0026-895X/01/6006-1201–1209$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 60:1201–1209, 2001

Vol. 60, No. 6
635/936157
Printed in U.S.A.

Human Bronchial Epithelial and Endothelial Cells Express α7 Nicotinic Acetylcholine Receptors

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Received October 12, 2000; accepted July 3, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The epithelial or endothelial cells that line the human bronchi and the aorta express nicotinic acetylcholine receptors (nAChRs) of α3 subtypes. We report here that human bronchial epithelial cells (BEC) and aortic endothelial cells (AEC) express also the nAChR α7 subunit, which forms functional nAChRs. Polymerase chain reaction and in situ hybridization experiments detected α7 subunit mRNA in cultured human BEC and AEC and in sections of rat trachea. The binding of radiolabeled α-bungarotoxin revealed a few thousand binding sites per cell in cultured human BEC and human and bovine AEC. Western blot and immunohistochemistry experiments demonstrated that cultured BEC and AEC express a protein(s) recognized by anti-α7 antibodies. Whole-cell patch-clamp studies of cultured human BEC demonstrated the presence of fast-desensitizing currents activated by choline and nicotine that were blocked reversibly by methyllycaconitine (1 nM) and irreversibly by α-bungarotoxin (100 nM), consistent with the expression of functional α7 nAChRs. In some cells, choline activated also slowly decaying currents, confirming previous reports that BEC express functional α3β4 nAChRs. Exposure of cultured BEC to nicotine (1 μM) for 3 days up-regulated functional α7 and α3 nAChRs, as indicated by the increased number of cells responding to acetylcholine and choline, with both fast-desensitizing currents, which were blocked irreversibly by α-bungarotoxin, and with slowly desensitizing currents, which are α-bungarotoxin–insensitive currents. The presence of α7 nAChRs in BEC and AEC suggests that some toxic effects of tobacco smoke could be mediated through these nicotine-sensitive receptors.

The nicotinic acetylcholine (ACh) receptors (nAChRs) are a family of ionotropic receptor proteins formed by five homologous or identical subunits and are involved in signal transduction between neurons and between nerves and muscle cells (Conti-Tronconi et al., 1994; Albuquerque et al., 1997; Lindstrom, 2000). Muscle nAChRs are formed by four types of subunits. In contrast, neuronal nAChRs include only two kinds of subunits, α and β, or just five copies of the same α subunit. Neurons express at least nine α (α2-α10) and three β (β2-β4) nAChR subunits: association of different α and β subunits results in a multitude of nAChRs that differ in their ion-gating and ligand-binding properties. Non-neuronal cells may express functional nAChRs (Conti-Fine et al., 2000). Human bronchial epithelial cells (BEC) and aortic endothelial cells (AEC) express functional nAChRs of the α3 subtype, which modulate cell shape and affect cell to cell contact (Macklin et al., 1998; Maus et al., 1998). Human skin keratinocytes express functional nAChRs of different subtypes, which include α3, α9, and possibly α7 nAChRs (Grando et al., 1995, 1996; Nguyen et al., 2000). These findings support the possibility that nAChRs modulate cellular functions other than synaptic transmission. The α7 nAChR subunit forms homo-oligomeric nAChRs with unique properties. They are more permeable to Ca^{2+} than other nAChRs (Vernino et al., 1994; Albuquerque et al., 1997). They desensitize quickly (Albuquerque et al., 1997); thus, only a limited amount of ions can go through them. Also, α7 nAChRs are activated by choline, a long-lived degradation product of ACh that might be their natural ligand (Papke et al., 1996; Albuquerque et al., 1997). This prolongs the action of ACh on the α7 nAChRs beyond the short-lived effects mediated by binding of ACh itself. In the central nervous system, the α7 nAChRs are

Supported by National Institute on Drug Abuse Center Grant DA11806 (to B.M.C.-F.) and United States Public Health Service Grant NS25296 and a grant from PRONEX, Brazil (to E.X.A.).

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ABBREVIATIONS: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptors; BEC, bronchial epithelial cells; AEC, aortic endothelial cells; α-BTX, α-bungarotoxin; RT-PCR, reverse transcription polymerase chain reaction; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; ChAT, choline-acetyl transferase; AcCoA, acetyl coenzyme A; KRB, Krebs-Ringer-bicarbonate; MLA, methyllycacotine.

An erratum has been published:
http://molpharm.aspetjournals.org/content/62/1/192.full.pdf

predominantly presynaptic, suggesting that they modulate synaptic transmission, in addition to their function in signal transduction (Radcliffe et al., 1999; Zarei et al., 1999). In embryonic muscle, α7 nAChRs appear before the synapses, and they may be involved in muscle development (Fischer et al., 1999). In this study, we investigated the expression of α7 nAChR subunit in human BEC and AEC, and the presence of functional, choline-sensitive nAChRs in cultured human BEC.

Materials and Methods

Cultures of BEC and AEC. Primary cultures of human BEC and AEC (BioWhittaker/Clonetics, Walkersville, MD) were seeded in T-25 culture flasks (Corning Inc., Corning, NY) and propagated as described previously (Macklin et al., 1998; Maus et al., 1998). When the cells reached 80 to 90% confluence, they were detached by mild trypsinization using 0.25% Trypsin/EDTA (BioWhittaker/Clonetics), and used for α-bungarotoxin (α-BTX) binding studies, or plated on glass cover slips for the in situ hybridization assays and for patch clamp studies. Before plating the cells onto glass coverslips (Fisherbrand Microscope Cover Glass, 12-mm circle; Fisher Scientific, Pittsburgh, PA), the cover slips were wiped with 70% ethanol and placed in a 24-well culture plate until dry. A small drop of medium containing 500 to 800 cells was spotted in the center of the coverslip. After 15 min, 1 ml of fresh medium was added slowly to the well. The cultures were grown until they reached confluence. We used a similar procedure for plating cells on 4-well glass slides (Nalge Nunc, Naperville, IL) for the immunofluorescence assays. For the Western blot experiments, the cells were collected by scraping the flasks with a cell scraper, to avoid the use of trypsin.

For one ^{125}I-α-BTX binding experiment, we used bovine AEC propagated from fresh bovine aortas obtained from a local slaughterhouse. After their dissection, sections of aorta were flushed several times with sterile cold medium (bronchial epithelial growth medium; BioWhittaker/Clonetics) containing 100 μg/ml penicillin and streptomycin, and flushed twice with the same medium containing 0.5 U/mg dispase (Boehringer-Mannheim, Indianapolis, IN). The sections were closed at both ends with sterile hemostat clamps and filled, using a sterile 1-ml tuberculin syringe with, dispase-containing medium. After overnight incubation at 4°C in a beaker of sterile cold medium, they were carefully opened and their surface scraped with a sterile spatula. The AEC thus obtained were rinsed with fresh medium, gently breaking up any cell clumps with a pipette, and transferred into a T-25 flask (Corning Inc.) pretreated with 30 μg/cm^2 collagen (Sigma-Aldrich, St. Louis, MO). The cells were cultured at 37°C in 5% CO_2. The medium was changed after 24 h, and every 2 days afterward. When the cells were confluent, we used 0.25 μg/ml trypsin/EDTA (BioWhittaker/Clonetics) to remove the cells, and we subcultured them using a 1 to 3 split, and a starting concentration of 0.3×10^6 cells/ml.

