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Tobacco-Specific Carcinogenic Nitrosamines

LIGANDS FOR NICOTINIC ACETYLCHOLINE RECEPTORS IN HUMAN LUNG CANCER CELLS

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ABSTRACT. Lung cancer demonstrates a strong etiologic association with smoking. Of the two most common histologic lung cancer types, small cell carcinoma (SCLC) is found almost exclusively in smokers, whereas peripheral adenocarcinoma (PAC) also develops in a significant number of nonsmokers. *N′*-Nitrosonornicotine (NNN) and 4(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), nicotine-derived nitrosamines, are potent lung carcinogens contained in tobacco products. Because of the structural similarity of NNN and NNK with nicotine, we hypothesized that these carcinogens are ligands for nicotinic acetylcholine receptors (nAChRs). Using cell lines derived from human small cell lung carcinoma and pulmonary adenocarcinoma with the site-selective ligands α-bungarotoxin (α-BTX) and epibatidine (EB) in receptor binding and cell proliferation assays, we found that SCLC expressed neuronal nicotinic receptors with high affinity to α-BTX, whereas PAC cells expressed nicotinic receptors with high affinity to EB. NNK bound with high affinity to α-BTX-sensitive nAChRs in SCLC cells, while NNN bound with high affinity to EB-sensitive nAChRs in PAC cells. The affinity of each nitrosamine to these receptors was several orders of magnitude greater than that of nicotine. NNK stimulated the proliferation of SCLC cells via this mechanism. Our findings suggest that NNK may contribute to the genesis of SCLC in smokers via chronic stimulation of the α-BTX-sensitive nAChR-subtype expressed in these cells. Both nitrosamines may also contribute to a host of nicotine-related diseases that are currently thought to be caused by the chronic interaction of nicotine with nAChRs expressed in a large spectrum of mammalian cells. BIOCHEM PHARMACOL **55**;9:1377–1384, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. tobacco-specific; nitrosamine; nicotinic receptor; α-bungarotoxin; epibatidine

Lung cancer and cardiovascular disease demonstrate a strong etiologic association with smoking [1, 2]. SCLC† and PAC are the most common types of lung cancer [1]. PAC also develops in a significant number of non-smokers [1]. By contrast, SCLC is found almost exclusively in smokers [1], rendering this histologic type of lung cancer tobacco-specific.

NNN and NNK, tobacco-specific nitrosamines, form from nicotine by nitrosation during the processing and storage of tobacco and in the mammalian organism [3]. The alkaloid nicotine is a tertiary amine consisting of a pyridine and a pyrrolidine ring (Fig. 1). NNN closely resembles nicotine in structure, whereas the formation of NNK from nicotine results in ring opening of the pyrrolidine ring [4] (Fig. 1). NNN and NNK are powerful carcinogens. This effect is thought to be initiated by the formation of promutagenic DNA-adducts from reactive metabolites [5]. Nicotine and both nitrosamines are absorbed rapidly by the lungs and enter the bloodstream to be distributed extensively to body tissues [6, 7]. The concentration of nicotine in the mainstream smoke of cigarettes is on average 5,000–10,000 times greater than that of NNK, whereas the concentration of NNN is about 2–3 times greater than that of NNK [3]. The blood concentration of nicotine in an individual who smokes, on an average, 2 packs of cigarettes a day ranges from 20 to 50 ng/mL [6]. Currently available technology precludes the direct measurement of NNN and NNK in the blood of a smoker, and the determination of hemoglobin adducts is used, instead, to assess the levels of exposure to these carcinogens [7]. Experiments in patas monkeys have revealed blood levels of 1.6 pg/mL of NNK after exposure to a dose of tritiated NNK equivalent to the amount of NNK contained in 2 packs of cigarettes [8]. The immediate blood levels of unmetabolized nicotine and this unmetabolized nitrosamine in smokers thus appear to reflect the relative proportions of these agents in cigarette smoke.

Neurotransmitter receptors of the neuronal nAChR family regulate a wide spectrum of mammalian cell and organ functions [9]. Individual members of this receptor family are identified by the binding of site-selective pharmacological ligands to receptor subunits. The snake venom

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† *Abbreviations:* SCLC, small cell carcinoma; NNN, *N′*-nitrosonornicotine; NNK, 4(methylnitrosamino)-1-(3-pyridyl)-1-butanone; EB, epibatidine; α-BTX, alpha-bungarotoxin; nAChR, nicotinic acetylcholine receptor; and PAC, peripheral adenocarcinoma.

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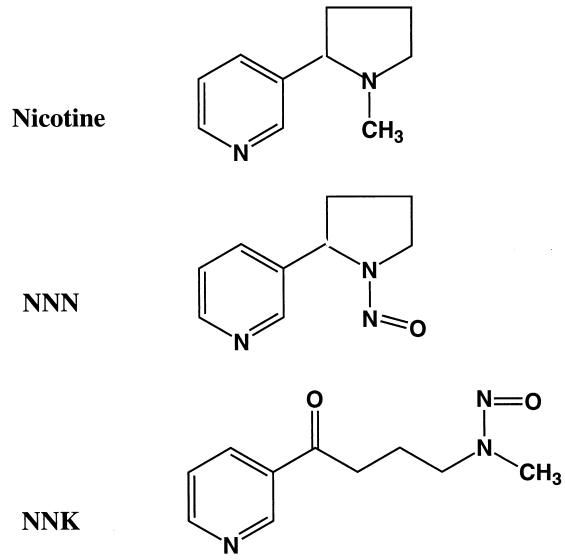

FIG. 1. Structures of nicotine and two carcinogenic nitrosamines, NNN and NNK. Nicotine is a tertiary amine consisting of a pyridine and a pyrrolidine ring. NNN and NNK form from nicotine by nitrosation during the processing and storage of tobacco and in the mammalian organism. The formation of NNN from nicotine involves nitrosation of the pyrrolidine ring under loss of a methyl group. NNK forms from nicotine by nitrosation under ring opening of the pyrrolidine ring.

