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$GABA_A$ receptors are expressed and facilitate relaxation in airway smooth muscle

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Abstract

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system and exerts its actions via both ionotropic ($GABA_A$) channels and metabotropic ($GABA_B$) receptors. $GABA_A$ channels are ubiquitously expressed in neuronal tissues, and in mature neurons modulate an inward chloride current resulting in neuronal inhibition due to membrane hyperpolarization. In airway smooth muscle (ASM) cells, membrane hyperpolarization favors smooth muscle relaxation. Although $GABA_A$ channels and $GABA_B$ receptors have been functionally identified on peripheral nerves in the lung, $GABA_A$ channels have never been identified on ASM itself. We detected the mRNA encoding of the $GABA_A$ α_4-, α_5-, β_3-, δ-, $\gamma_{1–3}$-, π-, and θ-subunits in total RNA isolated from native human and guinea pig ASM and from cultured human ASM cells. Selected immunoblots identified the $GABA_A$ α_4-, α_5-, β_3-, and γ_2-subunit proteins in native human and guinea pig ASM and cultured human ASM cells. The $GABA_A$ β_3-subunit protein was immunohistochemically localized to ASM in guinea pig tracheal rings. While muscimol, a specific $GABA_A$ channel agonist, did not affect the magnitude or the time to peak contractile effect of substance P, it directly concentration dependently relaxed a tachykinin-induced contraction in guinea pig tracheal rings, which was inhibited by the $GABA_A$-selective antagonist gabazine. Muscimol also relaxed a contraction induced by an alternative contractile agonist histamine. These results demonstrate that functional $GABA_A$ channels are expressed on ASM and suggest a novel therapeutic target for the relaxation of ASM in diseases such as asthma and chronic obstructive lung disease.

Keywords

RT-PCR; immunoblot; tachykinin; guinea pig; organ bath; histamine

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS). GABA acts at two distinct types of receptors, pentameric ligand-gated ionotropic $GABA_A$ channels and G protein-linked metabotropic ($GABA_B$)

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receptors. $GABA_A$ channels are ubiquitously expressed in neuronal tissues, and in mature neurons modulate an inward chloride current resulting in neuronal inhibition due to membrane hyperpolarization. $GABA_A$ channels are classically pentameric protein channels composed of combinations of subunit subtypes (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, θ), which dictate pharmacological and gating properties of this chloride channel in the mammalian brain. Although $GABA_A$ channels are ubiquitously expressed in the CNS, their expression in nonneuronal cells has received limited study. $GABA_A$ channels in the brainstem modulate cholinergic outflow to the lung (26, 37), and both $GABA_A$ channels and $GABA_B$ receptors have been pharmacologically identified on the presynaptic side of lung postganglionic parasympathetic nerves where they inhibit cholinergic nerve activity (14, 53, 55). Although we recently described the functional expression of $GABA_B$ receptors on airway smooth muscle cells (42), $GABA_A$ channels have not been previously identified on airway smooth muscle cells. Membrane hyperpolarization of the airway smooth muscle cell is known to reduce airway smooth muscle tone (45).

In the present study, we investigated the expression of $GABA_A$ channels in native guinea pig and human airway smooth muscle and cultured human airway smooth muscle cells and assessed the ability of a selective $GABA_A$ agonist to relax tachykinin- or histamine-induced airway smooth muscle contraction in guinea pig tracheal rings.

METHODS

Materials

Cells were cultured in SmGM-2 smooth muscle medium (Biowhittaker, Walkersville, MD). Lysates of human brain cerebral cortex or thalamus used as a positive protein control on immunoblots were obtained from BD Biosciences (Palo Alto, CA). Total RNA from whole human brain was purchased from Clontech (Mountain View, CA). Protease inhibitor cocktail III was purchased from EMD Biosciences (San Diego, CA). All other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

Cell culture

Primary cultures of human tracheal smooth muscle 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 (43). Cells were grown 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; Biowhittaker, Walkersville, MD) at 37°C in an atmosphere of 5% CO_2-95% air. For immunoblotting, cells were grown to confluency in T75 flasks. Cells were rinsed with cold PBS and scraped in cold PBS. Cells were pelleted (500 *g*, 10 min, 4°C), and cells lysed in 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 Na_3VO_4, 1 mM NaF]. Lysed cells were centrifuged (15,000 *g*, 15 min, 4°C), and the supernatant was subjected to protein analysis and then solubilized by heating at 95°C for 5 min in sample buffer (final concentrations: 50 mM Tris•HCl, pH6.8, 2.5% SDS, 6% glycerol, 2.5% 2-mercaptoethanol, bromophenol blue) and stored at −20°C.

Isolation of smooth muscle from human trachea and guinea pig for RNA and protein analysis

Studies were approved by Columbia University's Institutional Review Board and deemed not human subjects research under 45 CFR 46. Human trachea came from two sources. Snap-frozen tracheas obtained at autopsy from non-asthmatic adults within 8 h of death were

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obtained from the National Disease Research Exchange (Philadelphia, PA). Additional tracheae 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 on ice and submerged in cold (4°C) Krebs-Henseleit (KH) buffer (in mM: 118 NaCl, 5.6 KCl, 0.5 $CaCl_2$, 0.24 $MgSO_4$, 1.3 NaH_2PO_4, 25 $NaHCO_3$, 5.6 glucose, pH 7.4).

Adult male guinea pigs were deeply anesthetized by intraperitoneal pentobarbital (50 mg/kg), the chest cavity was opened, and the animal exsanguinated before the dissection of the trachea. The trachea or whole brain were surgically removed and placed in cold (4°C) KH buffer.

The exteriors of either the human or guinea pig trachea were meticulously dissected free of adherent connective tissue under a dissecting microscope. Tracheas were opened longitudinally along the anterior border, the epithelium was removed, and the airway smooth muscle between the non-contiguous ends of the cartilaginous tracheal rings was dissected free. Airway smooth muscle or whole brain was homogenized (Tekmar Ultra Turrax T25 high-speed homogenizer 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 [50 mM Tris, 10 mM HEPES, pH 7.4, 1 mM EDTA with a 1:200 dilution of protease inhibitor cocktail III (EMD Biosciences)] for total protein isolation. The homogenate was filtered through 125-μM Nitex mesh and centrifuged twice at 500 *g* for 15 min. The supernatant was transferred into new tubes and centrifuged at 50,000 *g* for 30 min at 4°C. The final membrane pellet was resuspended in the same buffer for protein concentration determinations and stored at −80°C.

RNA isolation and RT-PCR

Total RNA was extracted from freshly dissected native human or guinea pig airway smooth muscle, cultured human airway smooth muscle (HASM) cells, and guinea pig whole brain using TRIzol reagent (Ambion) according to the manufacturer's recommendations. Total RNA from whole human brain (Clontech, Mountain View, CA) 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 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, pH8.3, 75 mM KCl, 3 mM $MgCl_2$).

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 $GABA_A$ subunits (Tables 1 and 2). Two-step PCR (annealing and extension at same temperature) was performed for 1 min with a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA) for all PCR reactions at indicated temperatures (Tables 1 and 2) except for guinea pig $GABA_A$ θ for which annealing (60°C/30 s) and extension (70°C/30 s) conditions were used. PCR conditions for all reactions included an initial denaturation step at 94°C for 1 min followed by 40 cycles of denaturation (94°C for 10 s) and annealing/extension at indicated temperatures for 1 min. PCR products were electrophoresed on 5% nondenaturing polyacrylamide gel in 1×Tris, acetate, and 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).

