sites were transiently down-regulated early during nicotine exposure before rising to new equilibrium levels. Total numbers of I-Bgt binding sites in cell surface, intracellular or membrane fractions changed on exposure to nicotine in accordance with the sum of the effects on individual I-Bgt binding site components.

Abbreviated studies (not shown) examining time-dependent effects of 1 μM nicotine exposure on numbers of mouse muscle-type nAChRs in BC3H-1 cells indicated that a simple, monophasic increase with a rate constant of 0.10 ± 0.08/hr described an up-regulation to a maximum of 79.9 ± 8.6% above control levels of specific I-Bgt binding ($r^2 = .95$).
Saturation and Scatchard analysis of effects of chronic nicotine exposure on numbers of muscle-type nAChRs. To gain further insight into processes that underlie nicotine exposure-induced up-regulation of muscle-type nAChRs, I-Bgt saturation binding studies were conducted using membrane preparations from control cells or from cells treated for 48 hr with 1 mM nicotine. Saturation isotherms (fig. 4A) indicate that a finite number of total, Carb-sensitive or Carb-insensitive specific I-Bgt sites are expressed by control or nicotine-treated TE671/RD cells. Scatchard analyses indicated that I-Bgt binding in each case appeared to be to a single class of sites (fig. 4B). Moreover, effects of nicotine treatment were due to increases in maximum I-Bgt binding levels and not to changes in I-Bgt binding affinities. $B_{\text{max}}$ values for control preparations were 2451 ± 82 cpm for total I-Bgt binding, 1257 ± 92 cpm for Carb-sensitive I-Bgt binding and 1629 ± 197 cpm for Carb-insensitive I-Bgt binding, but increase 131% to 286% in these nicotine-treated preparations to 8655 ± 153 cpm for total I-Bgt binding, 4857 ± 118 cpm for Carb-sensitive I-Bgt binding and 3767 ± 125 cpm for Carb-insensitive I-Bgt binding. By contrast, $K_d$ values for control or nicotine-treated samples, respectively, are 0.99 ± 0.14 nM and 1.15 ± 0.12 nM for total I-Bgt binding, 1.09 ± 0.71 nM and 0.99 ± 0.10 nM for Carb-sensitive I-Bgt binding and 2.43 ± 1.02 nM and 1.82 ± 0.28 nM for Carb-insensitive I-Bgt binding.

Time dependence of nicotine-induced up-regulation of ganglionic nAChRs. Prolonged exposure to nicotine induces increases in numbers of I-Bgt binding sites (corresponding to human ganglionic nAChRs containing alpha-7 subunits; Lukas et al., 1993; Puchacz et al., 1994) in intracellular pools from SH-SY5Y cells. The increase can be fit to a two-phase process predominantly reflecting a slow ($\tau = 36.4$ hr), 237% increase that follows a faster ($\tau = 0.14$ hr) and more modest (23%) rise (fig. 5A; $r^2 = .94$). Time-dependent changes in cell surface I-Bgt binding sites on nicotine exposure reflect an initial ~34% loss of sites followed by a slower increase toward initial levels. Effects of nicotine treatment on numbers of I-Bgt binding sites in membrane preparations (containing both cell surface and intracellular pools of sites) reflect the weighted admixture of effects on each pool of sites alone ($r^2 = .96$).

The kinetics for increases in specific $[^3H]$ACh binding sites (corresponding to human ganglionic nAChRs containing alpha-3 and beta-4 subunits) reflects a process characterized by a rate constant of ~0.03/hr with a magnitude of 581% ($r^2 = .97$).

Abbreviated radioligand binding saturation studies and Scatchard analyses using SH-SY5Y cells (not shown) indicate that nicotine exposure induces changes in numbers of $[^3H]$ACh or I-Bgt binding sites corresponding to alpha-3 beta-4- or alpha-7 nAChRs, respectively, and not changes in $K_d$ values for radioligand binding.

Dose-dependent effects of nicotine exposure on nAChR numbers. The dose dependence of nicotine exposure-induced up-regulation of TE671/RD cell I-Bgt binding sites is illustrated in figure 6A. Levels of total, Carb-sensitive and Carb-insensitive I-Bgt binding after a 48-hr exposure to 1 mM nicotine were similar to those observed in time-depen-

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Fig. 4. Analysis of effects of nicotine treatment on TE671/RD cell I-Bgt binding site levels and $K_d$ values. Cells were treated under control conditions or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mM nicotine for 48 hr before being processed into membranes and subjected to I-Bgt binding assays at different concentrations of I-Bgt as described in Experimental Procedures. A, Specific I-Bgt binding (ordinate; specific I-Bgt bound, cpm per sample) is plotted as a function of free I-Bgt or with 1 mA.

