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Neuronal nicotinic receptors in non-neuronal cells: new mediators of tobacco toxicity? [1]

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Abstract

The nicotinic acetylcholine receptors are prototypic ionotropic receptors that mediate fast synaptic transmission. However, also non-excitable cells, and particularly the tegumental cells that line external and internal body surfaces, express acetylcholine receptors of neuronal type sensitive to nicotine. Bronchial epithelial cells, endothelial cells of blood vessels and skin keratinocytes express neuronal nicotinic receptors composed of α_3, α_5, β_2 and β_4 subunits, similar to those expressed in sympathetic ganglia, and neuronal nicotinic receptors composed of α_7 subunits. Neuronal nicotinic receptors in tegumental cells are involved in modulating cell shape and motility, and therefore in maintaining the integrity of the surfaces lined by those cells. Neuronal nicotinic receptors in non-neuronal tissues may modulate other functions, including cell proliferation and differentiation. Acetylcholine is synthesized, secreted and degraded by a variety of cells, including the tegumental cells that express neuronal nicotinic receptors. Thus, acetylcholine may function as a local ''hormone'' that is able to modulate cell functions that require fast adaptation to new conditions. The presence of neuronal nicotinic receptors sensitive to nicotine in tissues known to be involved in tobacco toxicity, like bronchi and blood vessels, raises the possibility that they mediate some of the toxic effects of smoking. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Nicotine; Nicotinic receptor, Non-neuronal; Lung; Blood vessel; Skin

1. Introduction

The nicotinic receptors for acetylcholine are prototypic ionotropic receptors that mediate fast synaptic transmission. They are a family of proteins formed by five homologous or identical subunits, arranged symmetrically around a central ion channel (Conti-Tronconi et al., 1994; Galzi and Changeux, 1995; Lindstrom, 1996; Maelicke, 1996; Albuquerque et al., 1997). Opening of the channel is triggered by the binding of acetylcholine (Conti-Tronconi et al., 1994; Galzi and Changeux, 1995; Lindstrom, 1996; Maelicke, 1996; Albuquerque et al., 1997). Different nicotinic receptors isotypes exist in muscle and neurons. Nicotinic receptors expressed in muscle are composed of four different types of subunit (α, β, γ or ε and δ), whereas in neurons they may include only two types of subunits (α and β) or five copies of the same α subunit (Conti-Tronconi et al., 1994; Galzi and Changeux, 1995; Lindstrom, 1995, 1996; Maelicke, 1996; Albuquerque et al., 1997). Neurons express at least eight different α subunits (α_2 to α_9) and three β subunits (β_2 to β_4): a large variety of neuronal nicotinic receptors results from the combinatorial association of different α and β subunits (Conti-Tronconi et al., 1994; Lindstrom, 1996; Maelicke, 1996; Albuquerque et al., 1997). The characteristics of neuronal nicotinic receptors comprised of different α and β subunits are described elsewhere in this issue. Nicotine activates neuronal nicotinic receptors, although its affinity for the different receptor subtypes can vary widely (Lindstrom, 1996; Albuquerque et al., 1997).

A variety of non-neuronal, non-excitable cells synthesize, release, and degrade acetylcholine (Sastry et al., 1981; Mark et al., 1983; Milner et al., 1985; Grando et al., 1993b; Rinner and Schauenstein, 1993). In humans, acetylcholine was detected in the surface epithelia of bronchi, mouth, small and large intestine, gall bladder, vagina and skin; in rat, in the skin and in the tracheal and intestinal

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epithelia (Klapproth et al., 1997). Human surface epithelia, such as the epithelial cells of human bronchi and small intestine, also contain choline acetyltransferase, the enzyme that synthesizes acetylcholine (Klapproth et al., 1997). In human placenta, the epithelial cells of the trophoblast release acetylcholine that might control the transport of choline and amino acids into the fetal circulation (Sastry, 1997; Sastry and Sadavongvivad, 1979; Sweiry and Yudilevich 1985; Klapproth et al., 1997). Also, non-epithelial surface cells, like vascular endothelial cells and pulmonary pleural cells, make acetylcholine (Klapproth et al., 1997). This suggests that acetylcholine might function in an autocrine or paracrine fashion as a local hormone, and modulate cellular functions in non-neuronal cells by acting on specific receptors. Several studies have shown that administration of acetylcholine may influence cellular functions in non-neuronal tissues (Grando et al., 1993b; Klapproth et al., 1997).

The *muscarinic* receptors for acetylcholine are metabotropic, and mediate longer-lasting effects on cellular functions than those resulting by activation of the nicotinic receptors (Eglen et al., 1996). Thus, if the acetylcholine secreted and released by non-neuronal tissues serves as a local hormone, the receptors involved in such actions might be muscarinic. Muscarinic receptors for acetylcholine are present in a variety of tissues (Brann et al., 1993). However, early pharmacologic studies suggested the presence of *nicotinic* acetylcholine receptors in cells such as lymphocytes, spermatozoa, and secretory cells of the pancreatic islets of Langerhans (Nelson et al., 1982; Ejiri et al., 1989). Also, some neurons express abundant nicotinic receptors outside the synapses (e.g., see Jacob and Berg, 1983; Loring et al., 1985), whose function is yet unknown. The presence of neuronal nicotinic receptors at locations unrelated to synaptic activity supports the hypothesis that acetylcholine may have cellular functions, other than transmission of nerve signals, that are mediated by nicotinic receptors.

That hypothesis has been verified by the studies reviewed here, which demonstrated that the cells that line external and internal surfaces in humans and other mammals express functional nicotinic acetylcholine receptors, similar to those expressed by neurons. We refer to those cells as tegumental cells: they include the epithelial cells that line the bronchi, the keratinocytes that cover the skin surface, and the endothelial cells that line the arteries. All those cells synthesize, secrete and degrade acetylcholine (Klapproth et al., 1997). Thus, they have all the components needed for modulating cellular functions through nicotinic cholinergic signaling. Neuronal nicotinic receptors comprising the α_3 subunit, similar to those expressed by sympathetic ganglia, are involved in modulating cell shape and motility of tegumental cells, and therefore in preserving the integrity of the surface they line. The α_3 nicotinic receptors, and other receptor subtypes comprising the α_7 subunit, may be involved in regulating also other cellular functions, including cell proliferation and differentiation.

Also embryonic myocytes (Corriveau et al. 1995; Romano et al. 1997; Fischer et al., 1999) and the thymus (Navaneetham et al., 1997) — an immune organ — express α_7 nicotinic receptors. The thymus expresses also nicotinic receptors comprising α_3 and α_5 subunits (Mihovilovic and Roses, 1991, 1993; Navaneetham et al., 1997), and nicotinic receptors of muscle type (Nelson and Conti-Tronconi, 1990; Geuder et al., 1992; Wheatley et al., 1992; Hara et al., 1993; Kaminski et al., 1993).