α-BTX binds with high affinity to a small subset of neuronal nAChRs [10], whereas the frog venom EB binds with high affinity to about 90% of the neuronal nAChR family [11]. Molecular cloning has revealed that the binding domains for α-BTX are α7 or α8 subunits [10]. Of these, α7 predominates in the brain, whereas α8 predominates in the retina [10]. Similarly, α4 subunits, which predominate in the brain, and α3 subunits, which predominate in the adrenal medulla, have been identified as the binding domains for EB [11]. Desensitization of neuronal nAChRs in response to chronic exposure to nicotine are implicated in the addictive effects of tobacco products [12]. The main cardiovascular effects of nicotine are mediated by stimulation of EB-sensitive neuronal nAChRs [2].

Recent studies have revealed that the proliferation of normal pulmonary neuroendocrine cells and human lung cancers with a neuroendocrine phenotype (SCLC and carcinoid) is regulated by a neuronal nAChR [13–16]. Studies in SCLC cells have shown that this receptor is α-BTX-sensitive and expresses the α7 subunit [14–16].

Based on the structural similarities between nicotine, NNN, and NNK (Fig. 1), we have addressed a potential direct interaction of these nicotine-derived nitrosamines with nicotinic acetylcholine receptors. Receptor binding assays with hamster lung homogenates have shown that NNK competed successfully with nicotine for nicotinic binding sites [17]. However, the receptor in question was not characterized, and it was unclear in what cells these receptors were expressed. Our current data show that NNK bound with high affinity to α-BTX-sensitive neuronal nicotinic receptors expressed in SCLC, while NNN bound with high affinity to EB-sensitive neuronal nicotinic receptors expressed in PAC. Binding of NNK to the α7 nAChR in SCLC stimulated cell proliferation, a factor that likely contributes to the fact that this cancer type develops almost exclusively in smokers [1].

MATERIALS AND METHODS

Cell Culture

The human lung cancer cell lines derived from SCLC (NCI-H69, NCI-H82) and PAC (NCI-H322, NCI-H441) were purchased at the American Type Culture Collection. The cells were maintained in an atmosphere of 5% CO_2/95% air at 37° in RPMI medium supplemented with fetal bovine serum (10%, v/v), L-glutamine (2 mM), nerve growth factor (50 ng/mL), and gentamycin sulfate (50 mg/mL).

Receptor Binding Assays

The SCLC cells were transferred into an environment of 10% CO_2/90% air 24 hr prior to the receptor binding assays to provide an environment identical to that used during the cell proliferation assays (see below). Each binding assay (saturation and competition experiments) was conducted as two replicate experiments with triplicate samples for the data points of each experiment. The population correlation between data from replicate experiments was tested by a paired *t*-test, which established a positive linear correlation between each replicate pair of experiments ($P < 0.01$). The data presented in the tables and figures each exemplify one of the replicate experiments. All cells were harvested, washed with PBS, and centrifuged at 200 *g* for 2 min. The cells were resuspended in PBS, homogenized with a Polytron for 20 sec, and placed into assay tubes to yield 500 μg protein/tube. Ligands were added in aliquots of 100 μL. The total volume of the reaction mixture per tube was 300 μL. Control experiments were conducted to determine at which time binding of the radioligand had reached a steady-state condition. The cell homogenate was incubated with [^{125}I]α-BTX (sp. act. 230 Ci/mmol, Amersham) for time intervals ranging from 30 min to 3 hr. Binding reached a plateau at 60 min when the reaction was conducted at 37°. For saturation analysis with [^{125}I]α-BTX, triplicate assay tubes were incubated for 90 min at 37° with concentrations of the radioligand from 5 to 100 nM. Nonspecific binding was determined by a 60-min preincubation with an excess concentration of nonradioactive α-BTX (1 μM). Tubes without radioligand served as negative controls. The reaction was terminated by separating bound from free ligand by centrifugation [18] (23,000 *g* for 5 min). Following three washes with PBS, bound radioactivity was determined by liquid scintillation spectrophotometry. The binding data were analyzed by nonlinear regression for single-site isotherms and linear regression after Scatchard transformation [18–20] using computer programs EBDA/Ligand for the Macintosh and Prism/Graphpad for the IBM.

Saturation analysis with [^{3}H]EB (48 Ci/mmol, Dupont) was conducted under identical conditions except that preincubation with (−)-nicotine tartrate (10 mM, Sigma) was used for the determination of nonspecific binding. For the competition assays, non-radioactive NNN, NNK (Chemsyn Science Laboratories), or nicotine was added to the reaction mixture at the concentrations indicated in Figs. 4 and 5 immediately followed by the addition of the radioligand under study. Following computerized analysis of binding data by nonlinear regression for a single class of binding sites, including calculation of EC_{50} values for each competing ligand, statistical analysis of EC_{50} values was by one-way analysis of variance and the unpaired *t*-test.

