Human and Rodent Bronchial Epithelial Cells Express Functional Nicotinic Acetylcholine Receptors

ARNO D. J. MAUS, EDNA F. R. PEREIRA, PETER I. KARACHUNSKI, ROBERT M. HORTON, DURAISWAMY NAVANEETHAM, KEVIN MACKLIN, WELLINGTON S. CORTES,[1] EDSON X. ALBUQUERQUE, and BIANCA M. CONTI-FINE[2]

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108 (A.D.J.M., P.I.K., R.M.H., D.N., K.M., B.M.C.-F.), Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455 (B.M.C.-F.), Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201 (E.F.R.P., W.S.C., E.X.A.), and Institute of Biophysics, Carlos Chagas Filho, and Department of Basic and Clinical Pharmacology, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21944, Brazil (E.X.A.)

ABSTRACT

We demonstrated previously that human skin keratinocytes express acetylcholine receptors (AChRs) sensitive to acetylcholine and nicotine, which regulate cell adhesion and motility. We demonstrate here that human and rodent bronchial epithelial cells (BECs) express AChRs similar to those expressed by keratinocytes and by some neurons. Patch-clamp experiments demonstrated that the BEC AChRs are functional, and they are activated by acetylcholine and nicotine. They are blocked by κ-bungarotoxin, a specific antagonist of the AChR isotypes expressed by neurons in ganglia. Their ion-gating properties are consistent with those of AChR isotypes expressed in ganglia, formed by α3, α5, and β2 or β4 subunits. Reverse transcription-polymerase chain reaction and *in situ* hybridization experiments demonstrated the presence in BECs of mRNA transcripts for all those AChR subunits, both in cell cultures and in tissue sections, whereas we could not detect transcripts for the α2, α4, α6, and β3 AChR subunits. The expression of α3 and α5 proteins in BEC *in vivo* was verified by the binding of subunit-specific antibodies to sections of trachea. Mecamylamine and κ-bungarotoxin, which are cholinergic antagonists able to block the ganglionic α3 AChRs, caused a reversible change of the cell shape of cultured, confluent human BECs. This resulted in a reduction of the area covered by the cell and in cell/cell detachment. The presence of AChRs sensitive to nicotine on the lining of the airways raises the possibility that the high concentrations of nicotine resulting from tobacco smoking will cause an abnormal activation, a desensitization, or both of the bronchial AChRs. This may mediate or facilitate some of the toxic effects of cigarette smoking in the respiratory system.

The use of tobacco is so widespread and the health hazards deriving from it are so substantial that it has been referred to as the "global tobacco epidemic" (Bartecchi, 1995). Chronic bronchitis, emphysema, and lung cancer occur frequently in tobacco smokers. They result from the direct effect of tobacco smoke, the components it contains, or both (Peto *et al.*, 1992; Bartecchi *et al.*, 1995; The Harvard Mental Health Letter, 1997). In the United States, the use of tobacco is responsible for one in every seven deaths. Among people 35 to 70 years old, cardiopulmonary diseases related to tobacco smoking account for one in every three deaths (Peto *et al.*, 1992; The Harvard Mental Health Letter, 1997).

Nic, although addictive and likely responsible for the sub-stance dependence resulting from tobacco use (Bock and Marsh, 1990), is considered to be one of the less dangerous components of tobacco smoke (Bartecchi *et al.*, 1995; The Harvard Mental Health Letter, 1997). It has been suggested that the amount of Nic to which one is exposed as a result of tobacco smoking may not pose a serious health risk (The Harvard Mental Health Letter, 1997). Low tar cigarettes have been considered an acceptable solution to satisfy the smoker's craving for Nic. Devices for aerosol delivery of Nic without tar-related carcinogens are actively developed and tested as safe alternatives to tobacco smoking. Nic is highly soluble in water, 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.

Nic binds to and activates the nicotinic receptors for ACh. These are a family of proteins formed by five homologous or identical subunits, arranged symmetrically around a central

ABBREVIATIONS: Nic, nicotine; ACh, acetylcholine; AChR, nicotinic acetylcholine receptor; AnTX, (+)-anatoxin-a; BEC, bronchial epithelial cell; Mec, mecamylamine; RT, reverse transcription; PCR, polymerase chain reaction; α-BTX, α- bungarotoxin; κ-BTX, κ-bungarotoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline.

ion channel (Conti-Fine *et al.*, 1994; Galzi and Changeux, 1995). Different AChR isotypes exist in muscle and neurons. Muscle AChRs are composed of four different types of subunit (α, β, γ or ϵ, and δ) (Conti-Fine *et al.*, 1994; Galzi and Changeux, 1995). Neuronal AChRs may include only two types of subunits (α and β) or five copies of the same α subunit (Conti-Fine *et al.*, 1994; Galzi and Changeux, 1995). Neurons express at least eight different α subunits ($\alpha 2$–9) and three β subunits ($\beta 2$–4) (Conti-Fine *et al.*, 1994; Galzi and Changeux, 1995). A large variety of neuronal AChRs results from the combinatorial association of different α and β subunits (Conti-Fine *et al.*, 1994; Galzi and Changeux, 1995).

Human skin keratinocytes express AChRs sensitive to ACh and Nic, similar to those expressed by ganglionic neurons, that compose the $\alpha 3$ subunit (Grando *et al.*, 1995). Skin keratinocyte AChRs are likely activated in an autocrine or a paracrine fashion by ACh synthesized and secreted by the keratinocytes (Grando *et al.*, 1993). They seem to regulate cell adhesion and motility because their block by ganglionic AChR-specific antagonists, such as Mec and κ-BTX, causes cell paralysis and cell/cell detachment (Grando *et al.*, 1995). Because of those findings and because of reports that ACh is synthesized and released by the bronchial epithelium (Wessler *et al.*, 1995; Klapproth *et al.* 1997), we investigated whether the epithelial cells that line the surface of the bronchial tree express functional AChRs sensitive to Nic.

Materials and Methods

Cell cultures. Primary cultures of human BECs (Clonetics/Bio-Whittaker, San Diego, CA) were seeded in T-25 culture flasks (Corning Glassworks, New York, NY) [10,000 cells/ml in 10 ml of culture medium (Clonetics/BioWhittaker)]. When the cells reached 80–90% confluence, they were detached from the plastic by mild trypsinization using 0.25% trypsin EDTA (Clonetics/BioWhittaker), according to the manufacturer's instruction. They were used for the [^{3}H]epibatidine binding assay, or plated onto glass coverslips in 24-well plates (Corning Glass works) for other experimental uses, or reseeded in T-25 culture flasks for further expansion. For plating the cells onto glass, coverslips (Fisherbrand Microscope Cover Glass, 12-mm circle; Fisher Scientific, Pittsburg, PA) were wiped with 70% ethanol and set in the well until dry. A small drop of medium containing 500–800 cells was placed in the center of the coverslip, and 1 ml of medium was slowly delivered to the well. The cultures were grown until they reached confluence.

Patch-clamp recording of single-channel and whole-cell currents. Currents were recorded from confluent cultured human BECs using standard patch-clamp techniques (Hamill *et al.*, 1981) and an LM-EPC-7 patch-clamp system (List Electronic, Darmstadt, Germany). Single-channel currents were recorded from either outside-out or cell-attached patches (Hamill *et al.*, 1981). The resistance of the recording pipettes was 6–8 MΩ. The test solutions to be applied to the cell-attached patches were placed in the recording pipette. For outside-out patches, we applied the test solutions with a perfusion system consisting of an array of glass capillary tubes. The 10-kHz signal output from the EPC-7 apparatus was transferred to a video cassette recorder using a pulse-code modulation device (PCM: Neurodata Neurorecorder DR-384, Neuro Data Institute, Pasadena, CA) for off-line analysis. The electrical signal was filtered at 3 kHz in a Bessel filter (eight-pole, -3 dB, Frequency Devices 902; Frequency Devices, Haverville, MA), digitized at 12.5 kHz, and analyzed with the IPROC-2 program (Axon Instruments, Foster City, CA). Open events were considered finished when the amplitude decreased to below 50% of the estimated mean single-channel amplitude. We obtained the time constants by fitting an exponential equation to

histograms of the channel dwell times using the NFITS program (Axon Instruments).

