Functional Expression of GABA_B Receptors in Airway Epithelium

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γ -Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system and exerts its actions via both ionotropic (GABA_A) and metabotropic (GABA_B) receptors. The GABA_B receptor is a dimer composed of R1 and R2 components and classically couples to the heterotrimeric G_i protein. In addition to their location on neurons, GABA and functional GABA_B receptors have been detected in peripheral tissue such as airway smooth muscle. We questioned whether airway epithelium expresses receptors that could respond to GABA. We detected the mRNA encoding multiple-splice variants of the GABA_BR1 and GABA_BR2 in total RNA isolated from native human and guinea pig airway epithelium and human airway epithelial cell lines (BEAS-2B and H441). Immunoblots identified the GABA_BR1 and GABA_BR2 proteins in both guinea pig airway epithelium and BEAS-2B cells. The expression of GABA_BR1 protein was immunohistochemically localized to basal mucin-secreting and ciliated columnar epithelial cells in guinea pig trachea. Baclofen inhibited adenylyl cyclase activity, induced ERK phosphorylation and cross-regulated phospholipase C, leading to increased inositol phosphates in BEAS-2B cells in a pertussis toxin-sensitive manner, implicating G_i protein coupling. Thus, these receptors couple to G_i and cross-regulate the phospholipase C/inositol phosphate pathway. The second messengers of these pathways, cyclic AMP and calcium, play pivotal roles in airway epithelial cell primary functions of mucus clearance. Furthermore, the enzyme that synthesizes GABA, glutamic acid decarboxylase (GAD65/67), was also localized to airway epithelium. GABA may modulate an uncharacterized signaling cascade via GABA_B receptors coupled to G_i protein in airway epithelium.

Keywords: G protein; adenylyl cyclase; mitogen-activated protein kinase; airway epithelium

γ -Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS). GABA acts at two distinct types of receptors, ligand-gated ionotropic GABA_A and G protein-linked metabotropic (GABA_B) receptors. Recently the expression of subunits of GABA_A receptors and the enzyme that synthesizes GABA (glutamic acid decarboxylase [GAD]) were identified in airway epithelium and found to play a pivotal role in mucous overproduction in asthma (1). However, the expression and coupling of the GABA_B receptor has never been described in airway epithelial cells. The GABA_B receptor is composed of two subunits (GABA_BR1 and GABA_BR2) and typically functions as a G_i protein-coupled receptor. The GABA_B receptor is fully func-

CLINICAL RELEVANCE

This is a novel demonstration of the functional coupling of the GABA_B receptor on airway epithelium to cyclic AMP and calcium signaling pathways, which in turn regulate a fundamental function of airway epithelial cells, mucociliary clearance.

tional only when both subunits are expressed and are linked in a heterodimeric assembly (2). In addition to their well-characterized expression and function on neurons, GABA and functional GABA_B receptors have been detected in peripheral tissues such as adrenal medulla, islets of Langerhans, placenta, and smooth muscle cells of the urinary bladder and uterus.

Airway epithelial cells play an integral part in airway function and disease (1, 3). A novel G_i-coupled receptor could have immense importance in epithelial cell function, since G_i regulation of cyclic AMP is pivotal in the central function of airway epithelial cells, mucociliary clearance. Moreover, in some cells liberated G_i α or G_i $\beta\gamma$ subunits cross-regulate phospholipase C β_1 , an enzyme that liberates inositol phosphates and increases intracellular calcium, which in turn regulates ion transport, mucin secretion, and ciliary beat frequency in airway epithelial cells—all components of a fundamental role of airway epithelial cells, mucus clearance.

Studies have shown that several subunits of GABA_A receptor are expressed in airway epithelium (1, 4), and activation of GABA_A receptor in airway epithelium induced goblet cell hyperplasia and mucus overproduction (1). We recently described the presence of GABA in airway epithelium and of GABA_B receptor in airway smooth muscle (5). In this previous study very dense immunohistochemical staining of GABA_BR1 was detected in the airway epithelium. These findings led us to hypothesize that there may be functional GABA_B receptors that could respond to GABA in airway epithelium.

In the present study, we investigated the expression of GABA_B receptors in native guinea pig and human airway epithelium and in cultured human airway epithelial cell lines (BEAS-2B and H441), and assessed the functional coupling of the GABA_B receptor to the G_i protein by demonstrating adenylyl cyclase inhibition, extracellular signal-regulated kinase (ERK) activation and crosstalk activation of phospholipase C. Furthermore, the expression of glutamic acid decarboxylase (GAD65/67) in airway epithelium was confirmed to identify one potential source of the endogenous ligand GABA in airways.