Cell Proliferation Assays

Similar to the receptor binding assays, each cell proliferation assay was conducted in two replicate experiments with triplicate samples per data point of each experiment, and the paired *t*-test established a positive linear correlation between each replicate pair of experiments ($P < 0.01$). The data shown in Table 2 and Fig. 6 exemplify the results of one of these replicate experiments. The cell proliferation assays were conducted using cell counts by hemocytometer and incorporation of [^{3}H]thymidine as previously described [13, 15]. Briefly, cells were seeded in RPMI medium without serum into 6-well plates (5×10^4 cells/mL, triplicate wells for each treatment group). NNK (30 nM) and α-BTX (100 nM) were added to the culture medium of SCLC cells at the start of the assay (time point 0) and on day 4, when the medium was replaced. Nicotine (100 nM–10 μM) and NNN (100 nM–10 μM) were added at identical time points to the culture medium of PAC cells. SCLC cells, which grow as floating aggregates, and PAC cells, which grow as monolayers, were dissociated into single-cell suspensions by the addition of trypsin/EDTA at the time intervals indicated in Fig. 6. Viable cells were identified by trypan blue dye exclusion and counted by hemocytometer. Cell viability was greater than 95% in all treatment groups at all time intervals. The observed effects on cell numbers were verified by an alternative cell proliferation assay using the incorporation of [^{3}H]thymidine as an endpoint. Cells were seeded into 96-well plates (5×10^3 cells/well, triplicate wells/treatment group). NNK, α-BTX, nicotine, and NNN were added as specified in Table 2. Tritiated thymidine (sp. act. 84 Ci/mmol, 0.5 μCi/well, Amersham) was added 48 hr before harvesting the cells with two washes onto filters with a cell harvester (Micro-mate, Packard). Radioactivity bound to the filters was counted by liquid scintillation spectrophotometry. Statistical evaluation of data was by one-way analysis of variance and the unpaired *t*-test. The proliferation assays with SCLC cells were conducted in an atmosphere of 10% CO_2, a condition that simulates the intrapulmonary environment of the diseased lung [21], and has been identified as a requirement for the activation of nAChR-mediated mitogenic responses [14, 15]. The proliferation assays with PAC cells were conducted in an atmosphere of 5% CO_2, a condition required to maintain viability of these cells, whereas 10% CO_2 causes 100% cytotoxicity [13, 15].

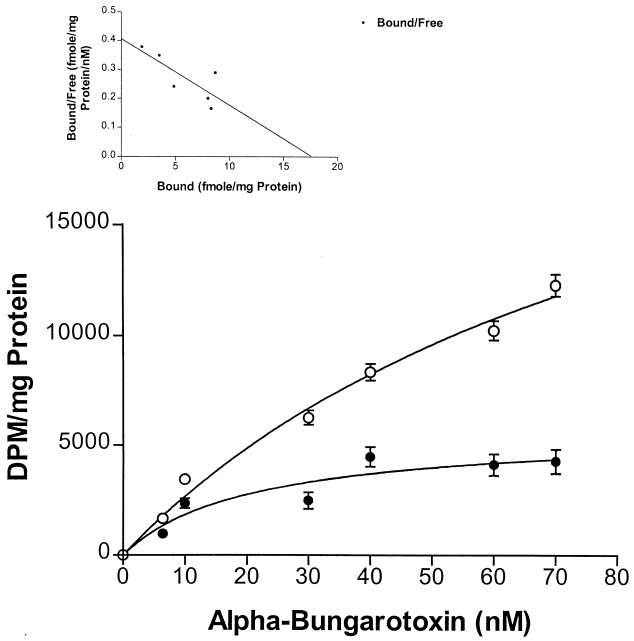

FIG. 2. Saturation curve of [^{125}I]α-BTX binding in SCLC cells exemplified in NCI-H69. Nonspecific binding was assessed by preincubation of the cell homogenate with nonradioactive α-BTX (1 μM) for 60 min at 37°. Specific binding (●) was determined by subtraction of nonspecific binding from total binding (○). Inset: Analysis of the binding data by Scatchard transformation. The linear curve suggests the presence of a single class of binding sites with a B_{max} of 17.69 fmol/mg of protein and a K_d of 11.44 nM. Accordingly, nonlinear regression analysis for single-site isotherms of the saturation curve yielded a close fit of the regression lines to the data points at the 95% confidence interval (goodness of fit for total binding, r^2 = 0.99; for specific binding, r^2 = 0.92). Saturation binding analysis with [^{125}I]α-BTX in NCI-H82 yielded a similar curve. No specific binding was detected with this ligand in the two PAC cell lines, NCI-H322 and NCI-H441. Data shown are means ± SEM of triplicate samples per data point from one of two replicate experiments.

RESULTS

Receptor binding assays with both SCLC cell lines (NCI-H69, NCI-H82) revealed that saturable specific binding of [^{125}I]α-BTX (Fig. 2) was inhibited by hexamethonium but not decamethonium (data not shown). Hexamethonium is a selective antagonist of neuronal nAChRs, whereas decamethonium is selective for muscle-type nAChRs [9]. The snake venom α-BTX binds exclusively to the α7 and α8 subunits, which are expressed in the brain and retina, respectively [10]. Accordingly, the nAChR subtype expressed in SCLC cells has been identified as a receptor comprised of α7 subunits [16]. Analysis of the binding data by Scatchard transformation (Fig. 2, inset) suggested that binding may be to a single class of receptors, although the data points did not exactly match the linear regression line.