Whole-cell currents were elicited by agonists applied onto the cells with a U-shaped glass tube (Alkondon and Albuquerque, 1993). The resistance of the recording pipettes was 2–4 MΩ. The cells were perfused at a rate of 1.5–3 ml/min with the bathing solution. Capacitance transients were minimized, and the series resistance was not compensated. Whenever changes in series resistance occurred, that experiment was discarded. We used the pCLAMP program (Axon Instruments) to analyze the currents on-line.

The composition of the external solution used to bathe the cells and to dilute agonists, antagonists, and test compounds was 165 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 5 mM HEPES, and 10 mM dextrose (pH 7.3; osmolarity 340 mOsm). The composition of the internal solution used for outside-out and whole-cell patches was 80 mM CsCl, 80 mM CsF, 10 mM EGTA, and 10 mM HEPES (pH 7.3; osmolarity 330 mOsm).

Assay of neuronal-type AChRs by binding of ^{3}H-labeled epibatidine. We verified the presence of neuronal-type AChR on confluent cultured human BECs by studying the binding of ^{3}H-labeled epibatidine. Epibatidine is a specific, high affinity ligand of several neuronal AChRs, including the $\alpha 3$ AChR subtypes (Gerzanich *et al.*, 1995; Wang *et al.*, 1996). We used the neuronal PC12 cell line, which expresses $\alpha 3$ neuronal AChR (Conti-Fine *et al.*, 1994), to identify the saturating concentration of epibatidine in our experimental system. For Scatchard analysis, samples containing 0.5–1 $\times$ 10^6 PC12 cells were incubated with increasing concentrations of [^{3}H]epibatidine (NEN, Boston, MA; 0.1–10 nM; specific activity 48 Ci/nmol). For BECs, because of the small number of cells that we could grow, we used single-dose [^{3}H]epibatidine binding assays. In this assay, we used suspensions of trypsinized human BECs (3×10^5 to 2×10^6 cells/sample in a final volume of 100 μl). For all and each condition, we set up samples at least in triplicate. The cells were incubated with 5–10 nM [^{3}H]epibatidine for 4 hr at 4° and harvested by vacuum filtration over Whatman GF/C filters or by centrifugation at $300 \times g$ for 3 min. The filters were counted by liquid scintillation counting. In each experiment, we determined the nonspecific binding by preincubating the cells with 10 μM nonradiolabeled epibatidine for 30 min at 4° before the addition of [^{3}H]epibatidine.

Detection of neuronal AChR subunits by RT-PCR assay. The results of the patch-clamp and [^{3}H]epibatidine binding experiments suggested the presence on BECs of AChRs of isotype or isotypes similar to those expressed by the neurons in sympathetic ganglia, formed by $\alpha 3$, $\alpha 5$, $\beta 2$, and $\beta 4$ subunits (Vernallis *et al.*, 1993; Conti-Fine *et al.*, 1994; Galzi and Changeux, 1995; Wang *et al.*, 1996). We investigated the presence of mRNA transcripts for AChR subunits by RT-PCR experiments using mRNA isolated from human BEC cultures and primers specific for the $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 2$, $\beta 3$, and $\beta 4$ AChR subunits and for actin as a positive control. As a positive control tissue, we used mRNA isolated from human brain (a generous gift of Dr. James Howard, University of North Carolina at Chapel Hill) or commercially available human brain cDNA (Quick Clone cDNA, Clontech Laboratories, Palo Alto, CA). As a negative control tissue, we used mRNA isolated from adult human muscle (a generous gift of Dr. James Howard). Adult muscle does not expresses neuronal AChRs but instead expresses the muscle AChR isotype, which includes the $\alpha 1$ subunit subtype and the homologous β, ϵ, and δ subunits. In the experiments that used muscle mRNA, we used primers specific for the $\alpha 3$, $\alpha 5$, $\beta 2$, and $\beta 4$ AChR subunits and for the $\alpha 1$ subunit as a positive control.

RNA was extracted using RNAzol (Tel-Test B, Friendswood, TX) and reverse transcribed using Superscript RNase H$^-$ Reverse transcriptase (GIBCO BRL, Gaithersburg, MD). Hot-start PCR (Horton *et al.*, 1994) was run for 35–36 cycles (for the BEC cDNA and brain cDNA) and for 40 cycles (for the muscle cDNA) at 95° for 15 sec, 55° (except for the $\beta 4$ primers, which were annealed at 52°) for 15 sec, and 72° for 45 sec plus 1 sec per cycle increase in a Perkin-Elmer Cetus (Norwalk, CT) 9600 thermal cycler. RT-PCR products were electophoresed on a 1% agarose/0.5% Synergel (Diversified Biotech,

Boston, MA)/0.5 × Tris/borate/EDTA gel containing 0.5 mg/ml ethidium bromide. A 123-bp DNA ladder (GIBCO BRL) was used as molecular weight standard.

Primers were designed (Horton *et al.*, 1996a) to amplify specific members of the AChR gene family using GenBank sequences. Their sequences and expected product size (in parentheses) were for actin, 5′-GCTCCGGCATGTGCAA-3′ and 5′-AGGATCTTCATGAGG-TAGT-3′ (542 bp); for α1, 5′-CGTCTGGTGGCAAAGCT-3′ and 5′-CCGCTCTCCATGAAGTT-3′ [580 and 505 bp for products that do or do not include the sequence encoded by exon P3A (Beeson *et al.*, 1990)]; for α2, 5′-CCGGTGGCTTCTGATGA-3′ and 5′-CAGATCAT-TCCAGCTAGG-3′ (466 bp); for α3, 5′-CCATGTCTCAGCTGGTG-3′ and 5′-GTCCTTGAGGTTCATGGA-3′ (401 bp); for α4, 5′-TGGGT-GAAGCAGGAGAGTGG-3′ and 5′-AGTCCAGCTGGTCCACG-3′ (346 bp); for α5, 5′-GATAATGCAGATGGACGT-3′ and 5′-TGATGG-TATGATCTCTTC-3′ (525 bp); for α6, 5′-GGCCTCTGGACAAGA-CAA -3′ and 5′-AAGATTTTCCTGTGTTCCC-3′ (413 bp); for β2,5′-CAGCTCATCAGTGTGCA-3′ and 5′-GTGCGGTCGTAGGTCCA-3′ (347 bp); for β3, 5′-AGAGGCTCTTTCTGCAGA-3′ and 5′-GCCA-CATCTTCAAAGCAG-3′ (354 bp); and for β4 5′-CTGAAACAG-GAATGGACT-3′ and 5′-CCATGTCTATCTCCGTGT-3′ (310 bp). The primers yielded products of the expected size when human brain cDNA (or, for the α1 subunit, human muscle cDNA) was used. We used non-reverse-transcribed RNA or mock cDNA (an empty tube subjected to the same reactions as the brain and BEC samples) as negative controls for template contamination at every step of the procedure.

Cloning and sequencing of RT-PCR products. First-strand cDNA was synthesized from human BEC RNA (Grando *et al.*, 1995). Primers (5′-CCAGTGGCCAGGGCCTCCAGA-3′ and 5′-TATG-CATCTTCCCTGGCCATCA-3′) were designed to amplify the full-sequence region encoding the mature α3 subunit protein. Hot-start PCR (Horton *et al.*, 1994) was used in 10 separate 100-µl reactions containing 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 µM concentration each dNTP, 12% sucrose, 250 nM concentration each primer, 1 µl of cDNA template, and ~2 units of *Taq* DNA polymerase. The reaction yielded a prominent band that was excised and reamplified using histidine tag-encoding T-linkers (Horton *et al.*, 1996b). The reamplified product was cloned directionally into the plasmid pT7–7 using *Nde*I and *Hin*dIII restriction sites in the linkers and sequenced using the fmol sequencing kit (Promega, Madison, WI).