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[Image 42x157 to 544x383]
dence studies (see fig. 2A). Significant increases in numbers of Carb-sensitive sites were evident at nicotine concentrations as low as 1 μM, and there was no evidence that up-regulation would plateau at nicotine concentrations as high as 3 mM. The results could be well fit (r^2 = 1.0) to a two-phase Hill equation for nicotine-sensitive processes with EC_{50} values of 1.3 μM (88% increase in sites) and 800 μM (441% increase in sites). A more modest increase (by 49% with an EC_{50} value of 1.6 μM) in numbers of Carb-insensitive I-Bgt binding sites was suggested by the data. The increase in numbers of total I-Bgt binding sites was described (r^2 = 0.91) by an admixture of the fits to Carb- and Carb-insensitive I-Bgt binding weighted according to initial contributions to the total of those pools of binding sites.

Up-regulation in numbers of muscle-type nAChRs in TE671/RD cells quantified based on specific [3H]ACh binding also showed nicotine dose dependence (fig. 6B). Levels of specific [3H]ACh binding after a 48-hr exposure to 1 mM nicotine were similar to those observed in time-dependence studies (see fig. 2B), were significantly above control levels at 1 to 10 μM nicotine and continued to rise through concentrations of nicotine as high as 5 mM. The results could be well fit (r^2 = 0.97; solid line in fig. 6B) to a single-phase Hill equation for a nicotine-sensitive process with an EC_{50} value of 550 μM (558% increase in sites) or to a two-phase Hill equation (r^2 = 0.96; dotted line in fig. 6B) using EC_{50} values of 1.3 μM (140% increase in sites) and 800 μM (285% increase in sites) derived from fits to the data for nicotine dose-dependent changes in Carb-sensitive I-Bgt binding (see fig. 6A).

Results of abbreviated studies (not shown) concerning nicotine dose-dependent effects on numbers of mouse muscle-type nAChRs from BC3H-1 cells indicated that a maximal increase of 92.5 ± 7.8% in numbers of specific I-Bgt binding sites occurred via a process characterized by an EC_{50} value of 195 μM (r^2 = 0.97).

Nicotine exposure for 48 hr at concentrations between 100 nM and 100 μM had little effect on numbers of specific I-Bgt binding sites from SH-SY5Y cells, but exposure to nicotine at 1 mM induced increases in numbers of I-Bgt binding sites similar to those seen in time-dependence studies (fig. 7A). Numbers of I-Bgt binding sites were even higher in cells treated with 2 mM nicotine but fell again at 3 to 5 mM nicotine (IC_{50} = 3.9 mM), perhaps reflecting a subtle cytotoxic effect on SH-SY5Y cells at these concentrations. Data were well fit (r^2 = 0.99) to the Hill equation, giving an EC_{50} value of 812 μM for a 180% up-regulation of SH-SY5Y I-Bgt binding sites.

Dose-dependence studies (fig. 7B) again showed dramatic up-regulation of [3H]ACh binding sites from SH-SY5Y cells sites after 48 hr of treatment with 1 mM nicotine. However, numbers of sites fell again at higher doses of nicotine (IC_{50} = 2.24 mM), as was observed for SH-SY5Y I-Bgt binding sites. Nevertheless, significant increases in numbers of specific [3H]ACh binding sites were evident after 48 hr of nicotine treatment at concentrations as low as 1 μM. The rise in [3H]ACh sites could be fit to a two-phase process with EC_{50} values of 6.0 μM (265% up-regulation) and 480 μM (771% up-regulation; r^2 = 0.97).

Fig. 5. Time-dependent effects of nicotine exposure on numbers of SH-SY5Y cell nAChRs measured using (A) I-Bgt binding assays or (B) [3H]ACh binding assays. The SH-SY5Y human neuroblastoma line were treated with 1 mM nicotine for the indicated periods of time (abscissa; hours) before being processed to membrane preparations and subjected to radioligand binding assays to quantify nAChRs (ordinate; specific radioligand binding sites per mg of membrane protein) values. Fits for data with f > 0 do not improve r^2 values and predict that <3% of the increase in numbers of I-Bgt binding sites occurs with a faster rate constant.
Effects of nicotine exposure on nAChR subunit mRNA levels. Northern blot analyses conducted to determine whether nicotine exposure altered steady state levels of nAChR subunit gene expression as mRNA in TE671/RD cells indicated no significant differences in expression of alpha-1, beta-1, gamma or delta subunits as a function of duration of exposure to 1 mM nicotine and certainly not an increase as might be suggested to account for up-regulation of nAChR radioligand binding sites (fig. 8A). A single study of nicotine dose-dependent effects at 48 hr of drug exposure (not shown) similarly revealed no changes in TE671/RD cell muscle-type nAChR subunit mRNA. Similarly, no effect on nAChR alpha-5, alpha-6, alpha-7, beta-2 or beta-4 subunit mRNA levels was seen in Northern analyses of preparations from SH-SY5Y cells treated with 1 mM nicotine for times as long as 72 hr (fig. 8B).