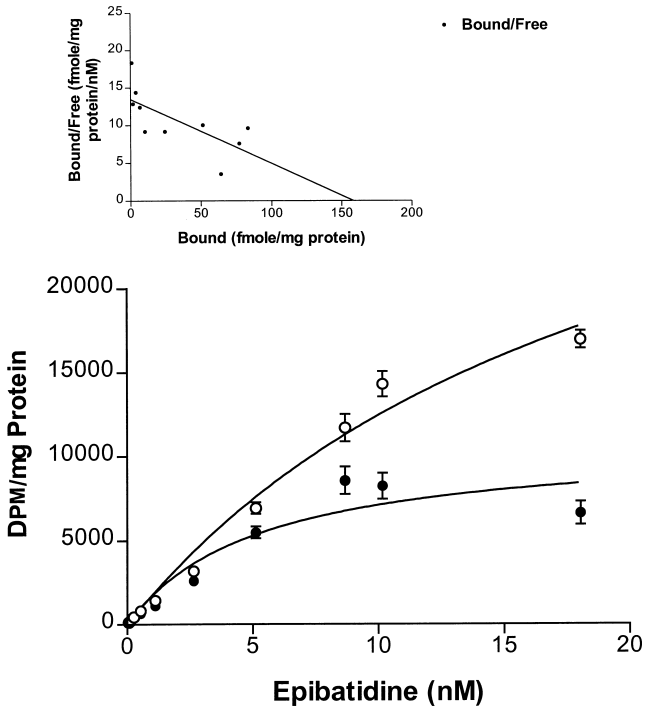

FIG. 3. Saturation curve with [^{3}H]EB in PAC cells exemplified in NCI-H441. Nonspecific binding was assessed by preincubation of the cell homogenate with nonradioactive (−)-nicotine hydrogen tartrate (10 mM) for 60 min at 37°. Analysis of the binding data by Scatchard transformation (inset) suggested the presence of a single class of binding sites with a B_{max} of 158.4 fmol/mg protein and a K_d of 11.76 nM. Nonlinear regression analysis for single-site isotherms of the saturation curves demonstrated a close fit of the data points to the regression lines (at the 95% confidence interval the goodness of fit for total binding (○) was $r^2 = 0.99$ and for specific binding (●), $r^2 = 0.94$). Saturation analysis with [^{3}H]EB in NCI-H322 yielded a similar curve. No specific binding was detected with this ligand in the two SCLC cell lines, NCI-H69 and NCI-H82. Data shown are means ± SEM of triplicate samples per data point from one of two replicate experiments.

Stronger evidence for a single class of binding sites came from the results of nonlinear regression for single-site isotherms of the saturation curves, which demonstrated a close fit of the data points to the regression lines (Fig. 2) No specific binding was detected in the two PAC cell lines (NCI-H441, NCI-H322) with [^{125}I]α-BTX, suggesting the absence of an α7 nAChR in this histologic cancer type. By contrast, saturation analysis with [^{3}H]EB revealed the expression of a neuronal nAChR with binding domains for this site-selective ligand (α4 or α3) in both PAC cell lines (Fig. 3), whereas the lack of specific binding in both SCLC lines with this radioligand indicated an absence of receptors with these subunits. Analysis of the binding data by nonlinear regression for single-site isotherms of the saturation curves (Fig. 3) and by Scatchard transformation (Fig. 3, inset) identified the binding sites as a single class of receptors.

Receptor binding assays in which ascending concentrations of NNN, NNK, and nicotine competed for nicotinic binding sites with [^{125}I]α-BTX in SCLC cells (Fig. 4) and with [^{3}H]EB in PAC cells (Fig. 5) revealed that each of the nitrosamines is a high-affinity ligand for one of these two nAChR subtypes. Analysis of the competitive binding curves by nonlinear regression, assuming a single class of binding sites, showed that affinities in competition for the [^{125}I]α-BTX binding sites in SCLC cells were NNK > nicotine > NNN (Table 1), whereas the affinities for the [^{3}H]EB binding sites in PAC cells were NNN > nicotine > NNK (Table 1). Analysis of EC_{50} values in the competition assay with [^{125}I]α-BTX by *t*-test revealed values of $P = 0.13$ for nicotine ↔ NNN, $P = 0.0001$ for nicotine ↔ NNK, and $P = 0.0001$ for NNK ↔ NNN. The respective P values for the competition assay with [^{3}H]EB were: $P = 0.0002$ (nicotine ↔ NNN), $P = 0.0001$ (nicotine ↔ NNK), and $P = 0.0001$ (NNK ↔ NNN). In keeping with its close structural similarity to nicotine, NNN shared the preference of nicotine for the EB-sensitive binding sites. The EC_{50} values (Table 1) indicated that the affinity of NNN for the α4 nAChR was about 60,500 times greater than that of NNK and 5000 times greater than that of nicotine. On the other hand, the affinity of NNK to the α-BTX-sensitive α7 nAChR was about 1300 times greater than that of nicotine and 3500 times greater than that of NNN.

Analysis of cell numbers over time by a hemocytometer showed that NNK significantly increased the number of SCLC cells over controls ($P < 0.001$, Fig. 6). This effect was inhibited completely by α-BTX ($P < 0.001$, Fig. 6). Assays using the incorporation of [^{3}H]thymidine as endpoint confirmed these results (Table 2) and identified the observed increases in cell number as a mitogenic response. These findings identify the observed binding of NNK to the α7 nAChR as a mitogenic stimulus for these cells. By contrast, neither NNN nor nicotine stimulated the proliferation of PAC cells (Table 2), suggesting that the EB-sensitive nAChR in these cells does not directly regulate cell proliferation. Stimulation of the α7 nAChR by NNK thus provides SCLC with a growth advantage over other cell types. As NNK is tobacco-specific and does not occur in an environment uncontaminated by tobacco products, this effect may directly contribute to the selective development of SCLC in smokers.