The widespread presence of both acetylcholine and a variety of nicotinic receptors at non-synaptic locations and in non-neuronal cells supports the possibility that acetylcholine has broad cellular functions mediated by activation of ionotropic nicotinic acetylcholine receptors. Acetylcholine might function both as a neurotransmitter and as a ''cytotransmitter'' that is able to modulate cellular functions towards fast adaptation to new conditions.

Usually, expression of acetylcholine is accompanied by that of acetylcholinesterase (Klapproth et al., 1997). Acetylcholinesterase degrades acetylcholine quickly, and prevents any long-range actions of acetylcholine. However, nicotinic receptors formed by the α_7 subunits are activated by choline, a degradation product of acetylcholine that might be the natural ligand for α_7 nicotinic acetylcholine receptors (Papke et al. 1996; Alkondon et al. 1997). The long half-life of choline would prolong the action of acetylcholine beyond the short-lived effects mediated by binding of acetylcholine itself to its receptors. The ability of choline to activate the α_7 nicotinic receptors blurs the distinction between the function of acetylcholine as a cytotransmitter and as a classic hormone.

Thus, acetylcholine and choline may be widespread and fundamental mediators of cellular interactions well beyond those mediated by synaptic contact. The presence of neuronal nicotinic receptors in a variety of non-neuronal cells suggests that acetylcholine may modulate cellular functions through the ionotropic neuronal nicotinic receptors described in this chapter. Also, it is possible that nicotine causes some of the toxic effects of tobacco usage in the bronchial and cardiovascular system through its binding to the nicotine-sensitive neuronal nicotinic receptors expressed by tegumental cells.

2. Neuronal nicotinic receptors in bronchial epithelial (BE) cells

That specific receptors for nicotine might be present on the bronchial and nasal epithelia of mice was proposed in 1980 to explain the finding that a nicotine derivative accumulated in those epithelia after its intravenous injection: this was attributed to receptor-mediated accumulation (Waddell and Marlowe, 1980). The rabbit tracheal mucous membrane contains high levels of acetylcholine (up to 14

μmol/g of tissue), of choline acetyltransferase and acetylcholinesterase (Sastry and Sadavongvivad, 1979). Isolated human bronchi synthesize and release acetylcholine (Wessler et al., 1995). Several lines of evidence indicate that the BE cells synthesize and secrete acetylcholine. Extracts of surface epithelial cells isolated from human bronchi contained acetylcholine, and human BE cells contained choline acetyltransferase (Klapproth et al., 1997). Since the bronchi are a primary target of the ill effects of tobacco smoking, we investigated whether the BE cells that line the surface of the bronchial tree express functional acetylcholine receptors sensitive to nicotine (Maus et al, 1998).

We used a variety of approaches to determine the presence and the structural and functional properties of nicotinic receptors in human and rodent BE cells. We used primary cultures of BE cells and also sections of trachea to verify the presence in vivo of the nicotinic receptors detected in the cell cultures. mRNA transcripts of nicotinic receptor subunits were detected by reverse- transcribed polymerase chain reaction (RT-PCR) and in situ hybridization approaches; the expressed subunit proteins were detected by the binding of specific antibodies. The assembly of different subunits into functional nicotinic receptors that are able to bind cholinergic agonists and antagonists and to transport ions was determined from the binding of radiolabeled cholinergic ligands, and by patch clamp investigations of the presence and properties of ion channels gated by nicotinic ligands.

The results of those studies consistently indicated that human and rodent BE cells express functional nicotinic receptors of neuronal types, similar to those expressed in parasympathetic ganglia, that were activated by nicotine. RT-PCR and in situ hybridization experiments indicated that BE cells, both in culture and in vivo, expressed mRNA encoding each and all the subunits that form neuronal nicotinic receptors of ganglionic type — α_3, α_5, β_2 and β_4 subunits (Figs. 1 and 2). On the other hand, they did not seem to express other constituent subunits of neuronal nicotinic receptors — α_2, α_4, α_6 and β_3 (Fig. 1). Neuronal nicotinic receptors formed by the α_3 subunit generally include the α_5 and the β_2 or the β_4 subunits: all those subunits are consistently expressed in ganglionic

