Functional expression of the GABA_B receptor in human airway smooth muscle

Yoko Osawa,1 Dingbang Xu,1 David Sternberg,2 Joshua R. Sonett,2 Jeanine D’Armiento,3 Reynold A. Panettieri,4 and Charles W. Emala1

Departments of 1Anesthesiology, 2Surgery, and 3Medicine, College of Physicians and Surgeons of Columbia University, New York, New York, and 4Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

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Osawa, Yoko, Dingbang Xu, David Sternberg, Joshua R. Sonett, Jeanine D’Armiento, Reynold A. Panettieri, and Charles W. Emala. Functional expression of the GABA_B receptor in human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 291: L923–L931, 2006. First published July 7, 2006; doi:10.1152/ajplung.00185.2006.—GABA is a major inhibitory neurotransmitter in the mammalian central nervous system and exerts its actions via both ionotropic (GABA_A/GABA_C) and metabotropic (GABA_B) receptors. Postsynaptically, GABA_B receptors stimulate adenylyl cyclase via the Gi protein, known to impair relaxation of airway smooth muscle. Functional expression of GABA_B receptors in airway smooth muscle would therefore be expected to modulate airway responsiveness. However, the expression and functional coupling of GABA_B receptors to Gi in airway smooth muscle itself have never been described. We detected the mRNA encoding multiple-splice variants of the GABA_B1 and GABA_B2 in total RNA isolated from native human and guinea pig airway smooth muscle and from RNA isolated from cultured human airway smooth muscle (HASM) cells. Immunoblots identified the GABA_B1 and GABA_B2 proteins in HASM and native and cultured airway smooth muscle. The GABA_B1 protein was immunohistochemically localized to airway smooth muscle in guinea pig tracheal rings. Baclofen, a GABA_B receptor agonist, elicited a concentration-dependent stimulation of [35S]GTPγS binding in HASM homogenates that was abrogated by the GABA_B receptor antagonist CGP-35348. Baclofen also inhibited adenylyl cyclase activity and induced ERK phosphorylation in HASM. Another GABA_B receptor agonist, SKF-97541, mimicked while pertussis toxin blocked baclofen’s effect on ERK phosphorylation, implicating Gi protein coupling. Functional GABA_B receptors are expressed in HASM. GABA may modulate smooth muscle cells of the urinary bladder and uterus. However, the expression and functional coupling of GABA_B receptors to Gi in airway smooth muscle has never been described.

It is known that GABA_B-specific agonists decrease airway responsiveness to various bronchoconstricting agents by modulating presynaptic acetylcholine release from parasympathetic nerves (6, 28). On the other hand, a GABA_B receptor agonist, baclofen, can worsen airway responses following the administration of methacholine to asthmatic patients (7). This paradoxical enhancement by baclofen of airway responsiveness led us to hypothesize that there may be postsynaptic (i.e., smooth muscle) GABA_B functional receptors that couple to the Gi protein, known to impair relaxation of airway smooth muscle (3, 23).

In the present study, we investigated the expression of GABA_B receptors in native guinea pig, human airway smooth muscle (HASM), and cultured HASM cells, and assessed the functional coupling of the GABA_B receptor to the Gi protein by demonstrating adenylyl cyclase inhibition and ERK activation.

MATERIALS AND METHODS

Cells were cultured in SmGM-2 smooth muscle medium (Cambrex, Walkersville, MD). [35S]GTPγS (1,250 Ci/mmol) was obtained from Perkin Elmer (Boston, MA). [α-32P]ATP (800 Ci/mmol) and [3H]cAMP (32 Ci/mmol) were obtained from MP Biomedicals (Irvine, CA). Human brain protein was obtained from BD Biosciences (Palo Alto, CA). All other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

Cell culture. Primary cultures of HASM cells were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings as previously described (20). The cells were grown to confluence on 24- (adenylyl cyclase) or 6-well (immunoblotting) plates in culture medium (SmGM-2 supplemented with 5% FBS, 5 µg/ml insulin, 1 ng/ml human fibroblast growth factor, 500 pg/ml human epidermal growth factor, 30 µg/ml gentamicin, and 15 ng/ml amphotericin B).

Address for reprint requests and other correspondence: C. W. Emala, Dept. of Anesthesiology, College of Physicians and Surgeons of Columbia Univ., 630 W. 168th St., P&S Box 46, New York, NY 10032 (e-mail: cwe5@columbia.edu).