Time-dependent effects of nicotine exposure on nAChR function. Function attributable to different nAChR subtypes was measured using carbamylcholine-stimulated $^{86}$Rb$^+$ influx (i) just after removal of medium, sometimes containing nicotine, used to pretreat cells (“0 min recovery”) or (ii) 5 min after removal of pretreatment medium (“5 min recovery”). The 5-min recovery period was previously determined to be adequate to allow nAChRs to recover from a quick-in-onset and quickly reversible phase of nAChR “desensitization” (Lukas, 1991), and thus served to operationally define nAChR function that reflected a persistent functional inactivation.

Temporal studies indicated that $^{86}$Rb$^+$ influx in TE671/RD cells is nearly completely lost (by 94.2 ± 5.5%) via a process characterized by a time constant of 30 sec when cells had been pretreated with 1 mM nicotine and not allowed to recover from desensitization (fig. 9A; “0 min recovery”). However, when cells were given 5 min to recover from nicotine pretreatment, more $^{86}$Rb$^+$ influx was observed, at least for short times of nicotine preexposure. Nevertheless, if nicotine preexposure continued for 60 min (or more; current data for times >60 min are not shown here, but see Lukas, 1991, for examples), $^{86}$Rb$^+$ influx levels again were negligible. Fits of the data ($r^2 = .96$) to a biphasic exponential decay indicated that a 45% loss of $^{86}$Rb$^+$ influx with a rate constant of 0.73/min ($\tau_r = .95$ min) and a 55% loss of function with a rate constant of 0.036/min ($\tau_s = 19.3$ min) could account for the results.

Other temporal studies indicated that $^{86}$Rb$^+$ influx in SH-SY5Y cells pretreated with 1 mM nicotine and not allowed to recover from desensitization is completely lost relative to untreated controls via two processes characterized by rate constants of 4.84/min ($\tau_r = 0.14$ min; ~60% of the loss) and 0.104/min ($\tau_s = 6.66$ min; ~40% of the loss; fig. 9B; “0 min recovery”). When cells were given 5 min to recover from nicotine pretreatment, more $^{86}$Rb$^+$ influx was observed, but more than 80% of $^{86}$Rb$^+$ influx remained lost if nicotine preexposure continued for 30 to 60 min (fig. 9B; “5 min recovery;” 18 ± 1% of control $^{86}$Rb$^+$ influx remained after 24 hr of nicotine pretreatment). Fits of the data ($r^2 = .96$) to a biphasic exponential decay indicated that a 56% loss of $^{86}$Rb$^+$ influx with a rate constant of 4.31/min ($\tau_r = 0.161$ min) and a 26% loss of function with a rate constant of 0.098/min ($\tau_s = 7.07$ min) could account for the results.
Dose-dependent effects of nicotine exposure on nAChR function. Dose dependencies for 1-hr nicotine pre-exposure-induced loss of TE671/RD cell nAChR function are illustrated in figure 10A. Function assessed just after removal of nicotine (“0 min recovery”) is almost entirely lost (half-maximally so at 700 nM nicotine). Consistent with temporal studies, function assessed 5 min after nicotine removal (“5 min recovery”) is also largely lost (by ~93%), half-
8.1% of control 86Rb influx of cell surface muscle-type nAChRs or followed by up-regulation to or beyond original levels of expression of nAChR subunit mRNA levels. Additional, principal findings of this study are (1) that chronic nicotine exposure induces transient down-regulation of nAChR subtypes, a human ganglionic nAChR subtype containing alpha-3 and beta-4 subunits, a human ganglionic nAChR containing alpha-7 subunits in intracellular pools, (2) that chronic nicotine exposure induces transient down-regulation followed by up-regulation to or beyond original levels of expression of cell surface muscle-type nAChRs or alpha-7 nAChRs, (3) that the potency with which chronic nicotine exposure exerts its maximal effects differs across alpha-1, alpha-3 beta-4 and alpha-7 nAChR subtypes, as do rates and magnitudes of the maximal “nicotine-induced nAChR up-regulation,” and (4) that these changes in nAChR numbers are not attributable to either transient or sustained changes in nAChR subunit mRNA levels. Additional, principal findings are (5) that nicotine exposure more potently, more rapidly and with nAChR-subtype specificity of action, induces two phases of functional loss for muscle-type nAChRs and alpha-3 beta-4 nAChRs, including a “persistent inactivation” that is distinct from classically defined “desensitization.” Table 1 summarizes key parameters from quantitative analysis of the data.