DISCUSSION

Nicotine is a classic agonist for all members of the nAChR family, and its many biological effects are mediated by ligand binding to the various subtypes of this receptor family. The structural similarities of nicotine with its two nitrosated derivatives, NNN and NNK, led us to hypothesize that these two carcinogenic nitrosamines are nicotinic receptor ligands. To test this hypothesis, we chose a pharmacological approach. Unlike molecular methods used for the analysis of receptor subunit expression [9, 10], the receptor binding assays with subunit-specific ligands employed by us allowed for the simultaneous identification of

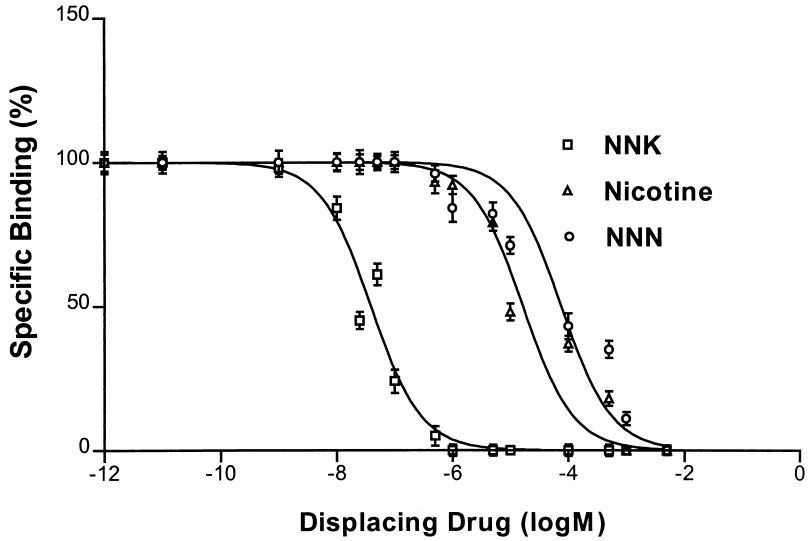

FIG. 4. Results of receptor binding assays in which ascending concentrations of NNK, NNN, and nicotine competed with [^{125}I]α-BTX (20 nM) for the α7 nicotinic binding sites in SCLC cells (NCI-H69). Analysis of the displacement data by nonlinear regression, assuming a single class of receptors (EBDA/Ligand for the Macintosh and Prism/Graphpad for the IBM) identified an EC_{50} value of 30.6 nM for NNK, thus identifying this nitrosamine as a high-affinity ligand for this nAChR subtype. Displacement of [^{125}I]α-BTX required substantially higher concentrations of nicotine and NNN (EC_{50} values of 40.4 and 106.9 μM, respectively, Table 1). Data shown are means ± SEM of triplicate samples per data point derived from one of two replicate experiments.

the receptor subtype(s) expressed and analysis of binding characteristics of the nitrosamines to these receptors.

Our data show that each of these nitrosamines is a selective high-affinity ligand for a different type of nAChR. NNK bound with high affinity to an α-BTX-sensitive α7 nAChR that was expressed selectively in SCLC but not PAC cells, whereas NNN bound with high affinity to an EB-sensitive nAChR that was expressed selectively in PAC cells. The concentration of nicotine in the mainstream smoke of cigarettes is on the average 5,000–10,000 times greater than that of NNK, whereas the concentration of NNN, in turn, is about 2–3 times greater than that of NNK [3]. The initial relative proportions of unmetabolized nicotine and nitrosamines in the blood of a smoker are likely similar [6, 7]. If all three of these nicotinic receptor ligands would bind with equal affinity to all nAChR subtypes in the mammalian body, the tobacco-specific nitrosamines would clearly be at a disadvantage in competition with nicotine for these binding sites. However, the selective high affinity of each nitrosamine for one nAChR subtype counteracts the prevalence of nicotine in the blood of smokers. The high selectivity of NNK for the α7 nAChR is enhanced even further by the fact that this nAChR subtype accounts for only about 10% of the neuronal nAChR family, which consists primarily of receptors expressing the EB-sensitive α4 subunit [10, 11]. Accordingly, our data suggest that the simultaneous exposure to nicotine and these two nitrosamines by tobacco products results in significant binding of NNK to members of the neuronal nAChR family expressing the α7 subunit and of NNN to receptors expressing EB-sensitive α4 or α3 subunits.