Assay of AChR subunit transcripts by *in situ* hybridization. We carried out *in situ* hybridization experiments (Cox *et al.*, 1984) using cultured human BECs and sections of rat trachea and probes specific for each of the AChR subunits detected by the RT-PCR experiments. The probes were transcribed *in vitro* from DNA clones (a generous gift of Dr. C. Lobron, University of Mainz, Mainz, Germany) and labeled with digoxygenin-UTP (Boehringer-Mannheim, Mannheim, Germany). The labeled single-stranded probes were hybridized to mRNA of the cell under conditions of high stringency of the hybridization. The conditions we used allowed the probes to bind only to their corresponding mRNA (Cox *et al.*, 1984). To detect the bound probe, anti-digoxygenin antibody coupled to alkaline phosphatase (Boehringer-Mannheim) was added. The NBT/BCIP mixture (Boehringer-Mannheim) was added as a substrate for alkaline phosphatase. The specificity of the binding of the probe is demonstrated by absence of the signal when the corresponding "sense" probe is used.

Detection of AChR subunit proteins in tissue sections by immunofluorescence. AChR subunit-specific antisera were obtained by immunizing individual rabbits with a mixture of peptides corresponding to unique sequence regions of a given AChR subunit (Bellone *et al.*, 1993). The peptides corresponded to nonconserved sequence regions of the α1, α3, α4, and α5 subunits: α1: residues 23–41, 154–172, 322–341, 343–362, and 423–437; α3: residues 23–41, 153–172, 319–338, 379–398, and 459–472; α4: residues 338–357, 391–410, 411–430, 431–45, 451–470, 471–490, and 491–510; and α5: residues 31–50 and 151–170. The peptides were coupled to keyhole limpet hemocyanin (0.5 mg/mg peptide) by reaction with a 5-molar excess of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide for 24 hr at room temperature, in a mixture of 1.5 volumes of 50 mM Na phosphate, 50 mM NaCl, 1 volume of water, and as much ethylene glycol as needed to keep the peptide in solution. After coupling, the mixture was dialyzed extensively against PBS (10 mM Na phosphate buffer, pH 7.4 containing 137 mM NaCl, and 2.7 mM KCl). Rabbits were injected several times with 0.5 mg of conjugate emulsified in complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for the subsequent injections. The presence of anti-peptide antibodies in the sera was verified by enzyme-linked immunosorbent assay (Lei *et al.*, 1993).

Fresh pieces of rat trachea were embedded with O.C.T. Compound Tissue-TEK (Miles Laboratories, Torrance, CA), frozen in liquid nitrogen, and stored at −70°. The frozen tissue was cryosectioned into 10-µm sections in the transverse direction using a Jung Frigocut 2800E Kryostat (Leica, Deerfield, IL). The sections were preincubated in PBS for 10 min, stained for 1 hr at room temperature with rabbit anti-AChR subunit antisera, washed, and incubated for 1 hr with fluorescein isothiocyanate-labeled anti-rabbit IgG (Sigma Chemical, St. Louis, MO), diluted 1:100 in 2% of bovine serum albumin-PBS. The sections were washed and viewed in a fluorescence microscope (Nikon Diaphot, Melville, NY). To prove the specificity of the staining, the antisera were preincubated with the AChR subunit peptide sequences used for immunization.

We tested the specificity of the antisera by investigating their binding to sections of rat muscle, which expresses the muscle AChR isotype. We identified the AChR-containing neuromuscular junctions by the binding of fluorescence labeled α-BTX. We processed fresh pieces of adult rat muscle, obtained sections, and stained them with the anti-α3 or anti-α5 antisera and with Texas Red-labeled α-BTX (Molecular Probes, Eugene, OR) as we described for the trachea.

Assay of the effects of cholinergic antagonists on the cell shape and cell/cell adhesion. We tested the effects of Mec (from 100 µM to 5 mM) and κ-BTX (~10 nM and ~1 µM) on cell shape and cell/cell adhesion in confluent human BEC cultures. In each experiment, we measured the area covered by three to five randomly chosen individual cells just before administering the drugs and at different intervals up to 20 min. We also measured the area covered by three to five randomly chosen individual control cells that received drug-free medium, at different intervals up to 20 min. Phase-contrast microscopic images were captured on a Macintosh Quadra 650 computer equipped with a LG3 NuBus frame grabber card (Scion Corporation, Fredrick, MD). Frame capture at fixed intervals was automated using the "Make movie to disk" macro of NIH Image (a public domain software available on the Internet at *http://rsb.info. nih.gov/nih-image/*; the macro is accessed under "Special, Load Macros, Movie Making"). Cell areas (in pixels) were determined by selecting "Area" under "Analyze, Options," tracing the outlines of the cells with the Freehand Selection Tool, and then selecting "Analyze, Measure." The results were analyzed with Microsoft Excel (Redmond, WA).

Results

Patch-clamp recording of single-channel and whole-cell currents induced by Nic in cultured human BECs. Nicotinic agonists applied to outside-out, cell-attached, and whole-cell patches from human BECs evoked nicotinic currents, indicating that BECs express functional AChRs. Application of the agonist AnTX (1 µM) to outside-out patches excised from three of 15 BECs elicited single-channel currents whose mean amplitude was ~1 pA at −80 mV (Fig. 1, *top*). Their open times were fitted by a double-exponential function. The short-lived channels had a mean open time of ~0.2 msec (τ_{fast}), and the long-lived channels had a mean open time of ~4.8 msec (τ_{slow}). The reversal potential for these nicotinic currents was close to 0

mV; thus, the conductance of the AChR channels expressed by the BECs is ~12 pS.

Application of nicotinic agonists to cell-attached patches allowed analysis of the nicotinic responses of the BECs with the intracellular contents intact. When the recording pipettes were filled with agonist-free external solution, no channel activity was detected in cell-attached patches on the BECs. We examined a total of 116 cells. All patches were silent when exposed to agonist-free external solution. When the pipettes were filled with external solutions containing either ACh (1 μM; in the presence of 1 μM atropine) or (−)-Nic (100 nM), we recorded single-channel currents from cell-attached patches in 10 of 116 cells. The frequency of channel activity induced by application of the nicotinic agonists to the cell-attached patches and the open times of the channels increased with hyperpolarization of the membrane (Fig. 1, *bottom*).

The frequency of single-channel activity elicited by nicotinic agonists was low in both outside-out and cell-attached patches. In only a few instances did we detect a high frequency of nicotinic single-channel activity. Consistent with those results, we could record whole-cell nicotinic currents from only seven of 31 patches tested. The BECs responded to ACh (1 μM and 1 mM) or Nic (0.1, 1, 10, and 100 μM) with slowly decaying whole-cell currents whose amplitude increased with the agonist concentrations (Fig. 2, A and B). The Nic-evoked currents were blocked by preincubation of the BECs for 15 min with 10 nM κ-BTX (Fig. 2C). The amplitude of the currents evoked by a saturating concentration of ACh was ~40 pA at −80 mV. We tested the responses to increasing concentrations of Nic from four BECs. Two cells responded to the highest concentration of Nic we used (100 μM) with currents that had very small amplitude (~15 pA at −100 mV). The other two BECs had much larger responses to 100 μM Nic (up to ~300 pA at −100 mV) (Fig. 2, B and C).

