

Differential Regulation of Nicotinic Acetylcholine Receptors in PC12 Cells by Nicotine and Nerve Growth Factor

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ABSTRACT

Neuronal nicotinic receptors in PC12 cells were measured by binding with [³H]epibatidine and in functional studies with agonist-stimulated ⁸⁶Rb⁺ efflux and [³H]norepinephrine release assays. Two subtypes of receptors labeled by [³H]epibatidine were found: one that was increased about 4-fold in cells grown for 2 to 4 days in the presence of nicotine and one that was increased 5-fold in cells grown for 2 to 4 days in the presence of nerve growth factor (NGF). The actions of the two treatments were superadditive, resulting in approximately a 13-fold increase in binding sites in cells grown in the combination of the two treatments. The pharmacology of the binding sites in the nicotine- and NGF-treated cells was compared with the pharmacology of defined $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nicotinic acetylcholine receptor (nAChR) subtypes heterologously expressed in human embryonic kidney 293 cells. Nicotine treatment predominantly increased a receptor with characteristics of an $\alpha 3\beta 2$ subtype,

whereas the NGF treatment exclusively increased a receptor with characteristics of an $\alpha 3\beta 4$ subtype. Nicotinic receptor-mediated function measured with the ⁸⁶Rb⁺ efflux assay was evident only in the NGF-treated cells, and it had a pharmacological profile that was, again, nearly identical to that of the heterologously expressed $\alpha 3\beta 4$ receptor subtype. Receptor function measured with the [³H]norepinephrine release assay was measurable in both nicotine-treated and NGF-treated cells; however, cytosine-stimulated [³H]norepinephrine release indicated that nicotine treatment increased an nAChR containing $\beta 2$ subunits, whereas NGF increased a receptor containing $\beta 4$ subunits. NGF treatment increased mRNA only for $\beta 4$ subunits in these cells, whereas nicotine treatment did not affect mRNA for any of the subunits measured. After withdrawal of the treatments, the receptors increased by nicotine were much less stable than those increased by NGF.

Neuronal nicotinic acetylcholine receptors (nAChRs) are found throughout the central nervous system as well as in autonomic ganglia and the adrenal gland, where they mediate cholinergic neurotransmission critical to the functions of the autonomic nervous system. These receptors are composed of two types of subunits, α and β . To date, nine neuronal α subunit genes ($\alpha 2$ – $\alpha 10$) and three β subunit genes ($\beta 2$ – $\beta 4$) have been found in vertebrate tissues. Different combinations of subunits compose subtypes of nicotinic receptors, all of which are ligand-gated cation channels that conduct Na⁺, K⁺, and Ca²⁺ when activated by the endogenous neurotransmitter acetylcholine or by nicotine or other nicotinic agonists. However, the different receptor subtypes have distinguishing

biophysical and/or pharmacological properties, including channel conductances, rates of desensitization and recovery, and sensitivity to drugs (for reviews, see Sargent, 1993; Colquhoun and Patrick, 1997; Lukas, 1998).

Certain subtypes of nAChRs are strongly regulated by nicotine. Previous studies have shown that chronic administration of nicotine increases nicotinic receptor binding sites in rat and mouse brain (Schwartz and Kellar, 1983; Marks et al., 1983; for review, see Gentry and Lukas, 2002), and a similar increase is found in human brains from people who smoked tobacco (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999). The $\alpha 4\beta 2$ nAChR subtype in rat brain is increased by chronic administration of nicotine (Flores et al., 1992), but not all subtypes are similarly affected, indicating that at the nicotine concentrations reached in vivo nAChR subtypes are differentially affected (Flores et al., 1997; Dávila-García et al., 2003).

In addition to native tissues, several model cell systems are useful for studying nAChRs. These include heterologous expression systems in *Xenopus* oocytes and transfected mammalian cell lines, as well as naturally occurring tumor-de-

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; NGF, nerve growth factor; A-85380, 3-(2(S)-azetidinylmethoxy)pyridine; EB, (±)-epibatidine; NE, norepinephrine; DH β E, dihydro- β -erythroidine; DMPP, 1,1-dimethyl-4-phenylpiperazinium; HEK, human embryonic kidney; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

rived cell lines. One of these is the PC12 cell line, which was developed from a transplantable rat adrenal pheochromocytoma by Greene and Tischler (1976). These cells exhibit several key physiological features of adrenal chromaffin cells and sympathetic neurons, such as synthesis, storage, and release of catecholamines (Greene and Tischler, 1976; Greene and Reine, 1977; Baizer and Weiner, 1985) and depolarization in response to acetylcholine (Dichter et al., 1977). Moreover, like sympathetic neurons, these cells express receptors for nerve growth factor (NGF), and in response to this neurotrophin they cease cell division and differentiate into sympathetic-like neurons, with a flattened cell body and extension of neurites (Greene and Tischler, 1976). Because PC12 cells originate from a native neuronal tissue, they provide a particularly good model for studying the regulation of nAChRs. Furthermore, these cells express mRNA encoding $\alpha 3$, $\alpha 5$, $\alpha 7$, $\beta 2$, and $\beta 4$ subunits of nAChRs (Rogers et al., 1992; Henderson et al., 1994; Takahashi et al., 1999), indicating that they have the capacity to express more than one subtype of receptor. Thus, it is possible to study the regulation of more than one nAChR before and after differentiation.

NGF treatment of PC12 cells increases nicotine-stimulated responses, including catecholamine release (Greene and Reine, 1977; Baizer and Weiner, 1985), ion flux (Amy and Bennett, 1983; Whiting et al., 1987), and whole cell currents (Ifune and Steinbach, 1990; Henderson et al., 1994). Furthermore, NGF has also been reported to increase (Rogers et al., 1992; Henderson et al., 1994; Hu et al., 1994; Takahashi et al., 1999; Nakayama et al., 2000) as well as decrease (Rogers et al., 1992) mRNA encoding certain nAChR subunits in PC12 cells. In addition, both nicotine and NGF treatment have been reported to increase [^3H]nicotine binding sites in these cells (Madhok and Sharp, 1992; Madhok et al., 1995). In the studies reported here, we compared the binding of [^3H]epibatidine ([^3H]EB), [^3H]cytisine, and [^3H]nicotine in PC12 cells and then investigated the effects of nicotine and NGF treatment alone and in combination on nAChR binding sites labeled by [^3H]EB in PC12 cells. We determined whether these two treatments affected the same or different receptor subtypes, characterized these subtypes with respect to their pharmacology, and determined how each treatment affected receptor functions. We then examined the effects of these treatments on mRNAs coding for nAChR subunits, and on the stability of the increased receptor binding sites after removal of the treatments.

Materials and Methods

Materials. Tissue culture medium and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum and horse serum were obtained from BioSource International (Camarillo, CA). [^3H]EB, [^3H](–)-nicotine, [^3H]cytisine HCl, and ^{86}Rb -chloride ($^{86}\text{Rb}^+$) were purchased from PerkinElmer Life Sciences (Boston, MA). [^{125}I]A-85380 was a generous gift from Drs. John Musachio and Hong Fan (Johns Hopkins University, Baltimore, MD). [^3H]Noradrenaline ([^3H]NE), [α - ^{32}P]CTP, and [γ - ^{32}P]ATP were purchased from Amersham Biosciences Inc. (Piscataway, NJ). Human recombinant NGF- β , nicotine bitartrate, and all other chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Cell Culture. PC12 cells were obtained from American Type Culture Collection (Manassas, VA). Frequent freeze downs of cells

were made to use cells of roughly the same passage number (10–30) for all experiments. Cells were grown in pH 7.4 growth medium consisting of Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin and maintained at 37°C and 7.5% CO_2 in a humidified incubator. Cells were split approximately every 4 to 5 days or when confluent. Cells were plated onto poly-D-lysine-coated flasks or 24-well plates and allowed to attach to plates overnight in a humidified incubator at 37°C and with 7.5% CO_2 . Cells in culture were treated with nicotine (100 μM), carbachol (1,000 μM), and/or NGF (50 ng/ml) for 2 to 120 h, as indicated. These concentrations were initially based on previous studies demonstrating that this concentration of nicotine increases heterologously expressed nAChRs in cell lines (Wang et al., 1998; Meyer et al., 2001) and that this concentration of NGF differentiates PC12 cells (Greene and Tischler, 1976; Greene and Rein, 1977), as well as our own preliminary studies with NGF. Untreated cells grown in parallel served as controls in all studies.

Receptor Binding. Radioligand binding experiments were carried out on untreated PC12 cells or cells cultured in the presence of nicotine, NGF, or the combination of nicotine and NGF for different periods of time. Cultured PC12 cells were collected from flasks by gentle scraping with 50 mM cold Tris-HCl buffer, pH 7.4, and centrifuged at 1,000 rpm (180g). The buffer was then decanted and the cells were stored at –80°C until used. To prepare tissue for binding, cells were resuspended in 50 mM Tris-HCl buffer, pH 7.4, homogenized with a Polytron homogenizer, and centrifuged at 35,000g for 10 min. The pellets were washed once in fresh buffer and then centrifuged again at 35,000g for 10 min. The membrane homogenate pellets were resuspended in 50 mM Tris buffer. Tissue aliquots (equivalent to ~200–300 μg of protein) were incubated with [^3H]EB or [^{125}I]A-85380 at 24°C for 2 h or 4 h, or with [^3H]cytisine or [^3H]nicotine in an ice-water bath for 2 h in a final assay volume of 250 μl or 500 μl . The concentrations of radioligands are indicated in the figure legends. Bound and free ligand were separated by vacuum filtration through Whatman GF/C filters treated with 0.5% polyethylenimine. The filter-retained radioactivity was measured by liquid scintillation counting. Nonspecific binding was determined by incubating membrane homogenates in the presence of 300 μM nicotine. Specific binding was defined as the difference between total binding and nonspecific binding. Membrane protein was measured by the bicinchoninic acid protein analysis method (Pierce Chemical, Rockford, IL).