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tericin B, Cambrex) at 37°C in 5% CO₂-95% air. In all studies, cell culture media was not changed for 72 h (conditioned media) before the beginning of treatment with GABA<sub>B</sub> agonists.

For analysis of ERK phosphorylation, cells were treated with GABA<sub>B</sub> agonists (baclofen or SKF-97541) for 5 min in 72-h conditioned culture medium. Pertussis toxin (100 ng/ml) was preincubated for 4 h prior to the addition of GABA<sub>B</sub> 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 (Calbiochem, San Diego, CA), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF) was added. Thereafter, the whole cell lysates were sonicated four times on ice for 15 s, and the protein concentration was determined. Cell lysates were solubilized by heating at 95°C for 5 min 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 smooth muscle from human trachea and guinea pig trachea.** Studies were approved by Columbia University’s Institutional Review Board and deemed not human subjects research under 45 CFR 46. Human trachea came from nonasthmatic adults within 8 h of death. Additional tracheas were obtained from discarded regions of healthy donor lungs harvested for lung transplantation at Columbia University. Lung transplant excess tissue was transported to the laboratory in cold (4°C) Krebs-Henseleit buffer (in mM: 118 NaCl, 5.6 KCl, 0.5 CaCl<sub>2</sub>, 0.24 MgSO<sub>4</sub>, 1.3 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 5.6 glucose, pH 7.4). Frozen tracheas were thawed in cold Krebs-Henseleit buffer.

Adult male guinea pigs were deeply anesthetized by intra-peritoneal pentobarbital, the chest cavity was opened, and the animal was exsanguinated before the dissection of the trachea. The trachea was surgically removed and placed in cold (4°C) PBS.

The exterior of either guinea pig or human trachea was dissected free of adhering connective tissue under a dissecting microscope. Tracheae were then cut open longitudinally along the anterior border. The tracheal epithelium was removed, and the airway smooth muscle between the noncontiguous ends of the cartilaginous tracheal rings was dissected free and 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 (Calbiochem, San Diego, CA), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF) was added. Thereafter, the whole cell lysates were sonicated four times on ice for 15 s, and the protein concentration was determined. Cell lysates were solubilized by heating at 95°C for 5 min 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.

**RNA isolation and RT-PCR.** Total RNA was extracted from freshly dissected native guinea pig or HASM, cultured HASM cells, and guinea pig whole brain using TRI Reagent (Ambion, Austin, TX) according to the manufacturer’s recommendations. Total RNA from whole human brain was purchased from Clontech and used as a positive control. Using the Advantage RT-for-PCR Kit (Clontech, Mountain View, CA), 1 μg of total RNA was reverse transcribed at 42°C for 1 h 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 MgCl<sub>2</sub>).

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, Mountain View, CA), and 0.4 μM of both sense and antisense primers (Sigma) for corresponding GABA<sub>B</sub>R subunits including four splice variants of human GABA<sub>B</sub>R1 (Table 1, Fig. 1). For the detection of the GABA<sub>B</sub>R1b splice variant, 20% glycerol was added in the PCR reaction mixture because of a GC-rich sequence of GABA<sub>B</sub>R1b. Two-step PCR (annealing and extension at same temperature) was performed with a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA). PCR conditions for all reactions included an initial denaturation step at 94°C for 1 min, 40 cycles of a denaturation step at 94°C for 10 s, and an annealing/extension step at 72°C for 1 min except for PCR amplification of human GABA<sub>B</sub>R1e, which used an annealing/extension step at 70°C for 1 min, and for amplification of human GABA<sub>B</sub>R1a/c, which used an annealing/extension time of 2 min. PCR products were electrophoresed on 5% nondenaturing polyacrylamide gel in 1× Tris, aceta, EDTA buffer. The gel was stained with ethidium bromide (Molecular Probes, Eugene, OR), visualized using ultraviolet illumination, and analyzed using Quantity One software (BioRad, Hercules, CA).