Assay of neuronal-type AChRs by binding of ³H-labeled epibatidine. Scatchard analysis of [³H]epibatidine binding to PC12 cells detected two populations of binding sites. Their K_d values were 70 pM (B_{max} = ~800 sites/cell) and 720 pM (B_{max} = ~3700 sites/cell) respectively, which are in the range of those described for [³H]epibatidine binding to neurons (Wang *et al.* 1996). We carried out [³H]epibatidine binding experiments with cultured confluent human BECs using a concentration of [³H]epibatidine (10 nM) that, based on the experiments with PC12 cells and pilot experiments with human BECs using a few increasing concentrations of [³H]epibatidine, we expected to be saturating. In four independent experiments carried out with different batches of [³H]epibatidine and of BECs, we found 500, 630, 1600, and 7800 binding sites/cell, respectively. Fig. 3 reports the results of a representative experiment.
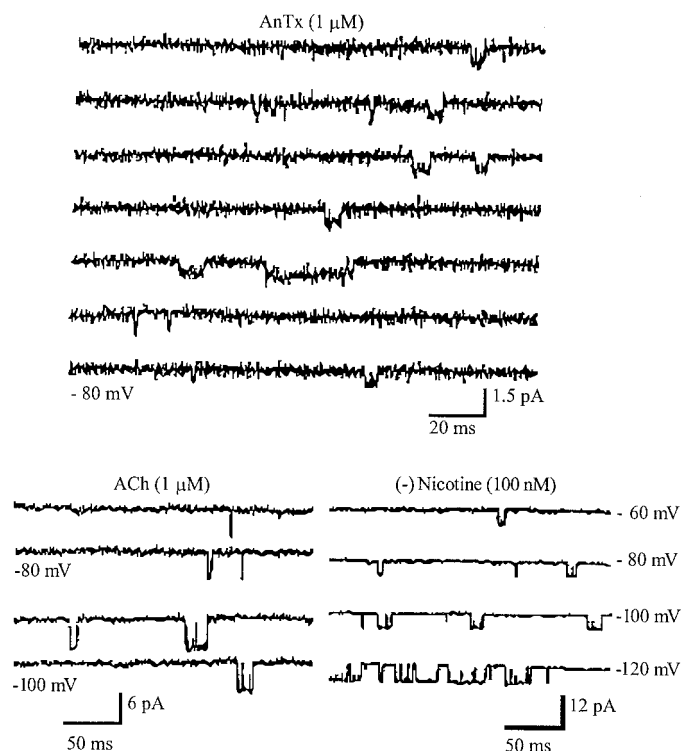

Fig. 1. Functional characterization by patch-clamp of the AChRs expressed by cultured human BECs: single-channel currents. *Top*, samples of AnTX-induced single-channel currents recorded from excised outside-out patches. *Bottom*, *left*, samples of ACh-induced single-channel currents recorded from a cell-attached patch. *Right*, samples of (−)-Nic-induced single-channel currents recorded from a cell-attached patch. The pipette potentials at which the currents were recorded is indicated next to the corresponding trace.
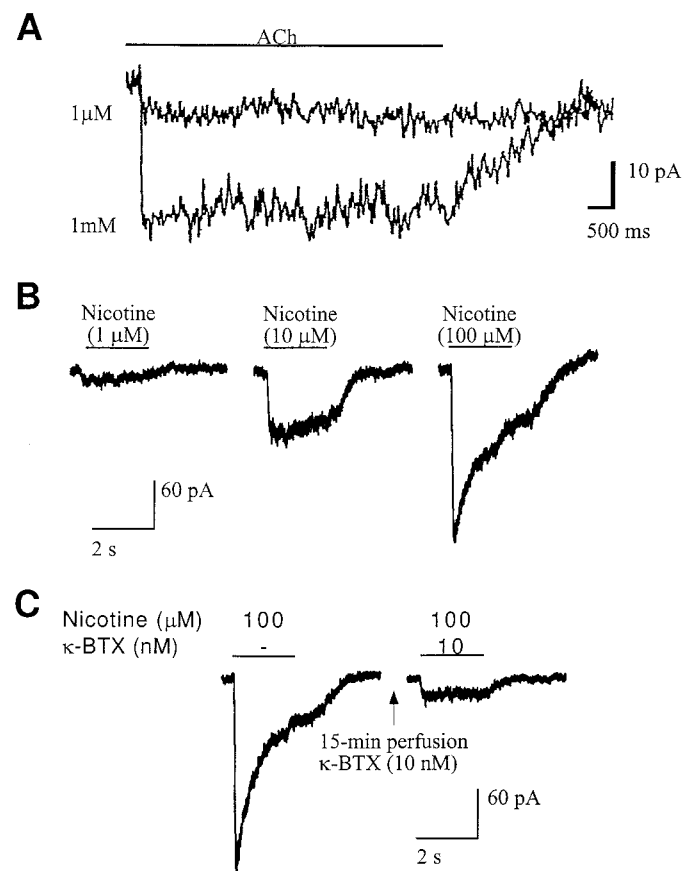

Fig. 2. Functional characterization by patch-clamp of the AChRs expressed by cultured human BECs: whole-cell currents and effect of κ-BTX. A, Samples of whole-cell currents evoked by 2-sec pulse application of ACh (1 μM and 1 mM) to a BEC held at −80 mV. B, Samples of whole-cell currents evoked by 2-sec pulse application of Nic (1, 10, and 100 μM) to a BEC held at −100 mV. C, Effect of κ-BTX on the nicotinic responses recorded from BECs. After recording the whole-cell current evoked by Nic (100 μM), a BEC was perfused for 15 min with external solution containing 10 nM κ-BTX. After exposure to κ-BTX, the sensitivity of the cell to Nic was tested again. More than 90% of the response was blocked by κ-BTX. Holding potential, −100 mV.

Detection of neuronal AChR subunit transcripts in cultured human BECs by RT-PCR: Verification of the identity of the α3 transcript. We used RT-PCR to investigate the presence of mRNA transcripts for AChR subunits in mRNA isolated from human BEC cultures. We used primers specific for the α2, α3, α4, α5, α6, β2, β3, and β4 AChR subunits. Fig. 4A reports the results of one of several consistent experiments. The primers for the α3, α5, β2, and β4 subunits yielded products of expected size. The β2 primers yielded a second product of higher molecular weight than expected, for both BEC and brain cDNA. This second band was especially prominent when we used BEC cDNA. We do not have any explanation at this point regarding the nature of the high molecular weight product obtained with the β2 primers. The α2, α4, α6, and β3 primers and the negative controls never yielded PCR products. When we used adult muscle cDNA, the α3, α5, β2, and β4 subunits did not yield detectable products even after 40 PCR cycles, whereas the α1 primers yielded two products of the expected size (Fig. 4B). The presence of two PCR products is due to the presence in adult human muscle of two isoforms of the α subunit. One of those isoforms includes a 75-bp sequence, encoded by an exon termed P3A, which is spliced out in the second isoform (Beeson *et al.*, 1990).

We cloned the entire region encoding the mature protein from a RT-PCR product obtained from human BEC cDNA using α3-specific primers. The clone had the size expected for the α3 subunit (1493 bp). Its partial sequencing yielded human α3 subunit sequences.

Detection of neuronal AChR subunit transcripts in cultured human BECs and in rat trachea by *in situ* hybridization. We wanted to verify that the subunit transcripts detected in the cell cultures were expressed *in vivo*. Toward this goal, we carried out *in situ* hybridization experiments using both cultured human BECs and sections of rat trachea. We used probes specific for each of the AChR subunits detected by the RT-PCR experiments. In cell cultures and in the epithelial layer of trachea sections, all probes yielded a clear and specific signal, which was absent when we used the corresponding "sense" probe (Fig. 5).

Detection of AChR subunit proteins in the BECs *in vivo* by immunofluorescence. Because we could not use patch-clamp approaches to demonstrate the expression of AChRs in intact tissue, we studied the presence of AChR subunit proteins in sections of rat trachea by immunofluorescence, using antisera specific for unique sequence regions of the α3 and α5 subunits.

Both antisera bound to rat BECs in sections of trachea (Fig. 6A). The BECs were identified by double immunofluorescence staining using an anti-centrin antibody (Salisbury *et al.*, 1986). The antisera stained virtually all cells, predominantly on the apical side of the epithelial layer. The binding of the anti-α3 and anti-α5 antisera was specific because it could not be detected if the subunit-specific antibody was omitted or substituted for the antisera specific for the α1 or α4 subunits. Also, it was blocked by preincubation of the antisera with synthetic peptides corresponding to the AChR subunit sequences they recognized (Fig. 6A). The peptides did not affect the signal of the control anti-centrin antibody.