Western blot analysis

Membrane lysates were electrophoresed (8–10% SDS-PAGE) and transferred to PVDF membranes. The PVDF membranes were blocked for 1 h at room temperature with 5%

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nonfat dry milk in TBS with 0.1% Tween 20 and were then probed with antibodies directed against $GABA_A$ α_4 (rabbit polyclonal 1:1,000; LS-C15, LifeSpan BioSciences, Seattle, WA), $GABA_A$ α_5 (rabbit polyclonal 1:500; GTX77692, Genetex, San Antonio, TX), $GABA_A$ $\beta_{2/3}$ (mouse monoclonal 1:250; MAB341, Chemicon, Temecula, CA), and $GABA_A$ γ_2 (rabbit polyclonal 1:1,000; AB5559, Chemicon) overnight at 4°C. After washing three times, membranes were incubated for 1 h at room temperature with HRP-conjugated secondary anti-rabbit antibodies (1:5,000; 81–6120, Zymed, South San Francisco, CA) or anti-mouse antibodies (1:5,000; NA931V, Amersham Biosciences, Arlington Heights, IL). 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).

Immunohistochemistry

Guinea pig tracheal rings were fixed with 10% formalin. 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 for 30 min at room temperature. Heat-mediated antigen retrieval was performed with 10 mM sodium citrate buffer, pH 6.0, for 30 min. Sections were blocked with 10% normal serum for 30 min. After washing with Tris-buffered saline solution with 0.1% Triton X-100 (TBST), an avidin biotin blocking kit (Vector Laboratories, Peterborough, UK) was used (in 10% serum in PBS) to block endogenous biotin. Slides were rinsed with TBST and incubated overnight at 4°C in primary antibody against $GABA_A$ $\beta_{2/3}$ (mouse, MAB341, Chemicon) at a concentration of 1:100 in 2% normal horse serum in TBST. A tracheal ring section was incubated with the appropriate isotype IgG (mouse IgG1) antibody as a negative control. Following overnight incubation at 4°C, slides were washed three times with TBST, and primary antibodies were detected using biotinylated anti-mouse antibodies (Vector Laboratories) at a concentration of 1:100. After incubation with ABC-HRP complex (Vector Laboratories) for 30 min, the antigen antibody complex was then visualized with the enzymatic reduction of 3,3-diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin, dried, and coverslipped using Polymount (Polysciences, Warrington, PA).

In vitro effects of $GABA_A$ agonist on guinea pig airway smooth muscle relaxation

All studies were approved by Columbia University's Institutional Animal Care and Use Committee. Force measurements were performed on closed guinea pig tracheal rings suspended in organ baths as previously described (30). Briefly, Hartley male guinea pigs (~400 g) were anesthetized with 50 mg/kg ip pentobarbital; the tracheas were removed promptly, dissected into closed rings composed of two cartilaginous rings from which mucosa, connective tissue, and epithelium were removed. Silk threads were tied to the rings such that the threads were at each end of the posterior aspect of the ring (lacking in cartilage), ~180° from one another. One thread was attached to a fixed point at the bottom of 4-ml organ baths (Radnoti Glass Technology, Monrovia, CA), and the opposing thread was attached to a Grass FT03 force transducer (Grass-Telefactor, West Warwick, RI) coupled to a computer via BioPac hardware and Acknowledge 7.3.3 software (Biopac Systems, Goleta, CA) for continuous digital recording of muscle tension. The rings were suspended in 4-ml organ baths filled to the top with 4 ml of KH buffer solution (composition in mM: 118 NaCl, 5.6 KCl, 0.5 $CaCl_2$, 0.2 $MgSO_4$, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 5.6 D-glucose) with 10 μM indomethacin (DMSO vehicle final concentration of 0.01%) added and gassed with 95% O_2 and 5% CO_2 at pH 7.4 at 37°C. The rings were equilibrated at 1 g of isotonic tension for 1 h with new KH buffer added every 15 min. Rings were pretreated with 10 μM capsaicin to induce contractions and deplete endogenous nonadrenergic, noncholinergic nerves of endogenous tachykinins. Following spontaneous relaxation (~20 min), baths and rings were

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washed with three buffer changes, resting tension was reset to 1 g, and all organ baths were treated with 1 μM tetrodotoxin (to block endogenous nerve activity). In experiments where substance P was the contractile agonist, the organ baths were also pretreated with 10 μM pyrilamine (to block potential effects of released histamine from resident mast cells in the preparation). In experiments where histamine was the contractile agonist, the organ baths were also pretreated with 1 μM atropine (to block histamine effects of stimulating cholinergic nerves). During the following contractile challenges, no further changes in buffer occurred, and the stated concentrations of substance P, histamine, muscimol, or gabazine represent the final concentrations of each reagent in the buffer within the organ baths.

Rings were then contracted with 1 μM substance P. Following the achievement of a stable contraction (typically 10 min), cumulatively increasing concentrations of the $GABA_A$-selective agonist muscimol (0.1 μM–1 mM) were incrementally added to the buffer in the baths at 7-min intervals. Control-contracted rings received vehicle (KH buffer) to serve as time controls for the muscimol-induced relaxation. In separate experiments, rings were pretreated with 100 μM gabazine ($GABA_A$-selective antagonist) 15 min before the addition of 1 μM substance P. Following the achievement of a stable contraction, a single concentration of the $GABA_A$-selective agonist muscimol (100 μM) was added to assess effects on airway smooth muscle force.

In additional separate experiments, guinea pig tracheal rings were contracted with 10 μM histamine. Following the achievement of a stable contraction (typically 10 min), muscimol (100 μM) was added to the organ baths. Control-contracted rings received vehicle (KH buffer) to serve as time controls for the muscimol-induced relaxation.

Statistical analysis

Statistical analysis was performed using repeated measures of ANOVA, followed by Bonferroni posttest comparison using Prism 4.0 software (GraphPad, San Diego, CA). Data are presented as means ± SE; $P < 0.05$ was considered significant.

RESULTS

RT-PCR analysis of $GABA_A$ channel subunits in airway smooth muscle

Initially, we assessed the expression of mRNA encoding $GABA_A$ channel subunits in at least three independent samples each of freshly isolated human and guinea pig airway smooth muscle and in primary cultures of human airway smooth muscle cells. Total RNA from whole brain was used as positive controls in each species. Freshly dissected native human and guinea pig airway smooth muscle expressed mRNA encoding multiple $GABA_A$ channel subunits (α_4, α_5, β_3, γ_2, γ_3, δ, π, and θ) (Figs. 1 and 2). Multiple other subunits (α_1, α_2, α_3, α_6, β_1, β_2, γ_1, ε) were not detected in freshly dissected airway smooth muscle from either human or guinea pig upper airways despite their detection in control brain RNA from both species (Figs. 1 and 2). Primary cultures of human airway smooth muscle cells expressed similar but not identical patterns of $GABA_A$ subunits. $GABA_A$ α_4-, α_5-, β_3-, γ_2-, δ-, and θ-subunits were also detected in cultured human airway smooth muscle, but $GABA_A$ γ_3 and π were not detected (Fig. 1, *D*, *E*, *I*, *K*, *P*). Moreover, $GABA_A$ γ_1, which was not detected in freshly isolated airway smooth muscle, was detected in primary cultures of airway smooth muscle (Fig. 1*J*). Additional semiquantitative experiments were performed for those subunits detected in fresh but not cultured human airway smooth muscle to determine if their mRNA detection was due to mRNA contamination from a minor cell type other than airway smooth muscle in these freshly dissected samples. RNA isolated from

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human brain and freshly dissected human airway smooth muscle contained similar quantities of mRNA encoding $GABA_A$ γ_3 (Fig. 1*M*) and $GABA_A$ π (Fig. 1*R*).