Detection of nAChR Subunit mRNA by Reverse Transcription Polymerase Chain Reaction. RNA extracted from cultured BEC and AEC using RNAzol (Tel-Test Inc., Friendswood, TX) was reverse transcribed using Superscript RNase H$^-$ Reverse Transcriptase (Invitrogen, Carlsbad, CA). Hot-start PCR (Horton et al., 1994) was run for 35 cycles at 95°C for 15 s, 55°C for 15 s, and 72°C for 45 s in a 9600 thermal cycler (PerkinElmer Life Sciences, Norwalk, CT). The PCR products were resolved by electrophoresis on a 1% agarose UltraPure (Invitrogen)/0.5× Tris/Borate/EDTA gel containing 0.5 mg/ml ethidium bromide. We used 100- or 123-bp DNA ladders (Invitrogen) as molecular mass standards.

The primer sequences and expected product size (in parentheses) were: actin, 5′-GCTCCGGCATGTGCAA-3′ and 5′-AGGATCTTCATGAGGTAGT-3′ (542 bp); α3 subunit, 5′ -CCATGTCTCAGCTGGTG-3′ and 5′-GTCCTTGAGGTTCATGGA-3′ (401 bp); α4 subunit, 5′-TGGGTGAAGCAGGAGAGTGG-3′ and 5′-AGTCCAGCTGGTCCACG-3′ (346 bp); α7 subunit, 5′-CCTGGCCAGTGTGGAG-3′ and 5′-TACGCAAAGTCTTTGGACAC-3′ (414 bp); α9 subunit, 5′-GTCCAGGGTCTTGTTTGT-3′ and 5′-ATCCGCTCTTGCTATGAT-3′ (403 bp); α10 subunit, 5′-CTGTTCCGTGACCTCTTT-3′ and 5′-GGAAGGCTGCTACATCCA-3′ (388 bp). The α3, α4, and α7 primers were designed to match the sequence of both human and rodent nAChR subunits, and they all yielded a product of the expected size when we used human brain cDNA (Fig. 1; Maus et al., 1998). We used also cDNA from human thymus that express the α3 and α7 subunits (Navaneetham et al., 1997) as positive control samples for the α3 and α7 primers (Fig. 1). For the α9 and α10 primers, we used cDNA of the human α9 and α10 subunits (a generous gift of Dr. Lawrence R. Lustig, Johns Hopkins University, Baltimore, MD) as positive control samples. The α9 and the α10 primers yielded a

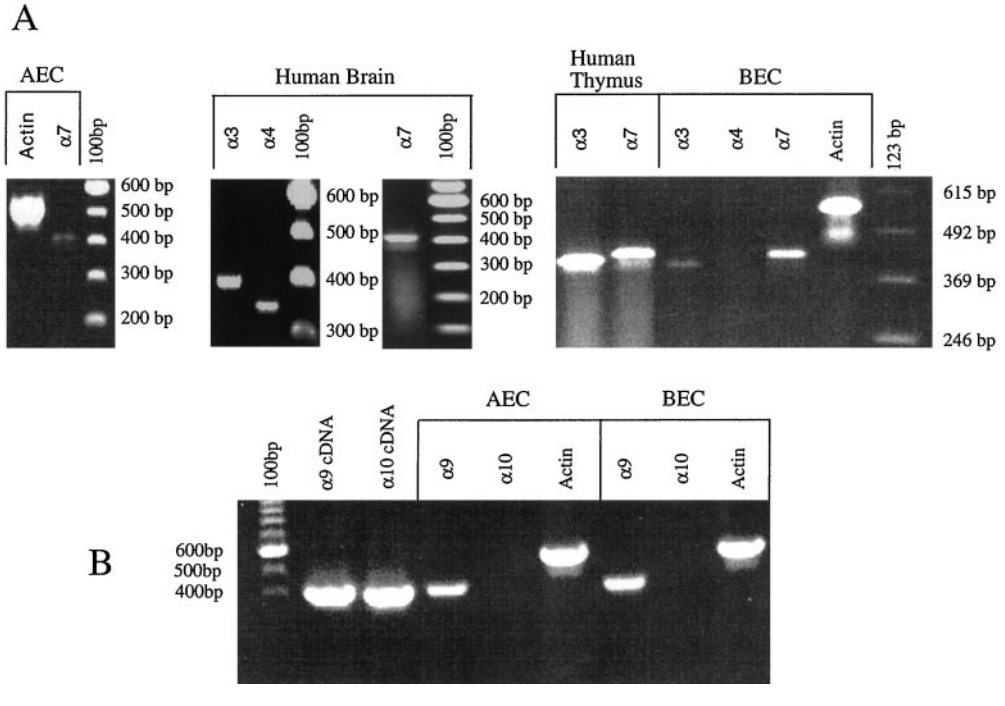

Fig. 1. A, detection of α7 nAChR subunit transcripts in cultured human AEC and BEC by RT-PCR. The α7 subunit primers yielded products of the expected size with both cell types. The primers for actin and for the α3 nAChR subunits also yielded products of the expected size, whereas the α4 subunit primers did not yield RT-PCR products. When using human brain cDNA, the α3, the α4, and the α7 primers yielded products of the appropriate size. When using thymus cDNA, which served as a second positive control, both the α3 and the α7 primers yielded products of the appropriate size. B. detection of α9 nAChR subunit transcripts in cultured human AEC and BEC by RT-PCR. The α9 subunit primers yielded products of the expected size with both cell types, whereas the primers for the α10 nAChR subunit did not yield RT-PCR products. When using the appropriate human α9 or α10 cDNA, both the α9 and the α10 primers yielded products of the appropriate size. See *Materials and Methods* for experimental details.

product of the appropriate molecular mass when we used the corresponding cDNA (Fig. 1). The actin primers served as a positive control for the quality of the cDNA.

Cloning and Sequencing of RT-PCR Products. We determined the sequence of the products we observed when using the α7 and the α9 primers, to verify that they were amplified from the α7 and the α9 templates. We purified (GeneClean II; Bio 101, Vista, CA) the RT-PCR products obtained with the α7 and the α9 primers, after they were resolved on agarose gel, and dissolved in 10 μl of H_2O. Two microliters of the dissolved product was cloned into a "TA" cloning vector (pCR II-TOPO TA Cloning, Version H; Invitrogen Corporation, Carlsbad, CA). The plasmids were purified (RPM kit; Bio 101) and sequenced at the Microchemical Facility of the University of Minnesota.