The peptide sequences we used as immunogens were selected because they are diverged in the different AChR subunits. However, given the homology of all the AChR subunit sequences, we cannot exclude that the sera might cross-react with other members of the AChR subunit family. Also, the use of denatured peptide sequences as immunogens might result in synthesis of antibody specific for structural features of the peptides other than the sequence of their side chains
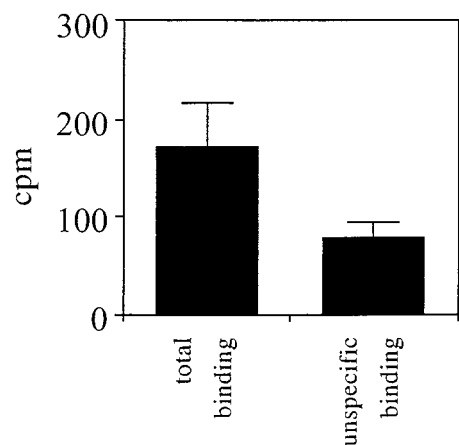

Fig. 3. Binding of [³H]epibatidine to cultured human BECs. Total and nonspecific binding of [³H]epibatidine to confluent BECs. We used replicates of seven samples, each containing 2×10^6 cells. The nonspecific binding was obtained by incubating the cultures with an excess of non-labeled epibatidine (10 μM) before the addition of 1 nM [³H]epibatidine. Preincubation with nonlabeled epibatidine significantly ($p < 0.0003$) reduced [³H]epibatidine binding. The specific binding, obtained by subtracting the nonspecific binding from the total binding, corresponded in this experiment to 630 binding sites/cell.
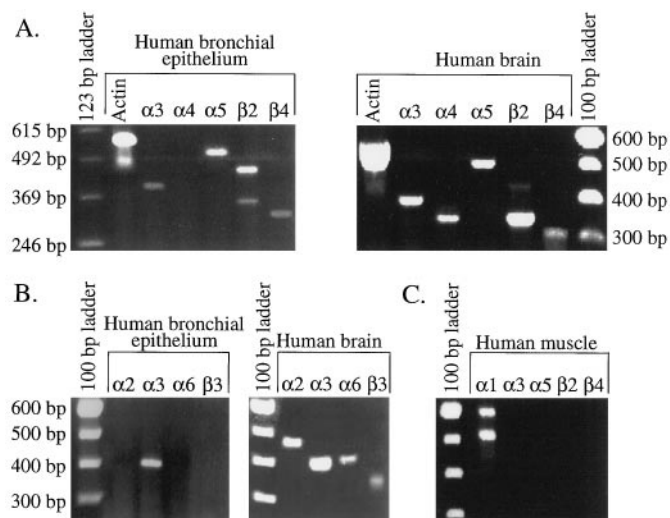

Fig. 4. Detection of α3, α5, β2, and β4 subunit transcripts in cultured human BECs by RT-PCR. A and B, RT-PCR experiments using cDNA from BECs, primer specific for the different neuronal AChR subunits, as indicated, and 35 PCR cycles. All primers yielded a PCR product of the expected size when cDNA from human brain was used. When human BEC cDNA was used, the primers for actin and for the α3, α5, β2, and β4 subunits yielded products of the expected size, whereas the primers for the α2, α4, α6, and β3 subunit did not. Primers for actin were used as a positive control for RT-PCR effectiveness. Mock cDNA did not yield any product band. C, RT-PCR experiments using cDNA from adult human muscle, primer specific for the different neuronal AChR subunits, as indicated, and 40 PCR cycles. Primers specific for the muscle α1 subunit were used as a positive control for RT-PCR effectiveness. The α3, α5, β2, and β4 subunit primers did not yield detectable products even after 40 cycles, whereas the α1 primers yielded two products of the expected size. They are due to the presence in adult human muscle of two α1 isoforms, one of which includes an additional 75-bp sequence.

(e.g., the peptide backbone or the amino or carboxyl termini) (Conti-Fine *et al.*, 1996). Such antibody might cross-react even outside the AChR subunit family. To test the specificity of the antisera we used, we tested their binding to section of adult rat muscle, which expresses at the neuromuscular junction a high concentration of the $\alpha1$ subunit, as well as the homologous β, ϵ, and δ subunit. We localized the synaptic junctions using the binding of fluorescence labeled α-BTX (Fig. 6B). Neither the $\alpha3$ nor the $\alpha5$ subunit-specific antisera stained the neuromuscular junctions (Fig. 6B).

Assay of the effect of cholinergic antagonists on the cell shape and cell/cell adhesion of cultured human BECs. We investigated whether the BEC AChRs modulate cell motility by testing the effects on confluent human BEC cultures of Mec (from 100 μM to 5 mM) and κ-BTX (10 nM and 1 μM). Mec and κ-BTX block ganglionic $\alpha3$ AChRs (Conti-Fine *et al.*, 1994; Galzi and Changeux, 1995). Figs. 7 and 8 report the results of representative experiments. Within 5 min after the application of either of those compounds, the BECs retracted their flat cytoplasmic flaps. Their cytoplasm became a thin layer around the nucleus, with reduction of the area covered by the cells, and the cells detached from each other. Both Mec and κ-BTX had this effect at all the concentrations used, although the extent and the rapidity of the effect increased with the concentration (Fig. 7). When we used 5 mM Mec or 1 μM κ-BTX, all cells responded to the drug (Fig. 7). Most cell responded to Mec even at the lowest concentrations we used, although the reduction in size was not as profound as that we observed for 5 mM Mec. When we used 10 nM κ-BTX, several cells, did not respond (Fig. 7).

We observed the cells for up to 20 min. During this time, the cell shrinkage observed using κ-BTX and 5 mM Mec was unchanged, whereas that induced by lower concentrations of Mec was reversible (Fig. 7). The effect of 5 mM Mec was also quickly reversible by washing (Figs. 7 and 8). Fig. 8 shows pictures of the same cells at different time intervals (as indicated inside each picture frame) after the administration of 5 mM Mec. The retraction of the cytoplasm around the nucleus increased during the first 5 min and then remained

constant. After replacing the medium containing Mec with normal medium, the cells extended their cytoplasm again.

Control cells that were not exposed to any drug had small, reversible fluctuations of their size, which did not exceed 20% (Fig. 7).

Discussion

Demonstration of functional AChRs in BECs. This study provides several lines of evidence suggesting that human and rodent BECs express functional AChRs similar to those expressed by some neurons, which can be activated by Nic. The presence of AChRs in BECs is demonstrated by the results of both structural and functional studies. First, the results of PCR and *in situ* hybridization experiments indicate that BECs, both in culture and *in vivo,* express mRNA encoding each of the subunits that contribute to AChRs of ganglionic type ($\alpha3$, $\alpha5$, $\beta2$, and $\beta4$ subunits), whereas they do not seem to express other constituent subunits of neuronal AChRs ($\alpha2$, $\alpha4$, $\alpha6$, and $\beta3$) (Figs. 4 and 5). Second, the binding of [^{3}H]epibatidine demonstrated the presence in cultured human BECs of a nicotinic cholinergic binding site (Fig. 3). Third, the binding of specific antibody to sections of trachea demonstrated that the $\alpha3$ and $\alpha5$ proteins are expressed on the BEC surface in the intact tissue (Fig. 6). Fourth, the patch-clamp experiments demonstrated that BECs express functional AChRs: they are activated by ACh and Nic, are blocked by κ-BTX, and have ion-gating properties similar to those of AChRs formed by $\alpha3$, $\alpha5$, and $\beta2$ or $\beta4$ subunits (Figs. 1 and 2).

That specific receptors for Nic might be present on the bronchial and nasal epithelia of mice was proposed in 1980 to explain the finding that a Nic derivative accumulated in those epithelia 24 hr after intravenous injection. This was attributed to receptor-mediated accumulation (Waddell and Marlowe, 1980). Another study that used anti-AChR antibodies also suggested the presence of $\alpha3$ and $\alpha5$ subunits in human bronchial epithelium (Zia *et al.*, 1997).

BEC AChRs are functionally similar to AChRs of ganglionic neurons. The ion-gating properties of the ACh- and Nic-activated ion channels measured in the patch-clamp experiments and their block by κ-BTX (Fig. 2B) are consistent with the properties of the AChR isotypes expressed by neurons of sympathetic ganglia (Figs. 1 and 2). Other properties of BEC AChRs are consistent with the functional characteristics of certain subtypes of neuronal AChRs (Conti-Fine *et al.*, 1994; Galzi and Changeux, 1995). First, the frequency of channel activity induced in BECs by application of the nicotinic agonists to the cell-attached patches increased with hyperpolarization (Fig. 1, *bottom*). Second, the open times of the BEC channels were prolonged by hyperpolarization of the membrane (Fig. 1, *bottom*).