**Immunoblotting.** Whole cell or tissue lysates were electrophoresed (8–10% SDS-PAGE) and immunoblotted using antibodies directed against GABA<sub>B</sub>R1 (rabbit 1:1,000, sc-14006; Santa Cruz Biotechnology, Santa Cruz, CA), GABA<sub>B</sub>R2 (rabbit 1:1,000, AB5848; Chemicon, Temecula, CA), and total or phospho-ERK (both rabbit 1:1,000, nos. 9102 and 9101, respectively; Cell Signaling Technology, Danvers, MA). Epitopes were visualized with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, 1:5,000, Santa Cruz Biotechnology) using ECL Plus (Amersham Biosciences) 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 are means ± SE.

**Immunohistochemistry.** Guinea pig tracheal rings were fixed using 10% formalin for 24 h at room temperature for GABA<sub>B</sub>R1 immunostaining and using 4% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 h at 4°C for GABA immunostaining. Tracheal rings were paraffin embedded, sectioned (5 μm), dewaxed in xylene, and rehydrated in a graded alcohol series to water. Endogenous peroxidase was blocked in 0.3% hydrogen peroxide. Heat-

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**Table 1. GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 primer sequences**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (from 5’ to 3’)</th>
<th>Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human R1ac</td>
<td>5’ CTG GCC AAC GCC TCC TGG ACA GAT ATG GAC</td>
<td>R1a: 1529</td>
</tr>
<tr>
<td></td>
<td>3’ AGT TTC TGT GAG AGG AAG CCG AAT GTC TGG AT</td>
<td>R1c: 1343</td>
</tr>
<tr>
<td>Human R1b</td>
<td>5’ GAT GGA GGC CCC TCC CCG GCA CCC TCT TA</td>
<td>272</td>
</tr>
<tr>
<td>Human R1e</td>
<td>5’ GCC CCC ATG GAC TAG ACT CCC CCC GCT TCT GA</td>
<td>321</td>
</tr>
<tr>
<td></td>
<td>3’ CCA GGG GGA AGA CAG CAG CTA AAG CCA GTG AG</td>
<td>R1a/b/e: 302</td>
</tr>
<tr>
<td>Human R2</td>
<td>5’ GGA CCT GGA TTC TCA CCG TGG GCT A</td>
<td>R1e: 150</td>
</tr>
<tr>
<td></td>
<td>3’ TGC GGC TGC CCG CTC GCT GTA</td>
<td>239</td>
</tr>
<tr>
<td>Guinea pig R1</td>
<td>5’ GCA AGA CAG TGG CCG AGG CCA GTG GTA</td>
<td>446</td>
</tr>
<tr>
<td>Guinea pig R2</td>
<td>3’ GCC CAG ACT AAA GCC CAG ACC CAG GAG CCA GA</td>
<td>348</td>
</tr>
</tbody>
</table>
mediated antigen retrieval was performed with 10 mM sodium citrate buffer, pH 6.0 for 30 min. An avidin biotin blocking kit (Vector Laboratories, Peterborough, UK) was used (in 10% serum in PBS) to block endogenous biotin. Slides were rinsed with PBS and incubated overnight at 4°C in primary antibody against GABA_B1 (rabbit, sc-14006, Santa Cruz Biotechnology) or GABA (mouse, MAB316, Chemicon) at a concentration of 1:250 or 1:50 in 2% serum in PBS, respectively. A tracheal ring section was incubated with the appropriate isotype IgG antibody as a negative control. A tracheal ring section was incubated with the appropriate antibody directed against human γ- and α-smooth muscle actin (1:10,000, mouse, MAB1522, Chemicon) to identify smooth muscle in trachea. Following overnight incubation at 4°C, slides were washed with PBS, and primary antibodies were detected using biotinylated anti-mouse or 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 (24). Briefly, confluent cultured HASM cells in 24-well plates were washed once with warm PBS (37°C). One hundred microliters of warm PBS were added to each well. Subsequently, 50 μl of 3x adenylyl cyclase buffer (13) with or without 100 μM bacaflon were 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 AMP, 7 mM MgCl2, 0.1 mM ATP (25 μCi/ml [α-32P]ATP), 0.1 mg/ml BSA, 50 U/ml creatine phosphokinase, and 7 mM phosphocreatine] in the presence of 10 μM forskolin, and plates were incubated at 37°C for 15 min. The reactions were terminated by the addition of 100 μl of stop buffer [50 mM HEPES, pH 7.5, 2 mM ATP, 0.5 mM cAMP (0.5 μCi/ml [3H]cAMP), 2% SDS], and newly synthesized [32P]cAMP was separated by sequential column chromatography over Dowex and alumina (24). Recovery of [3H]cAMP was used to correct for individual column recoveries, and radioactivity was quantitated by scintillation counting.