Assay of α7 Subunit Transcripts by in Situ Hybridization. We carried out in situ hybridization experiments using cultured human BEC and AEC and sections of rat trachea, and probes specific for the α7 nAChR subunits. The probes were transcribed in vitro from cloned and sequenced α7 PCR products obtained using thymus cDNA (Navaneetham et al., 1997). The probes were labeled with digoxigenin-UTP (Roche Molecular Biochemicals, Mannheim, Germany). The labeled single-stranded probes were hybridized to the cell mRNAs under high-stringency conditions, which allowed the probes to bind only to their corresponding mRNA (Maus et al., 1998). We used anti-digoxigenin antibody coupled to alkaline phosphatase to detect the bound probe (Roche Molecular Biochemicals), and the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate mixture (Roche Molecular Biochemicals) as a substrate for alkaline phosphatase. The specificity of the binding of the probes was demonstrated by absence of signal when we used the corresponding "sense" probe.

Assay of Binding of ^{125}I-Labeled α-BTX. We verified the presence of α7 nAChRs using the binding of ^{125}I-α-BTX to suspensions of cultured BEC and AEC, obtained by mild trypsinization of confluent cell cultures (Maus et al., 1998). Primary cultures of BEC and AEC grow slowly, and the fresh starting human material is scarce: because of the scarcity of these cells, we used single-dose binding experiments rather than dose-dependence curves. We used 0.5 to 2 $\times$ 10^6 cells/tube and set up the samples at least in triplicate. We determined the total binding by incubating the cells with 8 to 25 nM ^{125}I-α-BTX (in one experiment, 50 nM) for up to 48 h. The affinity of α7 nAChRs for α-BTX is 1 to 2 orders of magnitude lower than that of the heteromeric AChRs expressed by muscle and electric tissue [K_D = 2 nM for α7 nAChRs versus K_D = 0.1 nM or less for muscle type nAChRs (Lindstrom, 2000)]. We chose this range of concentrations because pilot experiments that employed increasing concentrations of ^{125}I-α-BTX indicated that, in our experimental conditions, 8 to 25 nM α-BTX allowed to reach binding equilibrium during the incubation time we used. In each experiment, we determined the nonspecific binding by preincubating aliquots of cells (in triplicate or more) with 10 μM unlabeled α-BTX for 2 to 12 h at 4°C. We spotted the cells on Whatman GF/B or GF/C filter disks (Whatman, Clifton, NJ) and washed them three times by vacuum filtration as we described previously (Macklin et al., 1998). Alternatively, we solubilized the cells in 1% Triton X-100, spotted them on Whatman DEAE filter disks, and washed them three times as described previously (Maus et al., 1998). The disks were counted by liquid scintillation. Either method yielded similar results.

Immunofluorescence Microscopy. We examined the presence on human BEC and AEC of proteins recognized by a rabbit antibody specific for the human α7 subunit (Santa Cruz Biotechnology, Santa Cruz, CA), using immunofluorescence microscopy.

Confluent BEC or AEC cultures grown on Labtek four-well slides (Nalge Nunc) were cooled on ice and washed in ice-cold PBS. The cells were fixed in 3% paraformaldehyde, 7% sucrose for 5 min, on ice, then washed three times for 5 min with ice-cold PBS. The rabbit anti-α7 antibody, diluted 1:200 in PBS, was added and incubated overnight at 4°C. The cells were washed three times for 5 min with ice-cold PBS, then incubated for 1 h at 4°C with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (a 1:200 dilution in PBS; Santa Cruz Biotechnology). The cells were washed, mounted in ProLong Antifade mounting media (Molecular Probes, Eugene, OR) and observed with a Nikon Eclipse 800 fluorescence microscope (Nikon Diaphot, Melville, NY).

We determined the unspecific binding in cultures incubated without the anti-α7 antibody, with or without purified rabbit IgG at concentrations comparable with or exceeding those used for the anti-α7 antibody.

Western Blots. For each experiment, we used confluent BEC or AEC grown in two 75-cm^2 flasks (Corning, Inc.) and collected by scraping the flasks with a cell scraper. As a positive control for expression of the α7 subunit, we used PC12 cells (American Type Culture Collection, Manassas, VA). The cells were rinsed twice in ice-cold PBS and solubilized in 100 μl of ice-cold PBS containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 1% SDS and freshly added phenylmethylsulfonyl fluoride (0.1 mg/ml; all reagents from Sigma Chemical Co., St. Louis, MO). We passed the cell lysate through a 21-gauge needle several times to shear the DNA, added 5 μl of 10 mg/ml phenylmethylsulfonyl fluoride, and incubated it on ice for 1 h. The solubilized extract was collected by centrifugation at 10,000 g for 10 min at 4°C, and a 5- to 10-μl sample was mixed with sample loading buffer (Laemmli, 1970), boiled for 5 min, and electrophoresed onto a 12% SDS-polyacrylamide gel, in a Mini-PROTEAN II system (Bio-Rad, Hercules, CA). The separated protein bands were transferred onto polyvinylidene difluoride membrane using a Bio-Rad Mini Trans-Blot cell. The polyvinylidene difluoride membrane was incubated in 10 mM Tris-HCL, pH 8.0, 150 mM NaCL, 5% milk, 0.1% Tween-20 (blocking buffer) for 1 h at room temperature, then overnight at 4°C in the primary antibodies (rabbit anti-human AChR α7 or goat anti-human AChR α7; Santa Cruz Biotechnology) appropriately diluted in blocking buffer. The membrane was washed three times for 10 min each with 10 mM Tris-HCL, pH 8.0, 150 mM NaCL (TBS), and 0.1% Tween-20, then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG or donkey anti-goat IgG, as appropriate; both from Santa Cruz Biotechnology). The membrane was washed three times for 10 min with TBS containing 0.1% Tween-20, and once for 5 min with TBS. The protein bands stained by the antibody were visualized on Kodak film using Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology).

Assay of Choline-Acetyl Transferase (ChAT). We examined whether cultured human BEC and AEC express the enzyme for ACh synthesis, using the enzymatic assay of Fonnum (1975) and [^{3}H]acetyl coenzyme A ([^{3}H]AcCoA; specific activity of 200 mCi/mmol; PerkinElmer Bioscience Products, Boston, MA) as acetyl donor. For each assay, we harvested the cells in three confluent T-75 culture flasks (Corning Inc.) by mild trypsinization (Maus et al., 1998), washed them once with culture medium (BioWhittaker/Clonetics), and resuspended them in 3.5-ml Krebs-Ringer-bicarbonate buffer (KRB buffer; Sigma-Aldrich) containing 10 mM glucose. The cells were put on ice and disrupted by sonication. We determined the protein concentration in the cell extract by the Lowry assay (Lowry et al., 1951). We added 0.5 ml of cell homogenate to 0.5 ml of incubation buffer [50 mM Tris/HCl, pH 8.0, 1 mM unlabeled AcCoA, 10 mM choline, 1 mM physostigmine (all from Sigma-Aldrich)] and 0.02 mM [^{3}H]AcCoA. Control vials (blanks) contained KRB buffer and incubation buffer. The vials were capped, vortexed, and placed in a CO_2 incubator at 37°C for 20 min. Five milliliters of freshly prepared scintillation cocktail [nine parts INSTA-Fluor (Packard Instrument Co. Inc., Downers Grove, IL), 1 part tetraphenylboron in n-butanol] was added to extract the newly synthesized [^{3}H]ACh from the aqueous phase. The ChAT activity was calculated by subtracting the counts-per-minute (c.p.m.) value of the blanks from the c.p.m. of the samples, and converting the result into the amount of ACh produced per milligram of protein per minute.