There is substantial evidence that nicotine exposure in vivo or in vitro induces up-regulation of radioligand binding sites corresponding to a predominant nAChR subtype in the brain (Marks et al., 1983; Schwartz and Kellar, 1983; Bencherif et al., 1995a; Collins and Marks, 1996). This up-regulation has been suggested to account for at least some of nicotine’s physiological effects (Wonnacott, 1990; Dani and Heinemann, 1996). Initially, we undertook the studies described here to test the hypothesis that muscle-type and ganglionic nAChRs would not be up-regulated on nicotine exposure. However, our results clearly are inconsistent with this hypothesis, suggesting that susceptibility to nicotine-induced up-regulation is a property of all nAChR subtypes. Nevertheless, doses of nicotine and durations of exposure required to induce up-regulation differ across nAChR subtypes. The magnitude of up-regulation also differs across nAChR subtypes. These findings are important because differences in the potency, rate and magnitude of nicotine-induced up-regulation of diverse nAChR subtypes may contribute to the spectrum of nicotine’s effects on the nervous system.
system and may help to explain features of nicotine dependence. For example, doses of nicotine in the range of those found in the plasma of human smokers (~300 nM; in rats, brain concentrations of nicotine are ~3-fold higher than plasma concentrations; see Rowell and Li, 1997) up-regulate alpha-4 beta-2 nAChRs in two different preparations by 50% to 100%, and maximal up-regulation of 100% to 500% occurs after treatment with 1–5 μM nicotine (Peng et al., 1994; Zhang et al., 1994; Bencherif et al., 1995a). The current studies indicate that there is no more than a 25% to 50% up-regulation of muscle-type, alpha-3 beta-4- or ganglionic alpha-7 nAChRs after 2-day treatment with 300 nM nicotine.

Fig. 10. Dose-dependent effects of nicotine exposure on function of nAChRs. Cells were pretreated with nicotine at the indicated concentrations (abscissa; molar; log scale) for 1 hr before being exposed to 86Rb+ influx assays (ordinate; specific 86Rb+ influx as a percentage of control values) as described in Experimental Procedures. Results are the mean (± range) for data from two-four experiments. A, TE671/RD cells: The solid line drawn through data points for specific 86Rb+ influx just after removal of nicotine (“0 min recovery,” •) are best fit ($r^2 = 1.0$) to equation 5 where $n = 0.75 ± 0.08$, $a = 3.2 ± 2.4$ and $c = −6.16 ± 0.07$ (pIC50 = −6.15). The solid line drawn through data points for specific 86Rb+ influx measured 5 min after removal of nicotine (“5 min recovery,” ☐) are best fit ($r^2 = 1.0$) to equation 5 where $n = −0.82 ± 0.07$, $a = 7.4 ± 1.6$ and $c = −6.10 ± 0.05$ (pIC50 = −6.03). Typically, total 86Rb+ influx was 5210 cpm and nonspecific influx was 250 cpm for samples containing ~220 μg of protein in ~22-mm-diameter wells. B, SH-SY5Y cells. The solid line drawn through data points for specific 86Rb+ influx just after removal of nicotine (“0 min recovery,” ○) are best fit ($r^2 = .95$) to equation 5 where $n = 0.50 ± 0.21$, $a = 0 ± 16.7$% and $c = −5.50 ± 0.47$. The solid line drawn through data points for specific 86Rb+ influx measured 5 min after removal of nicotine (“5 min recovery,” □) are best fit ($r^2 = 1.0$) to equation 5 where $n = −0.35 ± 0.05$, $a = 8.3 ± 7.7$% and $c = −5.28 ± 0.27$ (pIC50 = −5.01). Typically, total 86Rb+ influx was 730 cpm and nonspecific influx was 170 cpm for samples containing 100 to 120 μg of protein in ~22-mm-diameter wells.

TABLE 1. Parameters describing effects of nicotine exposure on nAChR numbers and function