Comparison of the ion-gating properties of the human BEC AChR channels with those of AChRs expressed in *Xenopus* oocytes (Conti-Fine *et al.*, 1994; Galzi and Changeux, 1995) verifies that the BEC AChRs should include $\alpha3$ and $\beta2$ subunits. Neuronal AChRs formed by the $\alpha3$ subunit generally include the $\alpha5$ and $\beta2$ or $\beta4$ subunits. All those subunits are consistently expressed in ganglionic neurons and in other neurons that express $\alpha3$ subunits (Vernallis *et al.*, 1993; Conti-Fine *et al.*, 1994; Wang *et al.*, 1996). The genes encoding the $\alpha3$, $\alpha5$, and $\beta4$ subunits are part of the same gene cluster in vertebrates, and they are expressed in highly restricted patterns (McDonough and Deneris, 1997, and references therein). In
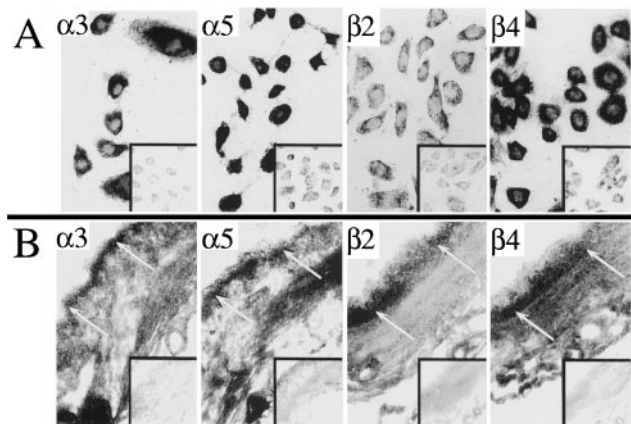

Fig. 5. Detection of $\alpha3$, $\alpha5$, $\beta2$, and $\beta4$ subunit transcripts in cultured human BECs and rat BECs in intact tissue by *in situ* hybridization. A, *In situ* hybridization experiments using cultured human BECs. The $\alpha3$, $\alpha5$, $\beta2$, and $\beta4$ subunit probes consistently yielded a specific strong signal. *Insets*, negative results obtained with the corresponding "sense" control probes. B, *In situ* hybridization experiments using rat trachea sections. The $\alpha3$, $\alpha5$, $\beta2$, and $\beta4$ subunit probes consistently yielded specific signals (*arrows*). Insets, negative results obtained with the "sense" control probes.

good agreement with the subunit composition found for the α3 AChRs physiologically expressed in neurons, the BEC AChRs also seem to include α3, α5, β2, and β4 subunits but not the α2, α4, α6, and β3 subunits (Figs. 4–6).

Numbers of AChRs expressed by BECs. We found excellent agreement between the conclusion of the structural and the patch-clamp studies regarding the likely subunit composition of the BEC AChRs. On the other hand, our attempts at measuring the number of AChRs expressed by cultured human BECs yielded variable results. [^{3}H]Epibatidine binding experiments yielded variable numbers of binding sites. The number of specific binding sites for [^{3}H]epibatidine is very small as compared with the nonspecific binding (Fig. 3). Thus, the [^{3}H]epibatidine binding assay is a qualitative verification of the presence of AChR on the BECs, rather than an accurate assessment of their number. Also, the data measuring whole-cell currents evoked by saturating concentrations of agonists in the electrophysiology experiments were variable and too scant to deduce a reliable estimate the number of functional AChR per cell. Still, the electrophysiology data suggest a lower number of receptors than those revealed by [^{3}H]epibatidine binding.

The electrophysiology experiments detected functional nicotinic AChR at low levels and in a few cells, whereas the *in*
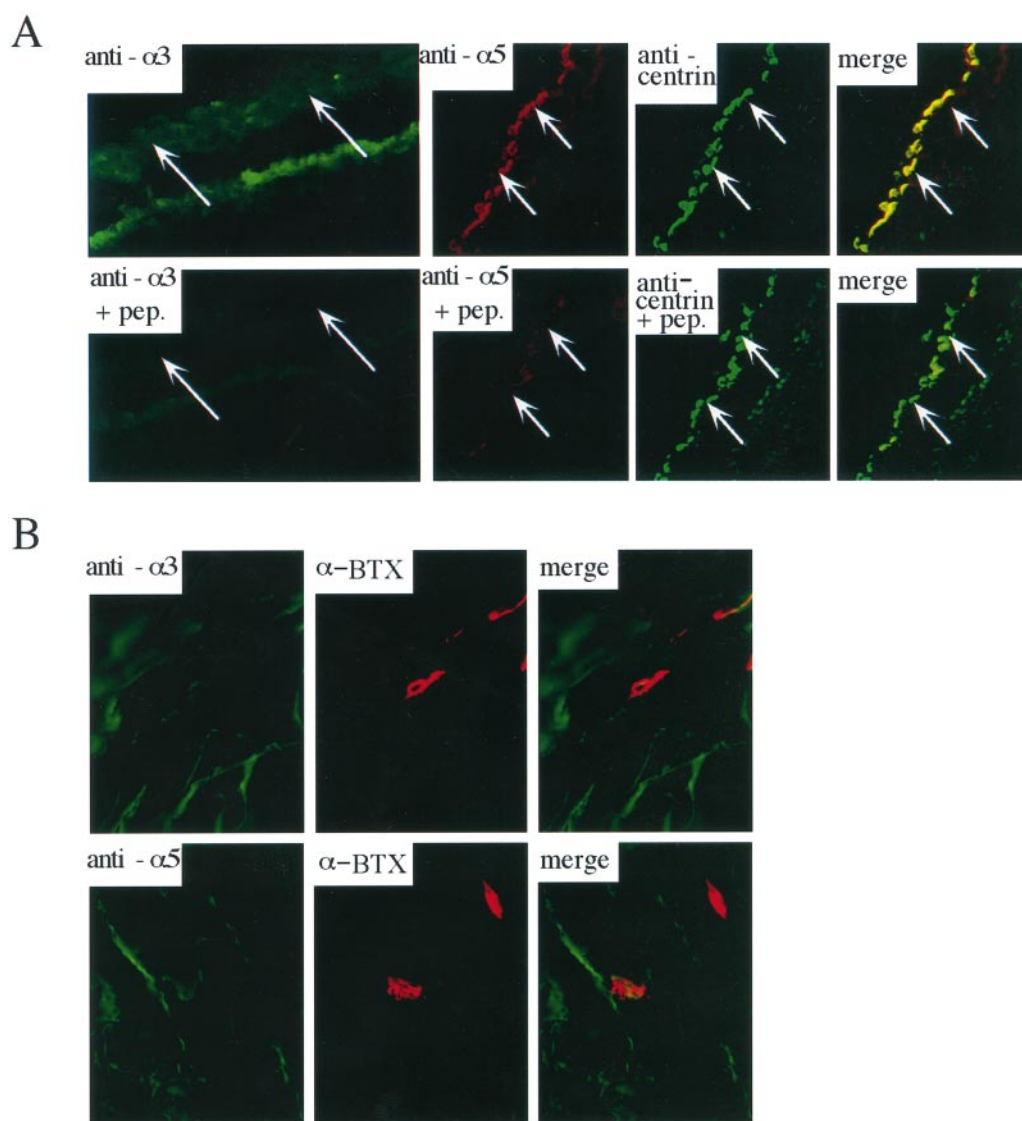