In cell proliferation assays, binding of NNK to the α-BTX-sensitive α7 nAChR was a potent mitogenic stimulus. These findings support the hypothesis that the neuronal α7 nAChR is an important regulator of SCLC [14–16]. NNK thus provides SCLC with a selective growth advantage over other cell types via this nAChR-mediated mechanism. As NNK is tobacco-specific and does not occur

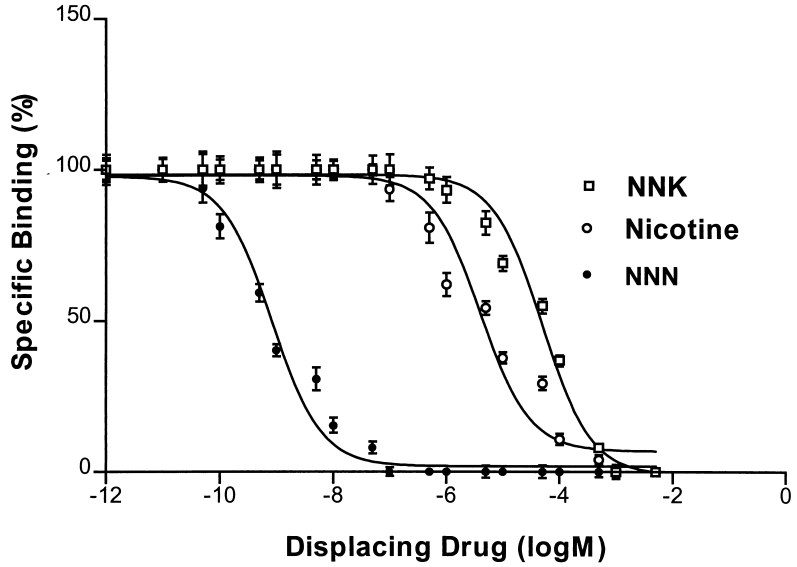

FIG. 5. Results of receptor binding assays in which NNN, nicotine, and NNK competed with [^{3}H]EB (20 nM) for the EB-sensitive nicotinic binding sites in PAC cells (NCI-H441). The curves are nonlinear regression lines for a single class of receptors. NNN demonstrated the highest affinity to this nAChR subtype as expressed by an extremely low EC_{50} (0.83 nM). The EC_{50} values for nicotine (4.27 μM) and NNK (50.2 μM) were significantly higher (Table 1). Data shown are means ± SEM of triplicate samples from one of two replicate experiments.

TABLE 1. EC_{50} values for nicotine, NNN, and NNK

Competing ligand	EC_{50} (μM)	
	Competition with [^{125}I]α-BTX in SCLC	Competition with [^{3}H]EB in PAC
Nicotine	40.4 ± 2.8	4.27 ± 1.0
NNN	106.9 ± 2.9	0.00083 ± 0.00004
NNK	0.0306 ± 0.003	50.17 ± 7.7

Analysis of all EC_{50} values in competition assays with [^{125}I]α-BTX by one-way analysis of variance revealed that the variation among EC_{50} means was significantly greater than expected by chance ($P < 0.0001$). Analysis of all EC_{50} values in competition assays with [^{3}H]EB showed that the variation among EC_{50} means was significantly greater than expected by chance ($P = 0.0002$). Data are mean values ± SEM of triplicate samples.

elsewhere in the human environment, this, in turn, explains why SCLC is a tobacco-specific histologic lung cancer type. By contrast, binding of nicotine or NNN to the EB-sensitive nAChR in PAC cells did not stimulate cell proliferation, suggesting that this nAChR subtype does not directly regulate the proliferation of PAC. Recent studies have shown that nicotine can inhibit apoptosis in some lung cancer cells by binding to a neuronal nAChR of an as yet unidentified subunit composition [22]. Further studies are needed to elucidate the function and exact subunit composition of this nAChR subtype in PAC cells.

Selective high-affinity binding of each of the two tobacco-specific nitrosamines to a defined subtype of the neuronal nAChR family introduces a novel mechanism of action of these carcinogens, a finding that impacts on diverse areas of science. Although it comes as no surprise that the nitrosated derivatives of nicotine act as nAChR ligands just like their parent compound, research on the mechanisms of carcinogenesis induced by NNN and NNK has not addressed this important aspect. Metabolic pathways of NNN and NNK as well as the interaction of reactive metabolites with DNA have been investigated extensively [3, 5, 7, 8, 23–26]. These studies culminated in the finding that the formation of the most prominent DNA-adduct (O^6-methylguanine) from reactive metabolites of these nitrosamines correlates with the expression of point mutations in the *K-ras* gene [25], an event implicated in the development and progression of many cancer types [26]. However, SCLC, which in the current study responded with cell proliferation to NNK, typically lacks point mutations in the *K-ras* gene [27] and is unable to metabolize nitrosamines *in vitro* [28]. In this context, our findings suggest that the initiation and progression of SCLC in smokers are independent of the formation of promutagenic DNA adducts from tobacco-specific nitrosamines and are instead mediated by the chronic selective stimulation of mitogenesis via ligand binding of NNK to the α7 nAChR. Although the unequivocal classification of NNK as an agonist for this receptor requires the demonstration of ion channel opening, the fact that the classic agonist nicotine also stimulates the growth of SCLC cells by binding to the α7 nAChR [13, 14, 16] suggests that this nitrosamine functions as an agonist.

In light of the close structural similarity of NNN with nicotine (Fig. 1), it is logical that this nitrosamine resembles nicotine in its binding characteristics to nAChR subtypes. The EB-sensitive nAChR to which both of these agents bound with high affinity has been implicated in the etiology of cardiovascular disease because of its regulatory role on blood pressure by the release of catecholamines from the adrenal medulla [2]. NNN may thus contribute to the development of this disease complex.