<table>
<thead>
<tr>
<th>Cell type and nAChR subtype</th>
<th>Measure</th>
<th>$\tau$ (hr)</th>
<th>Parameter</th>
<th>$EC_{50}/IC_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE671/RD (alpha-1 beta-1 gamma delta)</td>
<td>Intracellular Carb-sensitive I-Bgt binding</td>
<td>0.69/26</td>
<td>85/340</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intracellular Carb-insensitive I-Bgt binding</td>
<td>0.69/26</td>
<td>11/46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell surface Carb-sensitive I-Bgt binding</td>
<td>0.035/6.9/14</td>
<td>(50)/50/140</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell surface Carb-insensitive I-Bgt binding</td>
<td>0.035/0.34/23</td>
<td>(60)/80/50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Membrane [3H]ACh binding</td>
<td>0.36/26</td>
<td>60/360</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Membrane Carb-sensitive I-Bgt binding</td>
<td>0.36/26</td>
<td>140/290</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Membrane Carb-insensitive I-Bgt binding</td>
<td>0.36/26</td>
<td>40/340</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Membrane Carb-sensitive I-Bgt binding</td>
<td>0.36/26</td>
<td>88/440</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Membrane Carb-insensitive I-Bgt binding</td>
<td>0.36/26</td>
<td>0/58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Function</td>
<td>0.016/0.32</td>
<td>(45)/55</td>
<td>(0.79)</td>
</tr>
<tr>
<td></td>
<td>Membrane [3H]ACh binding</td>
<td>23</td>
<td>580</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Function</td>
<td>0.0027/0.12</td>
<td>(56)/26</td>
<td>(5.2)</td>
</tr>
<tr>
<td>SH-SY5Y (alpha-3 beta-4)</td>
<td>Intracellular I-Bgt binding</td>
<td>0.14/36</td>
<td>23/240</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell surface I-Bgt binding</td>
<td>1.1/90</td>
<td>270/770</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Membrane I-Bgt binding</td>
<td>6.9</td>
<td>8/100</td>
<td></td>
</tr>
<tr>
<td>BC3H-1 (alpha-1 beta-1 gamma delta)</td>
<td>Membrane I-Bgt binding</td>
<td>6.9</td>
<td>8/100</td>
<td></td>
</tr>
</tbody>
</table>
and maximal up-regulation occurs at concentrations of 1 mM or higher. Thus, at smokers' doses of nicotine, we predict that putative physiological effects mediated via nAChR up-regulation would be dominated by effects on alpha-4 beta-2 nAChRs in the brain, whereas any up-regulation-dependent effects at the nerve-muscle junction or in autonomic ganglia would be comparatively smaller.

The magnitude of nAChR numerical up-regulation varies across nAChR subtypes. The highest degree of up-regulation (to 700–900% of control levels) occurs for human ganglionic alpha-3 beta-4 nAChRs in total membrane fractions detected based on specific [3H]ACh binding. Numbers of muscle-type nAChRs in total membrane fractions measured by [3H]ACh binding were up-regulated to 500% to 600% of control levels. Up-regulation of alpha-7 nAChRs (to 250% of control levels) measured using I-Bgt binding sites in total membrane fractions was comparably smaller but still like the maximal level measured using I-Bgt binding sites in total membrane fractions measured by [3H]ACh binding were up-regulated to 500% to 600% of control levels. Up-regulation of alpha-7 nAChRs (to 250% of control levels) measured using I-Bgt binding sites in total membrane fractions was comparably smaller but still like the maximal level of up-regulation seen for alpha-4 beta-2 nAChRs. However, caveats in these analyses, which sound notes of caution in interpretation of binding assay or autoradiographic analysis results conducted using intact animals or membrane preparations from brain, other tissues or cultured cells, come from studies of I-Bgt binding subclasses from TE671/RD cells. First, there is a large intracellular pool (~75% of the total) of I-Bgt binding sites in TE671/RD cells, as well as a numerically smaller pool of cell surface I-Bgt binding sites. The current results show that effects of nicotine exposure on these different pools of sites differ, possibly compounded by movement of sites between intracellular and cell surface pools. Moreover, the existence in intracellular pools and on the cell surface of I-Bgt binding sites that differ in their sensitivity to blockade by small drugs such as Carb further identifies potential nAChR subclasses, which again display differing sensitivities to effects of nicotine exposure. Interexperimental variability in magnitudes of nAChR up-regulation, aside from complicating quantitative analysis of the results, complicated comparisons of effects of nicotine on these sites (and on [3H]ACh binding sites) unless all analyses were done using the same experimental material. These findings add layers of complexity to analysis of nicotine's effects and suggest that future studies will need to consider sample origin and processing methods in experimental design as well as microheterogeneity of, subcellular distribution of and interconversions between pools of radioligand binding sites.

