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Interaction of tobacco-specific toxicants with the neuronal α_7 nicotinic acetylcholine receptor and its associated mitogenic signal transduction pathway: potential role in lung carcinogenesis and pediatric lung disorders

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Abstract

Pulmonary neuroendocrine cells function as hypoxia-sensitive chemoreceptors, and they release peptides and biogenic amines that are important mediators of pulmonary neonatal adaptation. Some of these products additionally act as autocrine growth factors. Increased numbers of pulmonary neuroendocrine cells have been observed in several smoking-associated pediatric lung disorders such as bronchopulmonary dysplasia, cystic fibrosis, sudden infant death syndrome, and asthma. Disturbed pulmonary neuroendocrine function has been implicated in the etiology of this disease complex. One of the most common smoking-associated lung cancer types, small cell lung carcinoma, expresses phenotypic and functional features of pulmonary neuroendocrine cells. We, as well as others, have shown that the release of the autocrine growth factors 5-hydroxytryptamine (5-HT, serotonin) and mammalian bombesisn/gastrin releasing peptide (MB/GRP) by cell lines derived from human small cell lung carcinoma or fetal hamster pulmonary neuroendocrine cells are regulated by a neuronal nicotinic acetylcholine receptor comprised of α_7 subunits. In radio-receptor assays, nicotine and the nicotine-derived carcinogenic nitrosamines *N′*-nictrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) bound with high affinity to this receptor with affinitites NNK > nicotine > NNN. Binding of nicotine or NNK to the α_7 receptor resulted in calcium influx and overexpression and activation of the serine–threonine protein kinase Raf-1. In turn, this event lead to overexpression and activation of the mitogen activated (MAP) kinases extracellular signal regulated kinase 1 (ERK1) and extracellular signal regulated kinase 2 (ERK2) and stimulation of DNA synthesis accompanied by an increase in cell numbers in fetal pulmonary neuroendocrine cells and small cell carcinoma cells. Exposure of fetal pulmonary neuroendocrine cells for 6 days to NNK caused a prominant up-regulation of Raf-1. Our findings suggest that chronic exposure to nicotine and NNK in pregnant women who smoke may up-regulate the α_7 nicotinic receptor as well as components of its associated mitogenic signal transduction pathway, thus increasing the susceptibilities of the infants for the development of pediatric lung disorders. Similarly, up-regulation of one or several components of this nicotinic receptor pathway in smokers may be an important factor for the development of small cell lung carcinoma.

Keywords: α_7 Nicotinic receptor; Nicotine; Nicotine-derived nitrosamine; Small cell lung carcinoma; Pulmonary neuroendocrine cell; Mitogenic signal transduction

1. Introduction

This manuscript represents a review of our work during the past 3 years and includes new, unpublished data, as well as some data which have been previously published in a different context. Appropriate reference is given in the text and figure legends.

Pulmonary neuroendocrine cells are specialized lung cells which co-express features of neurons and endocrine cells (Becker, 1984). These cells are abundant in the neonate, and decrease rapidly in number by 12 months of age (Cutz, 1997; Cutz et al., 1985). Pulmonary neuroendocrine cells are hypoxia-sensitive chemoreceptors and are thought to be important mediators of pulmonary neonatal adaptation, particularly the onset of breathing (Cutz, 1997; Cutz et al., 1985). Pulmonary neuroendocrine cells demonstrate hyperplasia accompanied by elevated levels of their

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secretory products in a variety of pediatric pulmonary diseases, such as bronchopulmonary dysplasia (Johnson and Georgieff, 1989; Johnson et al., 1982; Cutz, 1997), cystic fibrosis (Johnson et al., 1988; Cutz, 1997), sudden infant death syndrome (Cutz et al., 1988, 1996; Johnson and Georgieff, 1989), and asthma (Stanislawski et al., 1981; Johnson and Georgieff, 1989; Aguayo, 1993). Functional abnormalities of these cells may contribute to the pathophysiology of this family of pediatric lung diseases because their peptide and biogenic amine secretory products modulate the tonus of bronchial and vascular smooth muscle, modulate neurotransmission, and stimulate the growth of epithelial lung cells and fibroblasts (Willey et al., 1984; Johnson et al., 1988; Seuwen et al. 1988; Sunday, 1988; Schuller and Hegedus, 1989; Rozengurth and Sinneth-Smith, 1990; Cutz, 1997). The most prominent product of pulmonary neuroendocrine cells for which effects on cell growth, smooth muscle tension and neuromodulation have been documented, is the biogenic amine 5-hydroxytryptamine (Cutz, 1997; 5-HT, serotonin). Research into the role of 5-HT in pediatric pulmonary disease has focused on its well-documented constricting effects on bronchial and vascular smooth muscle (Johnson et al., 1988; Lechin et al., 1996; Cutz, 1997). Although it has been known for some time that 5-HT stimulates the growth of fibroblasts, its growth stimulating effects on pulmonary neuroendocrine cells and small cell carcinima has been only recently discovered (Schuller, 1989; Schuller and Hegedus, 1989; Schuller and Orloff, 1998).

Epidemiologic studies have established that babies of mothers who smoke during pregnancy are at increased risk to develop sudden infant death syndrome (Cutz et al., 1988; Johnson and Georgieff, 1989; Malloy et al., 1992; Schoendorf and Kiely, 1992; Schellscheidt et al., 1997) and asthma (Hu et al., 1997; Oliveti et al., 1996). Moreover, both of these diseases as well as bronchopulmonary dysplasia and cystic fibrosis are exacerbated in newborns and infants exposed to second-hand smoke (Aguayo, 1993; Cutz et al., 1996; Knight et al., 1998). However, the underlying mechanisms of these effects are far from understood. Nicotine is a well-documented secretagogue for pulmonary neuroendocrine cells and causes exocytosis of their dense-cored cytoplasmic granules which are the storage site of peptides and serotonin (Lauweryns et al., 1977; Tabbasian et al., 1989; Cutz, 1997). Similarly, nicotine stimulated the release of 5-HT from human small cell lung carcinoma cells in culture (Codignola et al., 1994, Schuller and Orloff, 1998). As it is well established that the biological effects of nicotine are mediated by binding to nicotinic acetylcholine receptors, these findings suggest that the release of 5-HT is under autonomic control via members of the nicotinic acetylcholine receptor family.

Smoking has long been recognized as the single most important risk factor for the development of lung cancer (Weiss, 1983; Cook et al., 1993). While non-small cell lung carcinomas develop also in a significant number of non-smokers, small cell lung carcinoma is extremely rare in non-smokers (Weiss, 1983; Cook et al., 1993). Chronic non-neoplastic pulmonary disease (bronchitis/bronchiolitis, emphysema, chronic obstructive pulmonary disease, asthma) has more recently emerged as a risk factor for lung cancer in both smokers and non-smokers (Osann, 1991; Weiss, 1991; Park et al., 1995). In fact, a recent study has shown that elevated CO_2 levels comparable to those in the diseased lung stimulate the release of 5-HT, resulting in the activation of the MAP kinase cascade in SCLC (Merryman et al., 1997). Small cell lung carcinoma expresses phenotypic and functional characteristics of pulmonary neuroendocrine cells (Gazdar and Carney, 1984). All pulmonary neuroendocrine cells and small cell lung carcinoma cells have the ability to synthesize and release 5-HT (Becker, 1984; Gazdar, 1984), suggesting an important function of this biogenic amine in this histologic lung cancer type. This interpretation gains strong support from the recent finding that 5-HT is an autocrine growth factor for this cancer type as well as for pulmonary neuroendocrine cells (Schuller, 1989; Schuller and Hegedus, 1989; Schuller and Orloff, 1998; Codignola et al., 1994).

The tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N′*-nitrosonornicotine (NNN) are formed from nicotine by nitrosation (Fig. 1) during the curing of tobacco and in the mammalian organism (Fischer et al., 1990; Hecht and Hoffmann, 1990). NNK is a potent lung carcinogen in all animal species tested, and is thought to contribute significantly to the high lung cancer burden associated with smoking (Hecht and Hoffmann, 1990). On the other hand, NNN, which more closely resembles nicotine in structure than NNK (Fig. 1) is a weak carcinogen in laboratory animals. It is well established that reactive metabolites of

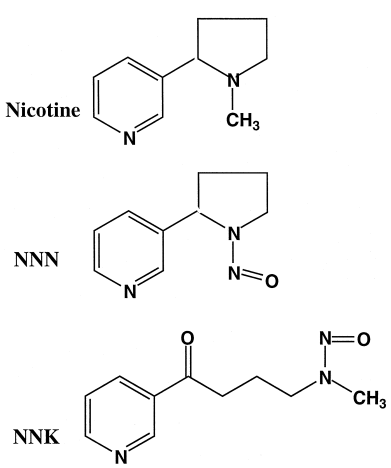

Fig. 1. Structure of nicotine and the two carcinogenic nitrosamines NNN and NNK: nicotine is a tertiary amine consisting of a pyridine and a pyrrolidine ring. NNN and NNK form from nicotine by nitrosation during the processing and storage of tobacco and in the mammalian organism. The formation of NNN 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.

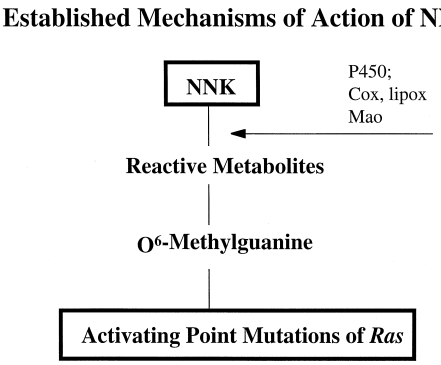

Fig. 2. The nicotine-derived nitrosamine NNK is the most potent carcinogen contained in tobacco products. It has been shown that NNK is metabolically activated by cytochrome P450 (P450), 5-lipoxygenase (Lipox), and monoamine oxidase (Mao). The reactive metabolites formed interact with the DNA molecule, thus forming DNA adducts. One of these adducts is O^6-methylguanine, which has been linked with the formation of activating point mutations in the Ki-*Ras* gene (Hecht and Hoffmann, 1990; Hecht et al., 1993a,b).

NNK or NNN may form DNA-methylating and pyridyloxobutylating products (Fig. 2), resulting in activating point mutations of the Ki-*ras* gene (Belinsky et al., 1988; Hecht et al., 1993a,b). With respect to the central role of *ras* as a signal transducer involved in mitogenic pathways of mammalian cells (Lange-Carter and Johnson, 1994), this event is thought to be primarily responsible for the development of lung tumors in response to NNK (Belinsky et al., 1988; Hecht et al., 1993a,b). However, point mutations of the *ras* gene have only been identified in non-small cell lung carcinomas whereas they are consistently absent in small cell lung carcinoma (Mitsudomi et al., 1991; Wagner et al., 1993). The α_7 nicotinic acetylcholine receptor and its associated mitogenic signal trasnduction pathway is emerging as an important growth regulator of pulmonary neuroendocrine cells and small cell lung carcinoma (Schuller, 1989; Schuller and Hegedus, 1989; Schuller and Orloff, 1998; Cattaneo et al., 1993, 1997; Codignola et al., 1994) and may be critically involved in the development of neoplastic and non-neoplastic pulmonary diseases.

2. Methods

2.1. Tissue culture

Cultures of fetal pulmonary neuroendocrine cells were established from fetal hamster lung periphery harvested on day 15 of gestation as previously described (Linnoila et al., 1993; Schuller, 1994). Following disaggregation of the tissue (100 mg) with 3 ml trypsin/EDTA for 60 min at 37°C, complete RPMI medium was added to yield 10^6 cells/ml, and 2 ml each of the resulting cell suspension was used to seed 6 T75 tissue culture flasks. The cultured cells were enriched to $>80\%$ of pulmonary neuroendocrine cells by selective growth for 5 days in an atmosphere of 10% CO_2 in RPMI medium supplemented with fetal bovine serum (10%, v/v), L-glutamine (2 mM), and gentamycin sulfate (50 mg/ml) (Biofluids, Rockville, MD) as previously described (Linnoila et al., 1993; Schuller, 1994). Cells were harvested when the monolayers were about 75% confluent. All studies were conducted on the primary cultures.

Cell lines NCI-H69 and NCI-H82 derived from human small cell lung carcinomas were purchased at the American Type Culture Collection (Rockville, MD). They were maintained under conditions identical to those used for the fetal pulmonary neuroendocrine cells.

2.2. Reverse transcription polymerase chain reaction (RT-PCR)

RNA was isolated from fetal pulmonary neuroendocrine cells and small cell carcinoma cells using guanidine isothiocyanate/cesium chloride ultracentrifugation (McDonald et al., 1987). Concentration of the RNA was determined by optical density at 260 nm. For the reverse transcription (RT) reaction, 2 μg RQ1 Rnase-free Dnase (Promega, Madison, WI) treated RNA, 1 μg Oligo dT_{12-18} primers (Gibco, Grand Island, NY) and nuclease free water was heated to 82°C for 3 min, then placed on ice. To this was added 0.5 mM each dGTP, dATP, dCTP and dTTP, 10 mM DTT, 40 U RNasin ribonuclease inhibitor (Promega), 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco) and 10× buffer (100 mM Tris–HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$) in a final volume of 20 μl. Following an incubation at 37°C for 1 h, the enzymes were heat inactivated for 10 min at 92°C. A reaction performed without the M-MLV served as negative control.

The PCR reactions for identification of the α_7 nicotinic receptor were performed with 5 μl (one-quarter) of the RT reaction. This aliquot was mixed with 0.2 mM dNTPs, 5 μl 10× PCR buffer (100 mM Tris–HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 1.25 U SuperTaq polymerase (Ambion, Austin, TX), 5% (2.5 μl) DMSO, a primer pair for β-actin used as an internal control (125 nM, Promega), and primers for the human α_7 nicotinic acetylcholine receptor (500 nM) and nuclease free water in a final volume of 50 μl. The human α_7 primers (30) were forward 5′-ctcctgcacgtgtccctgcaa-3′ and reverse 5′-tagagtg-tcctgcggcgcat-3′, which amplified a 651-bp fragment. Reactions were run on a MJ Research PTC-200 thermal cycler (Watertown, MA) with the following conditions: 1 cycle of 2 min at 94°C, 35 to 40 cycles of: 94°C, 20 s; 60°C, 20 s; 72°C, 45 s, with a final extension for 5 min at 72°C. Since the sequence for the hamster α_7 nicotinic acetylcholine receptor is not known, PCR primers derived from the human sequence were used to amplify cDNA in fetal hamster PNEC. The human primers amplified a 651-bp fragment from hamster cDNA. In order to determine if this sequence matched that of α_7, 611 bp of the α_7 fragment between

the PCR primers was sequenced using the primers employed to amplify the fragment by RT-PCR as well as primers derived from the sequence (Grando et al., 1996). Sequencing was conducted with an ABI Terminator Cycle Sequencing reaction kit on an ABI 373 DNA sequencer (Perkin-Elmer, Foster City, CA). Sequences were entered into DNASIS software (Hitachi, S. San Francisco, CA) and compared to published sequences for the α_7 nicotinic acetylcholine receptor in humans and mice.