Fig. 6. Detection of α3 and α5 subunit proteins in rat BECs in intact tissue by immunofluorescence. A, The anti-α3 and anti-α5 antisera yielded a clear stain of the epithelial layer in sections of trachea (*anti α-3 and anti α5*) (magnification, 400×). The BECs were identified by double immunofluorescence staining with an anti-centrin antibody (*anti centrin*). The signal resulting from the anti-α3 and anti-α5 antisera was blocked by preincubation with the corresponding peptides (*anti α3 + pep. and anti α5 + pep.*). Preincubation with the α3 or α5 peptides did not affect the signal of the control anti-centrin antibody (*anti-centrin + pep.*). *Merge*, the overlay of the images obtained for the same section using the anti-α5 antiserum and the anti-centrin antibody, in the absence of α5 blocking peptides (*top*) and in the presence of α5 blocking peptides (*bottom*). The overlay of the red and green signal resulting from binding of the two antibodies to the same epithelial cells results in the yellow color of the merged image. On the other hand, when the same overlay is used for antibody binding obtained in the presence of blocking peptides, only the green color of the anti-centrin antibody binding, which is not blocked by AChR peptides, is present in the merged image. B, The anti-α3 and anti-α5 antisera did not bind to the subunits of muscle AChR in sections of rat adult muscle (*anti α3 and anti α5*) (magnification, 400×). The neuromuscular junctions were identified by double immunofluorescence staining with α-BTX (*α-BTX*). *Merge*, the overlay of the images obtained for the same section using the anti-α5 or anti-α3 antisera, as indicated, and the α-BTX. The green signal observed with the anti-AChR subunit antisera, which is weak and diffused, as expected by nonspecific binding, never overlapped the red signal that indicated the presence of muscle AChR at the synaptic junctions.

situ hybridization experiments detected AChR mRNA in all of the cells (Fig. 5). Similarly, immunofluorescence localization of AChR subunit proteins revealed strong signals in virtually all cells in sections of trachea (Fig. 6). Finally, high concentrations of AChR antagonists caused retraction of cytoplasmic flaps and a decrease in the size of all BECs tested (Figs. 7 and 8).

The following considerations can reconcile the contrasts of the higher number of [³H]epibatidine binding sites compared with functional AChRs suggested from the whole-cell currents, and of the expression of AChR in all BECs suggested by the *in situ*, immunofluorescence, and functional experiments, whereas AChR function was detectable electrophysiologically in only a minority of cells. First, some [³H]epibatidine binding sites may not represent functional AChRs. Second, the whole-cell current recordings reflect the activation of AChRs that are reached by the agonists in the period of time while the agonist is being applied. Such time is several orders of magnitude shorter than the incubation period with [³H]epibatidine used in the binding assay (a few

seconds versus 4 hr). If the AChRs are located predominantly on the contact region between BECs, as it occurs in keratinocytes (Grando *et al.*, 1995), the probability that the agonists would quickly access these receptors is rather low. Also, the BEC surface is covered with a dense secretion, which may affect the diffusion of the agonists onto the BEC AChRs. This may explain why only a few cells responded to nicotinic agonists with whole-cell or single-channel currents. The following considerations support the possibility that although AChR function was detectable electrophysiologically in only a minority of cells, all BECs express functional AChR. First, the *in situ* hybridization detected AChR mRNA in all the cultured BECs (Fig. 5). Second, the immunofluorescence localization of AChR subunit proteins revealed a strong signal in virtually all cells in sections of trachea (Fig. 6). Third, high concentrations of the antagonists Mec and κ-BTX caused retraction of the BEC cytoplasmic flaps and a decrease in the size of all cells tested, whereas at lower concentrations some cells did not respond (Figs. 7 and 8), especially when we used a low concentration of κ-BTX [~10 nM, namely, the concentration that effectively blocked the response of the BEC ion channels to a high concentration of Nic in the patch-clamp experiments (Fig. 2)]. This may be due to the much larger size of κ-BTX than Mec (molecular weight 7200 and 204, respectively), which might hinder the diffusion of κ-BTX across the permeability barrier represented by the dense secretion coat that covers the BEC surface.

Only at first view is it surprising that we observed a rather large variation in the number of epibatidine binding sites/cell. In addition to the caveats of the [³H]epibatidine binding assay discussed above, studies on keratinocytes indicated that the level of AChR expression is greatly influenced by the degree of cell differentiation, and it increases sharply just after the cells reach confluence (Grando *et al.*, 1995). In human skin keratinocytes, the number of binding sites for κ-BTX varied between ~5500 sites/cell in cultured cells that had just reached confluence and ~35,400 sites in mature keratinocytes from human neonatal foreskins (Grando *et al.*, 1995). The variable amplitude of the whole-cell currents evoked by ACh and Nic in different BECs is consistent with variable levels of AChR expression of BECs in culture. The strong signal obtained with the anti-α3 and anti-α5 antisera in virtually all cells in sections of trachea (Fig. 6A) suggest that as it occurs for mature keratinocytes in human skin (Grando *et al.*, 1995), fully differentiated BECs in the intact tissue express higher levels of AChRs than in culture.

Possible physiological function of BEC AChRs. What is the physiological ligand for the BEC AChRs? Very high levels of ACh (up to 14 μmol/g of tissue) and of choline acetyltransferase and ACh esterase (the enzymes that synthesize and degrade ACh) are present in the rabbit tracheal mucous membrane (Sastry and Sadavongvivad, 1979). Isolated human bronchi synthesize and release ACh (Wessler *et al.*, 1995). Those findings could be explained by the cholinergic parasympathetic innervation of the airways. However, several lines of evidence indicate that BECs themselves synthesize and secrete ACh. ACh was found in extracts of surface epithelial cells isolated from human bronchi (Klapproth *et al.*, 1997). Human BECs contain the enzyme choline acetyltransferase, which synthesizes ACh. The presence of this enzyme was demonstrated by immunohistochemistry and Western blots of sections of human bronchi and by de-
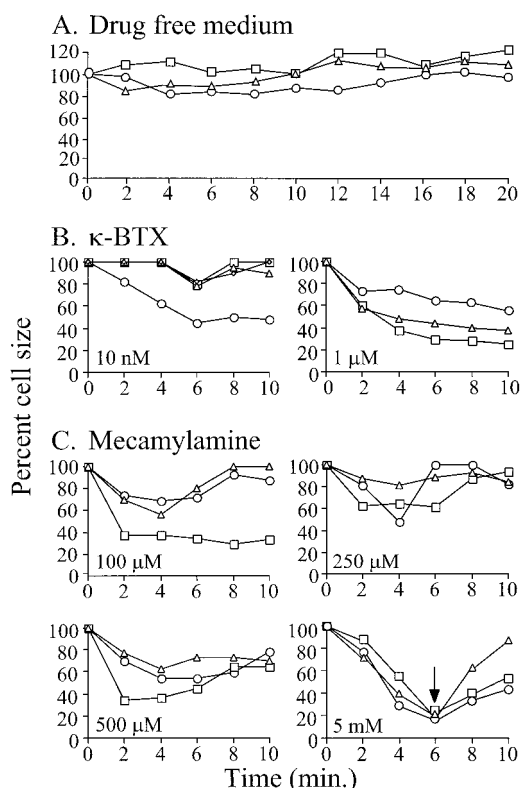

Fig. 7. Human BECs shrink under the influence of Mec. Area covered by individual randomly selected BECs after the addition of drug-free medium (A), medium containing different concentrations of κ-BTX, as indicated (B), or medium containing different concentrations of Mec, as indicated (C). The area covered by the individual cells is expressed as percent of the area covered by the same cell before the addition of the antagonist or of the drug-free medium. Cells that were not exposed to any drug had small, reversible fluctuations of their size, which did not exceed 20%. The cell area decreased after κ-BTX or Mec addition to an extent that increased with the concentration of the drug. With 5 mM Mec or 1 μM κ-BTX, all cells responded to the drug. Most cells responded to Mec at all the concentrations we used. When we used 10 nM κ-BTX, several cells did not respond. The reduction of the cell surface induced by the lower concentrations of Mec we used reversed spontaneously over the observation period. The effect of Mec was reversible even at the highest concentration we used because the cell area increases quickly after washout of Mec (*arrow* in the 5 mM plot of C).

tection of enzyme activity in isolated human BECs (Klapproth et al., 1997). In the current study, the effect of Mec and κ-BTX indicates that the BEC AChRs in cultures are normally being activated by endogenous agonist, as it occurs for the AChRs of skin keratinocytes.

Like in skin keratinocytes (Grando et al., 1995), the BEC AChR seems to be involved in maintenance of the flat shape of the BECs, which is necessary to form a continuous lining on the bronchial surface. This is supported by the finding that Mec and κ-BTX, which are specific antagonists of α3 AChRs, caused a reversible change of the cell shape of cultured, confluent human BECs (Figs. 7 and 8). This resulted in reduction of the area covered by the cell and in cell/cell detachment.