Preliminary studies conducted in our laboratory suggested that the α-BTX-sensitive α7 nicotinic receptor is expressed selectively in lung cells of a neuroendocrine phenotype (pulmonary neuroendocrine cells, carcinoids, SCLC). Our current data showed a lack of this receptor in

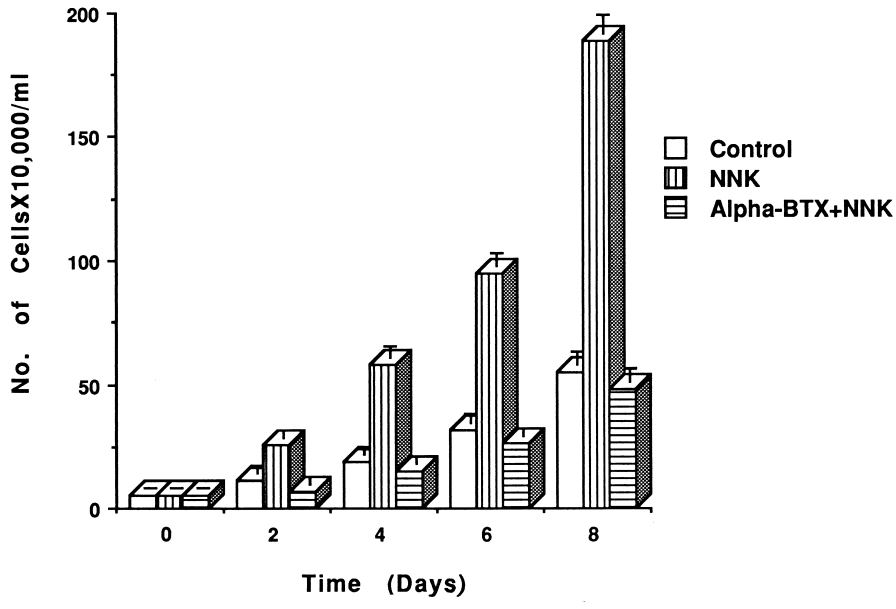

FIG. 6. Stimulation of SCLC cell proliferation over time by NNK. The stimulating effect of NNK was significant ($P < 0.001$ by unpaired *t*-test), an effect inhibited completely by α-BTX in excess of its saturation concentration (40 nM). These findings identify ligand binding of NNK to the α-BTX-sensitive neuronal nAChR, which expresses the α7 subunit as the mitogenic stimulus. Data shown are numbers of viable cells determined by hemocytometer. They were verified by assays using the incorporation of [^{3}H]thymidine as endpoint (Table 2). Data shown are means ± SEM of triplicate samples per data point from one of two replicate experiments. Control, open columns; NNK, vertically striped columns; α-BTX + NNK, horizontally striped columns.

TABLE 2. Results of thymidine incorporation assays

Cell line and treatment	[^{3}H]Thymidine incorporation (dpm)	Cell line and treatment	[^{3}H]Thymidine incorporation (dpm)
NCI-H69		**NCI-H82**	
Control	1640 ± 90	Control	1730 ± 80
NNK	5090 ± 150	NNK	6240 ± 130
α-BTX + NNK	1480 ± 70	α-BTX + NNK	1670 ± 40
α-BTX	1610 ± 100	α-BTX	1750 ± 40
NCI-H322		**NCI-H441**	
Control	1440 ± 20	Control	1300 ± 70
Nicotine	1400 ± 30	Nicotine	1300 ± 30
NNN	1420 ± 30	NNN	1280 ± 30

Data are mean values ± SD of triplicate samples. Incubation of cells with [^{3}H]thymidine was for 48 hr. One-way analysis of variance showed that the variation among means of all groups was significantly greater than expected by chance ($P < 0.001$) in both SCLC cell lines, whereas it was not significant ($P = 0.78$ and 0.5) in the two adenocarcinoma cell lines. The incorporation of [^{3}H]thymidine was significantly greater (t-test, $P < 0.001$) in both SCLC cell lines exposed to NNK than in the controls and after co-incubation with α-BTX and NNK. By contrast, neither nicotine- nor NNN-treated adenocarcinoma cells showed significant differences in the incorporation of [^{3}H]thymidine from controls (t-test, P values between 0.54 and 0.86).

PAC cells, which have a non-neuroendocrine phenotype. SCLC typically responds initially to conventional cancer therapy but demonstrates a high relapse rate accompanied by extensive metastasis and the development of multi-drug resistance. Utilization of the α7 nAChR as a tool for selective diagnostic imaging may significantly improve the early detection of this histologic cancer type and its metastases. Moreover, this receptor may be a promising target for the development of therapeutic strategies with selectivity for SCLC.