The PCR reactions for identification of voltage operated calcium-channels (Innis et al., 1990; Farrell, 1993, 1997) were performed with 5 μl of the RT reaction. To this aliquot was added dNTPs (0.2 mM each dNTP), PCR buffer, 5% DMSO, 1.25 U SuperTaq polymerase (Ambion), 500 nM of gene specific primers, or 100 nM of a cyclophilin primer pair (Ambion) as internal control (Finnegan et al., 1993), and nuclease free water to a final volume of 50 μl. The gene-specific primers for the L-type channels were forward 5′-GTG CCC TGC ACA CAG TAG TCG C-3′ and reverse 5′-GGC GCG GGC AGG TCG GCT GTT GG-3′ (bases 472–825, Genbank accession no. M76558, from a human neuroblastoma; Williams et al., 1992b) and amplified a 353-bp fragment. Primers for the N-type channels were forward 5′-GTC GCC AAC AGC TGG CCA GGA GC-3′ and reverse 5′-CGA AAG TGA GCG TGT CCT CAG GC-3′ (bases 6595–6986, Genbank accession no. M94172 from a human neuroblastoma; Williams et al., 1992a) and amplified a 391-bp fragment. Primers for the P-type channels were forward 5′-GCT TAT GTC TGT CTG CTG ATC G-3′ and reverse 5′-GAC AAA GAG ATT CAG CAT CAG-3′ (bases 1–345, Genbank accession no. S76537, from a human small cell lung carcinoma; Barry et al., 1995) and amplified a 345-bp fragment. The cylophilin primer amplified a 216-bp DNA product. Reactions were run on a MJ Research PTC-200 thermal cycler under the following conditions: 1 cycle of 2 min at 94°C, followed by 33 cycles of 94° for 60 s, 60°C (L-type primers), 51°C (N-type primers), or 56°C (P-type primers) for 90 s, and 72° for 90 s, with a final extension for 5 min at 72°C. In order to verify that the fragments amplified by the specific primers were homologous to the published Genbank sequences for the mRNA of the channel types under study, sequencing of the PCR fragments was conducted using both the forward and reverse primers with an ABI Terminator Cycle Sequencing reaction kit on an ABI 373 DNA sequencer (Perkin-Elmer). Sequences were entered into DNASIS software (Hitachi) to determine homology.

One half of the PCR reactions (25 μl) were run on a 1.5% agarose (Gibco) gel for 2.15 h at 75 V. A 100 bp DNA ladder (Gibco) was run on the same gel. The gels were imaged by ethidium bromide staining using a UVP (Upland, CA) 7500 gel documentation system.

For the RT reaction (Innis et al., 1990; Farrell, 1993, 1997), 2 μg DNase I-treated (Gibco BRC) RNA and 2 μl of oligo(dT) primers (Gibco) diluted in nuclease free water (total volume 10 μl) were heated to 82°C for 3 min, then placed on ice. To this was added dNTP mix (0.5 mM of each dNTP), 10 mM DTT, 40 U RNasin ribonuclease inhibitor (Promega), 200 U M-MLV reverse transcriptase (Gibco) and PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) in a final volume of 20 μl. Following an incubation at 37°C for 1 h, the reaction mixtures were heat inactivated for 10 min at 92°C. A reaction performed without the M-MLV served as negative control.

2.3. Radio-receptor assays

Radio-receptor assays were conducted as previously described (Schuller and Orloff, 1998; Plummer et al., 1999). Briefly, fetal hamster pulmonary neuroendocrine cells or human small cell lung carcinoma cells were washed twice with phosphate buffered saline (PBS), resuspended in PBS, and centrifuged at $200 \times g$ for 2 min. The pellet was resuspended in PBS, homogenized with a polytron for 20 s, and placed into assay tubes to yield 1 mg protein/tube (Bradford, 1976). Ligands detailed in the figure legend were added in aliquots of 30 μl with a total reaction volume of 300 μl/tube. Nonradioactive nicotine, NNN, or NNK at concentrations from 10 fM to 1 M were coincubated with [^{125}I]-alpha (α)-bungarotoxin (30 nM) for 90 min at 37°C. Nonspecific binding was determined by a 60-min preincubation at 37°C with nonradioactive α-bungarotoxin (1 μM). All reactions were terminated by separating bound from free radioligand by centrifugation ($23{,}000 \times g$ for 5 min; Hulme, 1990). The competition binding data were analyzed by computerized nonlinear regression for a single class of binding sites, including calculation of EC_{50} values (McPherson, 1985; Prism Graphpad for the Macintosh).

2.4. Flow cytometry

Fetal hamster pulmonary neuroendocrine cells or human small cell lung carcinoma cells were washed with PBS without calcium and magnesium, and placed into assay tubes (1×10^7 cells in 1 ml PBS/tube). The cell permeable ester forms of the calcium-indicator dyes Fluo-3 and Fura Red (Molecular Probes, Eugene, OR) were dissolved in DMSO and pluronic (Molecular Probes), and the dyes were added to the assay tubes at a final concentration of 5 and 10 μM, respectively. Following a 30-min incubation at 37°C in the dark in an atmosphere of 10% CO_2, the cells were centrifuged (3 min at $70 \times g$), rinsed in 1× PBS without calcium and magnesium and resuspended in 1 ml of flow cytometry buffer (150 mM NaCl, 5 mM KCl, 1.2 μM KH_2PO_4, 1.2 mM $MgSO_4$, 30 mM $CaCl_2$, 6 mM glucose, 25 mM HEPES, pH 7.4). The samples were filtered using 40 μm nylon strainers (Falcon, Franklin Lakes, NJ) and incubated at 37°C in the dark in an atmosphere of 10% CO_2 and used within 30 min. The wavelength emissions were measured using a flow cytome-

ter (FAC-SCAN, Becton Dickinson, Franklin Lakes, NJ) equipped with 480 nm argon-ion laser. The amount of fluorescence of Fluo-3 increases with increasing intracellular calcium whereas the Fura-Red fluorescence decreases (Novak and Rabinovitch, 1994). The Fluo3/Fura Red ratio thus provides a qualitative measure of changes in intracellular calcium. Baseline Fluo3/Fura Red ratios for each sample immediately prior to treatment were designated as 1.0 with subsequent alterations in the ratios being expressed as fractional changes. Multiple determinations of Fluo3/Fura Red were conducted at the time intervals indicated in the figure.