$^{86}\text{Rb}^+$ Efflux Assay. PC12 cells were plated at a density that would allow them to be 80 to 90% confluent on the day of the assay. Nicotine (100 μM), carbachol (1,000 μM), and/or NGF (50 ng/ml) was added to the cells 1 day after plating, and the incubation was continued for 48 h. On the day of an assay, $^{86}\text{RbCl}$ (1 $\mu\text{Ci}/\text{well}$) was added to the cells for 4 h at 37°C and 7.5% CO_2 (still in the presence of the nicotinic agonist and/or NGF). The $^{86}\text{RbCl}$ media were then aspirated and the cells were washed with HEPES buffer (15 mM HEPES, 140 mM NaCl, 2 mM KCl, 1 mM MgSO_4 , 1.8 mM CaCl_2 , 11 mM glucose, pH 7.4; 22°C, 1 ml/well) either three times over 6 min or four times over 60 min, as indicated. One milliliter of buffer with or without nicotinic agonists at the indicated concentration was then added to each well for 2 min. In some experiments, the nAChR channel blocker mecamylamine was added to the cells with the agonists. The $^{86}\text{Rb}^+$ released from the cells into the media was then collected and counted in a liquid scintillation counter. Cells were then lysed by adding 1 ml of 0.1 M NaOH to each well with mild shaking for at least 1 h before collecting the lysate and counting the amount of radioactivity left inside the cells after the efflux assay. The total amount of $^{86}\text{Rb}^+$ loaded in the cells in each well was calculated by adding the amount of $^{86}\text{Rb}^+$ released during efflux to the amount in the cell lysate. $^{86}\text{Rb}^+$ efflux was calculated as a percentage of the total amount of $^{86}\text{Rb}^+$ loaded per well (fractional release). Agonist-stimulated release was then expressed as a percentage of the basal

value, which was defined as the amount of $^{86}\text{Rb}^+$ released from cells in the absence of agonist.

[^3H]Norepinephrine Release. Cells were plated and allowed to attach overnight, as described above. The next day, with the cells 80 to 90% confluent, either nicotine (100 μM) and/or NGF (50 ng/ml) was added and the cells were incubated for 48 h. To prepare the cells for the [^3H]NE release assay, the medium was replaced with 0.5 ml of fresh culture medium containing 10 to 15 nM [^3H]NE, and the cells were incubated for 2 h in a humidified incubator at 37°C and 7.5% CO_2 . The media containing [^3H]NE was then aspirated from the wells and the cells were washed twice (1 ml/well) for 1 min with a modified Krebs' buffer (118 mM NaCl, 5 mM KCl, 2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 24 mM NaHCO_3 , 11 mM dextrose, and 2.5 mM CaCl_2) at 37°C. The cells were then washed four times for 15 min each time with culture medium and then two more times for 1 min with buffer at 37°C. After aspirating the last wash, either Krebs' buffer alone was added to cells for 2 min to measure basal release, or buffer containing nicotine (100 μM), carbachol (1,000 μM), or cytisine (100 μM) was added for 2 min to measure agonist-stimulated release. In some experiments, mecamylamine (10 μM) was added along with nicotine. After the 2-min release period, the incubation buffer was collected from each well and the amount of radioactivity was counted in a scintillation counter. NaOH (0.1 N, 1 ml/well) was added to each well and the plates were gently shaken for at least 1 h to lyse the cells. The cell lysate was collected and counted in a scintillation counter. The total amount of [^3H]NE loaded into the cells in each well was determined by adding the amount of [^3H]NE from the cell lysate to the amount of [^3H]NE released into the medium in each well. The [^3H]NE released under basal and stimulating conditions was calculated as a percentage of [^3H]NE loaded (fractional release), and agonist stimulated release was then expressed as a percentage of basal release.

mRNA Measurements. mRNA was measured by an RNase protection assay. PC12 cells were treated in culture with nicotine (100 μM) and/or NGF (50 ng/ml) for up to 87 h, and total cellular RNA was isolated using RNA-STAT-60 (Tel-Test Inc., Friendswood, TX). DNA templates for antisense riboprobes were prepared as described previously (Xiao et al., 1998). Antisense riboprobes for the α_2 , α_3 , α_4 , α_5 , α_6 , β_2 , and β_4 nAChR subunits were generated from DNA templates using T7 RNA polymerase and [$\alpha\text{-}^{32}\text{P}$]CTP. The RNase protection assays were carried out using the RPA II kit (Ambion, Austin, TX). Total RNA (20 μg) from the PC12 cell samples was hybridized overnight at 42°C with the subunit riboprobes and a riboprobe for rat GAPDH, which was used as an internal and loading control. After hybridization, non-protected fragments were digested with a combination of RNase A and RNase T1 for 30 min at 37°C. The processed samples were loaded onto a 6% acrylamide sequencing gel on a BRL model S2 system for 2 to 3 h at 55 W. The size in number of bases of the full-length probes and the protected fragments of the probe were as follows: α_2 , 421 and 332; α_3 , 308 and 231; α_4 , 497 and 408; α_5 , 411 and 380; α_6 , 462 and 396; β_2 , 328 and 266; and β_4 , 258 and 174. The protected and nonprotected fragments were visualized on X-ray film. The amount of radioactivity in each nAChR subunit band was then measured with a PhosphorImager (Amersham Biosciences Inc.) and normalized to the signal in the GAPDH band in each sample.

Statistical Analysis. Data were analyzed using the GraphPad Prism software package (GraphPad Software Inc., San Diego, CA) and compared statistically by either one-way or two-way analysis of variance followed by Newman-Keuls multiple comparison test. Binding competition data were fit using GraphPad to both a one-site and a two-site model based on nonlinear least-squares regression analysis, and an F test was applied to determine the best model. Statistical analysis of superadditivity of B_{max} for nicotine and NGF treatments was by the propagation of errors method (Bevington, 1969).

Results

Differentiation of PC12 Cells by NGF but Not Nicotine. The morphology of PC12 cells grown for 87 h in the presence of nicotine (100 μM), NGF (50 ng/ml), or the combination of nicotine plus NGF is shown in Fig. 1. Untreated PC12 cells (Fig. 1A) continue to divide and do not differentiate under these culture conditions; thus, they have a round morphology and show no neurite extensions. Cells treated with nicotine (Fig. 1B) show no evidence of differentiation and their morphology is indistinguishable from untreated cells. In contrast, as first reported by Greene and Tischler (1976), cells treated with NGF (Fig. 1C) cease dividing and differentiate into more elongated cells with neurites extending toward one another. Finally, cells treated with the combination of NGF and nicotine (Fig. 1D) display the morphological characteristics of NGF-differentiated cells.

Receptor Binding Site Measurements in PC12 Cells. Radioligand binding to nAChRs with high affinity for agonists (non- α_7 receptors) was initially compared using [^3H]EB, [^3H]cytisine, and [^3H](–)-nicotine in membrane homogenates from PC12 cells. Only [^3H]EB proved to be a suitable ligand for these receptors in our studies. For example, as shown in Fig. 2, at a saturating concentration of [^3H]EB (2 nM), specific binding to nAChRs in PC12 cell membranes represented ~90% of the total binding; whereas in contrast, binding measurements with both [^3H]cytisine and [^3H]nicotine, even at concentrations well below their dissociation constants for the nAChRs in these cells (see below), resulted in a low percentage (<30%) of specific binding because of high levels of nonspecific binding (Fig. 2). At higher ligand concentrations, nonspecific binding increased more than specific binding, and the low signal-to-noise ratio made it virtually impossible to use [^3H]cytisine or [^3H]nicotine to measure nAChR binding sites in these PC12 cells.

[^3H]Epibatidine Binding to nAChRs in PC12 Cells. Treatment of PC12 cells with nicotine, NGF, or the combination of the two markedly increased [^3H]EB binding in a time-dependent manner (Fig. 3A). Nicotine's peak effect was

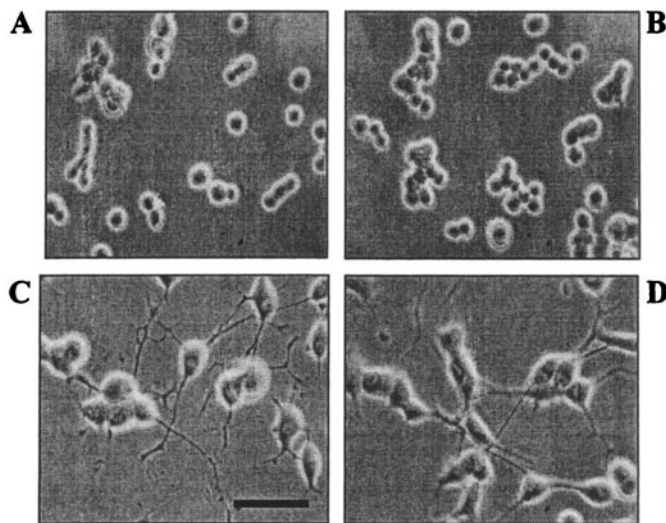


Fig. 1. Morphological differentiation of PC12 cells after treatment with nicotine, NGF or the combination of the two. Drugs were incubated with the cells in culture for 87 h. A, untreated cells. B, cells treated with nicotine (100 μM). C, cells treated with NGF (50 ng/ml). D, cells treated with the combination of nicotine and NGF. Scale bars, 50 μm .

seen within 17 h of its addition, whereas the peak effect of NGF was seen within about 37 h. The effect of treatment with the combination of nicotine and NGF on [3 H]EB binding seemed to be superadditive, and the peak effect occurred within 60 h (Fig. 3A). An additive or superadditive effect of nicotine and NGF could indicate that the two treatments increased a single nAChR subtype by different mechanisms

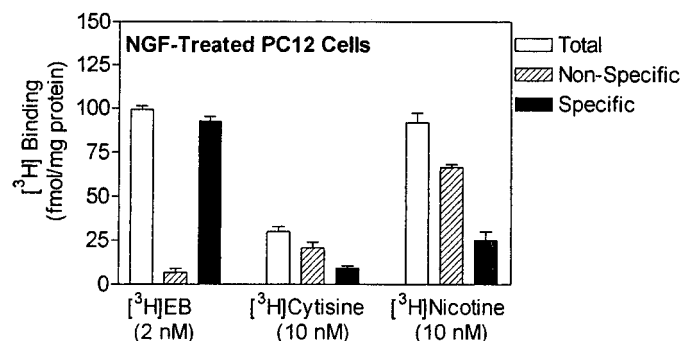


Fig. 2. Comparison of [3 H]epibatidine, [3 H]cytisine, and [3 H]nicotine binding to membrane homogenates from NGF treated PC12 cells. Receptor binding sites were measured with either [3 H]EB (2 nM), [3 H]cytisine (10 nM) or [3 H]nicotine (10 nM) in membrane homogenates from PC12 cells treated in culture with NGF (50 ng/ml) for 87 h. Binding assays were carried out as described under *Materials and Methods*. Open columns represent the total amount of binding, dashed columns represent non-specific binding measured in the presence of 300 μ M nicotine, and closed columns represent specific binding (total minus nonspecific). Data are the mean \pm S.E.M. from two independent experiments from separate cultures, each measured in duplicate.