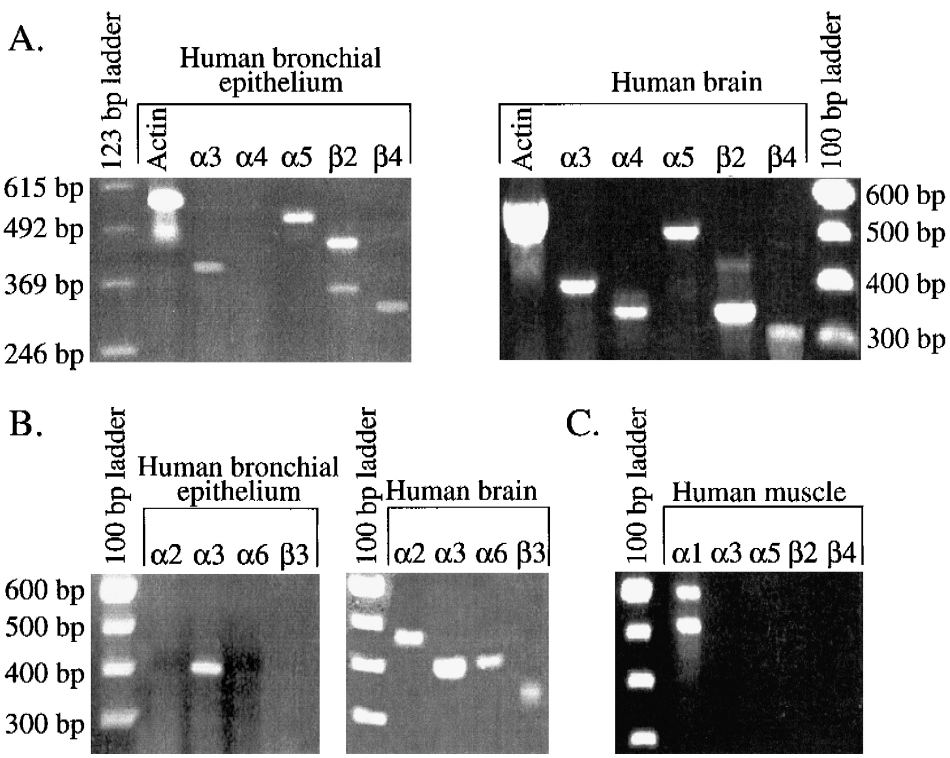

Fig. 1. Detection of α_3, α_5, β_2 and β_4 subunit transcripts in cultured human BE cells by RT-PCR. (A, B) RT-PCR experiments using cDNA from BE cells, primer specific for the different nicotinic acetylcholine receptors subunits, as indicated, and 35 PCR cycles. Primers for actin were used as a positive control for RT-PCR effectiveness. All primers yielded a PCR product of the expected size when cDNA from human brain was used. When human BE cells cDNA was used, the primers for actin and for the α_3, α_5, β_2 and β_4 subunits yielded products of the expected size, those of the α_2, α_4, α_6 and β_3 subunit did not. Mock cDNA did not yield any product band. (C) RT-PCR experiments using cDNA from adult human muscle, primer specific for the different nicotinic acetylcholine receptors subunits, as indicated, and 40 PCR cycles. Primers specific for the muscle α_1 subunit were used as a positive control for RT-PCR effectiveness. The α_3, α_5, β_2 and β_4 subunit primers did not yield detectable products even after 40 cycles, while the α_1 primers yielded two products of the expected size. They are due to the presence in adult human muscle of two α_1 isoforms, one of which includes an additional 75 bp sequence. Figure and legend reprinted with permission from Maus, A.D.J., Pereira, E.F.R., Karachunski, P.I., Horton, R.M., Navaneetham, D., Macklin, K., Cortes, W.S., Albuquerque, E.X., Conti-Fine, B.M., Mol. Pharmacol. 54 779–788, ©1998 by the American Society for Pharmacology and Experimental Therapeutics.

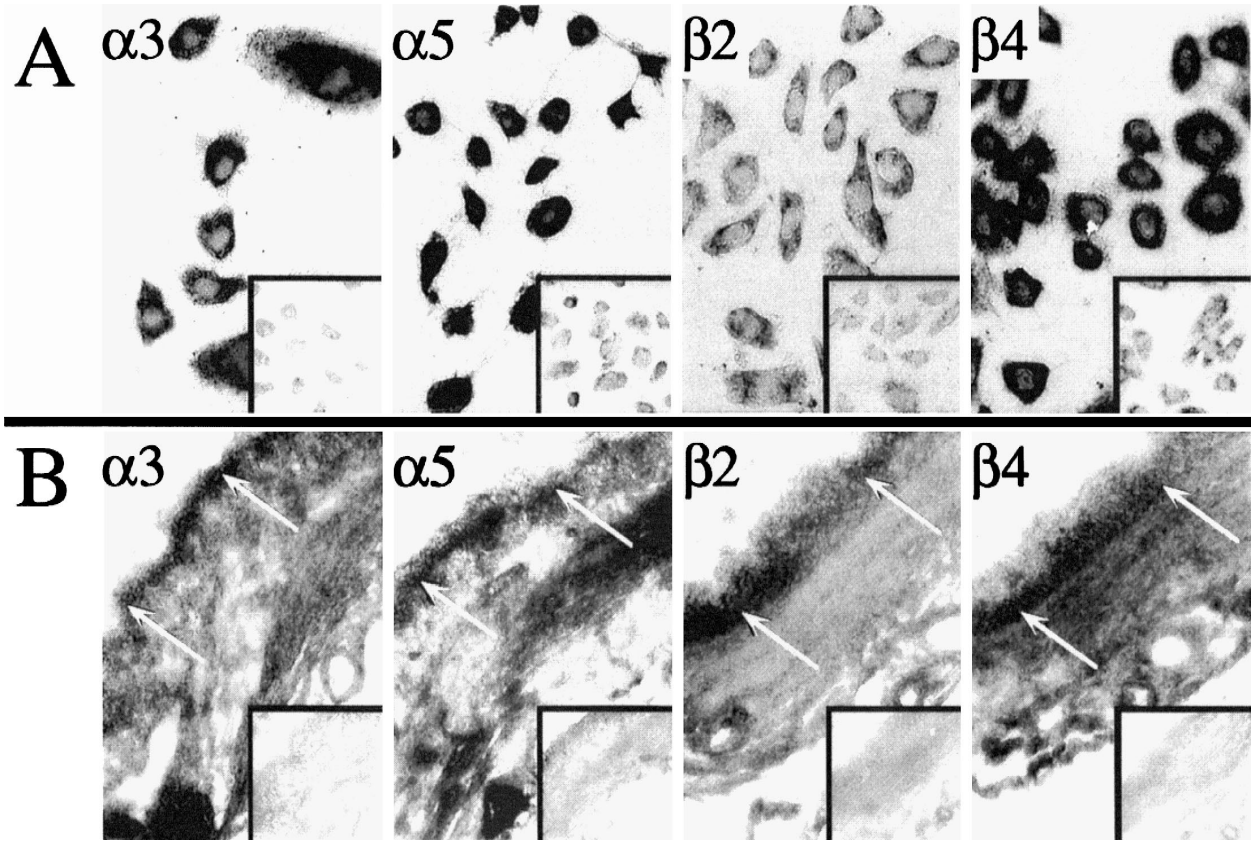

Fig. 2. Detection of α_3, α_5, β_2 and β_4 subunit transcripts in cultured human BE cells and rat BE cells in intact tissue by in situ hybridization. (A) In situ hybridization experiments using cultured human BE cells. The α_3, α_5, β_2 and β_4 subunit probes consistently yielded a specific strong signal. Inserts: negative results obtained with the corresponding ''sense'' control probes. (B) In situ hybridization experiments using rat trachea sections. The α_3, α_5, β_2 and β_4 subunit probes consistently yielded a specific strong signals. Inserts: negative results obtained with the ''sense'' control probes. Figure and legend reprinted with permission from Maus, A.D.J., Pereira, E.F.R., Karachunski, P.I., Horton, R.M., Navaneetham, D., Macklin, K., Cortes, W.S., Albuquerque, E.X., Conti-Fine, B.M., Mol. Pharmacol. 54 779–788, ©1998 by the American Society for Pharmacology and Experimental Therapeutics.

neurons and in other neurons that express α_3 subunits (Vernallis et al., 1993; Conti-Tronconi et al., 1994; Wang et al., 1996). The genes encoding the α_3, α_5 and the β_4 subunits are part of the same gene cluster in vertebrates (Boulter et al., 1990), and they are expressed in highly restricted patterns (McDonough and Deneris, 1997). Thus, the finding, that the nicotinic receptors expressed by the BE cells included α_3, α_5, β_2 and β_4 subunits but not the α_2, α_4, α_6 and β_3 subunits, is in good agreement with the subunit composition found for the α_3 nicotinic receptors physiologically expressed in neurons. To verify the identity of the α_3 product obtained by RT-PCR, we cloned the entire region encoding the mature α_3 protein from a RT-PCR product obtained from human BE cells cDNA using α_3-specific primers (Maus et al., 1998). The clone had the size expected for the α_3 subunit, and its partial sequencing yielded human α_3 subunit sequences. The binding of subunit-specific antibodies to sections of trachea verified that the α_3 and the α_5 proteins are expressed on the BE cells surface in the intact tissue (Fig. 3).