Two splice variants of the human $GABA_A$ α_4- and four splice variants of the human $GABA_A$ γ_2-subunit proteins are expressed in human tissues (28) (Fig. 3), and we used primer sets designed to distinguish between the two α_4 variants or to distinguish the γ_2L variant from the other three γ_2 variants (γ_2S/γ_2XL/γ_2 variant 3) (Figs. 1 and 3 and Table 1). We detected variants 1 and 2 of the human $GABA_A$ α_4-subunit in both freshly isolated and cultured human airway smooth muscle (Fig. 1*D*). Two variants of the $GABA_A$ γ_2-subunit were detected in both human (Fig. 1*K*) and guinea pig (Fig. 2*E*) freshly dissected airway smooth muscle, and the larger variant 1 (γ_2L) of the $GABA_A$ γ_2-subunit was detected in primary cultures of human airway smooth muscle (Fig. 1*K*).

Immunoblot analysis of $GABA_A$ channel subunits in airway smooth muscle

Lysates prepared from freshly dissected native human airway smooth muscle, freshly dissected native guinea pig airway smooth muscle, and cultured human airway smooth muscle cells were subjected to immunoblot analysis using selected specific anti-$GABA_A$ subunit antibodies (Fig. 4). Immunoreactive bands of appropriate molecular mass for $GABA_A$ α_4-, α_5-, β_3-, and γ_2-subunits were identified in airway smooth muscle. The antibody used to detect $GABA_A$ α_4 is capable of identifying two splice variants of α_4. A single band of the appropriate molecular mass for $GABA_A$ α_4 (~64 kDa) was identified in freshly isolated airway smooth muscle from human and guinea pig, primary cultures of human airway smooth muscle cells, and in guinea pig whole brain (Fig. 4*A*). The band for $GABA_A$ α_4 detected in cultured HASM cells migrated at a slightly lower-molecular-mass than the other samples, suggesting the possibility that a smaller splice variant was predominantly expressed in HASM. A single band of the appropriate molecular mass for $GABA_A$ α_5 (~55 kDa) was identified in whole human brain and freshly isolated human airway smooth muscle and in a separate series of experiments in human thalamus and primary cultures of human airway smooth muscle cells (Fig. 4*B*). The antibody used to detect $GABA_A$ $\beta_{2/3}$ identified a protein with an appropriate molecular mass of ~54 kDa in airway smooth muscle freshly dissected from human or guinea pig tracheal smooth muscle and primary cultures of human airway smooth muscle cells. In guinea pig brain, an additional protein band of slightly lower-molecular-mass was also identified, as has been shown previously in brain using different anti-$GABA_A$ β_3 antibodies (51, 56) including the antibody used in the present study (mouse monoclonal BD17) (20) (Fig. 4*C*). The antibody used to detect $GABA_A$ γ_2 recognizes the cytosolic loop region of two splice variants of $GABA_A$ γ_2. We resolved two immunoreactive bands of ~45 and ~47 kDa in whole human brain cortex and cultured human airway smooth muscle cells (Fig. 4*D*). In contrast, a single band corresponding to ~47 kDa was identified in freshly dissected airway smooth muscle from human and guinea pig trachea. Whereas the larger 47-kDa band corresponds to the expected molecular mass of $GABA_A$ γ_2L, the 45-kDa band could represent splice variants of $GABA_A$ γ_2S (25).

Immunohistochemical detection of $GABA_A$ β_3-subunit expression in guinea pig airway smooth muscle

To confirm the localization of $GABA_A$ protein to airway smooth muscle, immunohistochemistry was performed using a monoclonal antibody that recognizes both the $GABA_A$ β_2- and $GABA_A$ β3-subunit proteins in paraffin sections of guinea pig tracheal rings. Specific immunoreactivity was detected in the airway smooth muscle layer of the guinea pig trachea (indicated by brown color) with no staining in the negative control sections using a isotype-specific mouse IgG_1 control primary antibody (Fig. 5). Higher

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magnifications (×100 oil objective) revealed homogenous staining along muscle cells consistent with cell surface expression (Fig. 5, *bottom left*).

Functional studies of $GABA_A$ receptor function in intact guinea pig airway rings

Molecular identification of multiple subunits of $GABA_A$ receptors led us to question whether functional $GABA_A$ channels could modulate airway smooth muscle tone. Guinea pig tracheal rings suspended in organ baths and pretreated with capsaicin, tetrodotoxin, and pyrilamine demonstrated muscimol-induced relaxation in a concentration-dependent manner (0.1–100 μM) of a substance P-induced (1 μM) contraction (Fig. 6). Under identical conditions, the pretreatment of rings with the $GABA_A$ antagonist gabazine (100 μM) significantly attenuated the relaxation induced by 100 μM muscimol (Fig. 7). The pretreatment of guinea pig tracheal rings with 100 μM gabazine did not influence either the rate at which peak contraction with substance P was achieved (672 ± 30 s vs. 654 ± 43 s, in the absence or presence of gabazine pretreatment, respectively, $n = 6$) or the absolute magnitude of the peak contraction achieved with substance P (0.73 ± 0.10 g vs. 0.68 ± 0.11, in the absence or presence of gabazine pretreatment, respectively, $n = 6$).

In separate studies, the ability of muscimol to relax a different contractile agonist was assessed in guinea pig rings. Following a stable increase in muscle force achieved with 10 μM histamine, 100 μM muscimol significantly relaxed smooth muscle force in tracheal rings measured at 10 min (Fig. 8).

DISCUSSION

The primary findings of the present study include the first demonstration of $GABA_A$ subunit mRNA and protein expression in human and guinea pig airway smooth muscle cells and the ability of a $GABA_A$-selective agonist to relax a tachykinin- or histamine-induced contraction in airway smooth muscle.

GABA receptors include both ionotropic ($GABA_A$ channels) as well as metabotropic ($GABA_B$) receptors. We have recently demonstrated the expression and functional coupling of $GABA_B$ receptors to the G_i protein in human airway smooth muscle cells (42). The $GABA_A$ receptors are pentameric ligand-gated ion channels that conduct chloride currents, which in mature neurons results in hyperpolarization of the cell membrane impeding the effect of depolarizing (i.e., stimulatory) signals. $GABA_A$ receptors are classically pentamers composed of combinations of subunit subtypes (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, θ) that dictate pharmacological and gating properties of this chloride channel in the mammalian brain (8, 29, 34, 35). This diversity is further extended by splice variants for multiple subunit subtypes (6, 32). Despite the enormous combinational possibilities, most $GABA_A$ receptors in the CNS are composed of α-, β-, and γ-subunits in a 2:2:1 ratio (4, 12).

GABA receptors are ubiquitously expressed in the central nervous system, and the modulation of neuronal activity by GABA has been extensively studied. GABA receptors are also expressed in the peripheral nervous system where they also serve an inhibitory function (10, 57). In contrast, the native expression of GABA receptors in nonneuronal cells has received limited study. Initial attempts to survey the expression of GABA receptors outside the central nervous system relied on RT-PCR analysis of RNA isolated from whole peripheral organs (1, 9, 17, 41). Although these studies suggested ubiquitous expression of many GABA receptor subunits, it is unknown what specific cellular components of these tissues were expressing GABA receptor subunits. It is possible that these studies identified GABA receptor subunits expressed in peripheral nerves contained within these organs. More specific expression of GABA receptor subunits were identified in neuroendocrine cells including pancreatic β (54, 59), pituitary (48), and adrenal cells (44). Indirect

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pharmacological evidence suggested GABA receptor expression in vascular (22), bladder (19), uterine (2, 17, 21), and gut (47) smooth muscle in addition to the identification of GABA receptors in the peripheral nerves that innervate these tissues (5, 15, 46, 50). The expression of GABA receptors in these smooth muscles of gut, bladder, vascular, and uterine smooth muscle was inferred from pharmacological responses as opposed to a direct molecular identification of GABA receptors within the smooth muscle (2, 17, 19, 21, 22, 39, 47). Subsequently, subunits of GABA receptors have been demonstrated in heart (16), uterus (27, 40), kidney (49), liver (18, 52), and fibroblasts (24).