MATERIALS AND METHODS

Materials

The human bronchial epithelial cell line (BEAS-2B) (ATCC CRL-9609) and the human lung epithelial adenocarcinoma cell line (H441) (ATCC HTB-174) were obtained from ATCC (Manassas, VA). BEGM bronchial epithelial cell basal medium and growth supplements (for

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BEAS-2B cells) were purchased from Lonza (Walkersville, MD). RPMI basal media, fetal bovine serum, and L-glutamine (H441 cells) were purchased from Invitrogen. [³²P]-α-ATP (800 Ci/mmol), [³H]cAMP (32 Ci/mmol), and [³H]myo-inositol (20 Ci/mmol) were obtained from MP Biomedicals (Irvine, CA). Lysates of human brain cerebral cortex were used as positive protein controls on immunoblots and were obtained from BD Biosciences (Palo Alto, CA). Total RNA from whole human brain was purchased from Clontech (Mountain View, CA). Pertussis toxin and protease inhibitor cocktail III were purchased from EMD Biosciences (San Diego, CA). SKF97541 (also known as CGP35024) was obtained from Tocris Bioscience (Ellisville, MO). All other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated. We used R(+)-baclofen hydrochloride for all baclofen studies.

Cell Culture

The BEAS-2B cells were grown to confluence on 24- (adenylyl cyclase) or 6-well (immunoblotting) plates in culture medium (BEGM, supplemented with bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin-B, and retinoic acid as recommended by the manufacturer [Lonza]) at 37°C in an atmosphere of 5% CO₂-95% air. In all studies, cell culture medium was not changed for 72 hours (conditioned media) before initiating treatments with GABA_B agonists. For analysis of ERK phosphorylation, cells were treated with GABA_B agonists (baclofen or SKF97541) (100 μM) for 5 minutes in 72-hour conditioned culture medium. Pertussis toxin (100 ng/ml) was preincubated for 4 hours before the addition of GABA_B agonists. After treatment, cells were rinsed with cold phosphate-buffered saline (PBS), and ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1:200 dilution of protease inhibitor cocktail III [EMD Biosciences, San Diego, CA], 1 mM Na₂VO₄, 1 mM NaF) was added. After the whole cell lysates were centrifuged at 15000 × *g* for 15 minutes at 4°C, the supernatant was saved and the protein concentration was determined. Cell lysates were solubilized by heating at 95°C for 5 minutes in sample buffer (final concentrations: 50 mM Tris-HCl, pH 6.8, 2.5% SDS, 6% glycerol, 2.5% 2-mercaptoethanol, bromophenol blue) and were stored at -20°C.

Isolation of Airway Epithelium or Whole Brain from Guinea Pig

Studies were approved by the Institutional Animal Care and Use Committee (College of Physicians and Surgeons, Columbia University, New York, NY). Adult male guinea pigs were deeply anesthetized by intraperitoneal pentobarbital (100 mg/kg), the chest cavity was opened, and the animal was exsanguinated before the dissection of the trachea. The trachea or whole brain were surgically removed and placed in cold (4°C) buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, 1:200 dilution of protease inhibitor cocktail III; EMD Biosciences). The exterior of guinea pig trachea was meticulously dissected free of adherent connective tissue under a dissecting microscope. Tracheas were then cut open longitudinally along the anterior border. The tracheal epithelium was removed, and tracheal epithelium or whole brain was homogenized (Tekmar Ultra Turrax T25 high-speed homogenizer [Tekmar, Mason, OH] set at top speed for 30 s) in Trizol reagent (Ambion,

Austin, TX) for total RNA extraction according to the manufacturer's recommendations or in cold (4°C) buffer (10 mM Hepes, pH 7.4, 1 mM EDTA with a 1:200 dilution of protease inhibitor cocktail III). For protein samples the homogenate was filtered through 125-μm Nitex mesh and centrifuged at 1,000 × *g* for 10 minutes at 4°C. The supernatant was transferred into new tubes and centrifuged at 50,000 × *g* for 15 minutes at 4°C. The pellet was resuspended in the same buffer and centrifuged at 50,000 × *g* for 15 minutes at 4°C. The final pellet was resuspended in the same buffer and stored at -80°C.

Isolation of Airway Epithelium from Human Trachea

Studies were approved by Columbia University's Institutional Review Board (IRB) and deemed not human subjects research under 45 CFR 46. Discarded excess tracheal tissues from healthy donor lungs harvested for lung transplantation at Columbia University were transported to the laboratory in cold (4°C) Krebs-Henseleit buffer (in mM: NaCl 118, KCl 5.6, CaCl₂ 0.5, MgSO₄ 0.24, NaH₂PO₄ 1.3, NaHCO₃ 25, glucose 5.6, pH 7.4). The exterior of the human trachea were dissected free of adhering connective tissue under a dissecting microscope. Tracheae were then cut open longitudinally along the anterior border. The thin tracheal epithelial layer was meticulously removed with forceps under a dissecting microscope and was homogenized in cold (4°C) buffer (10 mM Hepes, pH 7.4, 1 mM EDTA with a 1:200 dilution of protease inhibitor cocktail III) using a Tekmar Ultra Turrax T25 high speed homogenizer set at top speed for 30 seconds. The homogenate was filtered through 125-μm Nitex mesh and centrifuged at 1,000 × *g* for 10 minutes at 4°C. The supernatant was transferred into new tubes and centrifuged at 50,000 × *g* for 15 minutes at 4°C. The pellet was resuspended in the same buffer and centrifuged at 50,000 × *g* for 15 minutes at 4°C. The final pellet was resuspended in the same buffer and stored at -80°C.