Assay of Acetylcholinesterase (AChE). We determined the presence of AChE in cultured human BEC and AEC, both intracel-

lular and secreted into the medium (Ellman et al., 1961). Two T75 culture flasks (Corning Inc.) with preconfluent human BEC or AEC were washed once with KRB buffer and incubated in KRB buffer for 24 h. The culture supernatant was used immediately to measure the AChE secreted into the medium. The cells were harvested with a rubber policeman, pooled, resuspended in 1 ml of ice-cold KRB buffer, and disrupted by sonication to release the intracellular AChE. We determined the AChE activity by measuring the absorbance at a wavelength of 412 nm in a solution containing acetylthiocholine as substrate and dithiobisnitrobenzoic acid, which yields a yellow color in the presence of thiocholine. The samples contained 0.5 ml of cell homogenate or medium of cell cultures, 20 μl of 0.075 M acetylthiocholine, and 100 μl of 0.01 M dithiobisnitrobenzoic acid in 0.1 M phosphate buffer, pH 8.0, in a total volume of 3 ml. Control samples (blanks) contained phosphate buffer instead of cell homogenate or supernatant. All regents and the KRB buffer were from Sigma-Aldrich. We converted the rates of enzymatic activity into enzymatic units (1 unit hydrolyzes 1 μmol of substrate per minute per milligram of protein to thiocholine and acetate).

Patch Clamp Recording of Whole-Cell Currents. We recorded macroscopic currents from preconfluent cultured human BEC using the standard whole cell mode of the patch-clamp technique (Hamill et al., 1981) and an LM-EPC-7 patch clamp system (List Electronic, Darmstadt, Germany). Signals were filtered at 1 to 2 kHz and directly sampled by a Pentium III computer using the pClAMP 6 program (Axon Instruments, Foster City, CA). The external solution bathing the BEC had the following composition: 165 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5 mM HEPES, and 10 mM dextrose (pH was adjusted to 7.3 with NaOH; osmolarity, 340 mOsM). We added atropine (1 μM) to the external solution to block muscarinic receptors. The internal solution had the following composition: 60 mM CsCl, 60 mM CsF, 10 mM EGTA, 22.5 mM CsOH, and 10 mM HEPES (pH adjusted to 7.3 with CsOH; 340 mOsM). The pipette resistance was 3 to 5 MΩ. The access resistance was $\leq$ 15 MΩ and we did not compensate for it. We used a standard U-shaped glass tube (U-tube) (Alkondon and Albuquerque, 1995) to apply agonists onto the cells. Antagonists were delivered to the cells via the U-tube (as admixtures with agonists) and/or by bath perfusion. To investigate changes in nAChR expression after exposure to nicotine, 1 μM nicotine was added to the culture medium for 3 days before testing the responsiveness of the cells to choline. For these experiments, we changed the medium with nicotine-containing medium on the first and the last day, before testing the cells.

Results

Detection of α7 Transcripts in Cultured Human BEC and AEC by RT-PCR. We used RT-PCR to investigate the presence of mRNA for the α7 nAChR subunit in cultured BEC and AEC. We used primers specific for the α3, α4, α7, α9, and α10 nAChR subunits and for actin. The actin and α3 subunit primers served as positive controls; the α4 subunit primers, which never yielded a PCR product using BEC cDNA (Maus et al., 1998), were used as negative controls. The primers for the α7, α3, and α9 subunits and those for actin always yielded products of the expected size both with BEC and AEC cDNA, whereas the α4 and α10 primers did not. The α3, α4, and α7 primers yielded products of the appropriate size when we used human brain cDNA, the α3 and α7 primers when we used human thymus cDNA, and the α9 and α10 primers when we used the appropriate (human α9 or human α10) cloned cDNA. Fig. 1 reports the result of representative experiments.

Cloning and sequencing of the RT-PCR products amplified using the α7 and the α9 primers yielded the sequences of the α7 and the α9 human nAChR subunits (GenBank accession numbers X70297 and NM_017581, respectively).

Detection of α7 Transcripts in Cultured Human BEC and AEC, and in Rat Trachea by in Situ Hybridization. To verify the presence of the α7 subunit transcript in the cell cultures, and to determine whether it was expressed also in vivo, we carried out in situ hybridization experiments using cultured, confluent human BEC and AEC or sections of rat trachea, and α7 specific RNA probes (Navaneetham et al., 1997). Fig. 2 reports the results of representative experiments. Cultured BEC and AEC showed a clear signal, which was absent when we used the corresponding 'sense' probe (Fig. 2, insets). The α7 specific RNA probe yielded a strong signal in the epithelial layer of the rat trachea (Fig. 2, arrows), which was greatly reduced when we used the 'sense' control probe.

^{125}I-α-BTX Binding. The presence of binding sites for α-BTX, a specific ligand of muscle and α7 nAChR subtypes (Lindstrom, 2000), would be consistent with the expression of the α7 nAChR subtype in BEC and AEC. We measured the ^{125}I-α-BTX binding to three different batches of BEC and AEC, in three independent experiments. Two batches of AEC were from human aorta and one from bovine aorta. All the batches of BEC were from human bronchi. We obtained 1,890, 1,860, and 11,000 ^{125}I-α-BTX binding sites/cell for the human BEC; 4,396 and 3,234 for human AEC; and 3,682 for the bovine AEC. Because of the limitations of the assay we used, these should be considered qualitative estimates of the presence of specific binding sites, rather than accurate measurements of their numbers.

Immunofluorescence and Western Blot Detection of Protein(s) Recognized by Antibodies Specific for the α7 Subunit. We determined the expression of α7 protein in BEC and AEC by examining the binding to cultured cells of a polyclonal rabbit IgG specific for the human α7 sequence (Santa Cruz Biotechnology). Fig. 3 reports the results of representative immunofluorescence experiments. The antibody stained weakly but consistently most BEC and AEC. No staining was detected if we omitted the anti-α7 antibody or if we substituted it with purified IgG from nonimmune rabbits (Sigma Aldrich).