$GABA_A$ channels in the brainstem modulate cholinergic outflow to the lung (26, 37), and both $GABA_A$ channels and $GABA_B$ receptors have been pharmacologically identified on the presynaptic side of lung postganglionic parasympathetic nerves where they inhibit cholinergic nerve activity (14, 53, 55). Conversely, GABA receptors have never been described in airway smooth muscle itself. It has been known for some time that $GABA_B$-specific agents decrease electrically field-stimulated airway constriction by modulating acetylcholine release from parasympathetic nerves (13, 14, 33, 53, 55). This is mediated by a presynaptic inhibition of acetylcholine release by $GABA_B$ receptors.

Previous studies have discounted a role for GABA receptors in the direct modulation of airway smooth muscle function, but these studies have typically been performed with GABA rather than a $GABA_A$-selective agonist (14, 53, 55). Simultaneous activation of smooth muscle $GABA_A$ and $GABA_B$ receptors may not allow for relaxation effects of $GABA_A$ receptors to be elucidated. Two previous studies have failed to show an effect of the $GABA_A$-selective agonist muscimol on basal tone (14, 53, 53) or acetylcholine-induced contraction (14) in guinea pig airway rings. However, these studies were limited to a single contractile agonist (acetylcholine) and more importantly did not address the potential ability of muscimol to relax contracted tissue as opposed to muscimol's ability to impair an initial contraction.

In neural tissues, activation of the $GABA_A$ receptor in mature neurons results in an inward chloride current and membrane hyperpolarization. In airway smooth muscle cells, membrane hyperpolarization is one mechanism that contributes to decreased intracellular calcium and decreased muscle tone (31). Hyperpolarization of the airway smooth muscle cell membrane by $GABA_A$-mediated chloride entry would be expected to contribute to a decrease in airway tone by reducing intracellular calcium, reducing the Ca^{2+}/calmodulin-dependent activation of myosin light chain (MLC) kinase, resulting in reduced phosphorylation of MLC, reduced interactions of actin and myosin, and less muscle contraction.

The current study demonstrates that airway smooth muscle cells express a limited repertoire of $GABA_A$ subunits and that there is remarkable agreement in subunit expression between the species studied (guinea pig and human). Identification of mRNA in freshly isolated human or guinea pig airway smooth muscle ensures that native expression of subunits is being examined. However, even with careful dissection, freshly isolated tissue is contaminated by other cell types that could give rise to cDNA products during sensitive RT-PCR amplification. Therefore, in all studies, we also evaluated the expression of $GABA_A$ subunits in established primary cultures of human airway smooth muscle cells that are homogenous populations of airway smooth muscle cells. Despite their cellular purity, cultured cell systems can result in an altered phenotype of protein expression. Despite these limitations, there was remarkable agreement but some differences between the expression of $GABA_A$ subunits in freshly isolated vs. cultured human airway smooth muscle. We detected mRNA encoding the α_4-, α_5-, β_3-, γ_2-, δ-, and θ-subunits in all sources of airway smooth muscle studied, including human and guinea pig freshly isolated airway smooth muscle and

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from cultured human airway smooth muscle cells. Additionally, multiple mRNAs encoding splice variants of the $GABA_A$ α_4-subunit were detected in freshly isolated vs. cultured airway smooth muscle.

In contrast, the expression of the γ_1-, γ_3-, and π-subunits differed between freshly isolated and cultured airway smooth muscle. The $GABA_A$ γ_3- and θ-subunits were expressed in freshly isolated airway smooth muscle from both human and guinea pig, but their expression did not persist in cultured human airway smooth muscle cells. One interpretation of these findings could be that the detection of these subunits was as a result of amplification from a nonmuscle cell type in the dissected tissue. To evaluate this possibility, equal amounts of total RNA from human brain (an abundant source of $GABA_A$ γ_3 and θ mRNA) and freshly dissected human airway smooth muscle were analyzed in parallel during increasing numbers of PCR cycles. If the $GABA_A$ γ_3 or θ cDNA products were arising from a minor cellular contaminant in the airway smooth muscle dissection, it would be expected that the cDNA product would be very small relative to the cDNA product arising from human brain where $GABA_A$ γ_3 and θ mRNA expression is extensive. This was done to make a relative, not an absolute, comparison between mRNA expression in brain vs. airway smooth muscle. Indeed, the relative band intensity of the cDNA product was equivalent from freshly dissected human airway smooth muscle and the brain control, suggesting that the mRNA expression arising from freshly dissected human and guinea pig airway smooth muscle was truly arising from the predominant cell type in the dissected tissue, smooth muscle. The decreased expression of the $GABA_A$ γ_3- and θ-subunits likely represents a phenotypic switch of these particular subunits during cell culture. In contrast, the $GABA_A$ γ_1-subunit was not identified in freshly isolated guinea pig or human airway smooth muscle but was readily detected in cultured airway smooth muscle cells. This raises the interesting possibility that there is a phenotypic switch from the $GABA_A$ γ_3- to the $GABA_A$ γ_1-subunit in cultured human airway smooth muscle cells, the functional consequence of which is unknown.

Identification of mRNA encoding multiple $GABA_A$ subunits in airway smooth muscle led us to investigate $GABA_A$ subunit protein expression by immunoblotting and immunohistochemistry where suitable antibodies were available. In agreement with our mRNA analysis, the $GABA_A$ α_4 protein was identified in airway smooth muscle from human and guinea pig airway smooth muscle, cultured airway smooth muscle cells, and the positive control guinea pig brain. The $GABA_A$ α_5 protein was identified in freshly dissected and primary cultures of human airway smooth muscle and in human brain thalamus controls. In addition to a protein with an appropriate molecular mass of 54 kDa, the antibody used to detect $GABA_A$ $\beta_{2/3}$ also identified a protein band of slightly lower molecular mass. Similar results with multiple different anti-$GABA_A$ β_3 antibodies (51, 56), including the antibody used in the present study (mouse monoclonal BD17) (20), have been obtained in brain yet the identity of this smaller immunoreactive band is unknown (20, 51, 56). The antibody used to detect $GABA_A$ γ_2 recognizes the cytosolic loop region of two splice variants of $GABA_A$ γ_2. The long splice variant (γ_2L) differs from the short isoform (γ_2S) by the presence of the eight-amino acid insert LLRMFSFK, which bears the PKC phosphorylation site, Ser343 (36, 38, 58). We resolved two immunoreactive bands of ~45 and ~47 kDa in whole human brain cortex and cultured human airway smooth muscle cells. In contrast, a single band corresponding to ~47 kDa was identified in freshly dissected airway smooth muscle from human and guinea pig trachea. Whereas the larger 47-kDa band corresponds to the expected molecular mass of $GABA_A$ γ_2L, the 45-kDa band could represent different splice variants of $GABA_A$ γ_2S (25, 28). The smooth muscle-specific expression of $GABA_A$ channels was further confirmed by the immunohistochemical detection of the $GABA_A$ β_2/β_3-subunit in guinea pig airway smooth muscle.