Nevertheless, several important conclusions derive from these studies of TE671/RD cell radioligand binding sites. First, time-dependent effects of nicotine exposure on Carb- and Carb-insensitive I-Bgt binding sites in intracellular pools (and, as a consequence of representing an admixture of Carb- plus Carb-insensitive sites, total I-Bgt binding sites) are well fit by the same rate constants (fig. 2), although the magnitude of the up-regulatory effect is larger for Carb-sensitive sites for both the fast (k2 = 1/hr; 85% vs. 11%) and slow (k1 = .03/hr; 341% vs. 46%) processes. Second, a slow (τs ~ 20 hr) process of up-regulation also describes increases in both Carb- and Carb-insensitive I-Bgt binding sites on the cell surface, although effects on Carb-sensitive sites are of larger magnitude (table 1). This suggests that a new equilibrium becomes established between cell surface and intracellular sites once the slower phase of up-regulation nears completion. Third, it can be inferred that there may be complex relationships between the transient down-regulation of cell surface sites (which evolves over ~30 min and therefore does not simply reflect blockade of I-Bgt binding by persistently bound nicotine), the fast phase of cell surface site up-regulation that essentially compensates for the transient loss of binding sites (but with different rates for Carb- and Carb-insensitive sites) and the fast rate of up-regulation of intracellular sites that cannot simply represent a conversion of nAChRs from low- to high-affinity agonist binding states as has been suggested for alpha-4 beta-2 nAChRs (Lippielio et al., 1987; Bhat et al., 1994; this is because these sites are detected based on I-Bgt binding rather than agonist binding). Further and more detailed studies of the fast phases of changes in nAChR numbers and distribution are planned.

Another important observation is that kinetics, dose dependency and magnitude of nicotine-induced up-regulation of muscle-type nAChRs are about the same whether measured using [3H]ACh or Carb-sensitive I-Bgt binding assays in TE671/RD cells when studies are done using the same total membrane preparations. This is yet another indication that both assays are measuring the same entity.

The current findings suggest that the faster process (τf ~ 0.4 hr) of up-regulation of Carb-sensitive I-Bgt binding sites has the same magnitude (~100% increase) as the process induced at lower nicotine concentrations (EC50 ~ 1.3 μM), whereas the slower up-regulatory process (τs ~ 1 day) has the same magnitude (~400%) as the process induced at higher nicotine concentrations (EC50 ~ 800 μM). Up-regulation of [3H]ACh binding sites representing alpha-3 beta-4 nAChRs on SH-SY5Y cells occurred as an apparently monophasic process characterized by a half-time of ~23 hr but had a two-phase nicotine dose dependence with EC50 values of 10 and 300 μM, respectively, producing minor and major increases in [3H]ACh binding levels and the only indication from the current study that there could be microheterogeneity in these sites. Up-regulation of surface or intracellular I-Bgt binding sites representing different subcellular pools of alpha-7 nAChRs on SH-SY5Y cells occurred as biphasic processes dominated by the slow up-regulation of intracellular sites characterized by a half-time of ~1.5 days. Nevertheless, and perhaps because of the dominance of this slow up-regulatory process, a single EC50 value of ~800 μM accounted for the dose dependence of nicotine-induced up-regulation of total membrane I-Bgt binding sites in SH-SY5Y cells. Collectively, these findings suggest the hypothesis that slow processes of nAChR up-regulation are induced via low affinity interactions of nicotine with its target or targets for muscle-type, alpha-3 beta-4 and alpha-7 nAChRs. Minor and faster phases of up-regulation are hypothesized to occur via processes induced at lower concentrations of nicotine.

Although numbers of cell surface I-Bgt binding sites corresponding to alpha-1 beta-1 gamma delta nAChRs on TE671/RD cells or alpha-7 nAChRs on SH-SY5Y cells eventually increased as nicotine exposure periods were lengthened, both classes of cell surface nAChRs were transiently down-regulated early after nicotine exposure. The kinetics for this down-regulation was too slow to reflect a simple blockade of nAChR radioligand binding sites, suggesting that this down-regulation might contribute to effects of prolonged nicotine exposure on nAChR function.

Northern blot analyses indicate that there is no significant change in levels of mRNA coding for any nAChR subunit (alpha-1, beta-1, gamma or delta) in TE671/RD cells; alpha-3,
alpha-5, alpha-7, beta-2, or beta-4 in SH-SY5Y cells; rat alpha-4 or beta-2 in cortical neurons in studies described by Bencherif et al., 1995a) either transiently or persistently during nicotine treatment or over a range of nicotine doses. This finding is surprising given that nicotine induces up-regulation of all nAChR subtypes tested to date, and such effects could have been neatly explained by actions at the nuclear level. Nevertheless, we conclude that changes in mRNA levels or nAChR subunit gene transcriptional activity are not involved in the nicotine-induced up-regulation or persistent inactivation of nAChRs for any combination of cell type and nAChR subtype. The need to focus on post-transcriptional processes toward identification of mechanisms involved in nAChR up-regulation is now clear, in part also because studies using M10 cells indicate that up-regulation occurs even when nAChR subunits are expressed from artificial promoters (Peng et al., 1994; Bencherif et al., 1995a).