The rabbit anti-α7 antibody is suitable also for detection of α7 protein in Western blots. Also, a goat polyclonal antibody specific for an α7 peptide sequence of the human α7 subunit is available (Santa Cruz Biotechnology), which recognizes the

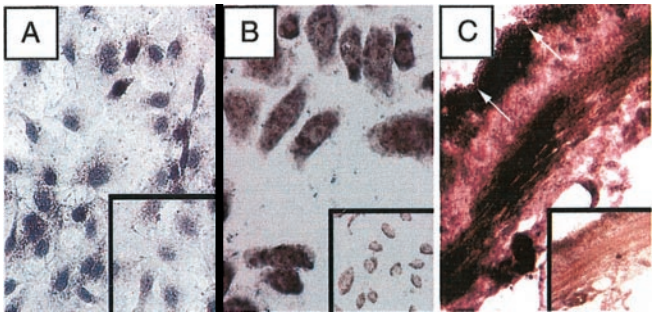

Fig. 2. Detection of α7 nAChR subunit transcripts in cultured human BEC (A) and AEC (B) and in rat trachea (C) by in situ hybridization. The α7-specific RNA probes yielded a clear signal in cultured BEC and AEC and in the epithelial layer of rat trachea (arrows in C), which was absent or greatly reduced when we used the corresponding 'sense' probe (insets). See *Materials and Methods* for experimental details.

denatured α7 in Western blots. We used both antibodies to probe by Western blotting several independent preparations of human AEC and BEC. Cultured PC12 cells served as positive controls of α7 expression. Fig. 4 reports the results of representative experiments.

When using extracts of different preparations of PC12 cells, the rabbit anti-α7 antibody recognized consistently a band of ~56 ± 1.6 kDa (n = 6), as expected for the α7 subunit, but also one or more bands of molecular mass 30 to 34 kDa, whose number and intensity varied in the different preparations: in some preparations they represented the majority of antibody staining. The goat anti-α7 antibody detected a band of ~55 kDa (n = 2).

In the BEC, both the rabbit and the goat anti-α7 antibodies recognized consistently only a sharp band of molecular mass ≈ 34 kDa (n = 2). In the AEC, the rabbit antibody recognized consistently a band of molecular mass 56.7 ± 1.7 kDa (n = 7), and one of 34.7 ± 0.9 kDa (n = 9), which was usually more prominent than the 57-kDa band. In some preparations, the rabbit antibody recognized also bands of molecular mass between 30 and 34 kDa and a faint band of ~90 kDa. In the AEC, the goat anti-α7 antibodies recognized consistently only a band of molecular mass ≈ 35 kDa.

Detection of ChAT and AChE activity. We next examined whether human cultured BEC and AEC have the key enzymes for the synthesis and degradation of ACh: ChAT and AChE. The results of those experiments, summarized in Table 1, indicated that both BEC and AEC contained ChAT and AChE, and secreted AChE in the culture medium.

Nicotinic Whole-Cell Currents Recorded from BEC. Eight of the 215 cultured human BEC we sampled (~4%) responded to 10 mM choline with currents that decayed to the baseline during the agonist pulse (Fig. 5A). The amplitude of these currents ranged from 15 to 40 pA. In addition, approximately 8% of the sampled cells responded to choline with currents that decayed to the baseline only after removal of the agonist (Fig. 5B). The fast decaying currents evoked by 10 mM choline were similar in amplitude and time course to the currents evoked in the same cells by 10 μM nicotine (n = 4; Fig. 5D), a nearly saturating agonist concentration for α7 nAChRs in hippocampal neurons (Alkondon and Albuquerque, 1995). They were blocked after 10-min perfusion of the BEC with physiological solution containing methyllycacotine (MLA, 1 nM; n = 3; Fig. 5D). The blockade was reversible after 15-min washing of the cells with MLA-free physiological solution (Fig. 5D). The choline-evoked fast decaying currents that were reversibly blocked by 1 nM MLA were blocked also after 15-min perfusion of the cells with physiological solution containing 100 nM α-BTX (n = 2); the blockade was irreversible after 30 min washing of the cells with α-BTX free medium (Fig. 5C). Therefore, the fast desensitizing currents that we recorded from the BEC had the characteristics of responses mediated by α7 nAChRs. In two of the eight cells, the choline-evoked fast-decaying currents had rising and decay phases comparable with those of type IA currents of cultured hippocampal neurons, which are mediated by α7 nAChRs (Alkondon and Albuquerque, 1995) (Fig. 5A, left trace). In the other cells, the rising and decay phases of the fast-decaying currents were somewhat slower than those of type IA currents evoked in cultured hippocampal neurons by application of nicotinic agonists via a U-tube (400 ± 101 ms, n = 6 for the BEC and ~30 ms for the hippocampal neurons). However, the decay-time constant of these BEC currents was faster than that of type IA currents evoked by U-tube application of nicotinic agonists to interneurons in hippocampal slices (~830 ms; Alkondon et al., 1999). The slower kinetics of the choline-evoked currents recorded from human BEC could be attributable to slow agonist diffusion in the extracellular matrix of the cells.

Chronic exposure to nicotinic agonists, including nicotine, up-regulates nAChRs (Lindstrom, 2000). To test whether nicotine affected the expression of BEC nAChRs, we exposed cultures of human BEC to 1 μM nicotine for three days, then washed them for 1 h with nicotine-free external solution and

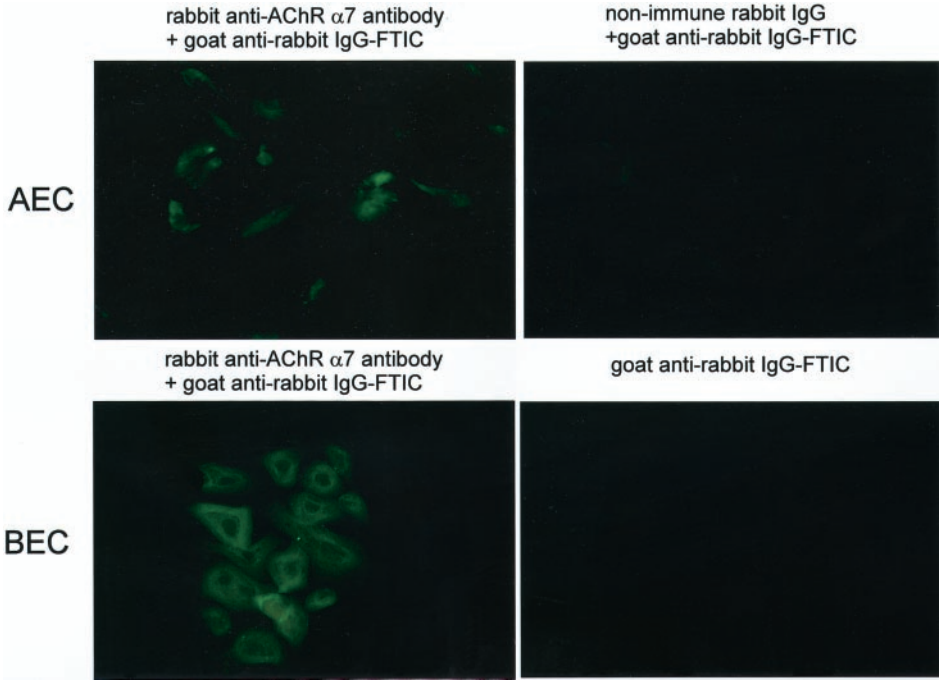

Fig. 3. Immunofluorescence detection of the binding of a rabbit antibody specific for the human α7 subunit to cultured human BEC and AEC, as indicated at left. The presence of antibodies bound to the cells is revealed by the binding of fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibody. Right, extent of nonspecific labeling obtained by omitting the anti-α7 antibody or by substituting it with a comparable concentration of nonimmune rabbit IgG. See text for experimental details.