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The molecular identification of $GABA_A$ subunits in airway smooth muscle cells led us to question whether these subunits formed functional channels. $GABA_A$ subunits in neuronal cells are activated by their endogenous ligand GABA as well as by synthetic selective ligands such as muscimol or 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-01 (THIP). Activation of neuronal $GABA_A$ channels in mature neurons results in an inward chloride current and hyperpolarization of the cell membrane. Hyperpolarization of the plasma membrane in airway smooth muscle cells is a component of airway muscle relaxation and is believed to be a partial mechanism by which classic relaxant agonists (i.e., β-adrenoceptor agonists) mediate their effects. Therefore, we questioned whether a $GABA_A$ agonist could potentiate the relaxation of intact airway smooth muscle. The spontaneous relaxation of tachykinin-contracted guinea pig airway smooth muscle was potentiated by the $GABA_A$-selective agonist muscimol in a concentration-dependent manner, and this effect was attenuated by the $GABA_A$-selective antagonist gabazine. However, 100 μM gabazine pretreatment did not completely block the effect of 100 μM muscimol, and there may be multiple reasons for this. It is possible that targets other than the $GABA_A$ receptor are involved in the muscimol effect; however, to date, no other nonspecific targets of muscimol have been identified in any tissue. Second, the efficiency with which gabazine penetrates tracheal rings suspended in organ baths is unknown, and its stability over time and the relative potency of gabazine (100 μM) to that of muscimol (100 μM) is unknown in this tissue. Moreover, there is evidence that gabazine may have selectivity for tonic but not phasic $GABA_A$ receptor activation (3, 7). Indeed, in this manuscript, we have identified the expression of the subunits in airway smooth muscle known to be required for tonic $GABA_A$ inhibition [α_4 and δ in the thalamus (11) as well as α_5 and δ in the cerebellum (23)] as well as subunits classically associated with phasic $GABA_A$ activation. Therefore, it is possible that our partial blockade by gabazine represents gabazine blockade of a subset of channels in our tissue that modulate tonic but not those that modulate phasic $GABA_A$ activity. This hypothesis will require further studies.

In summary, we demonstrate for the first time the molecular expression of multiple $GABA_A$ subunits in human and guinea pig airway smooth muscle. We demonstrate that a selective $GABA_A$ agonist can relax intact airway smooth muscle contracted with two different contractile agonists (substance P or histamine). These studies suggest a novel pharmacological target for the relaxation of airway smooth muscle.

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GRANTS

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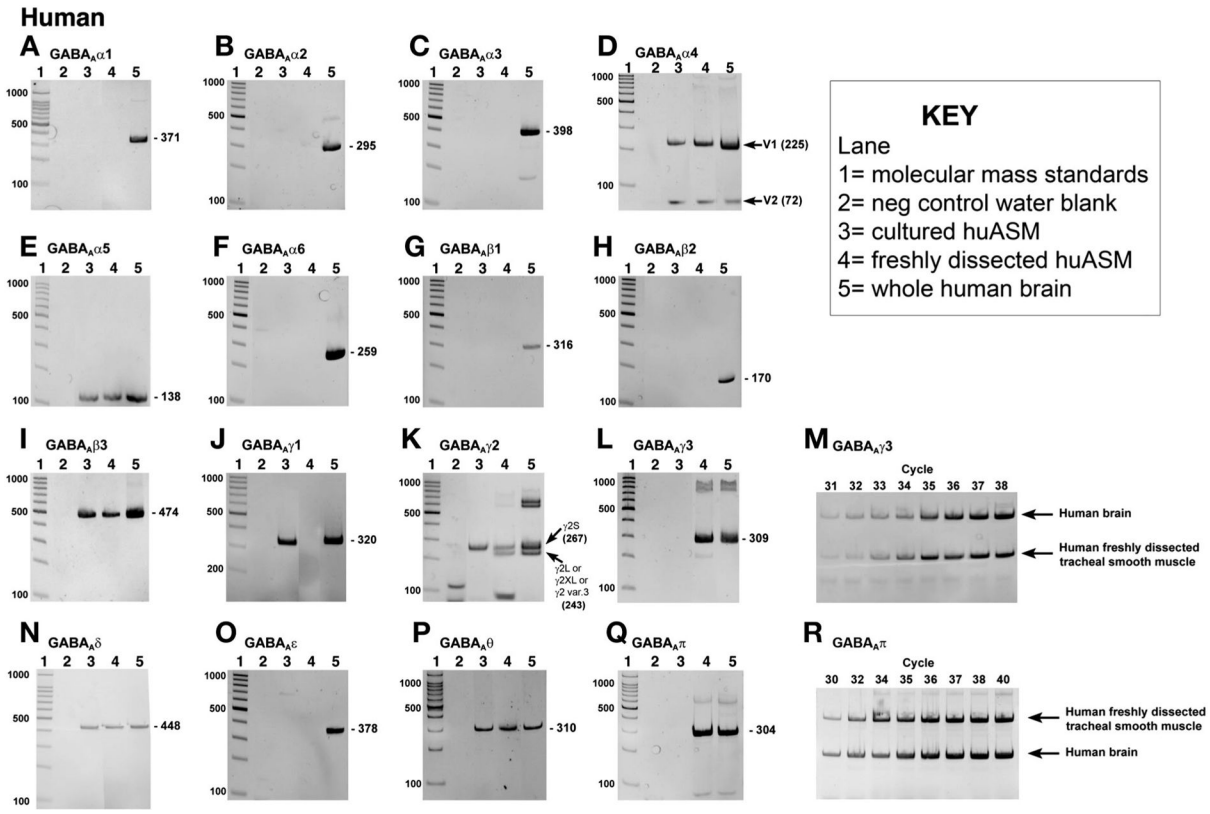

Fig. 1.

Representative gel images of RT-PCR analysis of total RNA using primers specific for each known $GABA_A$ channel subunit. Total RNA extracted from freshly dissected human tracheal airway smooth muscle or primary cultures of human airway smooth muscle (huASM) cells was analyzed. For 2 subunits (γ_3 and π), relative amounts of cDNA products from freshly dissected human airway smooth muscle or human brain were compared at increasing cycles of PCR amplification [31–38 (*M*) and 30–40 (*R*) cycles, respectively]. *Lanes 1*: bp standards; *lanes 2*: negative control water blanks; *lanes 3*: total RNA from primary cultured human airway smooth muscle cells; *lanes 4*: total RNA from freshly dissected human tracheal airway smooth muscle; *lanes 5*: total RNA from whole human brain. Messenger RNA encoding $GABA_A$ α_5, β_3, δ, and θ (*E*, *I*, *N*, and *P*) and 2 splice variants (V) for $GABA_A$ α_4 (*D*) and at least 2 splice variants for γ_2 (*K*) (also see Fig. 3) were detected in fresh and cultured human airway smooth muscle. $GABA_A$ γ_3 and π (*L* and *Q*) were detected in fresh human airway smooth muscle, and $GABA_A$ γ_1 (*J*) was detected in cultured human airway smooth muscle cells.

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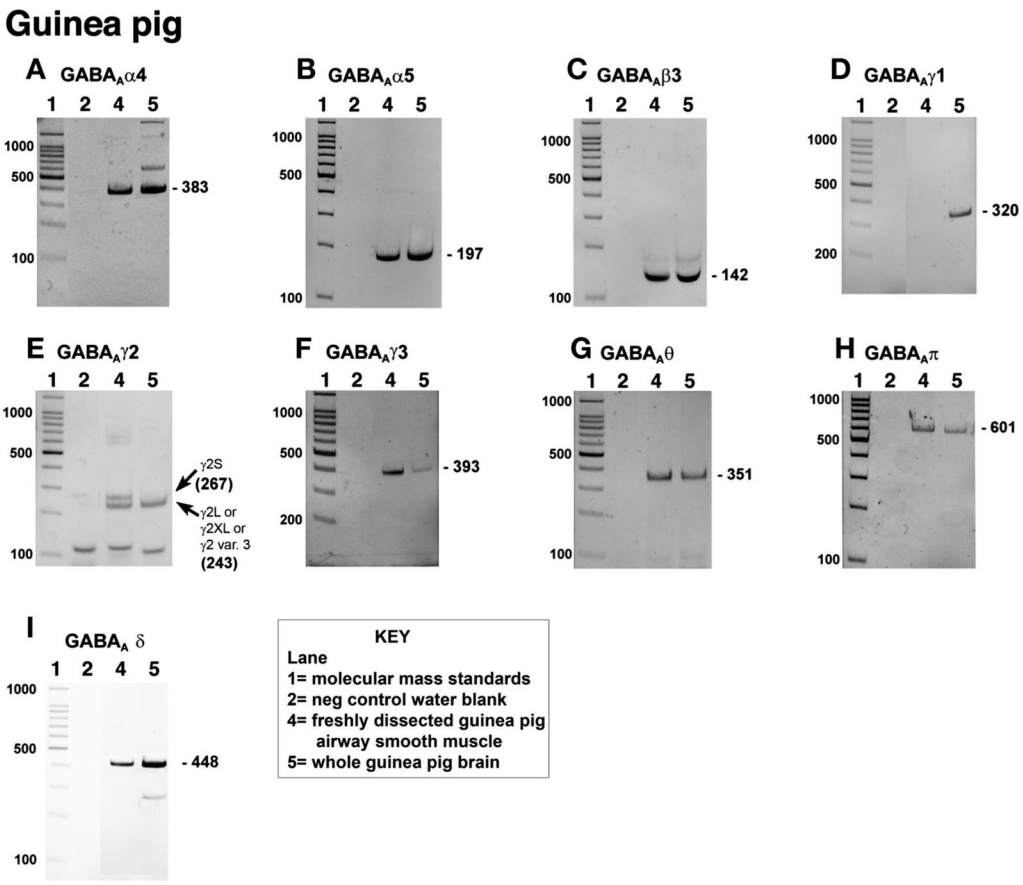