2.5. Analysis of released 5-HT

Analysis of released serotonin was conducted as previously described (Park et al., 1995) by an enzyme immunoassay using a serotonin enzyme-linked immunosorbent assay (ELISA) kit (ICN Pharmaceuticals, Costa Mesa, CA). The cells were washed twice with PBS and incubated in PBS with nicotine (±nicotine, 1 μM, Sigma Louisville, MO) or NNK (1 nM, Chemsyn Laboratories, Lenexa, KS) at 37°C, in 10% CO_2 for the time intervals indicated in Fig. 5. The incubation buffer was immediately used for analysis of secreted serotonin. Following derivatization of serotonin in the samples to *N*-acylserotonin by acetic acid anhydride in acetone (3%) for 15 min at 37°C, the precipitated protein was removed by centrifugation (10 min at 1500 × *g*), then 50 μl aliquots were withdrawn for ELISA. The ELISA assay was conducted according to instructions in the kit by incubation overnight at 4°C with biotinylated and nonbiotinylated serotonin antigen to allow for competitive binding. Following three washes in buffer, the wells were then incubated with the anti-biotin alkaline phosphatase (150 μL) for 2 h at room temperature. They were then washed three times and incubated for 60 min at room temperature with 200 μl substrate solution (*p*-nitrophenyl phosphate). Optical densities were read at 405 nm with a microplate reader (Biotek Instruments, Winooski, VT).

2.6. Determination of Raf-1 and ERK1/2 expression and activation

Small cell lung carcinoma cells or pulmonary neuroendocrine cells were washed with calcium-free PBS and incubated in low serum Dulbecco's modified eagles medium (0.1% bovine serum albumin, 210 mg L-glutamine, 500 units penicillin, and 500 μg streptomycin) for 24 h prior to the assays. Cells were then washed again in calcium-free PBS and reintroduced into fresh low serum medium 30 min prior to the assays. Following another wash in calcium free PBS, the cells were lysed with detergent (RIPA solution: 1 × PBS, 0.5% sodium deoxycholate, 1% NP4O, 0.1% SDS), containing protease inhibitors (10 μg/ml of 10 mg/ml phenylmethylsulfonyl fluoride, 30 μl/ml of 1.9 mg/ml apoprotein, and 10 μl/ml of 100 mM Na + orthovanadate). The lysates were passed three times through a 21 gauge needle with subsequent addition of phenylmethylsulfonylfluoride, (10 mg/ml) and incubation on ice for 30 min and centrifuged at 4° for 20 min at 14,000 rpm and the supernatant collected for protein expression assays. Expression levels of Raf-1 and ERK were determined by western blot using a polyclonal rabbit immunoglobulin G (IgG) for Raf-1 (200 μg/ml) or a polyclonal rabbit IgG for ERK1/2 (200 μg/ml, Santa Cruz Biolabs, Santa Cruz, CA) following electrophoretic transfer of the concentrated protein to nitrocellulose membranes. The membranes were incubated with the antibodies for 1 h at a dilution of 1:2000 in the blocking solution (Tris buffered saline with 0.05% Tween-20). Following three washes in Tris buffered saline, the membranes were then incubated for 1 h at room temperature with antirabbit IgG horse radish peroxidase (400 μg/ml, Santa Cruz Biolabs) at a dilution of 1:1000 in the blocking solution. The autoradiographic signals were monitored with a chemiluminescent detection system (Amersham, Arlington Heights, IL). Expression levels of Raf-1 or ERK protein were quantitated by densitometry using a compterized gel analysis software (SigmaGel™, SPSS, Chicago, IL). Activation of Raf-1 or ERK was determined in immuno-precipitation assays using a Raf-1 kinase cascade assay kit (Upstate Biotech., Lake Placid, NY) that measures the phosphotransferase activity of a kinase cascade reaction initiated by the active Raf-1 immunocomplex obtained from cell lysates. Following 2 h of incubation at 4°C in the immunoprecipitation buffer (50 mm Tris-base, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na_3VO_4, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 0.1 mM PMSF, 1 μg/ml apoprotein, 1 μg/ml leupeptin) containing 50% protein G agarose beads (Gibco, Gaithersburg, MD) coated with anti-raf-1 sheep polyclonal IgG (2 μg/500 mg lysate) which recognizes a 12 residue peptide that corresponds to amino acid residues 627–638 of the human raf-1 kinase, the immunocomplex was washed in immunoprecipitation buffer containing 0.5 M NaCl, centrigufed at 14,000 rpm, and washed in immunoprecipitation buffer containing 80 μl of dilution buffer (20 mM 3-[*N*-morpholino]propanesulfonic acid, pH 7.2, 25 mM beta-glycerol phosphate, 5 mM EGTA, 1 mM Na_3Vo_4, 1 mM dithiothreitol). The supernatant was removed, and the agarose beads resuspended in 20 μl of dilution buffer, 10 μl of a magnesium/ATP cocktail (500 mM ATP, 75 mM magnesium chloride in dilution buffer), 0.4 μg recombinant mouse non-active MAP kinase kinase 1, and 0.1 μg recombinant mouse non-active p42 MAP kinase (specific activity 1300 U/mg). The resulting mixture was incubated under constant agitation for 30 min at 30°C followed by centrifugation at 14,000 rpm for 15 s and separation of the immunocomplex-kinase reaction supernatant from the agarose beads. The supernatant (4 μl)

was added to 10 μl dilution buffer, 10 μl myelin basic protein, and 100 μCi [gamma-^{32}P]-ATP (Dupont-New England Nuclear, Boston, MA) that had been diluted 1:10 with a magnesium/ATP cocktail. The reaction mixture was incubated under constant agitation for 10 min at 30°C. P81 phosphocellulose squares were spotted with 25 μl of the reaction mixture followed by three washes with 0.75% phosphoric acid (Fisher, Pittsburgh, PA) and one wash with acetone. The squares were then placed into scintillation cocktail, and radioactivity was quantified by liquid scintillation spectrophotometry (Tri-Carb 2300 TR Scintillation counter, Packard, Meriden, CT).

3. Results

RT-PCR assays with the primer for the human α_7 nicotinic acetylcholine receptor revealed mRNA of this receptor in fetal hamster pulmonary neuroendocrine cells and human small cell lung carcinoma cells (Figs. 3 and 4). Sequence comparison of the 611 bp between the primers of the RT-PCR fragment from fetal hamster pulmonary neuroendocrine cells with the comparable sequence of human α_7 mRNA by a maximum matching program of DNASIS showed a matching percentage of 89%. A Blast search of Genbank using the sequence of the hamster RT-PCR fragment showed 96% homology with mouse α_7 (accession no. L37663) and 89% homology with human α_7 (accession no. Y08420). In support of these findings, radio-receptor assays with the site-selective ligand for the α_7 nicotinic acetylcholine receptor, [^{125}I]-α-bungarotoxin (specific activity 230 Ci/mmol, Amersham), yielded saturation binding curves in pulmonary neuroendocrine cells

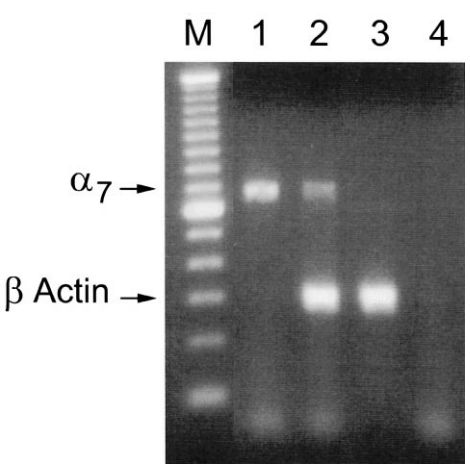

Fig. 3. Agarose gel showing α_7 nicotinic acetylcholine receptor expression in fetal hamster pulmonary neuroendocrine cells. Lane 1 — 500 nM α_7 primers; lane 2 — 500 nM α_7 and 125 nM β-actin primers; lane 3 — 125 nM β-actin primers; lane 4 — RT reaction without M-MLV and 500 nM α_7 primers and 125 nM β-actin primers (negative control). The bands were consistent with the expected sizes, 651 bp for the α_7 primers and 285 bp for the β-actin primers. The molecular weight marker is a 100-bp DNA ladder (from Plummer et al., 1999).