Even the highest concentration of Mec we used compared with those resulting from administration of Mec for therapeutic purposes (Tennant et al., 1983); however, all concentrations of Mec that we used were higher than those effective in whole-cell patch-clamp studies of α3 AChRs expressed in oocytes (Cachelin and Rust, 1995). In oocytes, 50 μM Mec completely blocked the α3 AChRs containing the β2 subunits, and 5 μM Mec completely blocked those containing the β4 subunits (Cachelin and Rust, 1995). Thus, even the lower dose of Mec that we used (100 μM) is 2–20-fold higher than expected from the studies in oocytes (Cachelin and Rust, 1995). Two considerations reconcile that difference. First, the patch-clamp studies on the effect of Mec on α3 AChRs used "denuded" oocytes, stripped of any extracellular coating. On the other hand, our experiments were performed with untreated BECs. Their dense coating of secretion likely forms a diffusion barrier. Second, in the studies on oocytes, the administered Mec did not have to outcompete any endogenously synthesized ACh. On the other hand, the bronchial tissue, and BECs in particular, contains large amounts of ACh (Wessler et al., 1995; Klapproth et al., 1997; Sastry and Sadavongvivad, 1979). It is likely that cultured BECs required a higher concentration of Mec for AChR block than oocytes, due to competition by endogenous ACh. Also, it is most likely that to achieve the extreme effects on the cell shape observed in experiments such as those of Figs. 7 and 8, all or most BEC AChRs must be blocked, even in the presence of endogenous ACh. The spontaneous reversibility of the reduction of the cell surface induced by the lower concentrations of Mec we used (Fig. 7) supports a competition by endogenous ACh.

Potential pathological consequences of BEC exposure to Nic. The characteristics of the BEC AChRs demonstrated here give clues to the pathological effects that might result from acute and chronic exposure to Nic. All AChR isotypes share the property of being desensitized after prolonged exposure to agonists (Conti-Fine et al., 1994; Galzi and Changeux, 1995). However, AChRs that contain the α3 subunit are much more resistant to desensitization than other AChR subtypes, such as the α4 and α7 subtypes (Olale et al., 1997). Human α3 AChRs can exist as hetero-oligomers of α3β2, α3β4, α3β2α5, and α3β4α5 subunits (Conti-Fine et al., 1994; Galzi and Changeux, 1995; Wang et al., 1996). In the α3 AChRs that include the α5 subunit, Nic acts as a full agonist and elicits responses as large as those obtained by full stimulation with ACh. In contrast, Nic acts only as a partial agonist on α3 AChR that do not include the α5 subunits (Wang et al., 1996). Furthermore, the presence of the α5 subunit increases the otherwise slow rate of desensitization of the α3 AChRs (Wang et al., 1996). The BEC AChRs likely include α3β2α5 and α3β4α5 AChR complexes; thus, they should be fully activated and then desensitized by Nic. Continued exposure to high Nic concentrations on the bronchial surface of cigarette smokers should desensitize the BEC AChRs, making them unable to respond to the endogenous ACh.

The following considerations support the contention that the Nic concentration present on the bronchial surface of tobacco smokers will likely result in desensitization of BEC AChRs. The K_d value for Nic of AChR isotypes formed by different combinations of α3, α5, β2, and β4 subunits has been determined by studies on AChRs expressed in oocytes or naturally occurring in neuroblastoma cells. Those studies indicated that α3 AChR complexes containing the β2 subunits have K_d values for Nic of 1–6 μM, and those containing the β4 subunit have values of 34–100 μM (Wang et al., 1996). On smoking days, an average of ~8 μM Nic is likely to be persistently present on the bronchial surface of tobacco smokers (Lindell et al., 1993). That concentration should quickly desensitize the BEC β2-containing AChRs and be adequate to desensitize the low affinity β4-containing AChRs. Dissociation of the bronchial epithelium caused by prolonged exposure to Nic and desensitization of the BEC α3 AChRs should cause or promote chronic bronchitis and facilitate the entry of the carcinogens present in tobacco smoke.

Prolonged exposure to high concentrations of nicotinic agonists of PC12 cells, which express α3 AChRs, results in long-lasting
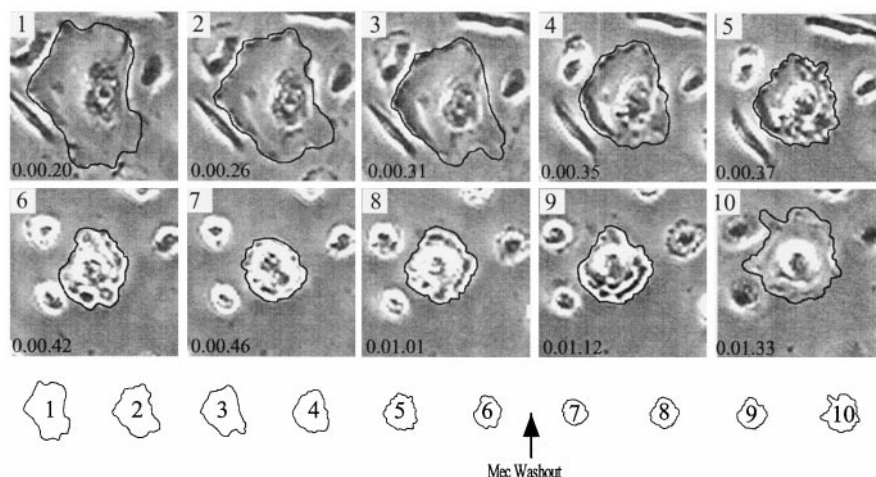
Fig. 8. Human BECs shrink under the influence of Mec. The effect of Mec on cultured BECs in the presence of 5 mM Mec and after washing (between *6 and 7*). Same cells at different time intervals are indicated inside each picture (hour, minutes, seconds) after Mec administration. Inside and below the pictures we report the outline of the largest cell in this field [note the different scale (2:1) of the outline below the pictures compared with the cell size in the actual pictures] to facilitate identification of the area occupied by the cell during the experiment.

inactivation of their AChRs (Lukas, 1991). However, a recent study directly demonstrated that α3 AChR isotypes are resistant to permanent inactivation by chronic exposure to Nic, whereas α4 and α7 AChRs are not (Olale *et al.*, 1997). Consequently, the chronic exposure to Nic of the BEC AChRs in tobacco smokers should not result in their permanent inactivation.

Neuronal α3 AChRs are permeable to Ca^{2+} (Costa *et al.*, 1994; Vernino *et al.*, 1994). Therefore, stimulation of the BEC AChRs due to acute exposure to Nic should cause an increase in intracellular calcium. This, if excessive, might lead to degenerative phenomena and cell death. For instance, increased AChR activity causes a Ca^{2+}-dependent degeneration of the motor end plate (Leonard and Salpeter, 1979, 1982). Also, in some congenital myasthenic syndromes, the AChR of skeletal muscle has increased open times, and muscle degeneration occurs (Engel *et al.*, 1993). In *C. elegans*, a mutation of a neuronal Ca^{2+}-permeable AChR causes prolonged AChR activation and cell death (Treinin and Chalfie, 1995). The neuronal death has been explained by inability of the cell to cope with the osmotic imbalance or Ca^{2+}-mediated toxicity that results from increased channel activity. Neurons may be more susceptible than muscle to the consequences of AChR hyperactivation (Treinin and Chalfie, 1995) because their small size would cause more intense osmotic imbalance. This may apply to the BECs, especially considering that the α3 AChRs desensitize slowly, and even more so when they do not include the α5 subunit (Alkondon and Albuquerque, 1993; Wang *et al.*, 1996; Olale *et al.*, 1997). This would represent another potential mechanism for Nic toxicity, resulting from acute exposure and hyperstimulation of the AChRs, as opposed to desensitization from chronic exposure.

Acknowledgments

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Send reprint requests to: Bianca M. Conti-Fine, M.D., Department of Biochemistry, University of Minnesota, 1479 Gortner Avenue, St. Paul, MN 55108. E-mail: conti@biosci.cbs.umn.edu