RNA Isolation and RT-PCR

Total RNA was extracted from freshly dissected native human and guinea pig airway epithelium, cultured BEAS-2B cells, cultured H441 cells, and guinea pig whole brain using Trizol Reagent (Ambion) according to the manufacturer's recommendations. Total RNA from whole human brain (Clontech) was used as a positive control. Using the Advantage RT-for-PCR Kit (Clontech), 1 μg of total RNA was reverse transcribed at 42°C for 1 hour in 20 μl including 200 units of Moloney murine leukemia virus reverse transcriptase, 20 units of RNase inhibitor, 20 pmol oligo(dT) primer, and 0.5 mM each of dNTP mix in reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂). PCR was performed by adding 5 μl of newly synthesized cDNA to a 45-μl reaction mixture yielding final concentrations of 0.2 mM of each dNTP, 1× Advantage 2 Polymerase Mix, PCR buffer (Clontech), and 0.4 μM of both sense and antisense primers for corresponding GABA_B receptor subunits and isoforms of glutamic acid decarboxylase (GAD) (Table 1). PCR was performed using a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA) at indicated temperatures and times (Table 1). PCR products were electrophoresed on 5% nondenaturing polyacrylamide gel in 1× Tris, acetate, EDTA buffer. The gel was stained with ethidium bromide (Molecular Probes, Eugene, OR), visualized using ultraviolet illumination, and analyzed using Quantity One software (Bio-Rad, Hercules, CA).

TABLE 1. PCR PRIMER SEQUENCES FOR GABA_B RECEPTOR SUBTYPES AND GAD ISOFORMS

Target	Sequence of Primer	Amplicon size (bp)	Denaturation (°C)	Annealing/Extension (°C)	Annealing/Extension time (min)	No. of PCR Cycles
GABA _B R1	FP: 5'-CAAGAAGATTGGCTACTATGACAGCACCAAGGATGA-3'	R1a/b/c: 302	94	Human: 72/72	1/1	40
	RP: 5'-CCAGGGGGAAGACAGCAGCTAAAGCCAGTGAG-3'	R1e: 150	94	Guinea pig: 60/72	0.5/0.5	40
GABA _B R2	FP: 5'-GTCCACCTCGGTACCAAGTGTGAACCA-3'	357	94	68/68	1/1	38
	RP: 5'-AGCCGACGCTGGATGTCTCTGAGAGT-3'	357	94	68/68	1/1	38
GAD65	FP: 5'-GAGTGGAGTGGAGAGGGCCAACTCTGTGAC-3'	488	Human: 96	Human: 68/68	1/1	45
	RP: 5'-TTGTGGTCCACTACTCCATCTTCTGGCTTAATC-3'	488	Guinea pig: 90	Guinea pig: 60/70	0.5/0.5	45
GAD67	FP: 5'-GACATGTGATTTTGATAAAGTGAATGAA-3'	375	Human: 96	Human: 68/68	1/1	45
	RP: 5'-CATCTGGTTCATCTCTGGAGTATACCT-3'	375	Guinea pig: 90	Guinea pig: 60/70	0.5/0.5	45

Western Blot Analysis

Whole cell or tissue lysates were electrophoresed (8–10% SDS-PAGE) and transferred to PVDF membranes. The PVDF membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk (for antibodies against GABA_B receptor) or bovine serum albumin (BSA) (for antibodies against total and phospho-ERK and β -actin) in TBS with 0.1% Tween 20, and were then probed with antibodies directed against GABA_BR1 (rabbit polyclonal 1:1,000, sc-14006; Santa Cruz Biotechnology, Santa Cruz, CA), GABA_BR2 (guinea pig polyclonal 1:5,000, AB5394; Chemicon, Temecula, CA), total ERK (rabbit polyclonal 1:1,000, #9102; Cell Signaling Technology, Danvers, MA), phospho-ERK (rabbit polyclonal 1:1,000, #9101, Cell Signaling Technology), or β -actin (mouse monoclonal 1:200, sc-8438; Santa Cruz Biotechnology) overnight at 4°C. After washing three times, membranes were incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibodies (1:5,000, NA934V; Amersham Biosciences, Arlington Heights, IL), with HRP-conjugated secondary anti-mouse antibodies (1:5,000, NA931V; Amersham Biosciences) or with HRP-conjugated secondary anti-guinea pig antibodies (1:5,000, AP193P; Chemicon). The signal from the immunoreactive bands was detected by ECL Plus (Amersham Biosciences) according to the manufacturer's recommendations and developed on film (Kodak Biomax light film; Kodak, Rochester, NY). Film was developed such that band intensities were within the linear range of film responses; and band intensities were quantified using Quantity One software (Bio-Rad). Data presented were means $\pm$ SEM values.