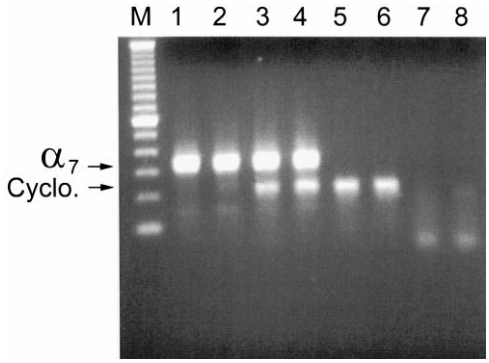

Fig. 4. Agarose gel showing α_7 nicotinic acetylcholine receptor expression in NCI-H-69 and NCI-H-82 cell lines derived from human small cell lung carcinomas. Lane 1 — 250 nM α_7 primers in NCI-H-69 cells; lane 2 — 250 nM α_7 primers in NCI-H-82 cells; lane 3 — 250 nM α_7 and 125 nM cyclophilin primers in H-69 cells; lane 4 — 250 nM α_7 and 125 nM cyclophilin primers in H-82 cells; lane 5 — 125 nM cyclophilin primers in H-69 cells; lane 6 — 125 nM cyclophilin primers in H-82 cells; lane 7 — RT reaction without M-MLV and 250 nM α_7 primers and 125 nM cyclophilin primers in H-69 cells (negative control); lane 8 — RT reaction without M-MLV and 250 nM α_7 primers and 125 nM cyclophilin primers in H-82 cells (negative control). The bands were consistent with the expected sizes, 336 bp for the α_7 primers and 216 bp for the cyclophilin primers. The molecular weight marker is a 100-bp DNA ladder.

(B_{max}: 15.5 fmol/mg, K_d: 31.4 nM; Plummer et al. 1999) and small cell carcinoma cells (B_{max}: 17.69 fmol/mg protein, K_d: 11.44 nM; Schuller and Orloff 1998). The tobacco-specific toxicants nicotine, NNN, or NNK competed with [^{125}I]-α-bungarotoxin for the nicotinic binding sites yielding one-site binding isotherms by nonlinear regression with affinities NNK > nicotine > NNN in human small cell carcinoma cells (Schuller and Orloff, 1998). Nicotine and NNK yielded similar one-site binding isotherms in pulmonary neuroendocrine cells with affinities of NNK > nicotine (Fig. 5).

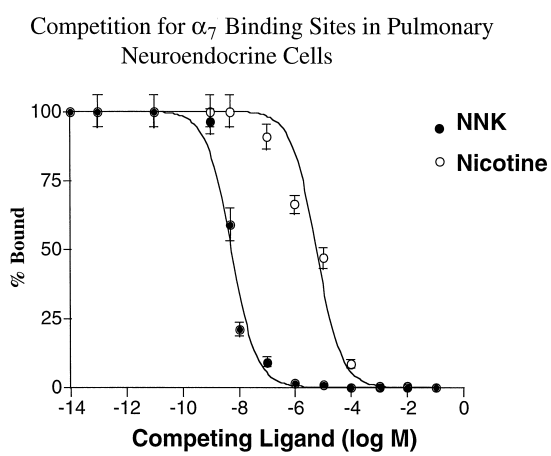

Fig. 5. Results of receptor binding assays in which ascending concentrations (1 fM–1 M) of nicotine or NNK competed for α_7 nicotinic binding sites with [^{125}I]-α-bungarotoxin (BTX) (30 nM). Analysis of the binding data by nonlinear regression for one site-binding isotherm yielded EC_{50} values of 5.5 nM for NNK and 6.0 μM for nicotine.

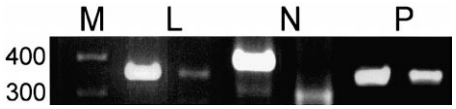

Fig. 6. RT-PCR for L-, N-, and P-type calcium-channels in NCI-H69 and pulmonary neuroendocrine cells. Messenger RNA for all three channel types were expressed in the small cell lung carcinoma line NCI-H69 (lanes 1, 3, 5) whereas mRNA from the pulmonary neuroendocrine cells (lanes 2, 4, 6) yielded a weak band for the L-type channel, a pronounced band for the P-type channel while mRNA for the N-type channel was non detectable. The bands were consistent with the expected sizes (353 bp for the L-type channels, 390 bp for the P-type channels, and 345 bp for P-type channels). ''M'' is a 100-bp DNA marker ladder.

RT-PCR assays for the identification of calcium-channel types revealed a prominent band for L-, N-, and P-type channels in small cell carcinoma cells (Fig. 6) whereas pulmonary neuroendocrine cells yielded a prominent band for the P-type channel, a weak band for the L-type channel, and no band for the N-type channel (Fig. 6).

Flow cytometric analysis demonstrated an increase in intracellular calcium in pulmonary neuroendocrine cells or small cell carcinoma cells exposed to NNK concentrations of 100 pM or 1 nM whereas a 1 μM concentration of nicotine was required to elicit calcium flux (Figs. 7 and 8). The response to NNK (1 nM) was completely inhibited by α-bungarotoxin (10 μM) in pulmonary neuroendocrine cells (Fig. 7) or small cell carcinoma cells (Fig. 8). These data are consistent with established functional characteristics of the α_7 nicotinic acetylcholine receptor as an ion channel with high permeability for calcium (Séguéla et al., 1994). The L-type Ca^{2+}-channel blocker nifedipine (10 nM), the N-type Ca^{2+}-channel blocker ω-conotoxin (10 nM), or the P-type Ca^{2+}-channel blocker ω-agatoxin (10 nM) also completely blocked the influx of calcium in small cell carcinoma cells (Fig. 8) while studies on their effects on this response in pulmonary neuroendocrine cells have not yet been completed. These findings indicate that all three types of voltage activated calcium-channels were activated by nicotine or NNK in small cell carcinoma cells,

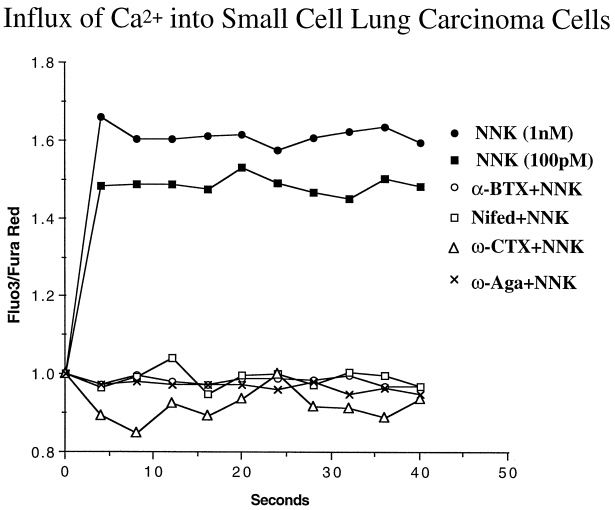

Fig. 8. Flow cytometric analysis of intracellular calcium levels over time in response to NNK in NCI-H69 cells. A significant ($p < 0.0001$) increase in intracellular calcium (Ca^{2+}) was observed with concentrations of NNK from 100 pM to 1 μM. This effect saturated at the 1 nM concentration (data from concentrations >1 nM not shown). The site-selective antagonist for α_7 nicotinic acetylcholine receptors, α-bungarotoxin (α-BTX, 10 μM) completely blocked this response. Similarly, blockers of calcium-channels of the L- (nifedipine, Nifed, 10 nM), N- (ω-conotoxin, ω-CTX, 10 nM), or P- (ω-agatoxin, ω-Aga, 10 nM) type completely inhibited calcium-influx in response to NNK (1 nM).