The current findings also demonstrate that there is not even a transient effect of nicotine on nAChR subunit transcript levels; relevant data reported elsewhere to date used quantitative in situ hybridization and found no change in mouse brain nAChR alpha-2, alpha-3, alpha-4, alpha-5 or beta-2 subunit mRNA levels after 10 days of chronic nicotine treatment (Marks et al., 1992; Pauly et al., 1996) or used Northern analysis and found no change in M10 cell or rat primary neuronal culture alpha-4 or beta-2 subunit mRNA levels after 2 to 3 days of nicotine exposure (Peng et al., 1994; Bencherif et al., 1995a), but a more detailed temporal study had not been done until our study. Finally, our results extend these observations to more nAChR subunit types and discount the possibility that up-regulation in numbers of heterooligomeric nAChR requires increased mRNA levels for only one nAChR subunit, expression of which may limit assembly and expression of the multi-subunit complex. It does not seem that nicotine treatment induces changes in nAChR subunit composition based on measures of mRNA levels, but protein chemical work to extend the level of this assessment should be done.

After earlier studies done by us and others (see overviews in Lukas, 1991; Lukas et al., 1996), we demonstrate that chronic nicotine exposure induces “persistent inactivation” (which is operationally, and we propose mechanistically, distinct from “desensitization”) of human muscle-type and ganglionic alpha-3 beta-4 nAChRs. Across these subtypes, persistent inactivation of alpha-3 beta-4 nAChRs occurs more rapidly for both fast and slow processes (τ ~ 0.16 and 7.2 min, respectively, compared with τt ~ 1.0 and 19 min, respectively, for muscle-type nAChRs) but is slightly less complete and requires higher concentrations of nicotine during a 1-hr exposure (~5 μM IC50 for alpha-3 beta-4 nAChRs compared with ~800 nM IC50 for alpha-1 beta-1 gamma delta nAChRs). Studies in progress are assessing whether a faster recovery from persistent inactivation, as we observed in PC12 cells (Lukas, 1991), might account for some of these differences. Nevertheless, these findings demonstrate that persistent inactivation occurs for all nAChR subtypes tested to date (see references cited in Lukas, 1991; Lukas et al., 1996, and Peng et al., 1994; Hsu et al., 1996, for other examples) but with different rates and nicotine dose dependencies.

The potencies and the rates with which nicotine induces persistent functional inactivation of diverse nAChR subtypes in vitro may be relevant to sequences and extents of nicotine effects on nAChRs and nervous system function in smokers or other tobacco users. For example, 20% to 50% persistent inactivation occurs for alpha-3 beta-4 nAChRs and muscle-type nAChRs over biologically relevant ranges of nicotine exposure (0.1–1 μM in plasma in humans or nicotine-treated rats; 3-fold higher in brain tissue; Rowell and Li, 1997), and greater effects are seen at concentrations of nicotine that are functionally equivalent to doses of acetylcholine found (0.1–1 mM), at least transiently, at active cholinergic synaptic clefts. Thus, the current findings suggest that there should be some effects of chronic nicotine exposure in smokers on neuromuscular junction and autonomic function, simply based on nicotine-induced persistent inactivation of muscle-type and ganglionic nAChRs. Temporal studies indicate that effects on autonomic nAChR function would occur more rapidly, well within the time taken by a typical smoker to consume a cigarette, whereas effects on muscle-type nAChRs would be slower to develop. However, nicotine induces maximal up-regulation of alpha-4 beta-2 nAChRs at doses (1–5 μM) much lower than those needed to maximally induce up-regulation of muscle-type or alpha-3 beta-4 nAChRs (~1 mM; compare Peng et al., 1994; and Bencherif et al., 1995a, with this study). Moreover, significant persistent inactivation of muscle-type or alpha-3 beta-4 nAChRs occurs at doses of nicotine (~300 nM) found in the plasma of smokers and much lower than those needed to induce maximal up-regulation of the corresponding binding sites. It is reasonable, then, to predict that persistent inactivation of alpha-4 beta-2 nAChRs will occur at concentrations of nicotine of <1 μM (Lukas et al., 1996), as has been substantiated by findings of Hsu et al. (1996). Extrapolating further, it is hypothesized that physiological effects of chronic nicotine exposure will be dominated by persistent inactivation of central (alpha-4 beta-2- or possibly other) nAChRs (Lukas et al., 1996). Sharp et al. (1987) and Hulihan-Giblin et al. (1990) provided findings indicating that persistent inactivation of at least some nicotine responses occurs in vivo.