Immunohistochemistry

Guinea pig tracheal rings were fixed using 10% formalin for 24 hours at room temperature. Tracheal rings were dehydrated, paraffin embedded, sectioned (5 μ m), dewaxed in xylene, and rehydrated in a graded alcohol series to water. Endogenous peroxidase was blocked in 0.3% hydrogen peroxide in PBS. Heat-mediated antigen retrieval was

performed with 10 mM sodium citrate buffer, pH 6.0 for 30 minutes. An avidin biotin blocking kit (Vector Laboratories, Peterborough, UK) and 10% serum in PBS was used to block endogenous biotin and nonspecific protein binding, respectively. Slides were rinsed with PBS and incubated overnight at 4°C in primary antibody against GABA_BR1 (rabbit polyclonal 1:250, sc-14006; Santa Cruz Biotechnology) or GAD65/67 (rabbit polyclonal 1:5,000, AB1511; Chemicon) in 2% serum in PBS. A parallel tracheal ring section was incubated with the appropriate isotype IgG antibody as a negative control. After overnight incubation at 4°C, slides were washed with PBS, and primary antibodies were detected using biotinylated anti-rabbit antibodies (Vector Laboratories) at a concentration of 1:100. The antigen antibody complex was then visualized by the enzymatic reduction of 3,3'-diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin and dried, and cover slides were mounted using Poly-mount (Polysciences, Warrington, PA).

Adenylyl Cyclase Assays

Adenylyl cyclase activity was measured as previously described (6). Briefly, cells in 24-well plates were washed once with warm PBS (37°C). In some experiments cells were pretreated for 4 hours with pertussis toxin (100 ng/ml) in cell culture media before washing with PBS. One hundred microliters of warm PBS was added to each well. Subsequently, 50 μ l of 3 $\times$ adenylyl cyclase buffer (6) was added directly to the wells (to achieve final concentrations of 50 mM HEPES, pH 8.0, 50 mM NaCl, 0.4 mM EGTA, 1 mM cAMP, 7 mM MgCl₂, 0.1 mM ATP [20 μ Ci/ml ³²P- α -ATP], 0.1 mg/ml BSA, 50 units/ml creatine phosphokinase, and 7 mM phosphocreatine) in the absence (basal activity) or presence of 10 μ M forskolin $\pm$ 100 μ M baclofen or 100 μ M SKF97541 and the plates were incubated at 37°C for 15 minutes. The reactions were terminated by the addition of 100 μ l stop buffer (50 mM HEPES, pH 7.5, 2 mM ATP, 0.5 mM cAMP [0.5 μ Ci/ml ³H-cAMP], 2% SDS) and synthesized ³²P-cAMP was separated and quantitated by sequentially column chromatography over dowex and alumina as described (7).

Figure 1. Representative gel images of RT-PCR analysis of total RNA using primers specific for GABA_B receptor subunits. (A) Splice variants (R1a/b/c [302 bp] and R1e [150 bp]) of GABA_BR1 were detected in human airway epithelium (Epi), human cultured bronchial epithelial cells (BEAS-2B), human lung epithelial adenocarcinoma cells (H441), guinea pig (GP) airway epithelium, and GP and human whole brain. (B) Messenger RNA encoding GABA_BR2 was detected in both human epithelial cell lines and in freshly isolated epithelium or whole brain from guinea pig and human. Images are representative of at least three independent RT-PCR analyses from each cell or tissue source.

Figure 2. Representative immunoblot analysis using protein prepared from (A, left) cultured human BEAS-2B cells (100 μ g), whole human brain (25 μ g), or (A, right) GP tracheal epithelium (Epi) (100 μ g), and GP brain (25 μ g). A larger splice variant (130 kD) corresponding to GABA_BR1a was detected in both brain-positive controls and in human BEAS-2B cells. A smaller splice variant (100 kD) that

could represent either GABA_BR1b or 1c was detected in both brain-positive controls and in freshly isolated GP epithelium but not in cultured human BEAS-2B cells. (B) Representative immunoblot using antibody directed against GABA_BR2 identified an immunoreactive band of appropriate molecular mass (120 kD) in cultured human BEAS-2B cells and in freshly isolated GP epithelium. Images are representative of at least 3 independent immunoblot analyses from each cell or tissue source.

Inositol Phosphate Assays

Synthesis of total ³H-inositol phosphates was measured in confluent BEAS-2B cells in 24-well tissue culture plates as described (6, 8). Briefly, after overnight loading with ³H-*myo*-inositol (10 μ Ci/ml, 20 Ci/mmol) in inositol-free and serum-free Dulbecco's modified Eagle's medium (Chemicon), plates were washed three times (37°C, 500 μ l Hanks' balanced salt solution with 10 mM LiCl). Incubation of cells with GABA_B receptor agonists (baclofen) in a final volume of 300 μ l at 37°C for 30 minutes was performed in the absence or presence of pertussis toxin (PTX; 4 h before the addition of baclofen) or 5 μ M U73122, a PLC inhibitor (30 min before the addition of baclofen). Reactions were terminated, and total [³H]inositol phosphates recovered by chromatography (6).