The subunits detected by the studies summarized above assemble into nicotinic receptors of neuronal type that are able to bind cholinergic ligands. This was verified using cultured human BE cells and the binding of [^{3}H]epibatidine (Maus et al., 1998). Epibatidine is a specific, high-affinity ligand of several neuronal nicotinic receptors, including the α_3 receptor subtypes (Gerzanich et al., 1995; Wang et al., 1996). Cultured human BE cells had specific binding sites for [^{3}H]epibatidine, in number that in different experiments ranged from 500 up to 7800 sites per cell. This variability is not surprising. First, the specific binding of [^{3}H]epibatidine to BE cells was small as compared to the non-specific binding: the [^{3}H]epibatidine binding assay is a qualitative verification of the presence of neuronal nicotinic receptors on the BE cells, rather than an accurate assessment of their number. Second, studies on skin keratinocytes (see below) indicated that the level of expression of α_3 neuronal nicotinic receptors was greatly influenced by the degree of cell differentiation, and in cultured keratinocytes it increases sharply just after the cells reach confluence (Grando et al., 1995a). Slight differences in the differentiation stage of the BE cell cultures used in the individual experiments may have resulted in different numbers of binding sites for [^{3}H]epibatidine.

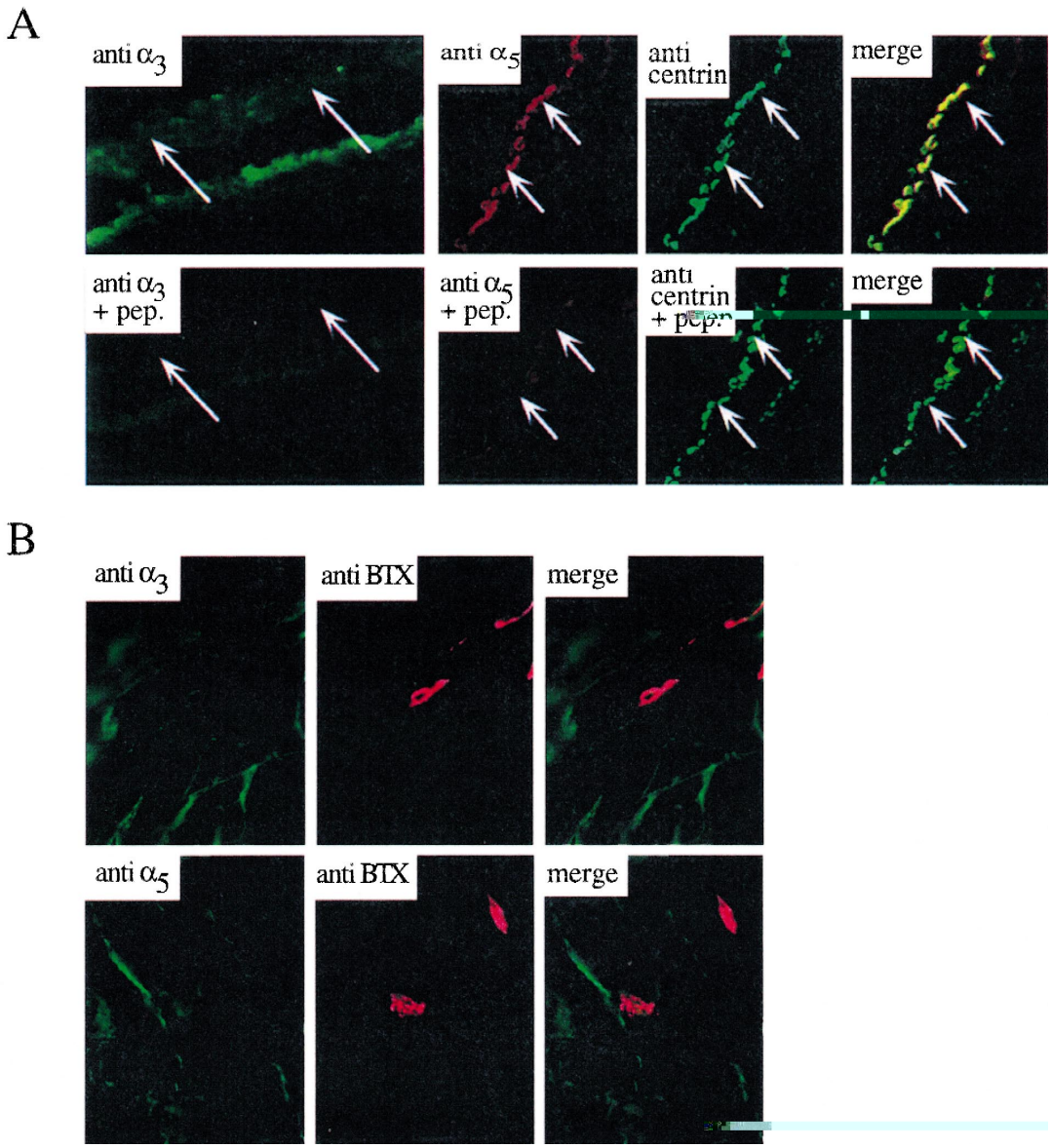

Fig. 3. Detection of α_3 and α_5 subunit proteins in rat BE cells in intact tissue by immunofluorescence. (A) The anti-α_3 and anti-α_5 antisera yielded a clear stain of the epithelial layer in sections of trachea (panels ''anti-α_3'' and ''anti-α_5'') (magnification: 400 ×). The BE cells were identified by double immunofluorescence staining with an anti-centrin antibody (panel ''anti-centrin''). The signal resulting from the anti-α_3 and anti-α_5 antisera was blocked by pre-incubation with synthetic α_3 and α_5 peptide sequences, corresponding to the epitopes recognized by the antisera (panels ''anti-α_3 + peptides'' and ''anti-α_5 + peptides''). Preincubation with the α_3 or the α_5 peptides did not affect the signal of the control anti-centrin antibody (panel ''anti-centrin + peptides''). The panels ''merge'' depict the overlay of the images obtained for the same section using the anti-α_5 antiserum and the anti-centrin antibody, in the absence of α_5-blocking peptides (top ''merge'' panel) and in the presence of α_5-blocking peptides (bottom ''merge'' panel). The overlay of the red and green signals resulting from binding of the two antibodies to the same epithelial cells results in the yellow color of the merged image. When the overlay is done for antibody binding obtained in the presence of blocking peptides, only the green color of the anti-centrin antibody, whose binding is not blocked by nicotinic receptor peptide sequences, is present in the merged image. (B) The anti-α_3 and anti-α_5 antisera did not bind to nicotinic acetylcholine receptors in sections of rat adult muscle (panels ''anti-α_3'' and ''anti-α_5'') (magnification: 400 ×). The neuromuscular junctions were identified by double immunofluorescence staining with an α-bungarotoxin (panel ''α-bungarotoxin''). The panels ''merge'' depict the overlay of the images obtained for the same section using the anti-α_5 or the anti-α_3 antisera, as indicated, and α-bungarotoxin: the weak and diffused green signal caused by non-specific binding of the anti-nicotinic receptor subunit antisera never overlapped the red signal that indicated the presence of muscle α_1 nicotinic receptors at the synaptic junctions. Figure and legend reprinted with permission from Maus, A.D.J., Pereira, E.F.R., Karachunski, P.I., Horton, R.M., Navaneetham, D., Macklin, K., Cortes, W.S., Albuquerque, E.X., Conti-Fine, B.M., Mol. Pharmacol. 54 779–788, ©1998 by the American Society for Pharmacology and Experimental Therapeutics.