Fig. 2.

Representative gel images of RT-PCR analysis of total RNA using primers specific for selected $GABA_A$ channel subunits using total RNA extracted from freshly dissected guinea pig tracheal airway smooth muscle. *Lanes 1*: bp standards; *lanes 2*: negative control water blanks; *lanes 4*: total RNA from freshly dissected guinea pig tracheal airway smooth muscle; *lanes 5*: total RNA from whole guinea pig brain. The label "*lane 3*" was omitted in the labeling of the panels to maintain parallel labeling with Fig. 1. Messenger RNA encoding $GABA_A$ α_4, α_5, β_3, γ_2, γ_3, δ, θ, and π (*A–C*, *E–H*) were detected in freshly dissected guinea pig airway smooth muscle.

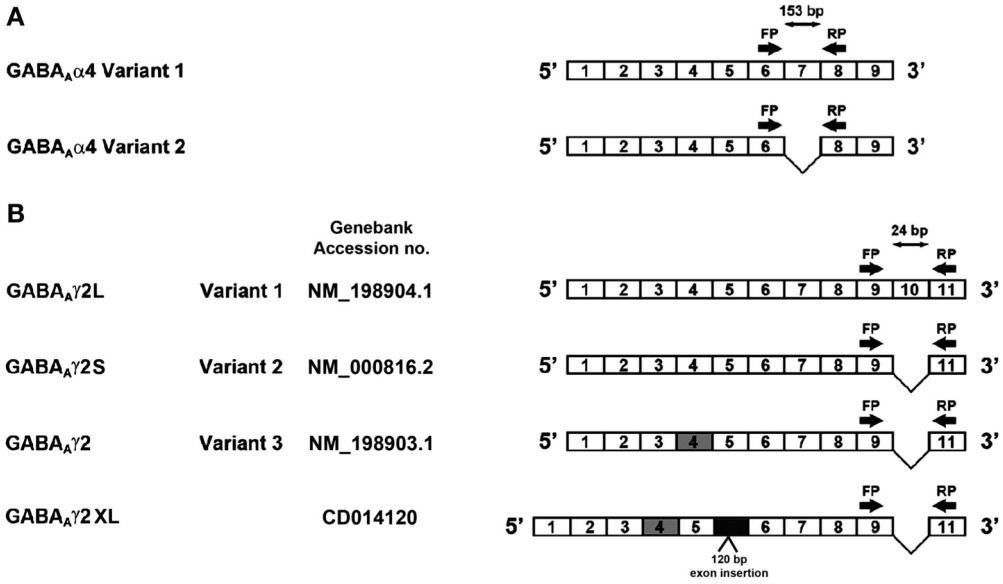

Fig. 3.

Diagram of PCR primer design for human GABA$_A$ α_4 and γ_2 variants. *A*: identical forward primers (FP) and reverse primers (RP) (Table 1) for GABA$_A$ α_4 yield PCR products of different sizes (225 and 72 bp, respectively) due to annealing to regions of exons 6 and 8 and the absence of exon 7 in variant 2. *B*: identical forward primers (FP) and reverse primers (RP) (Table 1) for GABA$_A$ γ_2 yield PCR products of different sizes (267 and 243 bp, respectively) due to annealing to regions of exons 9 and 11 and the absence of exon 10 in GABA$_A$ γ_2S, GABA$_A$ γ_2XL, and GABA$_A$ γ_2 variant 3. Four splice variants are known for human GABA$_A$ γ_2, and primers used distinguish γ_2S from the other 3 variants. GABA$_A$ γ_2XL has 120-bp insert encoding 40 amino acids between previously numbered exons 5 and 6 (28). Exon numbers are indicated by numbered boxes. Exon 4 in GABA$_A$ γ_2 variant 3 is shaded to indicate its sequence differences from GABA$_A$ γ_2 variants 1 and 2.

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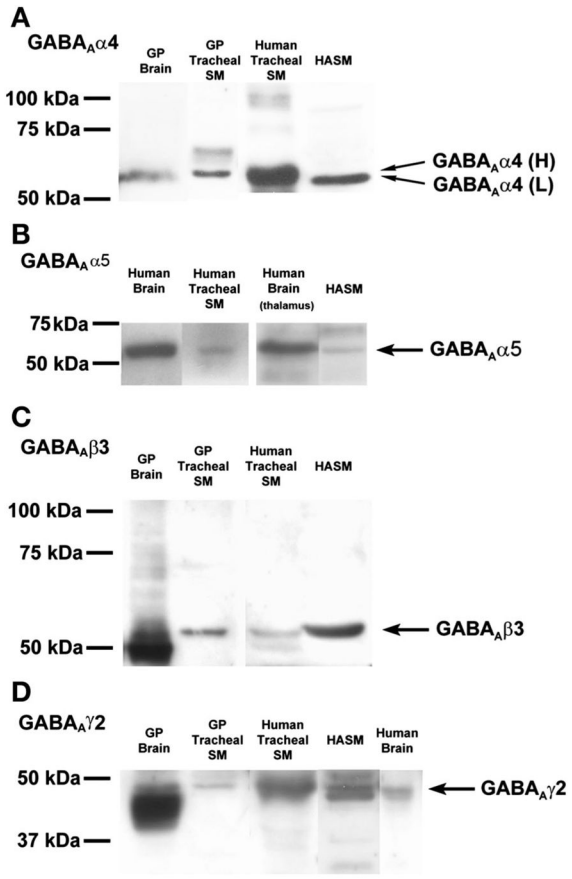

Fig. 4.

Representative immunoblot analyses using antibodies against $GABA_A$ subunits α_4, α_5, β_3, and γ_2 using protein prepared from cultured human airway smooth muscle (HASM) cells, human tracheal airway smooth muscle (SM), guinea pig (GP) tracheal SM, whole human or GP brain, or human thalamus. SM freshly dissected from human trachea (100 μg), primary cultures of HASM cells (100 μg), human or GP whole brain (25 μg), human thalamus (25 μg), or SM freshly dissected from GP trachea (50 μg) were analyzed. *A*: $GABA_A$ α_4 was detected in GP brain, fresh GP and human airway SM, and primary cultures of HASM cells. *B*: $GABA_A$ α_5 was detected in whole human brain, freshly dissected HASM, human thalamus, and primary cultures of HASM cells. *C*: $GABA_A$ β_3 is detected in GP brain, GP and human freshly dissected airway smooth muscle, and primary cultures of HASM cells. *D*: 2 closely resolving immunoreactive bands were identified in human brain and in cultured HASM cells representing $GABA_A$ γ_2L and $GABA_A$ γ_2S. A single band representing $GABA_A$ γ_2L was identified in freshly isolated GP and HASM.