Statistical Analysis

Statistical analysis was performed using repeated measures of ANOVA, followed by Bonferroni post-test comparison using Prism 4.0 software (GraphPad, San Diego, CA). Data are presented as mean $\pm$ SEM; $P < 0.05$ was considered significant.

RESULTS

RT-PCR Analysis of GABA_B Receptor Isoforms in Airway Epithelium

RT-PCR analysis demonstrated mRNA encoding the human GABA_BR1 protein (Figure 1A) and the GABA_BR2 protein (Figure 1B) in BEAS-2B cells, H441 cells, native human airway epithelium, guinea pig airway epithelium, and whole brain (positive controls) from both species. To date, four splice variants of the GABA_BR1 protein have been reported (GABA_BR1a, 1b, 1c, and 1e). In the present study primers for GABA_BR1 flanked the omitted exon of GABA_BR1e and therefore could distinguish 1e from other isoforms (i.e., 1a, 1b, and 1c). We observed two PCR products of predicted sizes corresponding to at least two splice variants of GABA_BR1, GABA_BR1a/b/c (302 bp) and GABA_BR1e (150 bp) in human airway epithelium, BEAS-2B cells, H441 cells, and guinea pig airway epithelium (Figure 1A). Messenger RNA encoding GABA_BR2 was also detected in

Figure 3. Representative immunohistochemical staining of GABA_BR1 in formalin-fixed GP trachea. (A and C) GABA_BR1 was localized in both basal mucin-producing and ciliated columnar epithelial cells within tracheal epithelium (original magnification: $\times 10$ and $\times 100$ objectives, respectively). (B and D) Isotype-specific negative control primary antibody (rabbit IgG) in a parallel section of GP trachea (original magnification: $\times 10$ and $\times 100$ objectives, respectively). Sections were counterstained with hematoxylin. Images are representative of at least three independent immunohistochemical analyses from GP trachea.

human airway epithelium, BEAS-2B cells, H441 cells, and guinea pig airway epithelium (Figure 1B). At least three independent tissue or cell sources were analyzed for each mRNA studied.

Immunoblot Analysis of GABA_BR Isoforms in Airway Epithelium

The antibody used for immunoblot analysis of GABA_BR1 recognizes the common COOH terminus region of three of the four known human isoforms of GABA_BR1 (i.e., GABA_BR1a, 1b, and 1c, but not 1e). Two immunoreactive bands of approximately 100 and 130 kD were identified in whole brain from both species (Figure 2A). Whereas the larger 130-kD band corresponds to the expected molecular mass of GABA_BR1a, the 100-kD band corresponds to the molecular mass of either GABA_BR1b or GABA_BR1c. A smaller splice variant of GABA_BR1 (GABA_BR1b or e) was predominant in guinea pig airway epithelium, while only the larger splice variant (GABA_BR1a) was identified in BEAS-2B cells (Figure 2A). An antibody raised against the GABA_BR2 protein identified a single immunoreactive band of 120 kD in protein samples from BEAS-2B cells and guinea pig tracheal epithelium (Figure 2B), consistent with the predicted size for GABA_BR2 (9). Since the secondary antibody to detect the GABA_BR2 primary antibody was an anti-guinea pig antibody, we performed control experiments in which the primary antibody was omitted. In these negative control studies no immunoreactive bands in the appropriate molecular weight range were detected (data not shown).

Immunohistochemical Analysis of GABA_BR1 Expression in Guinea Pig Trachea

To confirm the protein localization of GABA_BR1 to airway epithelium in guinea pig airways, immunohistochemistry was performed. Heavy immunoreactive staining was detected in guinea pig trachea within basal mucin-secreting and ciliated columnar epithelial cells with less intense staining detected in the airway smooth muscle and tracheal cartilage chondrocytes (Figures 3A and 3C). Representative results of an isotype-specific IgG primary antibody (negative control) are shown in Figures 3B and 3D.

Functional Coupling of the GABA_B Receptor to G_i Signaling Pathways in BEAS-2B Cells

Demonstration of GABA_B receptor mRNA and protein in airway epithelium led us to determine whether the receptor demonstrated classical coupling to the G_i protein by evaluating (1) pertussis toxin-sensitive agonist-induced inhibition of adenylyl cyclase activity, (2) pertussis toxin-sensitive agonist-induced activation of mitogen-activated protein kinase (MAPK ERK), and (3) G_i protein-mediated cross-regulation of phospholipase C activation of inositol phosphate synthesis.

Agonist-Induced Inhibition of Adenylyl Cyclase Activity in BEAS-2B Cells

GABA_B receptor-mediated inhibition of adenylyl cyclase activity via coupling to the G_i protein is well known in neurons (10). To examine whether GABA_B receptor agonists inhibit adenylyl cyclase activity in cultured BEAS-2B cells, we measured adenylyl cyclase activity in the presence or absence of either baclofen (100 μ M) or SKF97541 (100 μ M). Both GABA_B agonists significantly inhibited basal adenylyl cyclase activity (baclofen; $P < 0.05$, $n = 6$; SKF97541; $P < 0.01$, $n = 6$)

Figure 4. (A) Basal adenylyl cyclase activity in cultured BEAS-2B cells in the presence or absence of baclofen (100 μ M) or SKF97541 (100 μ M) ($n = 6$ experiments). Data represent means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ compared with control. (B) Forskolin (10 μ M)-stimulated adenylyl cyclase activity in cultured BEAS-2B cells in the presence or absence of baclofen (100 μ M) ($n = 13$). In some experiments, cells were pretreated with pertussis toxin (PTX) (100 ng/ml for 4 h pretreatment before baclofen treatment) ($n = 10$). Data represent means $\pm$ SEM. *** $P < 0.001$ compared with forskolin. In each experiment, values were determined in triplicate.