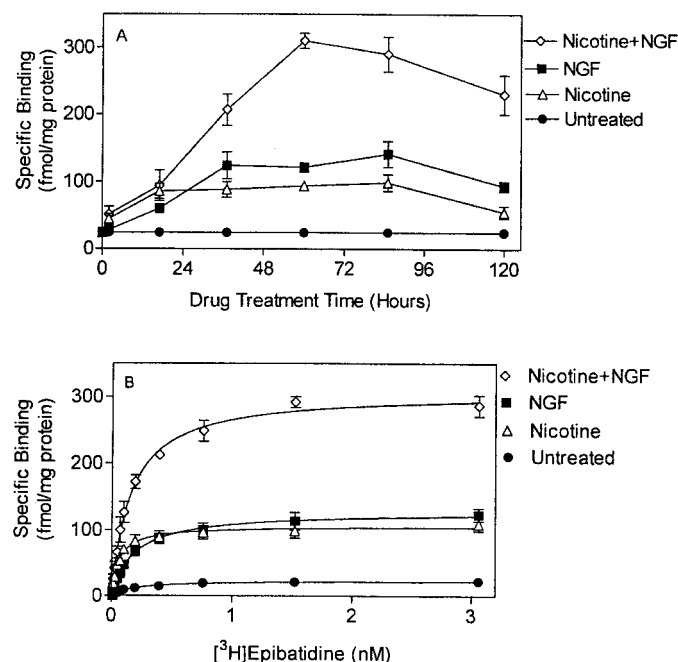


Fig. 3. The effect of treatment with nicotine, NGF or the combination of the two on [3 H]EB binding in membranes from PC12 cells. A, time course of effect. Specific binding with [3 H]EB (2 nM; 2-h incubation) was measured in membrane homogenates from untreated PC12 cells or cells treated for 2 to 120 h with nicotine (100 μ M), NGF (50 ng/ml), or the combination of the two. B, effects of treatment with nicotine, NGF, or the combination for 87 h on saturation binding in PC12 cell membrane homogenates. Specific binding was measured with [3 H]EB (6 pM–3 nM; 4-h incubation). Data are the mean \pm S.E.M. from three to four independent experiments. The data from these saturation binding studies were analyzed by nonlinear regression, and the K_d and B_{max} estimates are shown in Table 1.

or that [3 H]EB was labeling more than one nAChR subtype in these cells.

Saturation curves for [3 H]EB binding in PC12 cells grown in the absence or presence of nicotine, NGF or the combination of the two are shown in Fig. 3B, and a summary of the binding site density (B_{max}) and dissociation constants (K_d values) for [3 H]EB is given in Table 1. In untreated PC12 cells, the [3 H]EB binding site density was 24 fmol/mg protein and the K_d value was 0.27 nM. Treatment of the cells with nicotine for 87 h increased the density of binding sites by approximately 4-fold; in addition, the receptors in the nicotine-treated cells displayed higher affinity for [3 H]EB, as indicated by a significant decrease in the K_d value. Treatment of the cells with NGF increased the density of [3 H]EB binding sites by approximately 5-fold but did not significantly alter the K_d value. Treatment with nicotine and NGF in combination increased the density of sites by nearly 13-fold, which was significantly greater ($p < 0.001$) than the sum of the increases induced by the two treatments individually; the measured K_d value in cells treated with the combination was not significantly different from that in cells treated with NGF alone (Table 1).

The Pharmacology of nAChR Binding Sites in PC12 Cells. [3 H]EB has high affinity for all known heteromeric nAChRs, so it is unlikely to distinguish among them. Therefore, the pharmacological characteristics of the nAChR binding sites in membranes from PC12 cells grown in the absence or presence of nicotine, NGF, or the combination of the two were examined in binding competition assays against [3 H]EB. The competition curves for the nicotinic agonists A-85380, cytisine, nicotine, and carbachol, as well as the competitive antagonist dihydro- β -erythroidine (DH β E) are shown in Fig. 4, and the binding dissociation constants (K_i) derived from these curves are shown in Table 2. The rank order of apparent affinities for the drugs was not markedly different across the treatment groups. Thus, in each condition A-85380 was the most potent drug examined, carbachol was the least potent agonist, and the affinities of nicotine and cytisine were intermediate and similar to each other. The antagonist DH β E was generally 6- to 10-fold less potent than carbachol.

The K_i values for nicotine and cytisine in untreated and NGF-treated PC12 cells were ≥ 200 nM, whereas in cells treated with nicotine, the K_i values were 50 to 100 nM. These binding dissociation constants of 50 nM or higher explain why nicotine and cytisine are not very useful as radiolabeled ligands for the nAChRs in these cells. That is, at the high

TABLE 1

B_{max} and K_d values of [3 H]EB binding saturation curves from Fig. 3B. Data are presented as the mean \pm S.E.M. from four independent experiments.

| PC12 Cells | B_{max} fmol/mg protein | K_d nM |
|----------------|------------------------------|-------------------|
| Untreated | 24 \pm 1.8 | 0.27 \pm 0.04 |
| Nicotine | 105 \pm 11.7* | 0.07 \pm 0.01** |
| NGF | 129 \pm 12.3† | 0.21 \pm 0.01 |
| Nicotine + NGF | 310 \pm 18.6†‡ | 0.18 \pm 0.03 |

*, $p < 0.01$; †, $p < 0.001$; the B_{max} values in the treated groups were significantly greater than the value in the untreated group.

‡, $p < 0.001$; the B_{max} value of the group treated with the nicotine and NGF combination is significantly greater than the sum of the B_{max} values of the groups treated with nicotine and NGF alone.

** $p < 0.05$; the K_d value of the nicotine-treated group is significantly less than that of all other groups.

concentrations necessary to label more than a small fraction of the receptors, nonspecific binding would be very high, and the ratio of specific to nonspecific binding would be expected to be too low to provide an adequate signal, which is, in fact, what the data in Fig. 2 indicate.

The different treatments had clear and, in some cases, marked effects on the measured affinities of the receptors for certain drugs (Table 2) and on the shape and steepness of the inhibition curves (Fig. 4). Treatment of the cells with nicotine

resulted in a decrease in the K_i values (i.e., an increase in the apparent affinity) for all of the drugs examined (Table 2). This was most obvious for A-85380 (Fig. 4); thus, in membranes from untreated cells the A-85380 competition curve fit best to a model for two classes of binding sites, with one-third of the sites having a K_i value of 0.35 nM and two-thirds of the sites having a K_i value of 52 nM (Table 2). In contrast, in membranes from cells treated with nicotine the A-85380 competition curve was shifted to the left and fit best to a model for a single class of binding sites with a K_i value of 0.73 nM, which is similar to the value for the higher affinity site of the untreated cells (Table 2). In NGF-treated cells, the A-85380 competition curve also fit best to a model for a single class of binding sites, but in this case the K_i value was 69 nM, which is similar to the value for the lower affinity site of the untreated cells (Table 2). In cells treated with both nicotine and NGF, the A-85380 competition curve corresponded to a composite of the curves from the two individual treatments; thus, it fit a model for two classes of binding sites nearly equally divided between a high-affinity site with a K_i value of 1.2 nM and a lower affinity site with a K_i value of 87 nM (Fig. 4; Table 2).

Differences in the competition curves were also seen with the antagonist DH β E. In membranes from untreated cells, as well as from cells treated with NGF or the combination of nicotine and NGF, DH β E competition curves fit best to a single site with a K_i value between 20 and 50 μ M, indicating that most of the nAChRs in PC12 cells under these conditions have relatively low affinity for the antagonist. However, in membranes from cells treated with nicotine, the competition curve fit best to a model for two sites, in which 29% of the nAChRs had a relatively high affinity for DH β E (K_i of \sim 450 nM) and the remainder had a lower affinity, like that seen in untreated cells (Table 2).

The rank orders of binding affinities of these drugs for the nAChRs in PC12 cells across the treatment groups were similar; furthermore, as shown in Fig. 5, the affinities of the drugs at the receptors from both the nicotine-treated and the NGF-treated cells correlated highly ($r \geq 0.90$) with the affinities of these drugs at defined rat $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChR subtypes stably expressed in human embryonic kidney (HEK) 293 cells. However, the correlation in nicotine-treated cells was slightly higher with the $\alpha 3\beta 2$ receptors (Fig. 5A), whereas the correlation in NGF-treated cells was slightly higher with the $\alpha 3\beta 4$ receptors (Fig. 5B). More importantly, the lines of identity for these correlations indicated that the absolute values for the affinities of the receptors in nicotine-treated PC12 cells were nearly identical to those of the heterologously expressed $\alpha 3\beta 2$ receptor subtype, whereas the absolute values for the affinities of the receptors in the NGF-treated cells were nearly identical to those of the $\alpha 3\beta 4$ receptor subtype (Fig. 5, A and B).