RESULTS

RT-PCR analysis of GABA_B1 isoforms in airway smooth muscle. RT-PCR analysis demonstrated mRNA encoding four splice variants of the human GABA_B1 protein (Fig. 2, A–C) and the GABA_B2 protein (Fig. 2D) in both native human tracheal smooth muscle and cultured HASM cells. Several splice variants of the GABA_B1 protein are expressed in human tissues, and we used primer sets designed to distinguish between each of these GABA_B1 variants. The expression of mRNA encoding the GABA_B1a and 1c variants was examined using a common primer set and was distinguishable by virtue of the omitted exons in GABA_B1c, resulting in a smaller PCR product for GABA_B1c (1,343 bp) compared to GABA_B1a (1,529 bp; Fig. 2A). We identified PCR products corresponding to the expected size of GABA_B1a and GABA_B1c in both native human tracheal airway smooth muscle and cultured HASM cells (Fig. 2A). Additionally, mRNA encoding the splice variant GABA_B1b was detected in both native HASM and cultured HASM cells (Fig. 2B). We then confirmed the existence of GABA_B1e using primers designed to flank the omitted exons of GABA_B1e and therefore could distinguish 1e from other isoforms (i.e., 1a, b, and c). We observed two PCR products of predicted sizes corresponding to splice variants GABA_B1a, b, and c (302 bp) and GABA_B1e (150 bp) in human trachea and cultured HASM cells (Fig. 2C). In human brain, used as a positive control, GABA_B1e was detected as a faint band, mRNA encoding GABA_B2 was also detected in native and cultured HASM cells (Fig. 2D). Guinea pig airway smooth muscle also expressed mRNA encoding both the GABA_B1 and GABA_B2 protein (Fig. 2E).

Immunoblot analysis of GABA_B1 isoforms in airway smooth muscle. The antibody used for immunoblot analysis of GABA_B1 recognizes the common COOH terminus region of three of the four known human isoforms of GABA_B1 (i.e., GABA_B1a, b, and c, but not GABA_B1e). Two immunoreactive bands of ~100 and 130 kDa were identified in freshly isolated human and guinea pig tracheal smooth muscle or...
whole brain from both species (Fig. 3A). Whereas the larger 130-kDa band corresponds to the expected molecular mass of GABA<sub>B</sub>R1a, the 100-kDa band corresponds to the molecular mass of either GABA<sub>B</sub>R1b or GABA<sub>B</sub>R1c. In contrast, only the larger immunoreactive band corresponding to the GABA<sub>B</sub>R1a protein was identified in cultured HASM cells (Fig. 3A). An antibody raised against the GABA<sub>B</sub>R2 protein identified a single immunoreactive band of ~110 kDa in protein samples from whole human brain and both native and cultured HASM (Fig. 3B), consistent with the predicted size for GABA<sub>B</sub>R2. This antibody was weakly reactive using a protein sample from whole guinea pig brain (data not shown), and thus this antibody was considered not sufficiently reactive with guinea pig protein samples to analyze guinea pig airway smooth muscle expression.

**Immunohistochemical analysis of GABA<sub>B</sub>R1 and GABA expression in guinea pig trachea.** To confirm the protein localization of GABA<sub>B</sub>R1 to airway smooth muscle in guinea pig airways, immunohistochemistry was performed. GABA<sub>B</sub>R1 immunoreactivity in guinea pig trachea was detected in airway smooth muscle, airway epithelium, and tracheal cartilage chondrocytes (Fig. 4A) with no staining in the negative control studies with rabbit IgG (Fig. 4B). The identity of the airway smooth muscle layer (and vascular smooth muscle) was con-
firmed using an anti-α-smooth muscle actin antibody (Fig. 4C), and no staining was seen with control mouse IgG2a (Fig. 4D).

Next, we examined whether the endogenous ligand for the GABA<sub>B</sub> receptor, GABA, was expressed in guinea pig airways. GABA was localized to epithelium, chondrocytes, and an area immediately adjacent to airway smooth muscle with limited staining in small punctuate areas within the airway smooth muscle itself (Fig. 5, A and C). No specific staining was apparent within these structures using a negative control, mouse IgG1 (Fig. 5, B and D).