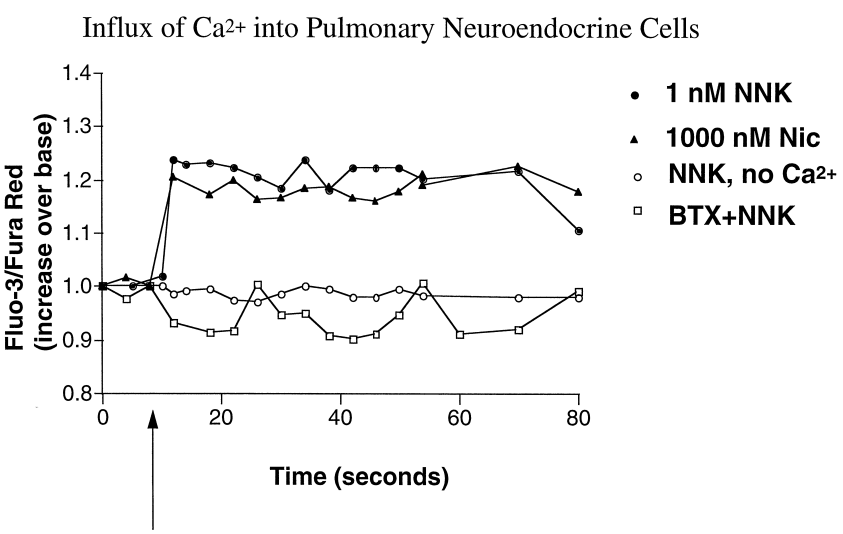

Fig. 7. Summary of flow cytometric data on the influx of calcium under various experimental conditions in pulmonary neuroendocrine cells. Nicotine (Nic) and NNK both caused an increase in intracellular calcium with substantially higher concentrations of nicotine being required to elicit this response than NNK. This response was completely inhibited by α-bungarotoxin (BTX), identifying binding of nicotine or NNK to the α_7 nicotinic receptor as the mediating event. Control experiments (exemplified with NNK) in calcium-free reaction buffer did not increase intracellular calcium, confirming that the observed response was due to influx of calcium from the extracellular environment. Experiments were conducted in duplicate with similar results. The current graph represents the results of one of these experiments. Base levels of intracellular calcium were established by three determinations prior to the addition of drugs (indicated by arrow).

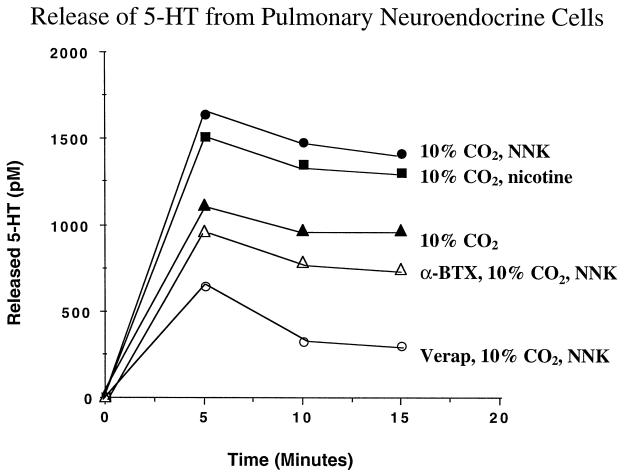

Fig. 9. Results of ELISA assay for the determination of serotonin (5-HT) released by the cells under various experimental conditions. Nicotine (1 μM) and NNK (1 nM) both increased the release of 5-HT above the levels observed with 10% CO_2 alone. The L-type calcium-channel blocker verapamil (Verap, 1 nM) inhibited this response below the levels observed with CO_2 alone while α-bungarotoxin (α-BTX, 30 nM) only inhibited the portion of this response caused by nicotinic receptor stimulation. Data points are mean values of triplicate data points (parts of these data are from Plummer et al., 1999).

thus supporting published data (Sher et al., 1990; Codignola et al., 1993) as well as our own findings by RT-PCR on the expression of calcium-channels in small cell lung carcinoma. Further studies with lower concentrations of individual channel blockers will need to clarify the relative contribution of each channel type to the observed intracellular increase in calcium as the concentrations (10 nM) used by us apparently nonspecifically blocked all three calcium-channel types.

Analysis of serotonin by ELISA revealed a pronounced and transient increase in released 5-HT within 5 min of exposure to nicotine or NNK over the levels of control pulmonary neuroendocrine cells (Fig. 9). The control cells also demonstrated constant basal release of 5-HT during the investigated time interval, but at a significantly ($p < 0.01$) lower level than cells exposed to NNK or nicotine. This reaction was caused by the re-introduction of cells into an atmosphere of 10% CO_2 for the incubations after having been washed with PBS in ambient air in the hood, and is in accordance with reports that hypoxia stimulates the release of 5-HT from pulmonary neuroendocrine cells via activation of a receptor which operates as an ion-channel (Lauweryns et al., 1977; Johnson et al., 1982; Cutz et al. 1985; Johnson and Georgieff, 1989; Youngsson et al., 1993). In support of this interpretation, we have recently shown that cell lines derived from human small cell lung carcinoma released 5-HT upon exposure to 10% CO_2 (Merryman et al., 1997). The site-selective antagonist for α_7 nicotinic receptors, α-bungarotoxin (30 nM) inhibited the release of 5-HT in response to NNK (Fig. 8) or nicotine (not shown) down to the level of control cells re-introduced into 10% CO_2. The L-type calcium-channel blocker, verapamil (1 nM) inhibited the release of 5-HT caused by NNK (Fig. 8) or nicotine (not shown) below the levels of control cells re-introduced into 10% CO_2, but did not yield a complete inhibition of the serotonergic response. These findings suggest that the response to nicotine or NNK was causd by binding of these ligands to the α_7 nicotinic acetylcholine receptor and that multiple calcium-channels were additionally activated. In support of this interpretation, our RT-PCR assays revealed mRNA for the P- and L-type calcium-channels in pulmonary neuroendocrine cells (Fig. 6).