(Figure 4A). Baclofen also significantly inhibited the forskolin-stimulated adenylyl cyclase activity ($P < 0.001$, $n = 13$), and this inhibition was blocked by 4 hours of pretreatment with pertussis toxin (100 ng/ml) ($n = 10$) (Figure 4B).

Agonist-Induced Activation of ERK in BEAS-2B Cells

Many G_i-coupled receptors are known to activate the ERK pathway (11). ERK activation was identified by increased phospho-ERK on immunoblots and expressed as a ratio of phospho/total ERK for each sample. Baclofen (100 μ M, 5 min) significantly increased phosphorylation of ERK (Figure 5, left; $n = 5$, $P < 0.05$). In separate experiments, a second selective GABA_B agonist, SKF-97541 (100 μ M, 5 min), also increased ERK phosphorylation (Figure 5, right; $P < 0.01$, $n = 5$). Four-hour pretreatment of cells with pertussis toxin (100 ng/ml) abrogated increases in ERK phosphorylation by either GABA_B agonist ($P < 0.01$ and $P < 0.05$ for pertussis toxin + agonist versus baclofen or SKF-97541 alone, respectively, $n = 5$) confirming the role of G_i in GABA_B receptor activation of ERK.

Figure 5. (A) Immunoblot analyses of phospho-ERK or total ERK expression in cultured BEAS-2B cells. Cells were treated with baclofen (100 μ M) (left) or SKF97541 (100 μ M) (right) for 5 minutes in the presence or absence of PTX (100 ng/ml for 4 h pretreatment before GABA_B selective agonist treatment). Representative immunoblots from five experiments are shown. (B) Relative band intensities of phospho-/total ERK from five separate immunoblots. The effects of baclofen (left) and SKF97541 (right) on ERK phosphorylation are given as percentage of control. Data represent means $\pm$ SEM. * P < 0.05, ** P < 0.01 compared with control. # P < 0.05, ## P < 0.01 compared with baclofen or SKF97541.

Agonist-Induced Stimulation of Inositol Phosphate Synthesis in BEAS-2B Cells

Classically receptors that couple to the G_q protein activate phospholipase C (PLC) increasing inositol phosphates and intracellular calcium. However, in some cells cross-talk to PLC β 1 from either G_i α or G_i $\beta\gamma$ subunits has been described (12–15). To examine whether GABA_B receptor agonists cross-talk through G_i to stimulate inositol phosphate synthesis in cultured BEAS-2B cells, we measured inositol phosphate synthesis in the presence or absence of baclofen (100 μ M). Baclofen significantly increased inositol phosphate synthesis. These increases were blocked by 4 hours of pretreatment with pertussis toxin (100 ng/ml) (n = 10) (Figure 6A) or by 30 minutes of pretreatment with the PLC inhibitor U73122 (5 μ M) (n = 11) (Figure 6B).

RT-PCR Analysis of GAD65 and GAD67 in Airway Epithelium

We questioned whether airway epithelium could be the source of the endogenous ligand, GABA, for the GABA_B receptor by assessing the presence of the mRNA encoding the enzyme that synthesizes GABA, glutamic acid decarboxylase (GAD). RT-PCR analysis demonstrated mRNA encoding GAD in BEAS-2B cells, H441 cells, native human airway epithelium, and guinea pig airway epithelium (Figure 7). We observed PCR products of predicted sizes corresponding to GAD65 and GAD67 in both human and guinea pig brain. While GAD67 was detected in human airway epithelium, BEAS-2B cells, and H441 cells (Figure 7B), GAD65 was detected in guinea pig airway epithelium (Figure 7A).

Immunohistochemical Analysis of GAD65/67 Expression in Guinea Pig Trachea

To confirm the protein localization of GAD65/67 to airway epithelium in guinea pig airways, immunohistochemistry was performed. GAD65/67 immunoreactivity in guinea pig trachea

was detected in airway epithelium (Figure 8A), with no staining in the negative control studies with rabbit IgG (Figure 8B).