Patch clamp experiments (Maus et al., 1998) demonstrated that human BE cells express functional nicotinic receptors with pharmacologic and ion gating properties similar to those of the most abundant type of neuronal nicotinic receptors expressed in sympathetic ganglia (Mathie et al., 1991), which contain α_3, α_5, β_4 and

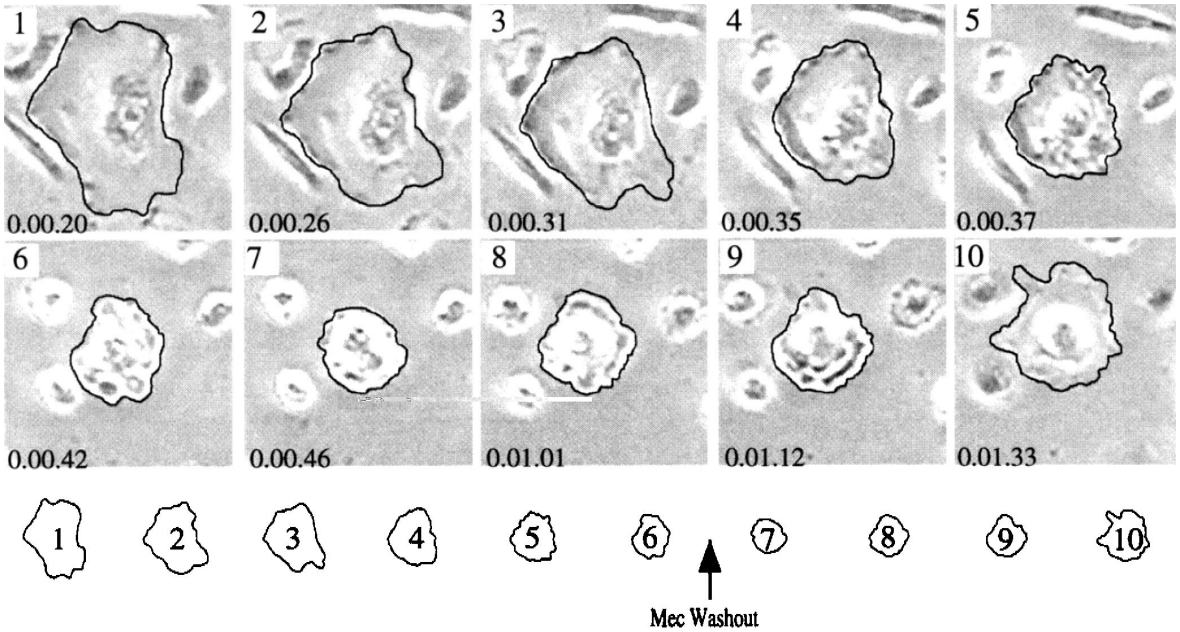

Fig. 5. Human BE cells shrink under the influence of mecamylamine. The pictures show the effect of mecamylamine on cultured BE cells in the presence of 5 mM mecamylamine and after washing (between pictures 6 and 7). They are pictures of the same cells at different time intervals, as indicated inside each picture (hour minutes seconds), after mecamylamine administration. Inside and below each picture, we report the outline of the largest cell in this field [notice the different scale (2:1) of the outline below the pictures, as compared to the cell size in the actual pictures] to facilitate identification of the area occupied by the cell during the experiment. Figure and legend reprinted with permission from Maus, A.D.J., Pereira, E.F.R., Karachunski, P.I., Horton, R.M., Navaneetham, D., Macklin, K., Cortes, W.S., Albuquerque, E.X., Conti-Fine, B.M., Mol. Pharmacol. 54 779–788, ©1998 by the American Society for Pharmacology and Experimental Therapeutics.

to be blocked simultaneously, even in the presence of endogenous acetylcholine.

Thus, BE cells express ''ganglionic'' neuronal nicotinic receptors that are involved in maintaining the flat shape of the BE cells necessary to form a continuous lining on the bronchial surface. The effects of mecamylamine and κ-bungarotoxin indicate that the nicotinic receptors in cultured BE cells are normally activated by an endogenous agonist. The physiologic ligand for the BE nicotinic receptors is likely acetylcholine that is synthesized by human BE cells.

3. Neuronal nicotinic receptors in skin keratinocytes

The keratinocytes that cover the skin are another type of epithelial cells that synthesize, release and degrade acetylcholine (Grando et al., 1993b). Cholinergic agents affect the shape and motility of cultured differentiated keratinocytes, their viability and rate of proliferation (Grando et al., 1993a,b). Those observations suggested that skin keratinocytes might express functional receptors for acetylcholine normally activated by acetylcholine produced by the keratinocytes themselves. Human keratinocytes express muscarinic acetylcholine receptors, and muscarinic agents affect some keratinocyte functions (Grando et al., 1993a,b, 1995b). The finding, that the effects of acetylcholine on tadpole keratinocytes could be mimicked by both muscarinic and nicotinic agents, first suggested that nicotinic acetylcholine receptors might be present in keratinocytes (Cox, 1993). More recent studies found direct evidence for nicotinic receptors of neuronal type in human keratinocytes, and gained information on their subunit structure, function and pharmacologic properties (Grando et al., 1995a, 1996).