[125 I]-A-85380 Binding Differentiates between nAChRs Increased by NGF and Nicotine. The data from Table 2 and Fig. 5 indicated that A-85380 has nearly 100-fold higher affinity in cells treated with nicotine than with NGF and, therefore, it distinguishes between the receptor subtypes differentially regulated by these treatments. This is consistent with previous data that indicated that A-85380 and [125 I]-A-85380 have much higher affinity for nAChRs containing $\beta 2$ subunits than those containing $\beta 4$ subunits (Mukhin et al., 2000; Perry et al., 2002; Xiao and Kellar, unpublished data). To test the hypothesis that

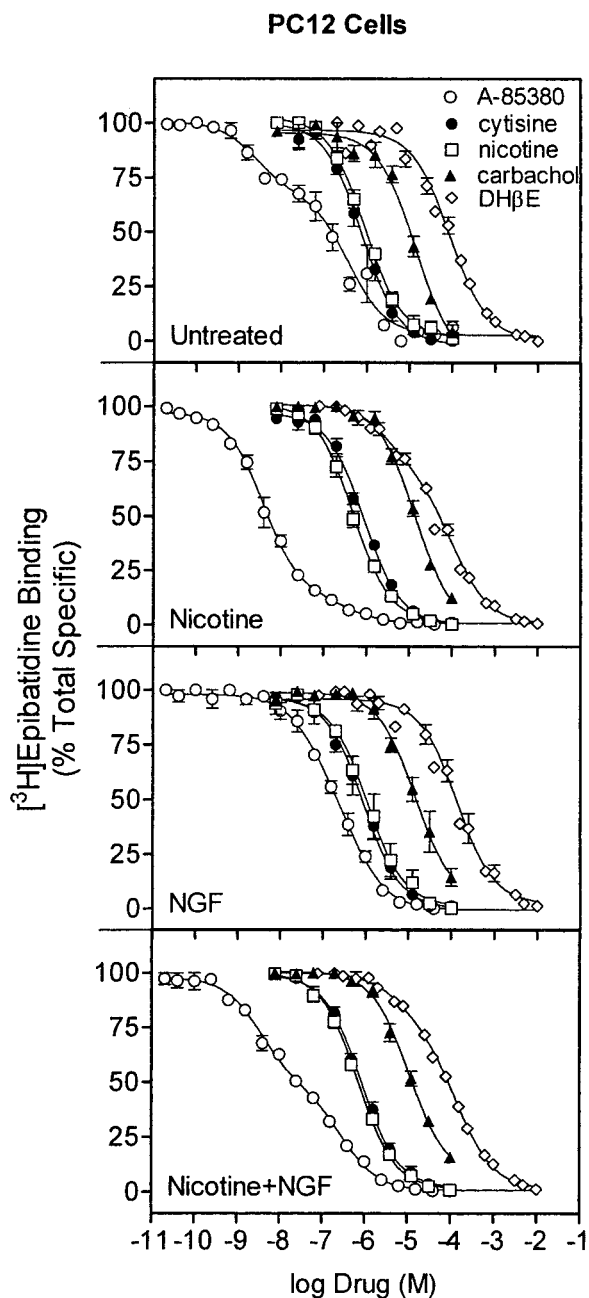


Fig. 4. The effects of nicotine, NGF, or the combination of the two on drug competition for nAChR binding sites in membrane homogenates from PC12 cells. Competition by drugs was measured in untreated PC12 cells or cells treated with nicotine (100 μ M), NGF (50 ng/ml), or the combination for 87 h. The concentration of [3 H]EB used for all experiments was \sim 500 pM. The drugs were added at the concentrations indicated and incubated for 4 h. Curves were fit for both one- and two-site competition curves. Data are the mean \pm S.E.M. from three independent assays. Values for K_i are given in Table 2.

nicotine and NGF predominantly affect different populations of nAChRs, we compared binding of [¹²⁵I]A-85380, which labels only those nAChRs containing $\beta 2$ subunits, to binding of [³H]EB, which labels both $\beta 2$ -containing and $\beta 4$ -containing receptors, in cells treated with nicotine, NGF, or the combination. As shown in Fig. 6, in untreated cells [¹²⁵I]A-85380 binding was ~66% of [³H]EB binding, suggesting that in these studies the untreated cells expressed ~66% $\alpha 3\beta 2^*$ and ~33% $\alpha 3\beta 4^*$ receptors (the * indicates that one or more other subunits could be part of the designated nAChR subtype). The analysis of untreated cells in Table 2, which was based on competition by A-85380 for [³H]EB binding sites, gave a value of ~33% for the $\alpha 3\beta 2^*$ subtype. This discrepancy represents a difference of only ~6 fmol/mg protein of the total density of ~20 fmol/mg protein in the untreated cells in these experiments, and it is probably caused by the imprecision inherent in the two different kinds of assays used.

In the nicotine-treated cells, the total number of nAChR binding sites labeled by [³H]EB was increased ~4-fold compared with untreated cells, whereas at the same time, [¹²⁵I]A-85380 binding also increased ~4-fold, and it was thus still ~64% of [³H]EB binding (Fig. 6). This result indicates that most (>60%) of the receptors increased by nicotine had high affinity for [¹²⁵I]A-85380, consistent with an $\alpha 3\beta 2^*$ receptor subtype. However, this analysis indicates that nicotine also increased the $\alpha 3\beta 4^*$ receptors.

In the NGF-treated cells, the total number of nAChR binding sites labeled by [³H]EB was increased more than 5-fold compared with untreated cells, whereas in contrast, the number of [¹²⁵I]A-85380 binding sites was virtually unchanged, indicating that NGF increased an $\alpha 3\beta 4^*$ receptor subtype exclusively. In cells treated with the combination of nicotine and NGF, the total number of nAChRs labeled by [³H]EB was increased approximately 14-fold, which was again greater than the sum of the increases elicited by the two treatments individually ($p < 0.01$). In contrast, the number of receptors labeled by [¹²⁵I]A-85380 was increased about 7-fold, to a level that was not significantly different from that seen after nicotine alone (Fig. 6).

The Agonist-Induced Increase of nAChRs Can Be Initiated at the Cell Surface. Nicotine may increase nAChRs through an action at the agonist binding site on the

extracellular region of the receptor or, because it is lipophilic and can cross cell membranes, it might act on intracellular targets to increase the receptors. To try to address the question of where agonists act to increase the receptors in PC12 cells, we treated cells with carbachol, a quaternary amine nicotinic agonist that does not cross cell membranes. As shown in Fig. 6, treatment of cells with carbachol for 48 h increased the number of nAChR binding sites labeled by [³H]EB and [¹²⁵I]A-85380 to at least the same extent as nicotine treatment. These data indicate that the agonist-induced increase in nAChRs can be initiated at the extracellular surface of the receptor and that it does not require a direct action at an intracellular target.

Functional Responses of nAChRs in Nicotine-Treated and NGF-Treated PC12 Cells as Measured by ⁸⁶Rb⁺ Efflux. The function of the nAChR in PC12 cells grown for 48 h in the absence or presence of nicotine, NGF, or the combination of the two was initially assessed by measuring ⁸⁶Rb⁺ efflux stimulated by four different nicotinic agonists, including cytosine, which discriminates between $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs in functional studies (Luetje and Patrick, 1991; Papke and Heinemann, 1994). As shown in Fig. 7A, nAChR function was not detected with this assay in untreated cells, nor in cells treated for 48 h with nicotine; however, in cells treated with NGF alone, function stimulated by each of the four agonists was clearly evident. The agonist-stimulated ⁸⁶Rb⁺ efflux in NGF-treated cells was completely blocked by the nAChR blocker mecamylamine (Fig. 7B).

Interestingly, in cells treated with the combination of nicotine and NGF, agonist-stimulated receptor function was not evident under these conditions (Fig. 7A). We thought it possible that under our standard cell washing conditions after the 48-h treatments, the absence of measurable nAChR function in cells grown in the presence of nicotine or the combination of nicotine and NGF was caused by desensitization of the receptors by residual nicotine. To test this possibility, the cells were washed for 60 min rather than the standard 6 min to more thoroughly remove the nicotine after the treatments and to allow the receptors more time to recover from any desensitization, if that were the case. After this 60-min wash-out, receptor function stimulated by each of the four agonists

TABLE 2

K_i values for nicotinic drugs in PC12 cells after the different treatments

The K_i values from competition binding curves shown in Fig. 4 were calculated using the Cheng-Prusoff equation and the K_d value for [³H]EB in each treatment group from Table 1. The concentration of [³H]EB used in these assays was 500 pM. Competition by nicotine, cytosine, and carbachol modeled to one site, whereas A-85380 and DH β E modeled to either one or two sites, depending on the treatment group. Where there were two sites, the fraction of high-affinity sites is reported in parentheses. Data are presented as the mean \pm S.E.M. from three independent experiments.

| Drugs | K_i | | | |
|---------------------------|-----------------------|---------------------|---------------------|---------------------|
| | Untreated | Nicotine | NGF | Nicotine + NGF |
| <i>nM</i> | | | | |
| One-site binding | | | | |
| Nicotine | 227 \pm 11 | 54 \pm 8 | 452 \pm 252 | 163 \pm 16 |
| Cytosine | 204 \pm 35 | 97 \pm 8 | 278 \pm 100 | 199 \pm 29 |
| Carbachol | 4,132 \pm 452 | 1,540 \pm 173 | 5,118 \pm 1,979 | 2,641 \pm 478 |
| One- and two-site binding | | | | |
| A-85380 | | | | |
| Site 1 | 0.35 \pm 0.07 (33%) | 0.73 \pm 0.04 | 69 \pm 7 | 1.2 \pm 0.5 (51%) |
| Site 2 | 52 \pm 11 | NA | NA | 87 \pm 17 |
| DH β E | | | | |
| Site 1 | 32,040 \pm 4,209 | 456 \pm 195 (29%) | 52,327 \pm 16,835 | 20,195 \pm 1,238 |
| Site 2 | NA | 12,589 \pm 2004 | NA | NA |

NA, data fit best to one binding site.

was, in fact, evident in cells treated with the combination of nicotine and NGF, as well as in cells treated with NGF alone (Fig. 7C). However, again no function was detected in cells treated with nicotine alone or in untreated cells (Fig. 7C). These results indicate that the 60-min washout procedure was sufficient to remove the nicotine from the cells treated with the combination of nicotine and NGF, allowing the function of the NGF-regulated receptors to emerge from nicotine desensitization.