DISCUSSION

The present study is the first to demonstrate that functional GABA_B receptors are expressed in both native human and guinea pig airway epithelium and cultured airway epithelial cells. Both GABA_BR1 and GABA_BR2 subunits were detected at the level of mRNA (by RT-PCR) and protein (by immunoblot) at appropriate molecular weights. Immunohistochemistry localized the GABA_BR1 subunit to airway epithelium in guinea pig tracheal rings. The GABA_B receptor agonist baclofen inhibited adenylyl cyclase activity and induced ERK phosphorylation, which was abrogated by pretreatment of cultured BEAS-2B cells with pertussis toxin, confirming classical coupling of the GABA_B receptor to the G_i protein signaling pathways in these cells. Furthermore, the enzyme GAD 65/67 that synthesizes GABA, the endogenous ligand for the GABA_B receptor, was also detected in airway epithelium at the level of mRNA (by RT-PCR) and protein (by immunohistochemistry).

Several studies indicate the possible expression of functional GABA_B receptors in peripheral and nonneuronal cells such as pancreatic β cells (16), adrenocortical cells (17), cardiomyocytes (18), chondrocytes (19), and osteoblasts (20). Furthermore, GABA_BR1 mRNA expression was detectable using RT-PCR analysis in many peripheral organs, including heart, spleen, lung, liver, intestine, kidney, stomach, adrenal gland, testis, ovary, and urinary bladder (21).

Recent evidence has shown that GABA_B receptors must exist as a heterodimer to form a functional G_i protein-coupled receptor in the plasma membrane (2, 22). The large N-terminal extracellular domain, in particular the GABA_BR1 subunit, is the site for ligand binding, whereas the GABA_BR2 subunit is crucial for effector coupling (23). The presence of both subunits in airway epithelial cells suggests that they can assemble

Figure 6. (A) Effects of 4 hours of pretreatment with PTX (100 ng/ml) on 100 μ M baclofen-induced inositol phosphate synthesis in BEAS-2B cells ($n = 10$). (B) Effects of PLC inhibitor (5 μ M U73122) on 100 μ M baclofen-induced inositol phosphate synthesis in BEAS-2B cells ($n = 11$). Data are shown as percentage of basal and represent means $\pm$ SEM. * $P < 0.05$ and *** $P < 0.001$ compared with basal. # $P < 0.05$ and ** $P < 0.01$ compared with baclofen.

functional GABA_B receptor heterodimers. Functional GABA_B receptors coupling through G_i to the inhibition of adenylyl cyclase or ERK phosphorylation were demonstrated using the GABA_B-selective agonists baclofen or SKF97541 in the absence or presence of pertussis toxin. To date, four different splice variants (GABA_BR1a, b, c, and e) of GABA_BR1 have been identified in humans. Indeed, we identified the expression of at least three splice variants in airway epithelium (GABA_BR1 a/b/c and e at the mRNA level and GABA_BR1 a or b/c at the protein level). At the present time, there are no confirmed GABA_BR2 splice variants (22).

Although we identified mRNA encoding of the GABA_BR1 subtype and the GABA_BR2 subtype in RNA isolated from freshly dissected human and guinea pig airway epithelium, RNA isolated from freshly dissected tissues invariably will contain some RNA from nonepithelial cells despite careful dissection. Therefore, to confirm the epithelial cell-specific expression of the GABA_BR1 and GABA_BR2 subunits, we analyzed RNA isolated from homogenous cultures of airway epithelial cells and confirmed the expression of the GABA_BR1 and GABA_BR2. Furthermore, immunohistochemistry in guinea pig tracheal rings localized the GABA_BR1 protein to both basal mucin-producing and ciliated columnar epithelial cells within the tracheal epithelium. The GABA_BR1 protein was also expressed in tracheal smooth muscle cells and chondrocytes as previously described (5, 19, 20) suggesting that multiple cell types in the airway may be responsive to endogenous GABA.

After demonstrating the mRNA and protein expression of GABA_B receptors in airway epithelium, we sought to confirm its coupling to G proteins in general and its specific classical coupling to the G_i protein. Three G_i-specific coupling pathways

were investigated using the GABA_B agonist baclofen. Inhibition of adenylyl cyclase is a well-known effect of G_i protein activation and is known to occur in airway smooth muscle in response to activation of several G_i-coupled receptors (e.g., M₂ muscarinic receptor and GABA_B receptor). Activation by phosphorylation of ERK is a ubiquitous signaling pathway after G_i activation, and pertussis toxin is a widely used tool to inactivate and implicate G_i proteins in signaling events. Indeed, baclofen inhibited adenylyl cyclase and activated ERK phosphorylation in a pertussis toxin-sensitive manner in BEAS-2B cells, confirming the coupling of GABA_B receptors to G_i proteins in these cells. The specificity of baclofen's effect at the GABA_B receptor in the present study is supported by the finding that another GABA_B agonist, SKF-97541, could mimic the baclofen's effect on ERK activation.

R-baclofen and SKF97541 (also known as CGP35024) are widely used selective agonists for the GABA_B receptor. The intrinsic activity of SKF97541 at the GABA_B receptor is nearly 10 times that of baclofen and 5 times that of GABA (24). These agonists do not have significant affinity for the GABA_A receptor (25–29).