We as well as others have shown that nicotine stimulates the proliferation of human small cell carcinoma cells by binding to the α_7 nicotinic acetylcholine receptor resulting in the release of 5-HT (Schuller, 1989; Cattaneo et al., 1993; Codignola et al., 1994; Schuller 1994). Moreover, we have shown that NNK activates an identical mitogenic loop in cells derived from this lung cancer type (Schuller and Orloff, 1998). In similar studies, we have shown that fetal pulmonary neuroendocrine cells react with an increase in DNA synthesis and cell number to nicotine or NNK via a mitogenic serotonergic loop regulated by the α_7 nicotinic receptor (Plummer et al., 1999). To further our understanding of the signal transduction events activated by this mitogenic loop, we are focusing our current efforts on the Raf-1-dependent MAP kinase cascade, which has been identified as a major growth regulating pathway in small cell lung carcinoma (Bunn et al., 1993; Heysley and Johnson, 1998). In accordance with a recent report on the activation of the MAP kinases ERK1/2 by nicotine in small cell lung carcinoma cells (Cattaneo et al., 1997), our Western blots revealed a pronounced induction of Raf-1 (74 kDa), ERK1 (44 kDa) and ERK2 (42 kDa) expression in small cell lung carcinoma cells and pulmonary neuroendocrine cells exposed to nicotine or NNK. As exemplified in Fig. 10, NNK was a more potent inducer of these kinases than nicotine in that a 1-μM concentration of nicotine was required to induce similar levels of expression as a 100 pM concentration of NNK. Analysis of

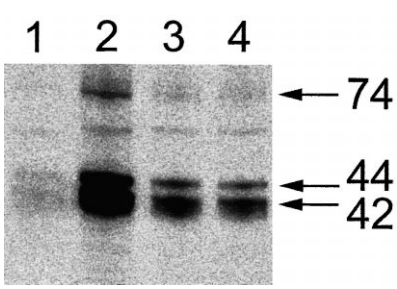

Fig. 10. Expression of protein levels of Raf-1 (74 kDa), ERK-1 (44 kDa), and ERK-2 (42 kDa) as determined by western blots in pulmonary neuroendocrine cells. Lane 1: untreated control; lane 2: 1 μM NNK for 120 min; lane 3: 100 pM NNK for 120 min; lane 4: 1 μM nicotine for 120 min.

NNK-induced expression levels of Raf-1 or ERK1/2 in the presence or absence of the antagonist for neuronal nicotinic receptors hexamethonium (1 μM), the site-selective antagonist for α_7 nicotinic receptors, α-bungarotoxin (20 nM), or the 5-HT uptake inhibitor, imipramine (1 μM), by densitometry demonstrated inhibition below control levels by imipramine and a 70–80% inhibition by each of the two nicotinic antagonists (Fig. 11). We believe that the incomplete inhibition by nicotinic antagonists as opposed to complete inhibition by imipramine reflects the fact that the portion of the autocrine growth factor 5-HT released in response to the CO_2 environment in the incubator was not inhibited by the receptor antagonists whereas imipramine blocked all 5-HT uptake (NNK-induced as well as CO_2-induced). A comparison of Raf-1 expression by densitometry in control pulmonary neuroendocrine cells, pulmonary neuroendocrine cells exposed to NNK (1 nM) for 6 consecutive days, and control small cell lung carcinoma cells revealed a similar degree of up-regulation in the NNK-treated pulmonary neuroendocrine cells as in the cancer cells (Fig. 12). This finding is in accord with the well-documented up-regulation of the Raf-1-dependent MAP kinase cascade in human small cell lung carcinoma (Heysley and Johnson, 1998), and suggests that chronic exposure to NNK in smokers may be an important contributing factor to this event.

Analysis of Raf-1 and ERK1/2 activation in pulmonary neuroendocrine cells exposed for 120 min to NNK (100 pM) demonstrated increased activation in the NNK exposed cells (Figs. 13 and 14). NNK-induced activation of Raf-1 was blocked by the protein kinase C inhibitor sphingosine (75 μM). The addition of 5-HT (100 pM) to the culture medium caused a similar degree of activation as NNK (Fig. 13). On the other hand, NNK-induced activation of ERK was blocked by the inhibitor of the MAP kinase kinase (MEK), PD98050 (100 μM, Fig. 14). As protein kinase C is an upstream activator of Raf-1 (Kolch et al., 1993) while MEK is the downstream effector of Raf-1 which activates ERK (Heidecker et al., 1992;

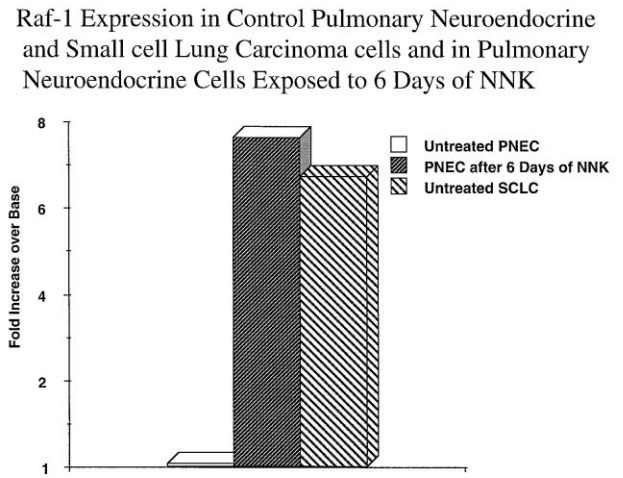

Fig. 12. Results of densitometric analysis of Raf-1 protein expression in untreated pulmonary neuroendocrine cells (PNEC), untreated cells from the human small cell lung carcinoma (SCLC) cell line NCI-H69, and pulmonary neuroendocrine cells exposed for 6 days continuously in vitro to NNK (1 nM). Note the prominent up-regulation of Raf-1 expression in small cell lung carcinoma cells and in pulmonary neuroendocrine cells exposed for 6 days to NNK. Base levels of Raf-1 expression in untreated pulmonary neuroendocrine cells were designated as 1.0 and levels in small cell lung carcinoma cells and NNK-treated cells are expressed as fold increase over untreated pulmonary neuroendocrine cells.

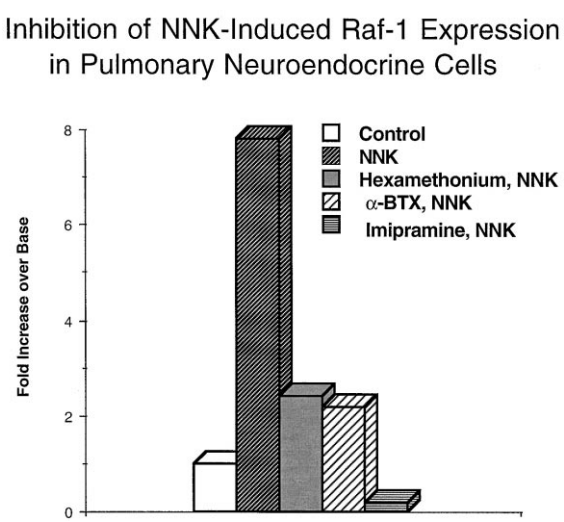

Fig. 11. Results of densitometric analysis of Raf-1 protein expression in pulmonary neuroendocrine cells. The pronounced increase in Raf-1 expression exposure (120 min) to NNK (100 pM) was significantly inhibited by the antagonist of neuronal nicotinic receptors, hexamethonium (1 μM), the site-selective antagonist of the α_7 nicotinic receptor, α-bungarotoxin (α-BTX, 20 nM), or the 5-HT uptake inhibitor, imipramine (1 μM).