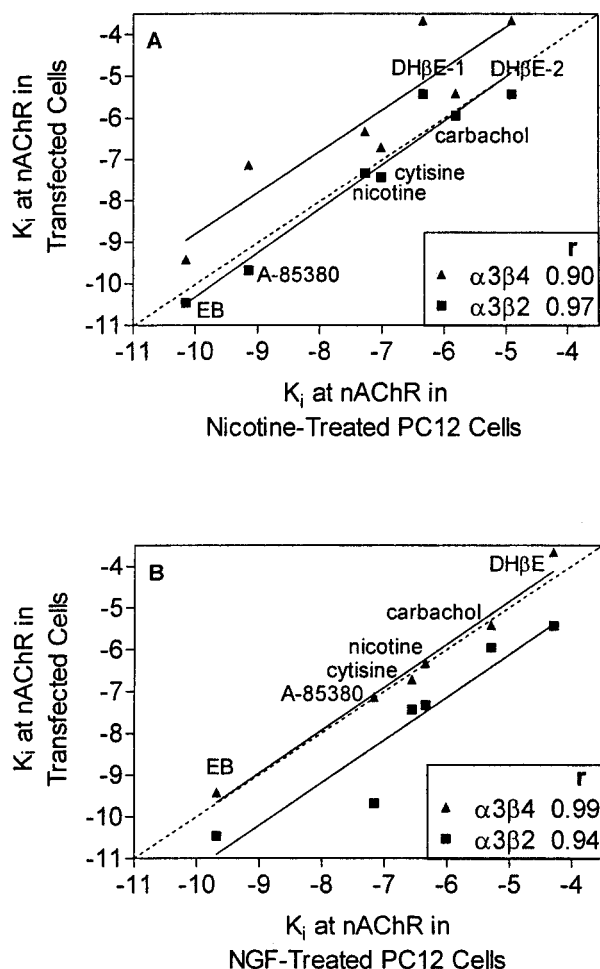


Fig. 5. Correlation of binding dissociation constants (K_i values) between receptors in PC12 cells treated with nicotine or NGF and defined $\alpha 3\beta 4$ and $\alpha 3\beta 2$ receptors in transfected cell lines. Data for nicotine-treated and NGF-treated PC12 cells were taken from Table 2. Data for $\alpha 3\beta 4$ transfected cells were taken from Xiao et al. (1998), and data for $\alpha 3\beta 2$ -transfected cells were taken from Y. Xiao and K. J. Kellar (unpublished data). The logs of the K_i values were used to generate the correlation plots, except for (\pm)-EB in which the K_d value from saturation binding in PC12 cells was used. Data were fit to a linear regression correlation and the Pearson correlation coefficient, r , was determined for each regression line. A, the correlation of K_i values for receptors in nicotine-treated PC12 cells. The correlation coefficient with the $\alpha 3\beta 4$ -transfected cells was 0.90 ($p < 0.05$). The correlation coefficient with the $\alpha 3\beta 2$ -transfected cells was 0.97 ($p < 0.0003$). In the nicotine-treated cells, DH β E competition modeled to two binding sites (Fig. 4; Table 2B), which are represented as DH β E-1, for the higher affinity site, and DH β E-2, for the lower affinity site. B, the correlation of K_i values for receptors in NGF-treated PC12 cells. The correlation coefficient with the $\alpha 3\beta 4$ transfected cells was 0.99 ($p < 0.0002$). The correlation coefficient with the $\alpha 3\beta 2$ transfected cells was 0.94 ($p < 0.005$). In each graph the dashed line represents the line of identity, which for nicotine-treated cells corresponded most closely to the $\alpha 3\beta 2$ -transfected cells and for NGF-treated cells corresponded most closely to the $\alpha 3\beta 4$ -transfected cells.

The data also suggest that the function of the nAChRs increased by exposure to nicotine in PC12 cells is not readily measured with the agonist-stimulated $^{86}\text{Rb}^+$ efflux assay under our conditions; or alternatively, because nicotine is lipophilic and might therefore be sequestered inside the cells, that there is still enough residual nicotine present even after the 60 min washout of the cells to maintain the receptors that are increased by nicotine in a desensitized state. To test this latter possibility, we examined agonist-stimulated $^{86}\text{Rb}^+$ efflux in cells treated for 48 h with carbachol, which increases nAChR binding sites to the same extent as nicotine (Fig. 6) but is more easily washed out of the cultures because it does not enter cells and it binds to the receptors with much lower affinity. As shown in Fig. 7, A and C, agonist-stimulated $^{86}\text{Rb}^+$ efflux was not detectable in these carbachol-treated cells after either a 6- or 60-min washout, suggesting that desensitization by residual agonist is not a likely explanation for the inability to measure nAChR mediated $^{86}\text{Rb}^+$ efflux in nicotine- or carbachol-treated cells.

The Pharmacology of $^{86}\text{Rb}^+$ Efflux Mediated by nAChRs. The concentration-response curves for seven nicotinic agonists to stimulate $^{86}\text{Rb}^+$ efflux in NGF-treated cells are shown in Fig. 8 and summarized in Table 3. Epibatidine was by far the most potent agonist examined. It was $>3,900$ times more potent than acetylcholine or carbachol and $>1,000$ times more potent than nicotine, cytosine, or DMPP. Interestingly, the second most potent agonist in this assay, although still about 230 times less potent than epibatidine, was A-85380. The E_{max} values for most of the agonists tested, including cytosine, were not significantly different from that of acetylcholine (Table 3), indicating that they were full agonists in this assay. However, DMPP seemed to be a weak partial agonist, producing a maximal response consistently much lower than that of acetylcholine and the other agonists

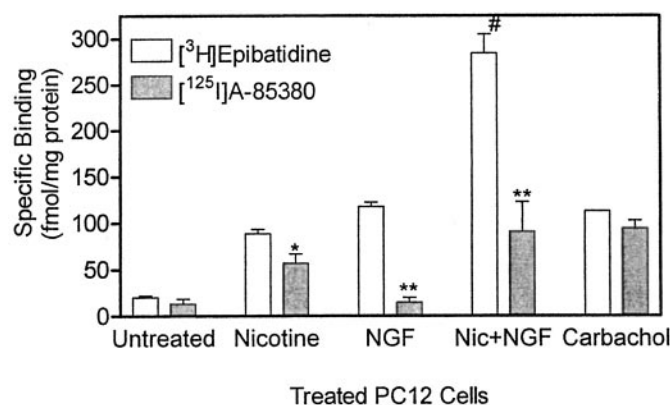


Fig. 6. Comparison of [^3H]epibatidine and [^{125}I]A-85380 binding in membrane homogenates from PC12 cells. PC12 cells were either untreated or treated with nicotine (100 μM), NGF (50 ng/ml), or the combination of nicotine plus NGF or carbachol (1,000 μM) for 48 h. Membrane homogenates were then incubated with either [^3H]EB (2.3 nM) or [^{125}I]A-85380 (3.6 nM) for 2 h. Data are the mean \pm S.E.M. from four independent experiments, except carbachol-treated cells, where $n = 2$. [^{125}I]A-85380 labeled significantly fewer binding sites than [^3H]EB in each treatment group except the carbachol group (*, $p < 0.05$; **, $p < 0.01$). Compared with untreated cells, binding of both ligands was significantly greater in all treatment groups ($p < 0.01$), except for binding of [^3H]A-85380 in the NGF-treated cells. Binding of [^3H]EB in the cells treated with the combination of nicotine and NGF was significantly greater than the sum of the binding values of the groups treated with nicotine and NGF alone (#, $p < 0.01$).