**Functional coupling of the GABA<sub>B</sub> receptor to G<sub>i</sub> signaling pathways in HASM.** Demonstration of GABA<sub>B</sub> receptor mRNA and protein in airway smooth muscle led us to determine whether the receptor demonstrated classical coupling to the G<sub>i</sub> protein by evaluating 1) agonist-induced enhanced [35S]GTP<sub>S</sub> binding, 2) agonist-induced inhibition of adenylyl cyclase activity, and 3) pertussis toxin-sensitive, agonist-induced activation of mitogen-activated protein kinase (MAPK ERK).

**Agonist-enhanced [35S]GTP<sub>S</sub> binding in native HASM.** Baclofen, a specific GABA<sub>B</sub> agonist, elicited a concentration-dependent stimulation of [35S]GTP<sub>S</sub> binding with a 152 ± 6% increase above basal values ($P < 0.001, n = 3$) at 1 mM baclofen (Fig. 6A). A GABA<sub>B</sub> antagonist, CGP-34358 (1 mM), blocked baclofen (300 μM)-induced increases ($P < 0.001, n = 3$) in [35S]GTP<sub>S</sub> binding (Fig. 6B). These results suggest that GABA<sub>B</sub> receptors are functionally coupled to a G protein in HASM.

**Agonist-induced inhibition of adenylyl cyclase activity in human cultured airway smooth muscle cells.** GABA<sub>B</sub> receptor-mediated inhibition of adenylyl cyclase activity via coupling to

![Image](http://ajplung.physiology.org/)

Fig. 4. Representative immunohistochemical staining of GABA<sub>B</sub>R1 (A) and α-smooth muscle (SM) actin (C) in formalin-fixed GP trachea. The GABA<sub>B</sub>R1 was localized in tracheal epithelium (Epi), tracheal SM, and cartilage (CL), whereas α-SM actin was localized in tracheal and vascular smooth muscle. B: isotype controls for GABA<sub>B</sub>R1. D: isotype control for α-SM actin. All sections were counterstained with hematoxylin (original magnification, ×100).
the G\textsubscript{i} protein is well known in neurons (12). To examine whether baclofen inhibits the adenylyl cyclase activity in cultured HASM cells, we measured 10 \textmu M forskolin-stimulated adenylyl cyclase activity in the presence or absence of 100 \textmu M baclofen. Baclofen significantly inhibited forskolin-stimulated adenylyl cyclase activity (\(P < 0.01, n = 8\); Fig. 7).

**Agonist-induced activation of ERK in cultured HASM cells.** Many Gi-coupled receptors are known to activate the ERK pathway via G protein \(\beta\gamma\)-subunits (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 \textmu M, 5 min) significantly increased phosphorylation of ERK (Fig. 8, left; \(n = 3\), \(P < 0.05\)). In separate experiments, a second selective GABA\textsubscript{B} agonist, SKF-97541, also increased ERK phosphorylation (Fig. 8, right; \(P < 0.01, n = 3\)). Four-hour pretreatment of cells with pertussis toxin (100 ng/ml) abrogated increases in ERK phosphorylation by either GABA\textsubscript{B} agonist (\(P < 0.05\) and \(P < 0.001\) for pertussis toxin + agonist vs. baclofen or SKF-97541 alone, respectively, \(n = 3\)) confirming the role of G\textsubscript{i} in GABA\textsubscript{B} receptor activation of ERK.

**DISCUSSION**

The present study is the first to demonstrate that functional GABA\textsubscript{B} receptors are expressed in both native human and guinea pig airway smooth muscle and cultured HASM cells. Both GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 subunits were detected at the level of mRNA (by RT-PCR) and protein (by immunoblot) at appropriate molecular weights. Immunocytochemistry localized the GABA\textsubscript{B}R1 subunit to airway smooth muscle in guinea pig tracheal rings. The GABA\textsubscript{B} receptor agonist baclofen significantly increased GTP\textsubscript{Y}S binding (implying G protein coupling) in a manner sensitive to the GABA\textsubscript{B} receptor antagonist CGP-35348. Baclofen also inhibited adenylyl cyclase

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**Fig. 5.** Representative immunohistochemical staining of GABA (A and C) and isotype control (B and D) in paraformaldehyde-glutaraldehyde-fixed GP trachea. GABA was localized in Epi, chondrocytes (CL), and connective tissue near the SM. Limited positive staining was also found in ASM [original magnification, \(\times 100\) (A and B) and \(\times 200\) (C and D)].