The physiological role of GABA_B receptors in airway epithelium and of GABA-ergic modulation of intercellular cAMP or ERK activation is at present unclear. Many of the protective and anti-inflammatory functions performed by airway epithelial cells are cAMP-dependent processes activated by the β 2-adrenergic receptor-adenylyl cyclase (β 2AR-AC) signaling cascade via the G_s α protein (30). In particular, activation of the β 2AR-AC system in human airway epithelial cells stimulates mucociliary clearance, water and electrolyte transport, and the release of bronchodilatory substances (e.g., nitric oxide) (30–34). In contrast, G_i α 2, which couples to the inhibition of adenylyl cyclase, was also distributed in guinea pig tracheal epithelial cells (35). In the present study, baclofen inhibited adenylyl cyclase activity in a pertussis toxin-sensitive manner, suggesting that GABA_B receptor activation in airway epithelium could inhibit cAMP-mediated airway protective and anti-inflammatory functions.

Mitogenic pathways are linked to MAPK cascades that can be activated by a variety of extracellular stimuli, including agonists for G protein-coupled receptors. G_i α 2 plays an important role in pathways that control cell differentiation and growth via activation of MAPK. In airway epithelial cells G_i α 2 regulates cell growth (36), and furthermore, MAP kinases including ERK regulate the expression of chemokines such as IL-8, IL-13, and IL-17, which is increased in the airways of patients with asthma (37, 38). In the present study, we found that baclofen induced ERK phosphorylation in BEAS-2B cells. This result was consistent with observations in HEK-293 cells transfected with GABA_BR1 and R2 subunits (39). Furthermore, baclofen can increase cell proliferation in a GABA_B receptor antagonist-sensitive manner in rat growth plate chondrocytes (19). These results suggest that activation of GABA_B receptor expressed in airway epithelial cells would theoretically favor airway epithelial cells growth through MAPK.

In addition to cyclic AMP, calcium is a second messenger important in the central functional role of airway epithelial cells; mucociliary clearance (40, 41). Intracellular calcium is increased by inositol phosphates after the activation of phospholipase C (PLC) by G_q-coupled receptors. However, evidence exists in multiple cell types that activated G_i α or G_i $\beta\gamma$ subunits can cross-activate PLC, thus increasing inositol phosphates and calcium (12–15). Therefore, we questioned whether GABA_B agonists could cross-talk through G_i to increase inositol phosphate levels. The GABA_B receptor agonist baclofen stimulated inositol phosphate synthesis, which was attenu-

Figure 7. Representative gel images of RT-PCR analysis of total RNA using primers specific for GAD65 and GAD67. (A) mRNA encoding GAD65 (488 bp) was detected in whole brain from both species and freshly isolated GP epithelium (Epi). (B) mRNA encoding GAD67 [375bp] was detected in whole brain from both species and in freshly isolated human epithelium and both human cultured epithelial cell lines (BEAS-2B and H441). Images are representative of at least three independent RT-PCR analyses from each cell or tissue source.

ated by pertussis toxin or an inhibitor of PLC demonstrating GABA_B receptor–G_i protein–PLC crosstalk in human airway epithelial cells.

GAD65/67 was also identified in airway epithelium at the mRNA and protein level. Human airway epithelium (BEAS-2B cells, H441 cells, and freshly dissected human airway epithelium) expressed mRNA encoding GAD67 but not GAD65. In contrast, guinea pig airway epithelium expressed mRNA encoding GAD65. Previously both GAD65 and GAD67 protein were identified in cultured BEAS-2B cells and primary cultures of human airway epithelial cells (1). Identification of both isoforms in BEAS-2B cells in this previous study as opposed to the detection of only the GAD67 isoform in the present study could reflect phenotypic regulation of expression of these isoforms in cell culture. These results suggest that airway epithelium could be the source of the endogenous ligand GABA for GABA_B receptors in airway.

The demonstration of the synthetic enzymes for GABA in the current and a previous study (1), and the expression of both

GABA_B and GABA_A (1) receptors in airway epithelial cells, suggests a previously unknown autocrine–paracrine GABAergic system exists in airway epithelial cells. The potential interplay between simultaneous activation of GABA_A and GABA_B receptors on the same epithelial cells is unknown. However, depolarization of membrane potential through GABA_A receptor activation was suggested to promote epithelial cell proliferation and mucus production (1). Similarly, activation of G_i through GABA_B receptor activation would be predicted to also favor proliferation after MAPK activation and decreased mucociliary clearance secondary to decreased cyclic AMP levels (30–34).

Further studies are required to identify the functional role of GABA_B receptors in the normal physiology and perhaps pathophysiology in airway epithelial cells. GABA may modulate an uncharacterized signaling cascade via GABA_B receptors expressed in airway epithelium. This signaling cascade could be a potential therapeutic target for modulation of airway epithelial cell functions.

Figure 8. Representative immunohistochemical staining of GAD65/67 in formalin-fixed GP trachea. (A) GAD65/67 immunostaining detected in tracheal epithelium (Epi). (B) Isotype-specific negative control primary antibody (rabbit IgG) in a parallel section of GP trachea. Both sections were counterstained with hematoxylin (original magnification: ×200). Images are representative of at least three independent immunohistochemical analyses from GP trachea.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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