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References

1. Weiss W, Epidemiology of lung cancer. In: *Comparative Respiratory Tract Carcinogensis* (Ed. Schuller HM), pp. 1–18. CRC Press, Boca Raton, 1983.
2. Benovitz NL, Nicotine and coronary heart disease. *Trends Cardiovasc Med* **1:** 315–321, 1991.
3. Hecht SS and Hoffmann D, Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. *Carcinogenesis* **9:** 875–884, 1990.
4. Fischer S, Spiegelhalder B, Eisenbarth J and Preussmann R, Investigation on the origin of tobacco-specific nitrosamines in mainstream smoke of cigarettes. *Carcinogenesis* **11:** 723–730, 1990.
5. Hecht SS, Carmella SG, Foiles PG, Murphy SE and Peterson LA, Tobacco-specific nitrosamine adducts. Studies in laboratory animals and humans. *Environ Health Perspect* **99:** 57–63, 1993.
6. Armitage AK, Dollery CT, George CF, Houseman TH, Lewis PJ and Turner DM, Absorption and metabolism of nicotine from cigarettes. *Br Med J* **4:** 313–316, 1975.
7. Carmella SG, Kagan SS, Kagan M, Foiles PG, Palladino G, Quart AM, Quart E and Hecht SS, Mass spectrometric analysis of tobacco-specific nitrosamine hemoglobin adducts in snuff dippers, smokers, and nonsmokers. *Cancer Res* **50:** 5438–5445, 1990.
8. Hecht SS, Trushin N, Reid-Quinn CA, Burak ES, Jones AB, Southers JL, Gombar CT, Carmella SG, Anderson LM and Rice JM, Metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in the patas monkey: Pharmacokinetics and characterization of glucuronide metabolites. *Carcinogenesis* **14:** 229–236, 1993.
9. Lindstrom J, Anand R, Peng X and Gerzanich V, Neuronal nicotinic receptor structure and function. In: *Effects of Nicotine on Biological Systems* (Eds. Clarke PBS, Quik M, Adlkofer F and Thurau K), pp. 45–50. Birkhäuser, Basel, 1995.
10. Couturier S, Bertrand D, Matter J-M, Hernandez M-C, Bertrand S, Millar N, Valera S, Barkas T and Ballivet M, A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX. *Neuron* **5:** 847–856, 1990.
11. Houghtling RA, Davila-Garcia MI and Kellar KJ, Characterization of (±)-[^{3}H]epibatidine binding to nicotinic cholinergic receptors in rat and human brain. *Mol Pharmacol* **48:** 280–287, 1995.
12. Marks MJ, Burch JB and Collins AC, Effects of chronic nicotine infusion on tolerance development and cholinergic receptors. *J Pharmacol Exp Ther* **226:** 806–816, 1983.
13. Schuller HM, Cell type-specific, receptor-mediated modulation of growth kinetics in human lung cancer cell lines by nicotine and tobacco-related nitrosamines. *Biochem Pharmacol* **38:** 3439–3442, 1989.
14. Quik M, Chan J and Patrick J, α-Bungarotoxin blocks the nicotinic receptor mediated increase in cell number in a neuroendocrine cell line. *Brain Res* **655:** 161–167, 1994.
15. Schuller HM, Carbon dioxide potentiates the mitogenic effects of nicotine and its carcinogenic derivative, NNK, in normal and neoplastic neuroendocrine lung cells via stimulation of autocrine and protein kinase C-dependent mitogenic pathways. *Neurotoxicology* **15:** 877–886, 1994.
16. Codignola A, Tarroni P, Cattaneo MG, Vicentini LM, Clementi F and Sher E, Serotonin release and cell proliferation are under the control of alpha-bungarotoxin-sensitive nicotinic receptors in small cell lung carcinoma cell lines. *FEBS Lett* **342:** 286–290, 1994.
17. Schuller HM, Castonguay A, Orloff M and Rossignol G, Modulation of the uptake and metabolism of 4-(methylnitro-

samino)-1-(3-pyridyl)-1-butanone by nicotine in hamster lung. *Cancer Res* **51:** 2009–2014, 1991.
18. Hulme EC, Receptor binding studies. In: *Receptor Biochemistry* (Ed. Hulme EC), pp. 303–315. Oxford University Press, Oxford, 1990.
19. McPherson GA, Analysis of radioligand binding experiments: A collection of computer programs for the IBM PC. *J Phamacol Methods* **14:** 213–228, 1985.
20. Martino-Burrows AM and Kellar KJ, [^{3}H]Acetylcholine and [^{3}H](−)nicotine label the same recognition site in rat brain. *Mol Pharmacol* **31:** 169–174, 1987.
21. Lambertson CJ, The atmosphere and gas exchange with the lungs and blood. In: *Medical Physiology* (Ed. Mountcastle VB), Vol. 2, pp. 1372–1398. C.V. Mosby Co., Saint Louis, 1974.
22. Maneckjee R and Minna JD, Opioids induce while nicotine suppresses apoptosis in human lung cancer cells. *Cell Growth Differ* **5:** 1033–1040, 1994.
23. Peterson LA and Hecht SS, O^6-Methylguanine is a critical determinant of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone tumourigenesis in A/J mouse lung. *Cancer Res* **51:** 5557–5564, 1991.
24. Hecht SS, Spratt TE and Trushin N, Evidence for 4-(3-pyridyl)-4-oxobutylation of DNA in F344 rats treated with the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N′*-nitrosonornicotine. *Carcinogenesis* **9:** 161–165, 1988.
25. Belinsky SA, Devereux TR, Stoner GD and Anderson MW, Activation of the K-*ras* protooncogene in lung tumors of mice treated with 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK) and nitrosodimethylamine (NDMA). *Cancer Res* **49:** 5303–5311, 1988.
26. Reynolds SH, Wiest JS, Devereux TR, Anderson MW and You M, Protooncogene activation in spontaneously occurring and chemically induced rodent and human lung tumors. In: *Comparative Molecular Carcinogenesis* (Eds. Klein-Szanto AJP, Anderson MW, Barrett JC and Slaga TJ), pp. 303–320. Wiley-Liss, New York, 1992.
27. Mitsudomi T, Viallet J, Mulshine JL, Linnoila RI, Minna JD and Gazdar AF, Mutations of *ras* genes distinguish a subset of non-small-cell lung cancer cell lines from small-cell lung cancer cell lines. *Oncogene* **6:** 1353–1362, 1991.
28. Falzon M, McMahon JB, Gazdar AF and Schuller HM, Preferential metabolism of *N*-nitrosodiethylamine by two cell lines derived from human pulmonary adenocarcinomas. *Carcinogenesis* **7:** 17–22, 1986.