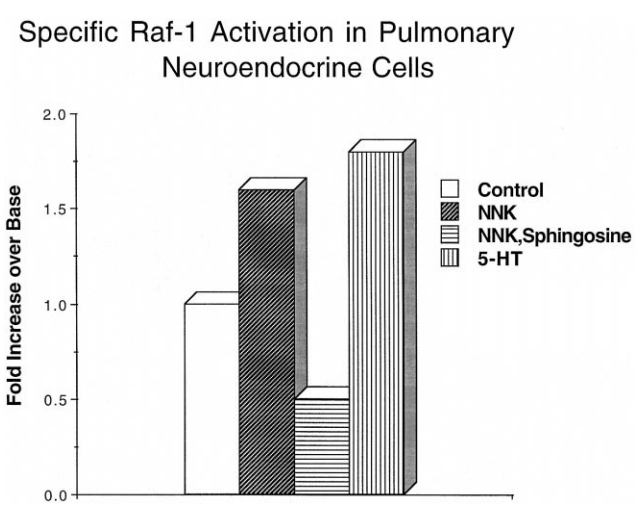

Fig. 13. Levels of specific Raf-1 activation in pulmonary neuroendocrine cells as determined by immunoprecipitation assays using a Raf-1 kinase cascade assay kit. NNK (100 pM for 120 min) or 5-HT (100 PM for 120 min) yielded similar levels of activation. Activation in response to INK was decreased below the levels of untreated control cells by the protein kinase C inhibitor, sphingosine (75 μM) which is one of several established upstream activators of Raf-1.

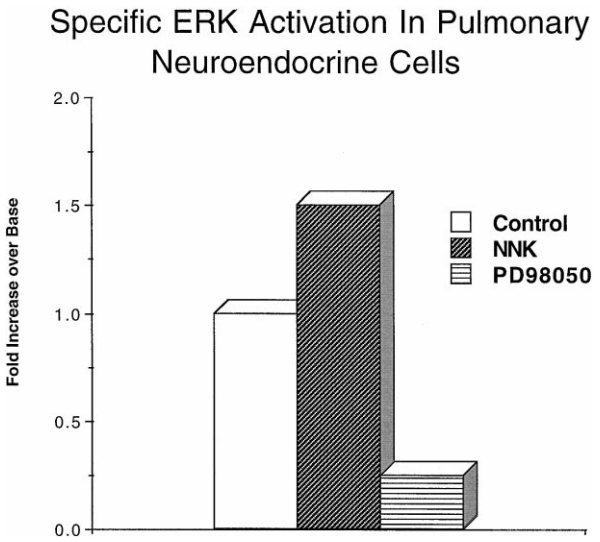

Fig. 14. Levels of specific ERK1/2 activation in pulmonary neuroendocrine cells as determined by immunoprecipitation assays using a Raf-1 kinase cascade assay kit. The response to NNK (100 pM for 120 min) was inhibited below the levels of untreated cells by the inhibitor of the MAP kinase kinase (MEK), PD98050 (100 μM), which is the downstream effector of Raf-1 that activates ERK1/2.

Lange-Carter et al., 1993), these findings further support our interpretation that the obeserved stimulation of the nicotinic receptor initiated mitogenic pathway by NNK is Raf-1 and ERK-dependent. The observed effect of exogeneously added 5-HT on Raf-1 additionally strengthens the interpretation that the release of this biogenic amine in response to nicotinic receptor stimulation by NNK is the trigger that activates this mitogenic cascade.

4. Discussion

The data presented in this review suggest that pediatric smoking-associated pulmonary diseases and small cell lung carcinoma may be caused by the direct chronic stimulation of an α_7 nicotinic acetylcholine receptor-initiated autocrine loop by nicotine or NNK. While it is well established that the Raf-1/MAP kinase cascade is a major mitogenic signal transduction pathway expressed in human small cell lung carcinoma (Bunn et al., 1993; Heysley and Johnson, 1998), the expression and function of this pathway in pulmonary neuroendocrine cells has not been previously described. Our data suggest that the in utero exposure of fetal pulmonary neuroendocrine lung cells to nicotine or NNK in cigarette smoke contributes to the development of pediatric lung disorders via two different mechanisms: (1) the direct effects of released 5-HT in response to α_7 receptor stimulation on bronchial and vascular smooth muscles and fibroblast growth, and (2) the indirect effects of 5-HT on pulmonary neuroendocrine cell numbers via activation of a Raf-1/MAP kinase pathway, resulting in yet more cells to synthesize and release 5-HT. A recent study in Rhesus monkeys has revealed increased pulmonary levels of α_7 nicotinic receptor immunoreactivity accompanied by an increase in the number of pulmonary neuroendocrine cells in neonates whose pregnant mothers were chronically exposed to nicotine (Sekhorn et al., 1999). These findings support the conclusions drawn from our in vitro data,that chronic stimulation of the α_7 nicotinic receptor by nicotine or NNK may up-regulate the α_7 receptor. This interpretation is consistent with reports that have documented up-regulation of nicotinic receptor expression in vitro (Pengh et al., 1997), and in adult humans (Breese et al., 1997) in response to chronic nicotine exposure. In addition to such changes in the nicotinic receptor itself, our data indicate that key elements (Raf-1, MAP kinases) of the mitogenic signal transduction pathway activated by α_7 receptor-mediated release of 5-HT are also overexpressed following acute or chronic exposure to nicotine or NNK. In assessing the biological significancce of these findings, it is important to consider that with every endpoint used by us for the assessment of α_7 receptor responsiveness (5-HT release, calcium-influx, overexpression/activation of Raf-1/MAP kinases, DNA synthesis, increase in cell number), significantly lower concentrations of NNK than nicotine were required to yield the observed responses. Pending on the brand, age, and storage conditions of cigarettes, cigarette smoke contains between 5000 and 30,000 times more nicotine than NNK (Fischer et al., 1990; Hecht and Hoffmann, 1990). As evidenced by a study in monkeys (Hecht et al., 1993a,b), these proportions of nicotine to NNK are mirror-imaged in the blood levels of these toxicants in smokers. If one additionally considers the recent finding (Schuller and Orloff, 1998) that NNK has a very low affinity to epibatidine-sensitive nicotinic receptors, which account for the vast majority of the nicotinic receptor family in mammalian cells (Lindstrom et al., 1995), and to which nicotine binds with high affinity (Lindstrom et al., 1995), it appears, that α_7 receptor-mediated effects commonly attributed to the nicotine in tobacco products may in fact be caused by NNK. Accordingly, the role of this extensively studied cancer causing agent for the genesis of pediatric pulmonary disease certainly deserves further investigation.

The observed stimulation of α_7 receptor-mediated 5-HT release, calcium-influx, overexpression/activation of Raf-1/MAP kinases and DNA synthesis as well as cell numbers in human small cell lung carcinoma cells in response to nicotine or NNK are in accord with recent publications on the effects of nicotine in cell lines derived from this histologic lung cancer type (Schuller, 1989, 1994; Cattaneo et al., 1993, 1997; Codignola et al., 1994). However, with respect to the above outlined higher sensitivity of the α_7 receptor and its ascociated serotonergic mitogenic pathway to NNK, the role of nicotine for the induction of this smoking-associated cancer type may have been overestimated. The data presented here clearly identify a novel

mechanism of action of NNK which is the most powerful cancer causing agent contained in tobacco products. The observed activation of a Raf-1/MAP kinase pathway by NNK-stimulated nicotinic receptor-mediated 5-HT release is of particular interest in that this pathway may activate the transcription factor c-*myc* (Heidecker et al., 1992; Lange-Carter et al., 1993). C-*myc* overexpresion/amplification is the molecular hallmark of human small cell carcinoma while being absent in non-small cell lung carcinoma (Mitsudomi et al., 1991; Lai et al., 1995) but has to this date not been experimentally induced by any known toxicant or carcinogen contained in tobacco products. Further studies are currently in progress to explore if NNK activates c-*myc* via the serotonergic Raf-1/MAP kinase pathwway in pulmonary neuroendocrine and small cell lung carcinoma cells.

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