tested their responsiveness to choline (10 mM) and ACh (1 mM). Choline evoked whole-cell currents in 9 of 17 randomly sampled cells. In four of those cells (23.5%), the currents evoked by 10 mM choline had the same amplitude as those evoked by 1 mM ACh, decayed to the baseline during a 2-s agonist pulse, and were irreversibly blocked by α-BTX (100 nM) (Fig. 6A). In one of these cells, we tested whether MLA could block the choline-evoked currents (1 nM): MLA completely blocked the current (Fig. 6C). In the other five cells (29%), choline evoked currents that decayed slowly and were not blocked by α-BTX (Fig. 6B). These results indicate that prolonged exposure of BEC to nicotine up-regulates functional α7 and non-α7 nAChRs.

Discussion

This study provides evidence that human BEC and AEC express functional α7 nAChRs. RT-PCR and in situ hybridization experiments indicated that BEC in culture and in vivo, and AEC in culture, expressed mRNA encoding the α7 nAChR subunit (Figs. 1 and 2). Cultured BEC and AEC expressed protein recognized by α7-specific antibodies (Figs. 3 and 4) and binding sites for ^{125}I-α-BTX. α-BTX binds also to

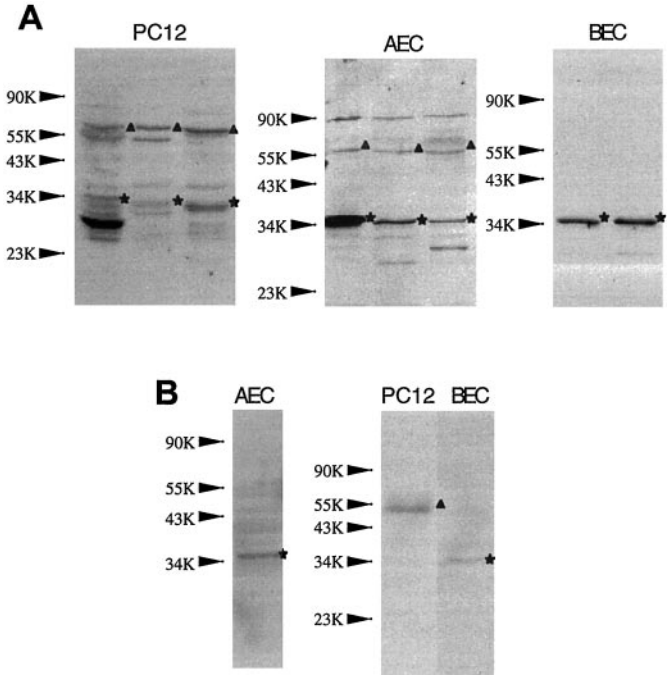

Fig. 4. Western blot detection of protein bands recognized by antibodies specific for the human α7 subunit and obtained in rabbit (A) or goat (B), in detergent extracts of PC12 cells and cultured human BEC and AEC. The arrows indicate the position of the molecular mass standards. The triangles and the stars indicate the position of bands of molecular mass of ~57 and 34 kDa that were frequently or consistently present in the cell extracts. See text for experimental details.

TABLE 1
ChAT and AChE in human cultured BEC and AEC

	BEC	AEC
Intracellular ChAT (mol of incorporated acetyl/mg of cellular protein/min)	2.91×10^{-12}	4.80×10^{-12}
Intracellular AChE (units/mg of cellular protein)	0.11	0.72
Secreted AChE (units/mg of cellular protein)	0.20	0.38

α9 nAChRs (Lindstrom, 2000), which may be expressed by human BEC and AEC (Fig. 1). However, the complexes of α-BTX with α7 nAChR are stable, those with α9 nAChRs are not: α-BTX–mediated block of α9 nAChRs is reversed after a 10-min wash (Elgoyhen et al., 1994). Because our assay of ^{125}I-α-BTX binding entailed three 10-min washes, the binding sites we detected were probably α7 nAChRs. Electrophysiological experiments indicated the presence in cultured BEC of fast decaying currents activated by choline and nicotine, and blocked reversibly by MLA and irreversibly by α-BTX (Figs. 5 and 6). Choline acted as a full agonist for those currents. All these properties are consistent with those of α7 nAChRs. The response to nicotine as an agonist and the irreversibility of the α-BTX block differentiate these currents from those mediated by α9 nAChRs. The fast rate of decay, the block by α-BTX, and the response to choline as a full agonist differentiate these currents from those gated by α3 nAChRs. A few BEC expressed choline-activated, slowly decaying currents (Figs. 5B and 6B), consistent with the reported presence of α3β4 nAChRs (Maus et al., 1998). The findings that bovine cultured AEC and rat BEC in situ expressed α7 subunit transcripts and ^{125}I-α-BTX binding sites suggests that BEC and AEC of all mammals express α7 nAChRs.

Western blots of BEC and AEC extracts demonstrated the presence of protein bands recognized by two different anti-α7 antibodies (Fig. 4). In AEC extracts, the antibodies recognized a protein band of the molecular mass expected for the α7 subunit (56.7 kDa), but also bands of lower molecular mass (≤34.7 kDa). In BEC extract, the antibodies recognized a single band of 34 kDa. A similar pattern of bands was observed in Western blots of PC12 cells and could be due to proteolytic degradation of the α7 subunit. nAChR subunits from other sources are prone to proteolytic degradation during their purification (Conti-Tronconi et al., 1994).

The numbers of α-BTX binding sites in human and bovine AEC were consistent in the three independent experiments (3770 ± 586 sites/cell), whereas in human BEC, they were the same in two experiments (1875 ± 21 sites/cell) and almost six times as much in a third experiment. The binding sites for α3 nAChR-specific ligands in human BEC, AEC, and skin keratinocytes had a similar variability, which in keratinocytes correlated with the degree of cell differentiation (Grando et al., 1995; Macklin et al., 1998; Maus et al., 1998). The variable number of α-BTX binding sites in cultured BEC may be related to their degree of differentiation. Also, the electrophysiology experiments detected nAChR function in only a fraction of the BEC; the variable number of α-BTX binding sites that we found might result from variable amounts of nAChR-expressing cells.