Are there any causal or mechanistic relationships between nicotine-induced up-regulation and persistent inactivation? Significantly, the time course for muscle-type or alpha-3 beta-4 nAChR up-regulation is much longer than the time course for persistent inactivation of the same nAChR subtype on chronic nicotine treatment. τ for the predominant component of nAChR up-regulation is of the order of 1 day rather than the minutes characteristic of τ for persistent inactivation, and numbers of nAChRs continue to climb for as long as 3 days of exposure to nicotine in some cases, long after persistent inactivation is maximal. On the other hand, it is possible that up-regulation represents some sort of compensatory response to the loss of functional nAChRs and that more time is required before a new equilibrium is reached in levels of intracellular and cell surface nAChRs due to the compensatory response than is needed to inactivate nAChRs. Moreover, the rate of the ~50% loss of muscle-type nAChRs from the cell surface after nicotine treatment (τ ~ 2 min) is within the range of rates of loss of function due to persistent inactivation (τ ~ 1 min for about half of the functional loss; τ ~ 19 min for the other half of the functional loss). Functional loss persists after days of nicotine exposure even when there is more than a compensatory up-regulation of cell surface (or intracellular) I-Bgt binding sites, but any new receptors that would appear on the cell surface would undergo the
process leading to initial loss of function as soon as they became exposed to nicotine in the medium if they were not already affected by intracellular nicotine. Hence, on their own, temporal studies do not suggest, nor do they disprove, that there are relationships between up-regulation and persistent inactivation.

At first glance, doses of nicotine required to induce persistent inactivation seem to be much lower than those needed to induce up-regulation. However, closer inspection indicates that concentrations of nicotine required to induce half-maximal persistent inactivation (~800 nM for muscle-type nAChRs and ~5 μM for alpha-3 beta-4 nAChRs; table 1) are similar to concentrations of nicotine required to half-maximally induce the minor phase (and possibly more rapid in onset) of nAChR up-regulation (~1.5 μM for muscle-type nAChRs and ~6 μM for alpha-3 beta-4 nAChRs). Treatment at still higher concentrations of nicotine induces a more substantial up-regulation of either alpha-1 beta-1 gamma delta nAChRs (~800 μM EC_{50}) or alpha-3 beta-4 nAChRs (~480 μM EC_{50}) that dominates those dose-response profiles and occurs for muscle-type nAChRs at nicotine doses far in excess of those needed (~100 μM) to produce maximal persistent inactivation. However, persistent inactivation of alpha-3 beta-4 nAChRs is still increasing even at 1 mM nicotine. Thus, on their own and as performed in this study (examining effects on up-regulation at 48 hr of drug treatment and on function at 1 hr of drug treatment), nicotine dose studies are not definitively for or against putative causal or mechanistic relationships between persistent inactivation and up-regulation.

In one sense, discussion about whether mechanisms of nicotine-induced up-regulation and persistent inactivation are related is a semantic issue. Clearly, some form of interaction of nAChRs with nicotine is likely to precipitate both effects, meaning that there might be some common steps in mechanisms for both processes. For example, it could be envisioned that nicotine binding to a site in the surface nAChR channel could promote a persistent functional block, whereas binding of nicotine to the same site on an intracellular subunit in an incompletely assembled nAChR could promote subunit assembly rather than degradation (perhaps by shielding a degradation signal sequence; Blount et al., 1990) leading to an increase in numbers of nAChR radioligand binding sites. However, downstream from nicotine binding, these two mechanisms are clearly different, and these distinctions would be even more clear if the sites of initial nicotine action on nAChRs can be distinguished. Further studies investigating dose/time dependencies in greater detail and using pharmacological approaches and drug pulse-chase protocols are in progress to determine whether there is some way to dissect mechanistic relationships between up-regulation and persistent inactivation. Among studies yet to be done are those investigating the nicotine dose dependence of transient down-regulation of cell surface muscle-type nAChRs to determine whether its IC_{50} matches with the ~800 nM IC_{50} for induction of persistent inactivation. Assays for cell surface alpha-3 beta-4 nAChRs need to be developed. Functional studies of alpha-7 nAChRs are needed before relationships to transient down-regulation of cell surface I-Bgt binding sites and later up-regulation of those and intracellular sites in SH-SY5Y cells can be evaluated.

Another prediction made in closing based on the current findings is that physiological effects of chronic nicotine exposure could be dominated by induction of persistent inactivation rather than activation of nAChRs. If so, then habitual use of tobacco products may be initiated and/or maintained to quiet potentially overactive nicotinic cholinergic signaling that might produce anxiety, depression, inattention and compromised cognitive function, thereby explaining the perceived beneficial effects of long-term nicotine exposure. The slowly evolving character of persistent inactivation offers an attractive explanation for the development of nicotine dependence and tolerance as well as the manifestation of unpleasant effects associated with nicotine withdrawal as nAChR function begins to return in the abstinence subject. Further work on effects of chronic nicotine exposure on function of the nAChR subtype found in pleasure/reward centers in the brain are warranted to ascertain whether activation or persistent inactivation of circuits containing those nAChRs occurs.

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Send reprint requests to: Dr. Ronald J. Lukas, Division of Neurobiology, Barrow Neurological Institute, 350 West Thomas Road, Phoenix, Arizona 85013. E-mail: rlukas@mha.chw.edu