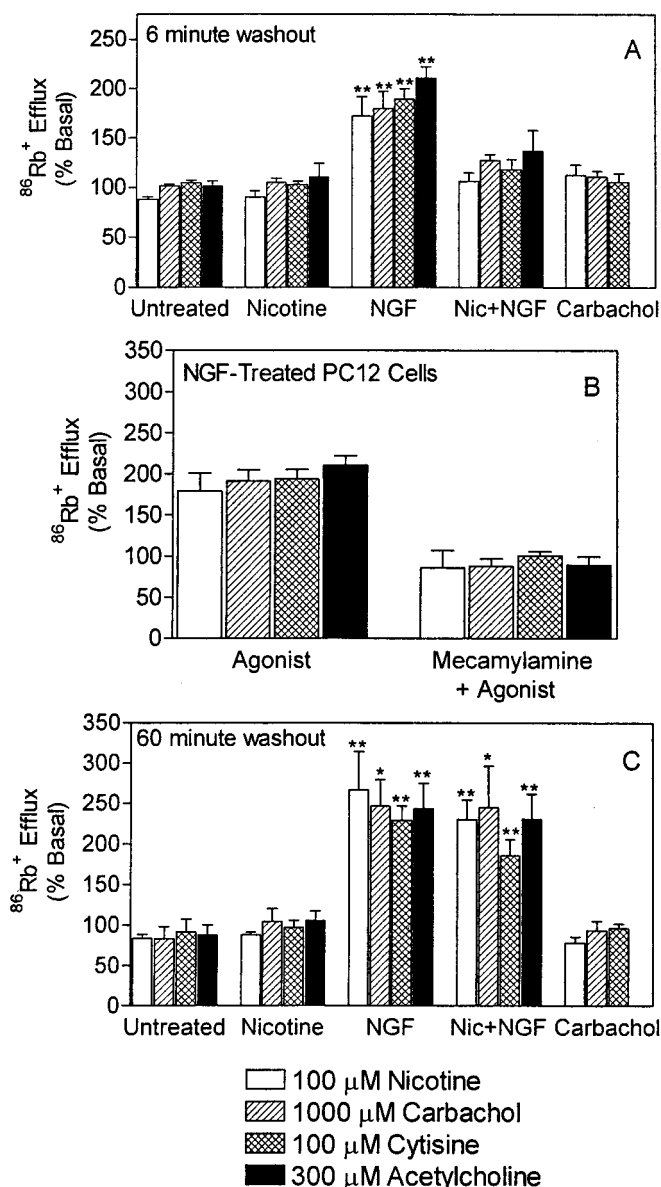


Fig. 7. $^{86}\text{Rb}^+$ efflux in PC12 cells treated with nicotine, NGF, the combination, or with carbachol. $^{86}\text{Rb}^+$ efflux was measured in untreated PC12 cells or cells treated with nicotine (100 μM), NGF (50 ng/ml), the combination, or with carbachol (1,000 μM) for 48 h. $^{86}\text{Rb}^+$ efflux stimulated by nicotine (100 μM), carbachol (1,000 μM), cytisine (100 μM), or acetylcholine (300 μM) was then measured as described under *Materials and Methods*. A, 6-min washout. After the 48-h treatment, cells were washed three times over 6 min before the 2-min stimulation with the agonists. No differences in basal efflux ($8.3 \pm 0.7\%$ of the total $^{86}\text{Rb}^+$ loaded) were seen across the treatment groups. B, mecamylamine block of agonist-stimulated $^{86}\text{Rb}^+$ efflux in PC12 cells treated with NGF (50 ng/ml) in culture for 48 h. After a 6-min washout, the agonists with or without the nicotinic receptor antagonist mecamylamine (10 μM) were added to NGF-treated cells for 2 min. C, 60-min washout. After the 48-h treatment, cells were washed four times over 60 min before the 2-min stimulation with the agonists. No differences in basal efflux ($7.1 \pm 0.4\%$ of the total $^{86}\text{Rb}^+$ loaded) were seen across the treatment groups. Values are expressed as percentage of basal efflux and are the mean \pm S.E.M. from three to four independent experiments measured in quadruplicate, except $n = 2$ for carbachol-treated cells in C. *, $p < 0.05$; **, $p < 0.01$; $^{86}\text{Rb}^+$ efflux significantly greater compared with untreated cells exposed to the same agonist.

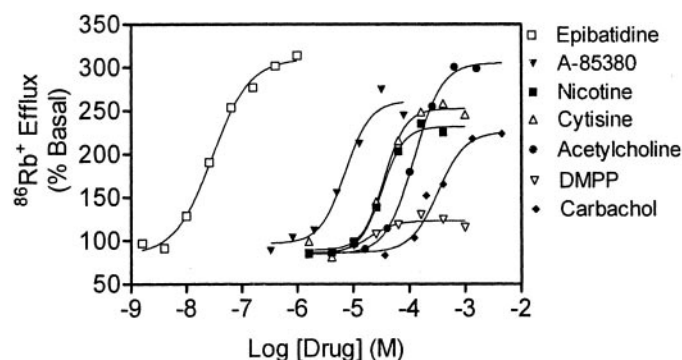


Fig. 8. Concentration-response relationships for nicotinic agonists stimulating $^{86}\text{Rb}^+$ efflux from NGF-treated PC12 cells. PC12 cells were treated in culture with NGF (50 ng/ml) for 48 h, and $^{86}\text{Rb}^+$ efflux stimulated by the drugs shown was then measured. Data are mean values from three independent experiments measured in quadruplicate. Data were fit to a sigmoidal concentration-response relationship using GraphPad. Standard errors of the mean were omitted for clarity. The EC_{50} and E_{max} values for each nicotinic agonist are shown in Table 3.

TABLE 3

EC_{50} and E_{max} values for agonist-stimulated $^{86}\text{Rb}^+$ efflux in NGF-treated cells

Data are derived from the curves shown in Fig. 8 and are presented as the mean \pm S.E.M. from three independent experiments.

| Drug | EC_{50} | E_{max} |
|---------------|------------------|------------------|
| | μM | % of Basal |
| Epibatidine | 0.03 ± 0.003 | 311 ± 37 |
| A-85380 | 7.0 ± 1.2 | 260 ± 5 |
| DMPP | 32 ± 17.1 | 125 ± 10^a |
| Nicotine | 33 ± 1.5 | 232 ± 23 |
| Cytisine | 42 ± 8.6 | 256 ± 21 |
| Acetylcholine | 118 ± 23 | 307 ± 24 |
| Carbachol | 379 ± 119 | 238 ± 43 |

^a E_{max} for DMPP is significantly less than that for acetylcholine ($p < 0.01$).

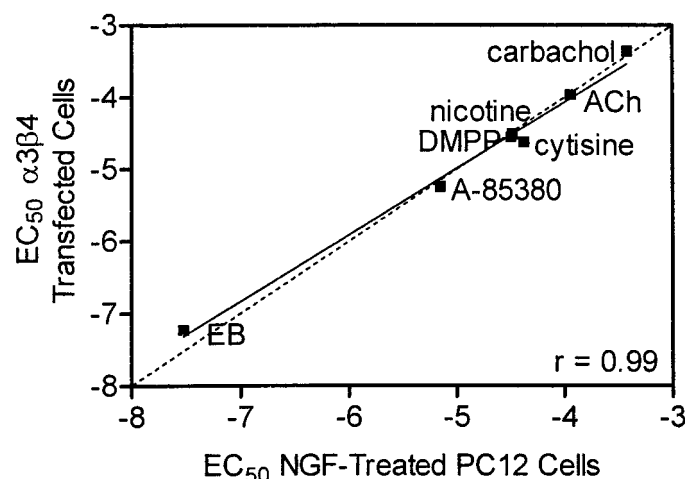


Fig. 9. Correlation of EC_{50} values for agonist-stimulated $^{86}\text{Rb}^+$ efflux in NGF-treated PC12 cells and transfected cells expressing defined $\alpha 3\beta 4$ receptors. The logs of the EC_{50} values were used to generate the correlation plot. Data for NGF-treated PC12 cells were taken from Table 3. Data for transfected cells expressing defined $\alpha 3\beta 4$ receptors were taken from Meyer et al. (2001). Data were fit to a linear regression and the Pearson correlation coefficient, r , was 0.99 ($p < 0.0001$). The dashed line represents the line of identity.

($p < 0.01$) and only 25% above its baseline values (Fig. 8; Table 3).

The EC_{50} values for these seven nicotinic agonists span four orders of magnitude. We compared the EC_{50} values of these drugs for stimulating $^{86}Rb^+$ efflux in NGF-treated PC12 cells to their values at the defined $\alpha 3\beta 4$ nAChR expressed in HEK293 cells (Xiao et al., 1998; Meyer et al., 2001). As shown in Fig. 9, there was a high correlation ($r = 0.99$; $p < 0.0001$) between the EC_{50} values of these seven drugs at the nAChR in NGF-treated PC12 cells and the heterologously expressed $\alpha 3\beta 4$ receptor. Moreover, as shown by the line of identity, the absolute values for the potencies of these drugs in the two systems were nearly identical. These data, together with the data from the binding studies (Fig. 5B), indicate that the nAChR increased by NGF is an $\alpha 3\beta 4^*$ subtype.

Functional Responses of nAChRs in Nicotine-Treated and NGF-Treated PC12 Cells as Measured by [3H]NE Release. One of the important physiological roles of nAChRs is to mediate catecholamine release in adrenal chromaffin cells, and, in fact, nAChR function in PC12 cells has long been assessed by measuring agonist-stimulated [3H]catecholamine release (Greene and Rein, 1977; Baizer and Weiner, 1985). This assay provides a measurement of nAChR function that, although downstream from the receptor itself, might be more sensitive than the $^{86}Rb^+$ efflux assay for certain nAChR subtypes.

PC12 cells were treated with nicotine, NGF or the combination for 72 h and then [3H]NE release was measured in response to a 2-min exposure to either nicotine (100 μM), carbachol (1,000 μM), or cytisine (100 μM). As shown in Fig. 10, in untreated cells agonist-stimulated [3H]NE release was not measurable, probably because the low number of nAChRs in untreated cells is below the threshold for this assay. However, in cells treated for 72 h with either nicotine, NGF, or the combination of the two, both nicotine and carbachol stimulated [3H]NE release (Fig. 10). Cytisine, in contrast, stimulated [3H]NE release in the cells treated with NGF and the combination of nicotine plus NGF, but not in the cells treated with nicotine alone (Fig. 10). In each condition where nicotine-stimulated [3H]NE release, mecamylamine could block

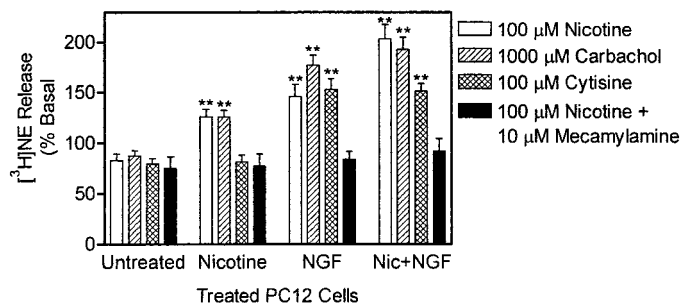


Fig. 10. [3H]Norepinephrine release from PC12 cells treated with nicotine, NGF, or the combination for 72 h. PC12 cells were either untreated or treated with nicotine (100 μM), NGF (50 ng/ml), or the combination for 72 h before being loaded with [3H]NE (15 nM; 0.5 μCi /well) and prepared for release measurements. The cells were then stimulated for 2 min with either nicotine (100 μM), carbachol (1,000 μM), cytisine (100 μM), or nicotine plus mecamylamine (10 μM), and the released [3H]NE was collected and counted. The data are expressed as a percentage of the basal release, measured in the presence of buffer alone, and are the mean \pm S.E.M. of four to eight independent experiments. **, $p < 0.01$, [3H]NE release significantly greater compared with the same stimulating drug in the untreated cell group.

it completely (Fig. 10). Thus, although treatment of PC12 cells with either nicotine or NGF results in agonist-stimulated [3H]NE release, the efficacy of cytisine in NGF-treated cells again suggests that NGF increases an $\alpha 3\beta 4^*$ receptor subtype; in contrast, the absence of efficacy of cytisine in nicotine-treated cells suggests that nicotine increases an $\alpha 3\beta 2^*$ subtype.