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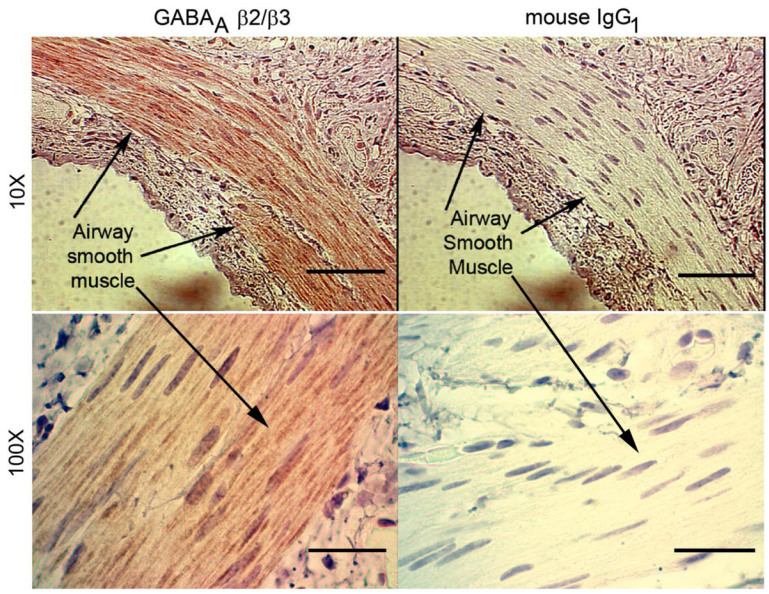

Fig. 5.

Representative immunohistochemical staining of $GABA_A$ channel β_3-subunit in formalin-fixed guinea pig trachea. *Left*: heavy immunoreactivity (brown stain) is localized to the airway smooth muscle layer of the guinea pig trachea. *Right*: anti-mouse IgG_1 isotype negative control in serial section of guinea pig trachea. *Bottom left*: immunohistochemical staining is distributed along airway smooth muscle cells. Bars at *top* represent 100 μm; bars at *bottom* represent 10 μm. All sections were counterstained with hematoxylin.

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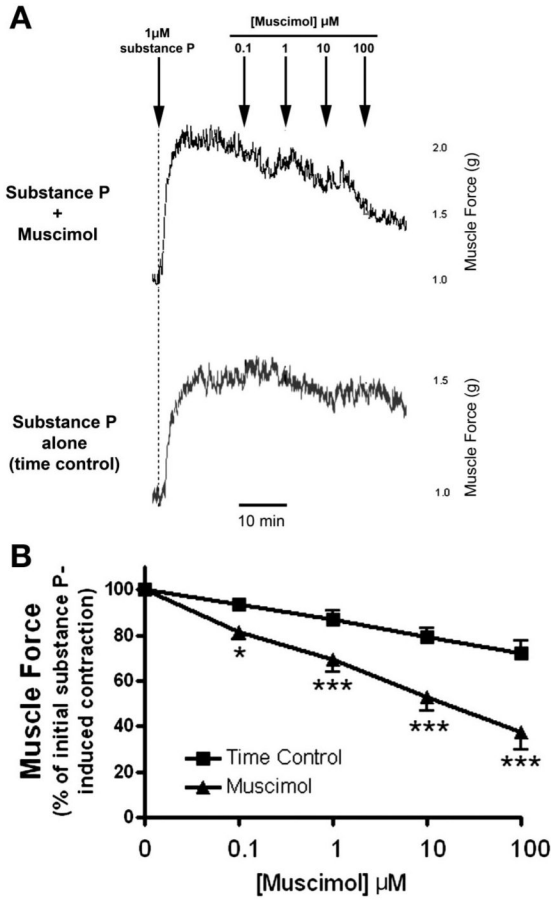

Fig. 6.

The $GABA_A$ agonist muscimol, in a concentration-dependent manner, relaxes substance P-induced contractions in guinea pig tracheal rings. *A*: representative tension tracing in guinea pig tracheal ring illustrating relaxation of substance P (1 μM) contraction by the $GABA_A$-selective agonist muscimol (0.1–100 μM). *B*: muscimol (0.1–100 μM) significantly relaxed a substance P-induced contraction. Means ± SE. $*P < 0.05$ and $***P < 0.001$ compared with control, $n = 8$.

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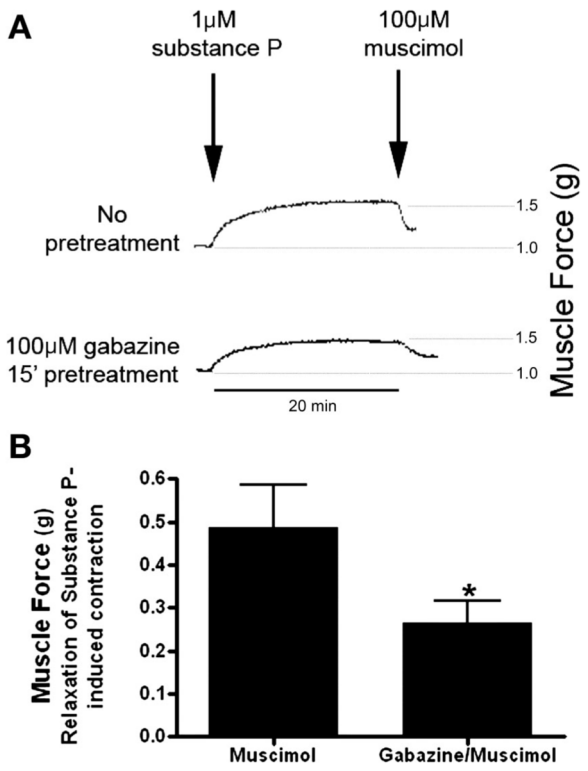

Fig. 7.

A: representative tension tracing in guinea pig tracheal ring illustrating relaxation of substance P (1 μM) contraction by the $GABA_A$-selective agonist muscimol (100 μM) and attenuation of relaxation by pretreatment with the $GABA_A$-selective antagonist gabazine (100 μM). *B*: pretreatment with the $GABA_A$-selective antagonist gabazine (100 μM) significantly attenuates the relaxation induced by muscimol (100 μM). $*P < 0.05$, $n = 6$.

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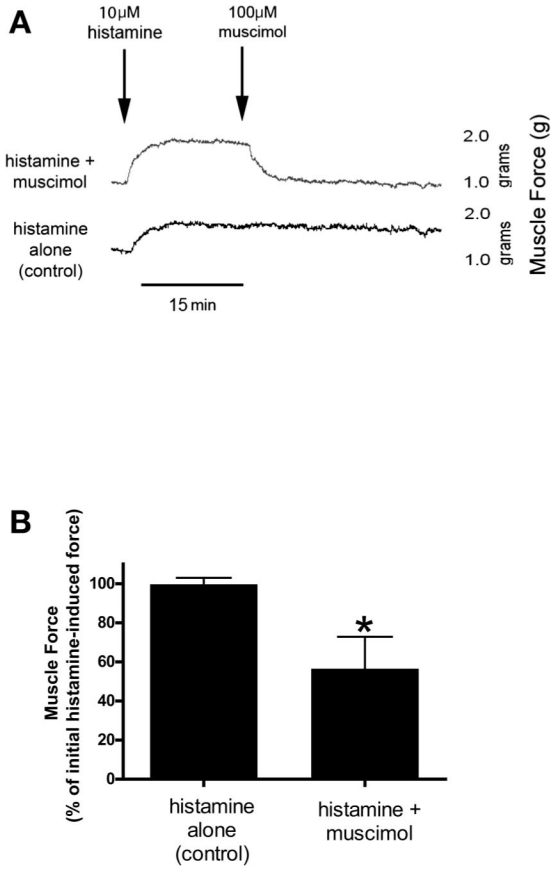

Fig. 8.