**Fig. 6.** A: concentration-dependent stimulation of [\(^{35}\)S]GTP\textsubscript{Y}S binding by baclofen in native human tracheal smooth muscle. Tissue membranes (50 \mu g of protein) were incubated with the indicated concentrations of baclofen for 30 min. \(*P < 0.05, ***P < 0.001\) compared with control. B: effect of CGP-35348 (1 mM) on the stimulation of [\(^{35}\)S]GTP\textsubscript{Y}S binding by baclofen. Tissue membranes were incubated with 300 \textmu M baclofen in the presence or absence of 1 mM CGP-35348 for 30 min. Data are expressed as percentage of basal [\(^{35}\)S]GTP\textsubscript{Y}S binding and are the means \(\pm\) SE of 3 experiments. Each experimental value was determined in triplicate. ***\(P < 0.001\) compared with control. ###\(P < 0.001\) compared with baclofen. Bacl, baclofen; cont, control.
activity and induced ERK phosphorylation, which was abrogated by pretreatment of cultured HASM cells with pertussis toxin, confirming classical coupling of the GABAB receptor to G\(_i\) protein signaling pathways in these cells.

Several studies indicate the possible expression of functional GABAB receptors in peripheral and nonneuronal cells such as pancreatic beta cells (4), adrenocortical cells (18), cardiomyocytes (16), chondrocytes (27), and osteoblasts (9). Furthermore, GABABR1 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 (5). Although mRNAs encoding GABABR1a and 1b have been reported in RNA isolated from whole lung (5), the present study is the first to demonstrate the expression of both the GABABR1 and GABABR2 localized to airway smooth muscle.

Recent evidence has shown that GABAB receptors must exist as a heterodimer to form a functional Gi protein-coupled receptor in the plasma membrane (2). The presence of both subunits in airway smooth muscle cells suggests that they can assemble functional GABAB receptor heterodimers. We demonstrated that baclofen was effective in activation of G protein as well as inhibition of adenylyl cyclase, thus showing that functional GABAB receptors are expressed in HASM. To date, four different splice variants (GABABR1a, b, c, and e) of GABABR1 have been identified in the human. Indeed, we identified not only the expression of major splice variants (GABABR1a and 1b) but also minor variants 1c and 1e in HASM. The GABABR1e splice variant encodes a truncated protein lacking the transmembrane and intracellular domains and is known to be present in a variety of peripheral human tissues. At the present time, there are no confirmed GABABR2 splice variants (2). The GABABR1e is suggested to compete for heterodimerization with the GABABR2 subunit and affect the formation of functional GABAB receptors in a dominant negative manner (26). In the present study, a high concentration of baclofen was needed to achieve significant stimulation of \([\text{35S}]\text{GTP}\gamma\text{S}\) binding. This may be due to the low expression of GABAB receptors in airway smooth muscle relative to brain. However, the expression of the GABABR1e subunit in airway smooth muscle may decrease the number of functional GABAB receptors.

Although we identified mRNA encoding all four known splice variants of the GABABR1 subunit and the GABABR2 subunit in RNA isolated from freshly dissected human and guinea pig airway smooth muscle, RNA isolated from freshly dissected tissues invariably will contain some RNA from nonmuscle cells despite careful dissection. Therefore, to confirm the smooth muscle cell-specific expression of the GABABR1 and GABABR2 subunits, we analyzed RNA isolated from homogenous cultures of HASM cells and confirmed the expression of the same splice variants of GABABR1 and GABABR2 specifically in airway smooth muscle cells. Furthermore, immunohistochemistry in guinea pig tracheal rings localized the GABABR1 protein to the airway smooth muscle layer. In HASM, the GABABR1 antibody we used for immunoblot analysis reacted strongly and apparently nonspecifically with a 50-kDa protein making its use for immunocytochemis-
try in HASM unreliable. Interestingly, the GABABR1 protein was also abundantly expressed in tracheal epithelial cells and chondrocytes. Whereas the identification of the GABAA receptor on chondrocytes has been previously described (9, 27), the identification of the GABAB receptor on airway epithelium is novel and suggests that multiple cell types in the airway may be responsive to endogenous GABA.