The presence of neuronal nicotinic receptor subunit mRNA transcripts, and of expressed receptor subunit proteins, was demonstrated by RT-PCR experiments using subunit-specific primers, and by the use of antibodies specific for different receptor subunits (Grando et al., 1995a). These experiments clearly demonstrated that human skin keratinocytes express α_3, β_2 and β_4 neuronal nicotinic receptor subunits: antisera specific for the α_3 and β_2 subunits produced a staining pattern that was predominantly intercellular, as expected from binding to a membrane protein (Fig. 6f).

Usually, expression of α_3 subunits is accompanied by that of the α_5 subunit (Vernallis et al., 1993; Wang et al., 1996; McDonough and Deneris, 1997). An earlier study (Grando et al., 1995a) did not investigate whether skin keratinocytes expressed the α_5 subunit. A later study (Grando et al., 1996) found that α_5 sequences could be amplified by RT-PCR from cultured keratinocytes, suggesting that the corresponding subunit protein was expressed by these cells. The electrophysiologic studies summarized below found that skin keratinocytes expressed nicotinic receptors with ion gating properties compatible with the presence of ganglionic type neuronal nicotinic

neuronal nicotinic acetylcholine receptors: ganglionic-type neuronal nicotinic receptors can transport Ca^{2+}, in addition to Na^{+} (Decker and Dani, 1990; Adams and Nutter, 1992; Mulle et al., 1992a; Vernino et al., 1994). Furthermore, skin keratinocytes may express the α_7 subunit (Grando et al., 1996) that forms homooligomeric nicotinic receptors that transport Ca^{2+} (Seguela et al., 1993; Castro and Albuquerque, 1995). The finding, that nicotine increased the uptake of Ca^{2+} by isolated human keratinocytes in a concentration-dependent manner, supports the possibility that keratinocyte receptors sensitive to nicotine may transport Ca^{2+} (Grando et al., 1996). Activation of the keratinocyte α_3 and α_7 nicotinic receptors might generate intracellular Ca^{2+} signals able to influence a variety of cellular functions, including motility. Neuronal nicotinic receptors control the outgrowth of neuronal processes thorough Ca^{2+} influx in a process that may be relevant to mechanisms for specific synaptic formation (Lipton et al., 1988; Zheng et al., 1994) and which is reminiscent of the changes in keratinocyte motility induced by activation of the keratinocyte nicotinic receptors.

4. Human vascular endothelial cells express functional neuronal nicotinic receptors

The findings summarized above, and the presence of acetylcholine in a variety of surface cells, suggested that expression of nicotinic receptors of neuronal type and the use of nicotinic cholinergic signaling to modulate cellular functions might be general properties of tegumental cells related to their function, not to their embryological origin.

To test this hypothesis, we searched for nicotinic receptors of neuronal type in human endothelial cells (Macklin et al., 1998). These are not epithelial cells, yet they line large internal body surfaces, including that of blood vessels. Vascular endothelial cells express muscarinic acetylcholine receptors whose stimulation induces dilation of the vessel through release of nitric oxide (Furchgott and Zawadzki, 1980).

Similar to the studies summarized above, we used both structural and functional approaches to determine the presence and the properties of neuronal nicotinic receptors in primary cultures of human and bovine aortic endothelial cells (Macklin et al., 1998). We used primary cell cultures that had been passaged for a limited number of times, and which formed confluent monolayers with endothelial morphology (Imke et al., 1991). Thus, it was reasonable to assume that any expression of nicotinic receptors that we could detect would not be caused by lack of differentiation. Also, our previous studies on BE cells and skin keratinocytes (Grando et al., 1995a; Maus et al., 1998) indicated that expression of nicotinic receptors in those cultured cells reflected that of the corresponding cells in vivo.

That study (Macklin et al., 1998) demonstrated that vascular endothelial cells expressed functional nicotinic receptors of neuronal type sensitive to nicotine. Detection of mRNA transcripts of nicotinic receptor subunits was obtained by in situ hybridization using probes specific for the neuronal nicotinic acetylcholine receptor subunits previously detected in human BE cells and keratinocytes — the α_3, α_5, β_2 and β_4 subunits — and confluent cultures of human endothelial cells. All probes yielded clear and specific signals (Fig. 7). Thus, the endothelial neuronal nicotinic acetylcholine receptors appear to include α_3, α_5, β_2 and β_4 subunits, in good agreement with the subunit composition found for the α_3 neuronal nicotinic receptors physiologically expressed in neurons (Conroy et al., 1992; Vernallis et al, 1993; Wang et al., 1996).

We verified that the different subunits assembled into functional neuronal nicotinic receptors from the binding of [^{3}H]epibatidine and in patch clamp experiments (Macklin et al., 1998). [^{3}H]epibatidine specifically bound human and

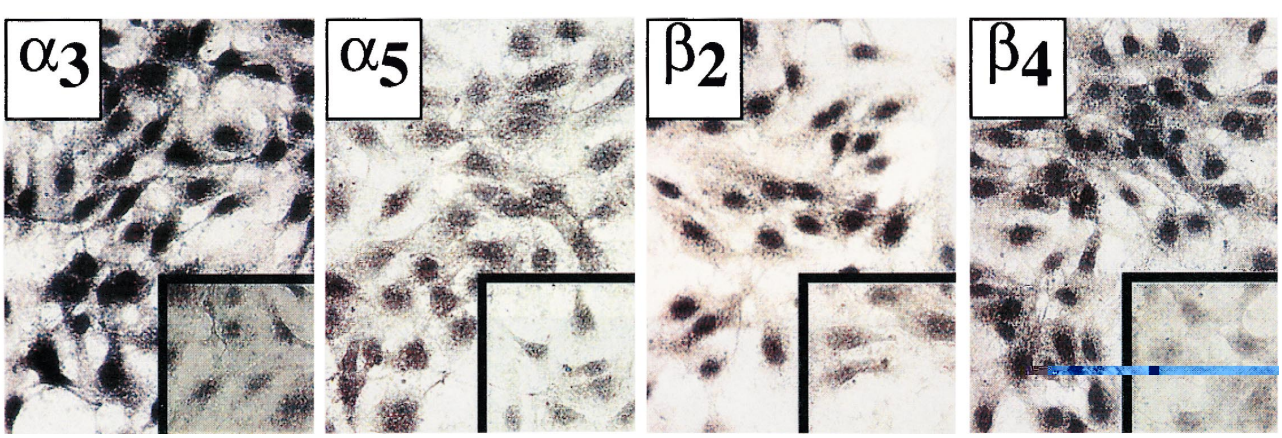

Fig. 7. Detection of α_3, α_5, β_2 and β_4 subunit transcripts in cultured human aortic endothelial cells by in situ hybridization. The α_3, α_5, β_2 and β_4 subunit probes consistently yielded a specific strong signal. Insets: negative results obtained with the corresponding ''sense'' control probes. Figure and legend reprinted with permission from Macklin, K.D., Maus, A.D.J., Pereira, E.F.R., Albuquerque, E.X., Conti-Fine, B.M., J. Pharmacol. Exp. Ther. 287 435–439, ©1998 by the American Society for Pharmacology and Experimental Therapeutics.