Human BEC and AEC express transcripts for the α9 nAChR subunit (Fig. 1) which can form homomeric nAChRs with unique pharmacological properties; they are blocked by nicotine, by antagonists of type A γ-aminobutyric acid, glycine, and type 3 serotonergic receptors, and by atropine (IC_{50} ~1 μM) (Elgoyhen et al., 1994; Rothlin et al., 1999; Verbitsky et al., 2000). Our inclusion of 1 μM atropine in the solution bathing the BEC would still permit their partial activation. α9 nAChRs are activated by choline (Verbitsky et al., 2000). However, their block by α-BTX is quickly reversible (Elgoyhen et al., 1994). Also, while rodent α9 nAChRs are very sensitive to MLA (IC_{50} = 1.1 nM; Verbitsky et al., 2000),

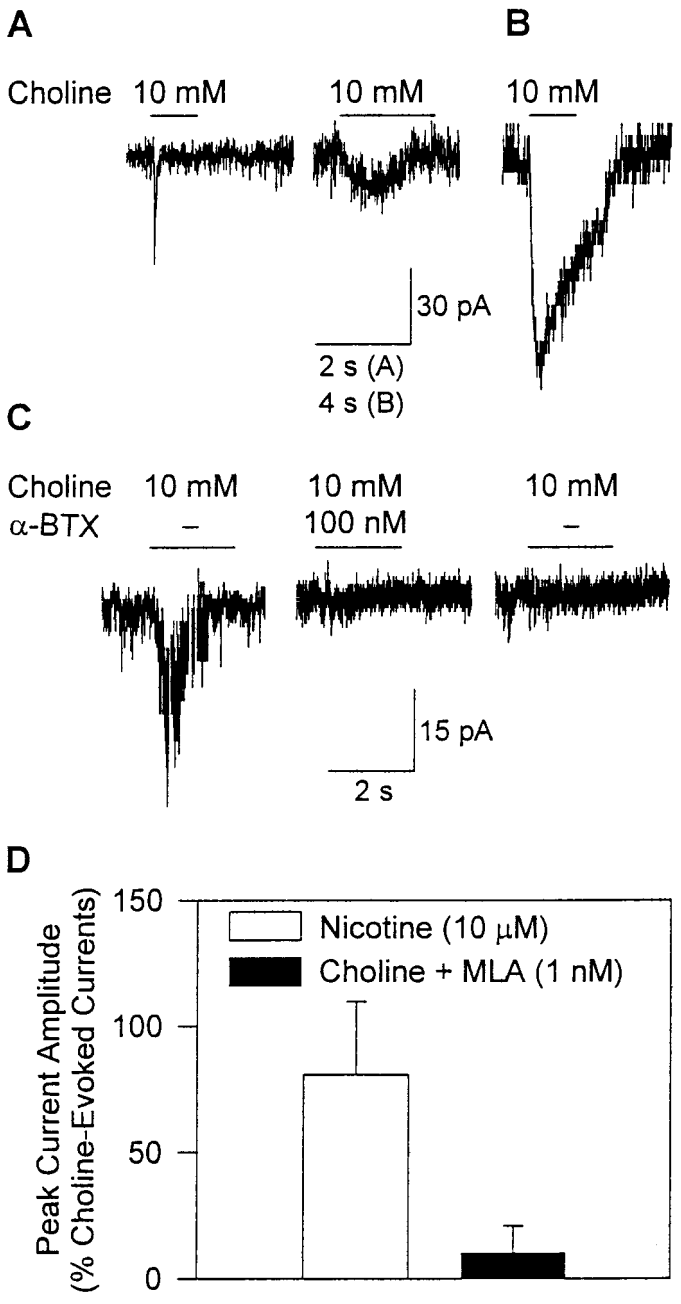

Fig. 5. Characterization of nicotinic responses recorded from human BEC in culture. A, sample recordings of whole-cell currents evoked by U-tube application of choline (10 mM) to two cultured BEC. Both currents decay to the baseline within the agonist pulse. However, they have different kinetics. The left sample is representative of responses recorded from 2 of 215 sampled cells. The right sample is representative of responses recorded from another six cells. B, sample recording of a choline-evoked whole-cell current obtained from a cultured BEC that decayed to the baseline only after removal of the agonist. C, sample recordings of a fast-decaying current evoked by choline under control condition (left trace), after 15-min perfusion with external solution containing α-BTX (100 nM; middle trace) and after 30-min wash with α-BTX-free external solution (right trace). D, graph quantifying the agonistic effect of nicotine and the antagonistic effect of MLA on BEC cells that responded to choline with fast-desensitizing currents. The amplitudes of fast-decaying currents evoked by choline were taken as 100% and used to normalize the amplitudes of fast-decaying currents evoked by a subsequent pulse of nicotine (the interval between pulses was 1 min) or by a pulse of choline-plus-MLA after the cells had been perfused for 10 min with MLA (1 nM)-containing physiological solution. Graph and error bars represent mean and S.E.M. of results obtained from four cells for nicotine and three cells for MLA. Membrane potential, −60 mV.

human α9 nAChRs are not blocked by MLA at concentrations of 100 nM (Besnard et al., 1999). The fast-decaying BEC currents were activated by nicotine, blocked irreversibly by α-BTX, and blocked by 1 nM MLA, and were therefore unlikely to have been α9 nAChRs. The absence of noticeable α9-nAChR mediated currents might be related to lack of expression of the α10 subunit in cultured BEC and AEC. The α10 subunit, which is highly homologous to the α9 subunit, may form heteromeric α9α10 nAChRs (Elgoyhen et al., 2001), which gate much more robust ACh-induced currents than those gated by homomeric α9 nAChRs (Oliver et al., 2001). Homomeric α9 nAChRs expressed in the BEC may have yielded whole-cell currents below the level of detectability of the experimental conditions we used. Other tissues transcribe the α9, but not the α10 gene (Elgoyhen et al., 2001).

Without a prolonged exposure to nicotine, only 4% of BEC expressed levels of α7 nAChR detectable by whole-cell recording. Some BEC may have expressed α7 nAChRs even when we could not record choline-evoked, fast-decaying currents.