Effect of Nicotine and NGF on nAChR Subunit mRNA. To investigate the mechanisms related to the differential regulation of nAChR subtypes by nicotine and NGF, the mRNAs encoding the $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\alpha 6$, as well as $\beta 2$ and $\beta 4$ nAChR subunits were measured by RNase protection assays. The amount of each nAChR subunit mRNA was measured and normalized to the amount of GAPDH mRNA in the same sample via phosphorimaging (Fig. 11A).

As has been reported in previous studies of PC12 cells (Rogers et al., 1992; Henderson et al., 1994; Takahashi et al., 1999), mRNAs coding for $\alpha 3$, $\alpha 5$, $\beta 2$, and $\beta 4$ nAChR subunits were present in these cells, whereas mRNAs for $\alpha 2$, $\alpha 4$, and $\alpha 6$ subunits were not detected. In all cases, there was more $\alpha 3$ subunit mRNA than $\alpha 5$ or either of the β subunit mRNAs, and $\beta 2$ mRNA exceeded $\beta 4$ mRNA (Fig. 11A); in fact, in cells not treated with NGF $\beta 4$ mRNA was barely detectable.

The effects of the treatments on nAChR subunit mRNA were expressed as a percentage of that in untreated cells. Nicotine treatment did not significantly alter the amount of any of the subunit mRNAs measured; whereas, NGF treatment increased $\beta 4$ subunit mRNA by about 2-fold without significantly changing the amount mRNAs for the $\alpha 3$, $\alpha 5$, or $\beta 2$ subunits (Fig. 11B). Treatment of the cells with the combination of nicotine and NGF also produced a significant increase in mRNA for the $\beta 4$ subunit, again without altering the other mRNAs.

Stability of nAChRs Increased by Nicotine or NGF Treatment. The nicotine-induced increase in apparent $\alpha 3\beta 2^*$ nAChRs without an increase in the mRNAs coding for either nAChR subunit suggests that the increase in the binding and function of these receptors in PC12 cells is not linked to increased de novo synthesis of subunits. Alternative mechanisms that could account for the increase in binding sites include nicotine-induced post-translational changes in nAChRs or the subunit proteins themselves that lead to an increase in high-affinity agonist binding sites (Bencherif et al., 1995; Ke et al., 1998; Wang et al., 1998) or an increase in receptor stability (i.e., a decrease in the rate of degradation of the receptors) (Peng et al., 1994).

To begin to explore these possibilities, we examined the stability of the nAChRs labeled by [3H]EB. Cells were treated with nicotine, NGF, or the combination of the two for 48 h and then washed thoroughly and incubated in fresh medium free of nicotine or NGF for 2 to 48 h (the washout time) before collection. We then compared [3H]EB binding to nAChRs in washed membranes from these cells and from control cells, which were incubated with the nicotine and/or NGF for the same time period but not subjected to the washout incubation. As shown in Fig. 12A, in control cells [3H]EB binding in cells treated with nicotine or NGF was 4- to 6-fold higher than in the untreated cells, similar to what was seen previously (Fig. 3; Table 1). However, in cells treated with nicotine and then subjected to a 24-h washout incubation, [3H]EB binding fell significantly to levels approaching those in untreated cells (Fig. 12A). In contrast, in cells treated with NGF

and then subjected to the same 24-h washout incubation, [^3H]EB binding was not significantly different from the binding in cells in which the NGF was not removed (Fig. 12A). In cells treated with the combination of nicotine and NGF, [^3H]EB binding was increased ~15-fold, but after the 24-h washout incubation, binding fell to levels similar to those in cells treated with NGF alone (Fig. 12A).

The time course of the fall of [^3H]EB binding in cells after incubation in nicotine-free medium is shown in Fig. 12B. There was a very rapid initial fall in measurable nAChR binding sites after removal of the nicotine from the cells, followed by a much slower decline. The decrease was best

described by two functions, suggesting two phases of the decline in measurable binding sites. The first phase had an apparent half-time ($t_{1/2}$) of ~0.9 h, whereas the second phase was much slower, with a $t_{1/2}$ estimated at ~81 h (Fig. 12B).

Discussion

PC12 cells have provided an important model system for the study of nAChRs for more than 25 years. The studies presented here indicate that these cells express at least two functional nAChR subtypes that can be reliably measured with [^3H]EB, but not with [^3H]cytisine or [^3H]nicotine. Treatment of the cells with nicotinic agonists markedly increases the density of one receptor subtype predominantly, whereas treatment with NGF markedly increases the density of another receptor subtype exclusively. The predominant nAChR binding site in the cells treated with nicotine has pharmaco-

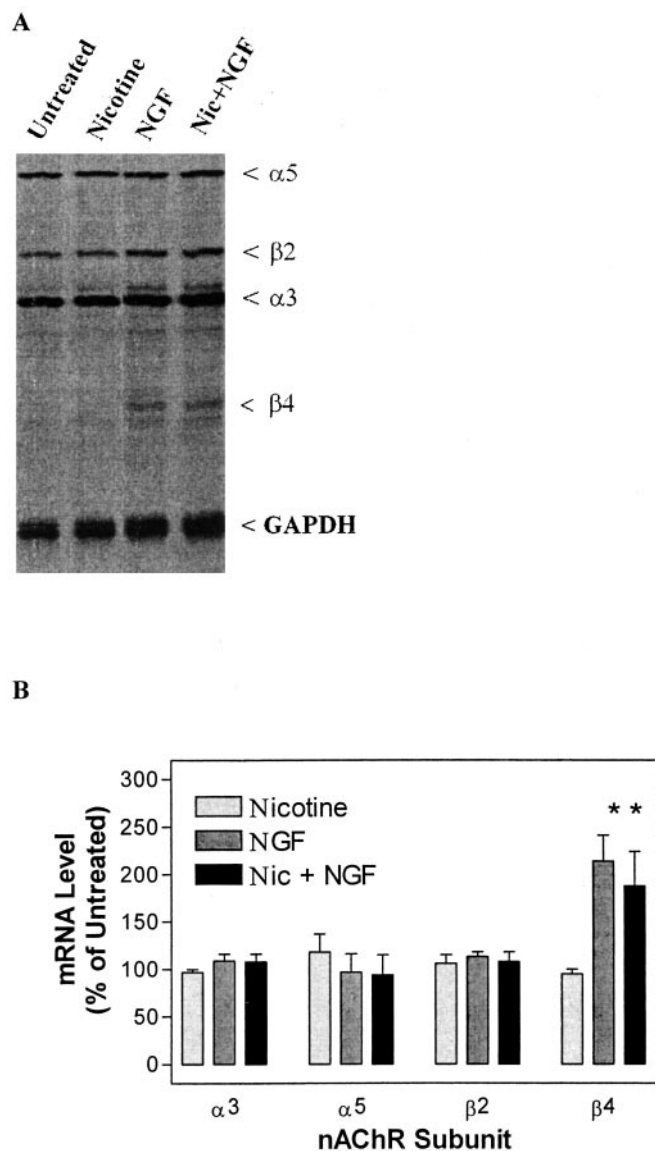


Fig. 11. nAChR subunit mRNA measurements in PC12 cells treated with nicotine, NGF, or the combination for 87 h. Subunit mRNAs were measured by an RNase protection assay as described under *Materials and Methods*. A, representative image showing results of one RNase protection assay. Note that the $\beta 4$ mRNA is visible to the eye only in samples from cells treated with NGF or the combination of nicotine and NGF. B, comparison of relative expression level of subunit mRNAs in treated cells. The mRNAs were measured by phosphorimaging and normalized to the GAPDH signal in each lane. The mRNA signal for each subunit across treatment groups was then compared with that from untreated cells. The $\beta 4$ mRNA signal in the cells treated with NGF or the combination was significantly greater than that in the untreated cells (*, $p < 0.05$).

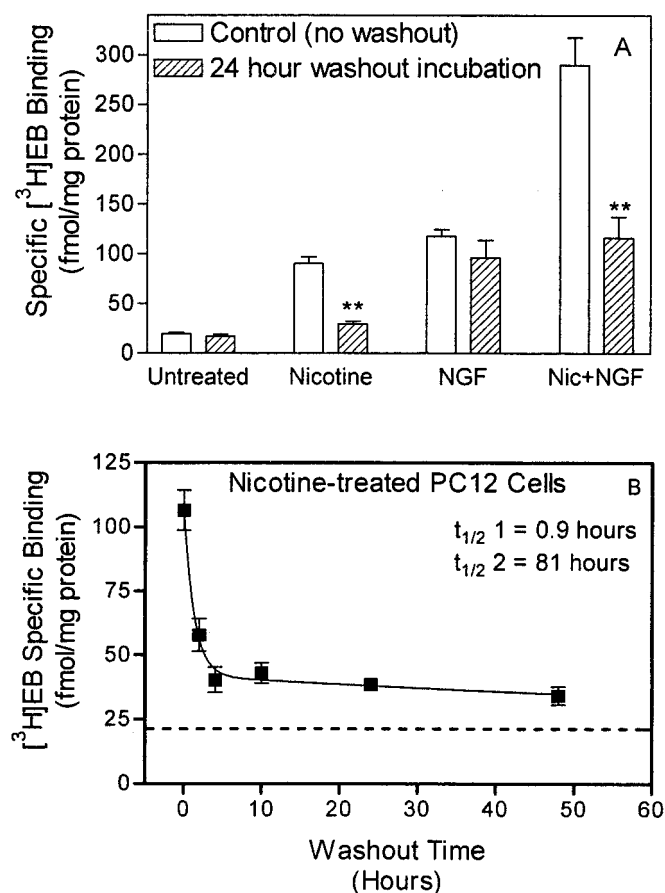


Fig. 12. Stability of nAChRs increased by nicotine and NGF treatments. A, PC12 cells were either untreated or treated with nicotine (100 μM), NGF (50 ng/ml), or the combination for 48 h in culture. The drugs were then removed and cells were washed and incubated in fresh media for 24 h (the washout) before collection. Control cells were incubated with nicotine and/or NGF for the same time period but not subjected to the washout incubation. Receptor binding was measured with [^3H]EB (2 nM). In cells treated with nicotine or the combination of nicotine and NGF, binding in cells subjected to the 24 h washout was significantly less than in control cells (no washout), **, $p < 0.01$. In contrast, binding in the NGF-treated cells 24 h after washout was not significantly different from that in control cells. B, PC12 cells were treated with nicotine (100 μM) for 48 h and then washed to remove nicotine and incubated in fresh medium without nicotine for 2 to 48 h. The decrease in [^3H]EB binding was calculated by nonlinear regression and modeled to two phases with the indicated $t_{1/2}$. All data are the mean \pm S.E.M. from three independent experiments.