The $GABA_A$ agonist muscimol (100 μM) relaxes histamine-induced contractions in guinea pig tracheal rings. *A*: representative tension tracing in guinea pig tracheal ring illustrating relaxation of histamine (10 μM) contraction by the $GABA_A$-selective agonist muscimol (100 μM). *B*: percent of initial muscle force induced by 10 μM histamine 10 min after the addition of 100 μM muscimol in guinea pig tracheal rings. $*P < 0.05$ compared with control, $n = 6$.

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Table 1

Primer sequences for human $GABA_A$ channel subunits

Primers (human) Target	GenBank Access No.	Sequence of Primer	Amplicon Size, bp	Annealing/ Extension (°C) (total 1 min)
$GABA_A$ α_1	NM_000806	FP: 5′-GAAGAGAAAGATTGGCTACTTTGTTATTCAAACAT-3′ RP: 5′-GAGCGTAAGTGTTGTTTTTCTTAATAAGAGGAT-3′	371	68/68
$GABA_A$ α_2	NM_000807	FP: 5′-TCTGCCCTAATTGAATTTGCAACTGTTAATTACTT-3′ RP: 5′-CTATTCTGGACATTCTGTCAATTTTGCTAACACTG-3′	295	70/70
$GABA_A$ α_3	NM_000808	FP: 5′-CACTTCCATCTCAAGCGAAAAATTGGCTACTTTGT-3′ RP: 5′-CCCACGATGTTGAAGGTAGTGCTGGTTTTCT-3′	398	70/70
$GABA_A$ α_4	NM_000809	FP: 5′-CAAACCGTATCAAGTGAAACCATCAAATCAAT-3′ RP: 5′-GCTTAGTGTGGTCATGGTGAGGACAGTTGTTAT-3′	variant 1: 225 variant 2: 72	70/70
$GABA_A$ α_5	NM_000810	FP: 5′-GCAGACGGTGGGCACTGAGAACA-3′ RP: 5′-GATAAGATCACGGTCATTATGCAGGGAAGGTA-3′	138	68/68
$GABA_A$ α_6	NM_000811	FP: 5′-CAGTGACAATATCAAAAGCTACTGAACCTTTGGAA-3′ RP: 5′-AATCCTGCAAATGCAACTGGGAAGAGAA-3′	259	70/70
$GABA_A$ β_1	X14767	FP: 5′-TCGCACTAGGAATCACGACGGTGCTTA-3′ RP: 5′-GAGCCACTCGTCTCATTCCGGATTT-3′	316	70/70
$GABA_A$ β_2	S77553	FP: 5′-GCTGCCAGTGCCAACAATGAGAAGA-3′ RP: 5′-TGGGGGTCCATCGTATACAGAGAGAAA-3′	170	70/70
$GABA_A$ β_3	M82919	FP: 5′-TCACAACTGTGCTGACAATGACAACCATCAAC-3′ RP: 5′-TAATTTTGAGCTGTGAAGACCTCCTCCGTAGA-3′	474	72/72
$GABA_A$ γ_1	NM_173536	FP: 5′-CTTTCCCATGGATGAACATTCCTGTCCACTGGAATTTT-3′ RP: 5′-CAGGCACTGCATCTTTATTGATCCAAAAAGACACCC-3′	320	70/70
$GABA_A$ γ_2	NM_198904 (variant 1) NM_000816 (variant 2) NM_198903 (variant 3) CD014120	FP: 5′-AGGTCTCCTATGTCACAGCGATGGATCTCT-3′ RP: 5′-GACACTCATAGCCGTACTCTTCATCTCTCTCT-3′	γ2L: 267 γ2S: 243 γ2 variant 3: 243 γ2XL: 243	70/70
$GABA_A$ γ_3	NM_033223	FP: 5′-CGCTGATGGAGTATGCCACCCTCAACTACTATT-3′ RP: 5′-GCCCTTTCCTCCAGGATCCTGATTTACATTCT-3′	309	70/70
$GABA_A$ δ	NM_000815	FP: 5′-GCAGTTCACCATCACCAGCTACCGCTTCAC-3′ RP: 5′-GACAATGGCGTTCCTCACGTCCATCTCT-3′	448	70/70
$GABA_A$ ε	NM_004961	FP: 5′-CACATGCTCAGATTTCCAATGGATTCTCACTCTT-3′ RP: 5′-CAACGTGGTCATGGTCAGAACAGAGGTGAT-3′	378	70/70
$GABA_A$ θ	NM_018558	FP: 5′-GGGAAGGACGATTACTAGCAAGGAGGTGTATT-3′ RP: 5′-CACAAAGAACAAGCACACGAGGATATAGATATCAA-3′	310	72/72
$GABA_A$ π	NM_014211	FP: 5′-GAACCTGCATTGGAGTGACGACCGTGTTA-3′ RP: 5′-CTGGAAATTTCAATGCTGGCAAAGCTGATCT-3′	304	72/72

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Table 2

Primer sequences for guinea pig $GABA_A$ channel subunits

Primers (guinea pig) Target	Sequence of Primer	Amplicon Size, bp	Annealing/ Extension (°C) (total 1 min)
$GABA_A\ \alpha_4$	FP: 5′-GTTTGCTCGATGGTTATGACAACAGGCTGCGT-3′ RP: 5′-CATGGGAAAATCCACCAATCTCATGGGACACT-3′	383	70/70
$GABA_A\ \alpha_5$	FP: 5′-TCGGCCCGGTGTCAGACACAGAGATGGAATAC-3′ RP: 5′-GGGTGTGGTCATGTTGTGCGCGATAGACTTCTT-3′	197	70/70
$GABA_A\ \beta_3$	FP: 5′-ATGTACCTCATGGGCTGCTTCGTCTTCGTGTTC-3′ RP: 5′-TTGAACGGTCATTCTTTGCCTTGGCTGTCT-3′	142	70/70
$GABA_A\ \gamma_1$	FP: 5′-CTTTCCCATGGATGAACATTCCTGTCCACTGGAATTTT-3′ RP: 5′-CAGGCACTGCATCTTTATTGATCCAAAAAGACACCC-3′	320	70/70
$GABA_A\ \gamma_2$	FP: 5′-AGGTCTCCTATGTCACAGCGATGGATCTCT-3′ RP: 5′-GACACTCATAGCCGTACTCTTCATCTCTCTCT-3′	γ2L: 267 γ2S: 243 γ2 variant 3: 243 γ2XL: 243	66/66
$GABA_A\ \gamma_3$	FP: 5′-GACCTGGACAGATAGTCGCCTTCGATTCAACAGCACAA-3′ RP: 5′-TCTGTGGTGTTTCTGAGGCCCATGAAGTCAAACTGATA-3′	393	70/70
$GABA_A\ \delta$	FP: 5′-GCAGTTCACCATCACCAGCTACCGCTTCAC-3′ RP: 5′-GACAATGGCGTTCCTCACGTCCATCTCT-3′	448	70/70
$GABA_A\ \theta$	FP: 5′-GCTTGTTCCCTGGATCTGCATAAATTCCCTATGGAC-3′ RP: 5′-GTCACCCTGGCTGCAGAGGAATCATAGTTCATCCAA-3′	353	60/70 (30 s each)
$GABA_A\ \pi$	FP: 5′-GGAAACACGCTCATCCGCCTCTTCTCCAA-3′ RP: 5′-CCATCTGCTGTAACGAACTGTAGTGAGCAACTGCATA-3′	601	65/65