type (Corriveau et al., 1995; Romano et al., 1997; Fischer et al., 1999). A recent study (Fischer et al., 1999) also suggests that expression of α_7 nicotinic receptors in embryonic mammalian muscle occurs in parallel with developmental processes that involve cell migration and synapse formation — processes known to involve redistribution of Ca^{2+} (Mulle et al., 1992; Romano et al., 1997). During rat muscle development, the expression of α_7 nicotinic receptors was highest during muscle cell migration and end plate formation, and ceased after birth when the muscle was fully developed and innervated (Fischer et al., 1999). During early fetal development, α_7 nicotinic receptors were expressed in the outer layers of muscle cell clusters, suggesting that they might be involved in the control of migration and pathfinding (Fischer et al., 1999). Thus, α_7 nicotinic receptors might play a role in muscle development, and perhaps in formation or stabilization of the synapses.

Also the thymus expresses α_7 mRNA transcripts (Navaneetham et al., 1997), which likely correspond to the α-bungarotoxin binding sites demonstrated on the so-called thymus epithelial cells (Hohlfeld and Wekerle, 1994). Cholinergic agents stimulated protein synthesis in thymus epithelial cells, and this effect was blocked by α-bungarotoxin (Tominaga et al., 1989). The thymus expresses α-bungarotoxin binding sites also on the thymus myoid cells, so-called because they have characteristics reminiscent of myotubes; the α-bungarotoxin sites of myoid cells are likely related to the presence of nicotinic receptors of muscle type (Conti-Tronconi et al., 1994; Hohlfeld and Wekerle, 1994). The thymus expresses also α_3, α_5 and β_4 subunits, which are expressed by thymocytes (Mihovilovic and Roses, 1991, 1993; Navaneetham et al., 1997). The thymus contains many parasympathetic nerve terminals, which are in close proximity to the epithelial cells in the subcapsular region, and to the thymocytes in the corticomedullary region (Micic et al., 1992). The acetylcholine released by the parasympathetic terminals might interact with α_3 nicotinic receptors on thymocytes and α_7 nicotinic receptors on thymus epithelial cells: the latter receptors would be activated also by the choline released upon acetylcholine degradation.

6. Tegumental neuronal nicotinic acetylcholine receptors: new mediators of tobacco toxicity?

The use of tobacco is so widespread and the health hazards derived from it are so grave that it has been referred to as the ''global tobacco epidemic'' (Bartecchi et al., 1995). Cardiopulmonary diseases related to tobacco smoking account for one in every three deaths among people 35–70 years old (Peto et al., 1992; The Harvard Mental Health Letter, 1997). In the United States, the use of tobacco is responsible for one in every seven deaths (The Harvard Mental Health Letter, 1997). Atherosclerotic cardiovascular disease, chronic bronchitis, emphysema and lung cancer occur frequently in tobacco smokers: those diseases result from the direct effect of tobacco smoke and/or the components it contains (Peto et al., 1992; Bartecchi et al., 1995; The Harvard Mental Health Letter, 1997).

Nicotine, although addictive and likely responsible for the substance dependence that results from the tobacco use (Bock and Marsh, 1990), is considered a less dangerous component of tobacco smoke (Bartecchi et al., 1995; The Harvard Mental Health Letter, 1997). Nicotine is very water-soluble, and its concentration in the saliva of tobacco smokers can be very high (an average of 8 μM during ''smoking days'') (Lindell et al., 1993). Comparable concentrations are likely present on the bronchial and lung surface. Substantial concentrations of nicotine are present also in the blood of smokers (up to 70 ng/ml, i.e., approximately 0.5 μM; Russel et al., 1980). Yet, it has been suggested that the amount of nicotine to which one is exposed as a result of tobacco smoking may not pose a serious health risk (The Harvard Mental Health Letter, 1997). Thus, low-tar cigarettes have been considered an acceptable solution to satisfy the smokers' craving for nicotine, and devices for aerosol delivery of nicotine without tar-related carcinogens are actively developed and tested, as safe alternatives to tobacco smoking.

The findings, that the epithelial cells lining the bronchi and the endothelial cells lining the arteries express neuronal acetylcholine receptors sensitive to nicotine involved in the modulation of cellular functions that include cell motility, raise the possibility that tobacco toxicity in the cardiovascular and respiratory systems may be facilitated or even triggered by direct action of nicotine on those neuronal nicotinic receptors.

The characteristics of the α_3 neuronal nicotinic receptors expressed by BE cells and by vascular endothelial cells give clues to the pathological effects that might result from their acute and chronic exposure to nicotine. All nicotinic acetylcholine receptor isotypes share the property of being desensitized after prolonged exposure to agonists (Conti-Tronconi et al., 1994; Galzi and Changeux, 1995). However, α_3 neuronal nicotinic receptors are more resistant to desensitization than other receptor subtypes, such as those containing the α_4 and the α_7 subunits (Olale et al., 1997). Human α_3 neuronal nicotinic receptors can exist as heterooligomers of $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_3\beta_2\alpha_5$ and $\alpha_3\beta_4\alpha_5$ subunits (Conti-Tronconi et al., 1994; Galzi and Changeux, 1995; Wang et al., 1996). Nicotine is a full agonist for α_3 neuronal nicotinic receptors that include the α_5 subunit, and elicits responses as large as those obtained by full stimulation with acetylcholine. In contrast, nicotine is only a partial agonist for α_3 neuronal nicotinic receptors that do not include the α_5 subunits (Wang et al., 1996). Furthermore, the presence of the α_5 subunit increases the otherwise slow rate of desensitization of the α_3 nicotinic receptors (Wang et al., 1996). The α_3 neuronal nicotinic

receptors of BE cells and endothelial cells likely include $\alpha_3\beta_2\alpha_5$ and $\alpha_3\beta_4\alpha_5$ nicotinic receptors complexes: thus, they should be fully activated by nicotine. Also, continued exposure to the high concentrations of nicotine present on the bronchial surface of cigarette smokers should desensitize these nicotinic receptors, making them unable to respond to the endogenous acetylcholine.