logical characteristics consistent with an $\alpha 3\beta 2^*$ receptor subtype. That is, in receptor binding competition assays its affinities for the drugs examined are nearly identical to those of the defined $\alpha 3\beta 2$ receptor subtype expressed in HEK293 cells. In contrast, the predominant nAChR binding site in NGF-treated cells has the pharmacological profile of an $\alpha 3\beta 4^*$ receptor subtype. Thus, its affinities for the drugs examined in the binding competition assays are virtually identical to those of the defined $\alpha 3\beta 4$ receptor subtype expressed in HEK293 cells. This assignment of subtypes is reinforced by the finding that in cells treated with nicotine or carbachol an increase in receptor binding sites could be measured not only with [^3H]EB but also with [^{125}I]A-85380, which has high affinity for nAChRs containing $\beta 2$ subunits but not those containing $\beta 4$ subunits (Mukhin et al., 2000; Perry et al., 2002), whereas in contrast, in cells treated with NGF, the increased receptors could be measured only with [^3H]EB.

An increase in nAChR binding sites similar to that induced by nicotine is seen in cells treated with carbachol. Because carbachol is a quaternary amine that does not readily cross cell membranes, this finding indicates that the increase in receptors induced by nicotinic agonists is initiated at the cell surface, rather than at an intracellular site. Furthermore, it suggests that the increased receptors are, or were at some point in their cycle, on the cell surface.

The increase in nAChR binding sites measured in cells treated with the combination of nicotine and NGF is significantly greater than the sum of the increases induced by the two treatments separately, suggesting interacting mechanisms. The nicotine-induced increase in nAChR binding sites in PC12 cells is not accompanied by an increase in mRNA, and thus it may result from a post-translational action of nicotine, as has been suggested to occur in brain from animals chronically exposed to nicotine (Marks et al., 1992; Bencherif et al., 1995; Ke et al., 1998; Wang et al., 1998). In contrast, NGF increases the mRNA encoding certain nAChR subunits (Rogers et al., 1992; Henderson et al., 1994; Takahashi et al., 1999), suggesting that it induces synthesis of some subunit proteins (see below). Thus, the superadditive effect on nAChR binding sites in cells treated with the combination of nicotine and NGF may result from nicotine acting through post-translational mechanisms that affect NGF-induced $\alpha 3\beta 4^*$ receptors, as well as the $\alpha 3\beta 2^*$ receptors that seem to be independent of NGF.

PC12 cells treated with NGF alone consistently displayed agonist-stimulated nAChR function measured by $^{86}\text{Rb}^+$ efflux; moreover, as with the ligand binding studies, the potencies of nicotinic agonists to stimulate receptor function in NGF-treated cells are virtually identical to their potencies at defined rat $\alpha 3\beta 4$ nAChRs expressed in HEK293 cells. Together, these data suggest that if another subunit, such as $\alpha 5$, is a component of the receptor induced by NGF in PC12 cells, it does not have an obvious influence on the pharmacology of the receptor as measured in radioligand binding assays or the $^{86}\text{Rb}^+$ efflux assay. This is consistent with studies in *Xenopus* oocytes in which the coexpression of the $\alpha 5$ subunit with $\alpha 3$ and $\beta 4$ subunits had little effect on the binding affinity for epibatidine or the responses to acetylcholine or nicotine (Wang et al., 1996). Similarly, the coexpression of the $\alpha 5$ subunit with $\alpha 3$ and $\beta 2$ subunits in oocytes had little effect on the binding affinity for epibatidine, although it

did increase acetylcholine sensitivity and the efficacies of nicotine and DMPP (Wang et al., 1996; Gerzanich et al., 1998).

In contrast to the consistent function measured with the $^{86}\text{Rb}^+$ efflux assay in NGF-treated cells, we did not detect agonist-stimulated nAChR function in untreated cells. Although the $^{86}\text{Rb}^+$ efflux assay used here has many advantages, it is not the most sensitive assay for nAChR function, and the number of receptors in untreated PC12 cells (20–24 fmol/mg protein, measured by binding in membranes) may be below the threshold for measurable function with this assay. However, we also failed to detect receptor function in cells treated with nicotine or carbachol for 48 h, in which the receptor binding site density in membranes was increased by ~4-fold, to a level comparable with that in NGF-treated cells; moreover, function was not detected with the standard assay even in cells treated with the combination of nicotine and NGF, where the nAChR density was increased by ~13-fold. One explanation for the absence of function in cells treated with nicotine or carbachol may be that the receptors were desensitized by residual agonist not removed by the standard (6-min) washout procedure. Consistent with this possibility, when the washout procedure was increased to 60 min, agonist-stimulated function was elicited in the cells treated with the combination of nicotine and NGF; however, function still could not be measured in the cells treated with nicotine or carbachol alone.

There are several possible explanations for the absence of nAChR function as measured by $^{86}\text{Rb}^+$ efflux in cells treated with nicotine alone. One is that the channel properties of the receptors increased by agonist treatment may be unfavorable for measurement with this $^{86}\text{Rb}^+$ assay; i.e., the receptor may desensitize and the channel may close too rapidly to provide a signal. A second possibility is that nicotine may bind to the prevailing receptors with an affinity high enough that it does not completely dissociate during the washout procedures, thus keeping the receptors in a desensitized state. Although both of these possibilities are consistent with properties of an $\alpha 3\beta 2^*$ nAChR, which is also consistent with the pharmacology of the [^3H]EB binding sites observed in nicotine-treated PC12 cells, some evidence argues against the second possibility. First, no function was detected even after the 60-min washout of nicotine; and second, no function was detected with the $^{86}\text{Rb}^+$ efflux assay in cells in which nAChRs were increased to the same extent by a 48-h treatment with carbachol, which washes out of cells much quicker than nicotine (Meyer et al., 2001).

Receptor function was, in fact, seen in nicotine-treated PC12 cells when measured with an agonist-stimulated [^3H]NE release assay, which reflects a downstream effect of receptor activation. Although agonist-stimulated [^3H]NE release was not measurable in untreated cells with this assay, it was clearly evident in the cells in which the receptors were increased by treatment with nicotine, NGF or the combination. This indicates that the increased receptors in the nicotine-treated cells, like those in the NGF-treated cells, are indeed functional. Moreover, in agreement with the ligand binding assays, the pharmacology of the agonist-stimulated [^3H]NE release indicated that nicotine treatment predominantly increased an $\alpha 3\beta 2^*$ receptor subtype, because cytosine was inactive as an agonist compared with nicotine or carbachol. In contrast, cytosine seemed to be a full agonist in

NGF-treated cells, again indicating that the receptor increased by NGF is an $\alpha 3\beta 4^*$ subtype.

It is thus interesting to note that these data indicate that catecholamine release in PC12 cells can be mediated by both the $\alpha 3\beta 2^*$ and $\alpha 3\beta 4^*$ receptors. This suggests that both of these nAChR subtypes might function in adrenal chromaffin cells, perhaps under different conditions (i.e., basal and physiological release versus release induced by smoking).

The absence of mRNA changes in the cells treated with nicotine is consistent with previous reports that chronic nicotine treatment of mice in vivo or of the human neuroblastoma cell line SH-SY5Y in culture increases the density of nAChRs but not the mRNA for any of the subunits measured, including $\alpha 3$, $\alpha 4$, $\alpha 7$, and $\beta 2$ (Marks et al., 1992; Pauly et al., 1996; Peng et al., 1997). Other mechanisms that could lead to the nicotine-induced increase in binding sites include 1) conversion of nAChRs from a state with low affinity for agonists to one with high affinity (Bencherif et al., 1995); 2) an increase in assembly of existing subunits (Ke et al., 1998; Wang et al., 1998); or 3) a decrease in the rate of degradation of the receptors (Peng et al., 1994). Our studies here in PC12 cells could be consistent with any of these mechanisms, but the rapid decrease in [3 H]EB binding sites after removal of nicotine ($t_{1/2} < 1$ h) seems to be most consistent with an action of nicotine to convert receptors to a high-affinity state or to stabilize them by slowing degradation, either by inhibiting enzymatic hydrolysis of subunits or inhibiting disassembly of the subunit complex. It is also possible that more than one mechanism is involved. This clearly is an important question for further study.

In contrast to nicotine treatment, NGF did increase mRNA for the $\beta 4$ subunit, but not for any other subunit. The $\beta 4$ subunit mRNA is the least abundant of the nAChR subunits mRNAs in these cells, which suggests that its presence may be the limiting factor in the formation of $\alpha 3\beta 4^*$ receptors. NGF increases the transcriptional activity of the $\beta 4$ subunit promoter (Hu et al., 1994), which could account for the increase in $\beta 4$ mRNA found in our studies. Interestingly, however, NGF increased $\beta 4$ mRNA only 2-fold in our studies, whereas $\alpha 3\beta 4^*$ receptors were increased about 5-fold. This suggests that the mRNA increased by NGF is very efficient or that additional mechanisms are involved in the NGF-induced increase in receptors.

In conclusion, we have shown here that PC12 cells express at least two types of nAChRs with high affinity for agonists and that nicotine and NGF differentially increase the number and function of these receptors. The receptors increased by nicotine have pharmacological characteristics of an $\alpha 3\beta 2^*$ nAChR subtype predominantly, whereas those induced by NGF have characteristics of an $\alpha 3\beta 4^*$ subtype exclusively.

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