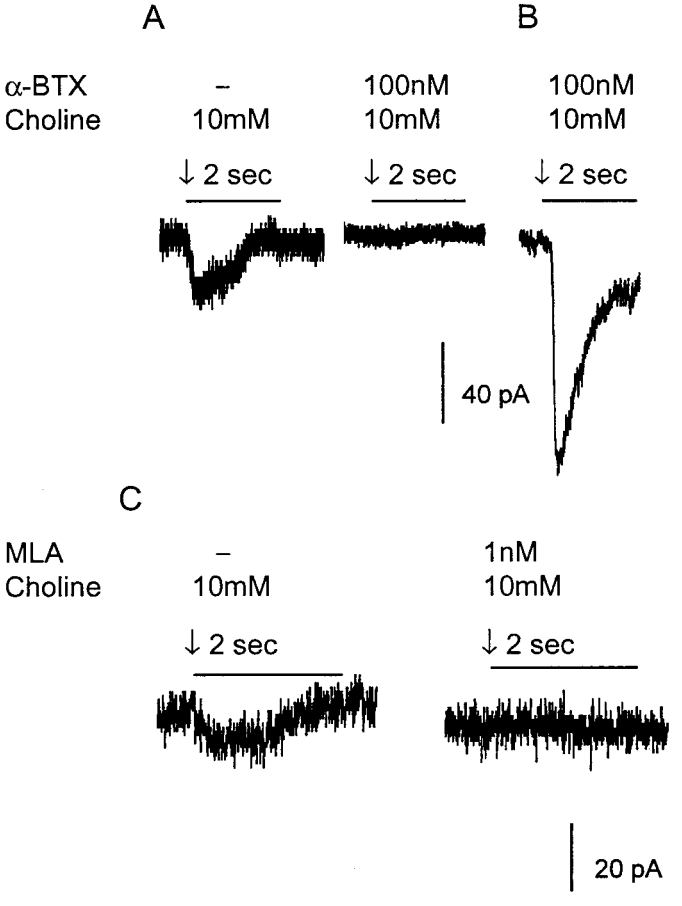

Fig. 6. Nicotinic responses recorded from human BEC in cultures treated with nicotine. The currents were induced with pulses of 10 mM choline, which lasted 2 s for the experiments reported in A and B and 1 s for the experiment reported in C. A, sample recordings of choline-evoked currents recorded from 4 of 17 BEC sampled randomly from cultures that were treated with 1 μM nicotine for 3 days. The currents evoked by 2-s choline pulses and that decayed to the baseline within the agonist pulse were blocked irreversibly after 20-min perfusion with external solution containing α-BTX (100 nM). B, in the continuous presence of α-BTX, slowly decaying currents evoked by 2 s pulses of choline could be recorded from 5 of the 17 BEC sampled randomly from nicotine-treated cultures. C, the fast-decaying current evoked by choline (in this experiment, using a 1-s pulse) was blocked by 1 nM MLA. Membrane potential, −60 mV. See *Materials and Methods* for experimental details.

The ability of α7 nAChRs to respond to choline and to desensitize quickly could have contributed to the scarcity of detectable functional α7 nAChRs in human BEC: the ACh and choline produced by the BEC may stimulate and desensitize the α7 nAChRs. Furthermore, the BEC extracellular matrix may impede removal of ACh and choline from the BEC surface, and facilitate α7 nAChR desensitization. This possibility is supported by the finding that choline-evoked currents with the pharmacological profile expected for α7 nAChR-mediated responses had rising and decay phases slower than those of α7 nAChR-mediated currents in cultured hippocampal neurons (Alkondon and Albuquerque, 1995): slow agonist diffusion in the BEC extracellular matrix could account for the slower kinetics of activation and inactivation of their nicotinic currents. BEC express scarce numbers of ^{125}I-α-BTX binding sites, and we might have missed the responses of cells where the agonist reached only a fraction of their α7 nAChRs.

Prolonged exposure to nicotine increased the number of BEC that showed nicotinic responses, both α-BTX-sensitive (4–23.5% of the cells sampled) and α-BTX-insensitive (8–29% of the cells sampled). This indicates that nicotine increases the number of functional nAChRs/cell to levels that allowed easier detection of their responses. However, it should not be considered a measure of the increase in nAChRs, which may be lower; chronic exposure of neurons to nicotine caused small increases of α7 nAChRs (Marks et al., 1985; Peng et al., 1997). Prolonged exposure to nicotine increased the number of hippocampal neurons expressing functional $\alpha4\beta2$ nAChRs (Almeida et al., 2000), and the numbers of α4, α3, and α7 nAChRs in brain, and in neuronal or nAChR-transfected cell lines (Flores et al., 1992; Peng et al., 1997; Wang et al., 1998).

BEC and AEC contained ChAT and AChE, in amounts (Table 1) comparable with those detected in cultured human keratinocytes (Grando et al., 1993). ACh and ChAT have been shown in the epithelia of human and rat trachea and bronchi and in vascular endothelial cells (Klapproth et al., 1997). ACh secreted by BEC and AEC, or its metabolite choline, are probably physiologic ligands for the α7 nAChRs. Still, choline availability may not require secretion and degradation of ACh, because it may depend instead on blood levels and transporter activity. We did not detect ACh in the cell culture medium, using a mass spectrometry assay (not shown). However, this does not exclude that BEC and AEC secreted amounts of ACh below the detection limit of the assay we used (~30 pmol of secreted ACh/10^6 cells).

The finding that in addition to BEC and AEC, other tegumental cells (Conti-Fine et al., 2000) have all the components needed for nicotinic cholinergic signaling suggests that by acting on specific nAChRs, ACh and choline might function as local "cytotransmitters" and modulate cellular functions. The α7 nAChR are good candidates as mediators of long-lasting, "hormonal" functions of ACh, because they can be activated by choline long after ACh cleavage by AChE and they are very permeable to Ca^{2+} (Seguela et al., 1993; Albuquerque et al., 1997); changes in intracellular Ca^{2+} may have a variety of metabolic effects.

A previous study has found α7 transcripts and α-BTX binding sites in the cells surrounding the large airways and the blood vessels and other cell types in monkey fetal lungs (Sekhon et al., 1999); consistent with the up-regulation by nicotine of choline-evoked currents reported here, nicotine administration to the mother increased expression of α7 subunit and binding of α-BTX in fetal lung. Also, small-cell lung carcinomas express α-BTX binding sites and α7 nAChRs, which might modulate cell growth (Chini et al., 1992; Quik et al., 1994; Codignola et al., 1996).

The presence of α7 nAChRs in BEC and AEC suggests that some of the deleterious effects of smoking on the bronchi and the blood vessels may be mediated by activation of the nicotine-sensitive α7 nAChRs.

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Correction to "Human Bronchial Epithelial and Endothelial Cells Express $\alpha 7$ Nicotinic Acetylcholine Receptors"

In the above article [Wang Y, Pereira EFR, Maus ADJ, Ostlie NS, Navaneetham D, Lei S, Albuquerque EX, and Conti-Fine BM (2001) *Mol Pharmacol* **60:**1201–1209], Figure 2, A and B, are reproduced from photographs obtained in early experiments that were published previously in the proceedings of the meeting "Neuronal Nicotinic Receptors" in a special issue of the *European Journal of Pharmacology* [Conti-Fine BM, Navaneetham D, Lei S, and Maus ADJ (2000) Neuronal nicotinic receptors in non-neuronal cells: new mediators of tobacco toxicity? *Eur J Pharmacol* **393:**279–294]. The authors have acknowledged that the same photographs were used inadvertently. They are, however, representative of micrographs obtained from other in situ hybridization experiments performed more recently in the authors' laboratory.

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