The endogenous ligand for GABAB receptors, GABA, has also been identified in many peripheral tissues, especially in endocrine organs such as the pituitary, pancreas, testis, gastrointestinal tract, ovary, placenta, uterus, and adrenal medulla (10). Peripheral GABA has been suggested to act not only as a neurotransmitter or neuromodulator in the autonomic nervous system but also as a hormone or trophic factor in nonneuronal tissue (19). To identify the existence and localization of GABA in airway, we performed immunohistochemical analysis using a specific antibody against GABA in guinea pig trachea. In the present study, we detected GABA immunoreactivity in the connective tissue near the smooth muscle as well as tracheal epithelium and cartilage chondrocytes. This result suggests that GABA may bind to GABAB receptors in airway smooth muscle, although the origin of GABA in airway is still unclear.

After demonstrating the mRNA and protein expression of GABAB receptors in airway smooth muscle, we sought to confirm its coupling to G proteins in general and its specific classical coupling to the G1 protein. Activation of GTP binding is a standard measure of receptor coupling to heterotrimeric G proteins, and indeed the GABAB receptor agonist baclofen enhanced GTP binding in HASM. Two G-specific coupling pathways were investigated using the GABAB receptor agonist baclofen. Inhibition of adenylyl cyclase is a well-known effect of G1 protein activation and is known to occur in airway smooth muscle in response to activation of several G1-coupled receptors (e.g., M2 muscarinic receptor). Activation by phosphorylation of ERK is a ubiquitous signaling pathway following G1 activation, and pertussis toxin is a widely used tool to inactivate and implicate G1 proteins in signaling events. Indeed, baclofen inhibited adenylyl cyclase and activated ERK phosphorylation in a pertussis toxin-sensitive manner in HASM cells, confirming the coupling of GABAB receptors to G1 proteins in these cells. The specificity of baclofen’s effect at the GABAB receptor in the present study is supported by the finding that the antagonist CGP-35348 blocked the effect of baclofen in GTPγS binding and by the finding that another GABAB agonist, SKF-97541, could mimic the baclofen’s effect on ERK activation.

The physiological role of GABAB receptors in airway smooth muscle and of GABA-ergic modulation of intercellular cAMP or ERK activation is at present unclear. Because cAMP is known to induce relaxation of airway smooth muscle, an inhibitory effect of baclofen on adenylyl cyclase suggests that GABAB receptor activation in airway smooth muscle could inhibit cAMP-mediated relaxation. A well known example of a G1-coupled receptor that modulates airway smooth muscle relaxation is the M2 muscarinic receptor. The M2 muscarinic receptor couples to G1, inhibits adenylyl cyclase, and is known to inhibit β2-adrenoceptor-induced smooth muscle relaxation (25), whereas M2 muscarinic receptor antagonists are known to facilitate isoprenaline- and forskolin-mediated relaxation of acetylcholine-induced contraction of airway smooth muscle (8). These findings support the idea that the GABAB receptor could modulate airway smooth muscle tone via activation of the G1 protein. Consistent with this, GABAB receptors have been implicated in modulation of contractility in the rabbit uterus (22), where the receptors appear to be nonneuronal, and are most likely expressed in smooth muscle cells.

Baclofen can increase cell proliferation in a GABAB receptor antagonist-sensitive manner in rat growth plate chondrocytes (27). In the present study, we found that baclofen induced ERK phosphorylation. This result was consistent with observations in HEK-293 cells transfected with GABABR1 and R2 subunits (1). The requirement for ERK activation in HASM mitogenic signaling pathways has been well established (21). Since hyperplasia and hypertrophy of smooth muscle is considered to contribute to airway hyperresponsiveness in asthma (30), stimulation of GABAB receptors coupling to ERK activation would theoretically favor HASM cell proliferation and be associated with airway hyperresponsiveness. Further investigations are required to identify the physiological and possibly pathophysiological role of GABAB receptors in airway smooth muscle cells.

Although the source of the endogenous ligand GABA for GABAB receptors in airway is unclear at present, GABA may modulate an uncharacterized signaling cascade via GABAB receptors expressed in airway smooth muscle. This signaling cascade could be a target for new therapeutic interventions in controlling airway tone.

GRANTS

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REFERENCES