The following considerations support the contention that the nicotine present on the bronchial surface of tobacco smokers will likely desensitize the α_3 receptors on BE cells. The α_3 receptor complexes containing the β_2 subunits have K_d for nicotine of 1–6 μM, those containing the β_4 subunit of 34–100 μM (Wang et al., 1996). On smoking days, an average of ~8 μM nicotine is likely persistently present on the bronchial surface of smokers (Lindell et al., 1993). That concentration should quickly desensitize the β_2-containing α_3 nicotinic receptors, and be adequate to desensitize the low-affinity β_4-containing α_3 nicotinic receptors. Dissociation of the bronchial epithelium caused by prolonged exposure to nicotine, and desensitization of the BE cells α_3 nicotinic receptors, may cause or promote chronic bronchitis, and facilitate the entry of the carcinogens present in tobacco smoke.

Continued exposure to nicotine in the blood may slowly desensitize also the α_3 neuronal nicotinic receptors of endothelial cells that contain the β_2 subunit, and make them unable to respond to the endogenous acetylcholine. If neuronal nicotinic receptors have a role in the maintenance of the integrity of the endothelial cell layer lining the blood vessels, chronic exposure to nicotine may affect this function, and facilitate the atherosclerotic lesions of the arteries that are common in people who smoked cigarettes for many years.

Neuronal α_3 nicotinic receptors are permeable to Ca^{2+} (Costa et al., 1994; Vernino et al., 1994). Therefore, stimulation of the nicotinic receptors on BE cells and endothelial cells after acute exposure to nicotine should cause an increase in intracellular Ca^{2+}. This, if excessive, might lead to degenerative phenomena and cell death. For instance, increased nicotinic receptors activity in muscle caused a Ca^{2+}-dependent degeneration of the motor endplate (Leonard and Salpeter, 1979; 1982). In some congenital myasthenic syndromes, the muscle nicotinic receptor has increased open times, and muscle degeneration occurs (Engel et al., 1993). In *Caenorhabditis elegans*, a mutation of a Ca^{2+}-permeable neuronal nicotinic receptor caused prolonged receptor activation and cell death: the neuronal death was explained by inability of the cell to cope with the osmotic imbalance or Ca^{2+}-mediated toxicity that results from increased channel activity (Treinin and Chalfie, 1995). Neurons may be more susceptible than muscle to the consequences of nicotinic receptor hyperactivation because their small size would cause more intense osmotic imbalance. This may apply to the BE cells and the vascular endothelial cells, especially considering that the α_3 nicotinic receptors desensitize slowly, and even more so when they do not include the α_5 subunit (Alkondon and Albuquerque, 1993; Wang et al., 1996; Olale et al., 1997). This would represent another potential mechanism for nicotine toxicity, resulting from acute exposure and hyperstimulation of the α_3 neuronal nicotinic receptors, as opposed to desensitization from chronic exposure.

The BE cells and the vascular endothelial cells express also the α_7 subunit, which forms nicotinic receptors that transport Ca^{2+} very effectively (Seguela et al., 1993; Castro and Albuquerque, 1995). However, α_7 nicotinic receptors desensitize very quickly (Peng et al., 1994), and they are exquisitely sensitive to inactivation by nicotine (IC_{50} of 2.8 nM) (Olale et al., 1997). Thus, their activation by nicotine in smokers might be less important than that of the α_3 neuronal nicotinic receptors in causing an excessive increase in intracellular Ca^{2+}, and Ca^{2+}-mediated toxic effects. However, tobacco smoking might increase lung morbidity mediated by α_7 neuronal nicotinic receptors as a result of exposure to nicotine during prenatal life. The α_7 nicotinic receptors are expressed by a variety of cells in fetal lung, and nicotine administration to pregnant monkeys increased the expression of α_7 nicotinic receptors in the fetal lung, and the number of certain cell types that bear α_7 nicotinic receptors (type II alveolar cells and neuroendocrine cells) (Sekhon et al., 1999). Nicotine administration to pregnant monkeys altered the lung development in the fetuses: it caused lung hypoplasia and reduction in the structural complexity and the surface of the alveoli (Sekhon et al., 1999). Those findings strongly support the possibility that nicotine consumption by the mother will alter fetal lung development by affecting the function of nicotinic receptors on non-neuronal cells in the developing lung.

Effects similar to those described in monkeys likely occur in human infants whose mothers smoke during pregnancy. Smoking during pregnancy impairs the development of the fetal lungs and results in altered lung function in the children of smoking mothers (Taylor and Wadsworth, 1987; Hanrahan et al., 1992; Tager et al., 1993; Cunningham et al., 1994). Children of mothers who smoke have increased frequency of respiratory diseases (Taylor and Wadsworth, 1987; Tager et al., 1993). Infants born of mothers who smoke have decreased respiratory flow rates and decreased pulmonary ventilation at functional residual capacity (Hanrahan et al., 1992; Tager et al., 1993). A study of pulmonary function in children 8–12 years old found reduced forced expiratory flow in children whose mothers smoked during pregnancy, confirming the earlier studies on younger children and suggesting that the pulmonary deficit continued into adolescence (Cunningham et al., 1994). The increased frequency of lower respiratory illness and the reduced lung function correlated with prenatal, not postnatal, maternal smoking (Tager et al., 1993; Cunningham et al., 1994). Also in rats, exposure of the mother to cigarette smoke during pregnancy and lactation caused reduction in the lung development and

emphysema-like changes in the pups (Collins et al., 1985; Maritz et al., 1993; Maritz and Thomas, 1995). Preliminary results suggest that developing lung alveoli and fetal pulmonary macrophages may express also α_3 nicotinic receptors, and perhaps also muscle-type nicotinic receptors containing the α_1 subunits (Sekhon et al., 1999). Thus, the ill effects of maternal exposure to nicotine on development of fetal lungs may be mediated by multiple nicotinic receptor targets. Those findings strongly suggest that many negative effects of maternal smoking on fetal lung development and on the respiratory function and health of children are mediated by nicotine.

Finally, the presence of α_7 nicotinic receptors in small cell lung carcinomas, and the finding that the growth of cell lines derived from these tumors was inhibited by α_7-specific antagonist (Chini et al., 1992; Quik et al., 1994; Codignola et al., 1996) suggest that stimulation of α_7 receptors by nicotine in smokers which harbor small cell lung carcinomas may facilitate the cancer growth.

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