Endogenous γ-Aminobutyric Acid Modulates Tonic Guinea Pig Airway Tone and Propofol-induced Airway Smooth Muscle Relaxation

George Gallos, M.D.,* Neil R. Gleason, M.D.,† Laszlo Virag, Ph.D.,‡ Yi Zhang, M.D.,‡ Kentaro Mizuta, D.D.S., Ph.D.,§ Robert A. Whittington, M.D.,|| Charles W. Emala, M.D.||

Background: Emerging evidence indicates that an endogenous autocrine/paracrine system involving γ-aminobutyric acid (GABA) is present in airways. GABA_A channels, GABA_B receptors, and the enzyme that synthesizes GABA have been identified in airway epithelium and smooth muscle. However, the endogenous ligand itself, GABA, has not been measured in airway tissues. The authors sought to demonstrate that GABA is released in response to contractile agonists and tonically contributes a prorelaxant component to contracted airway smooth muscle.

Methods: The amount and cellular localization of GABA in upper guinea pig airways under resting and contracted tone was determined by high pressure liquid chromatography and immunohistochemistry, respectively. The contribution that endogenous GABA imparts on the maintenance of airway smooth muscle acetylcholine-induced contraction was assessed in intact guinea pig airway tracheal rings using selective GABA_A antagonist (gabazine) under resting or acetylcholine-contracted conditions. The ability of an allosteric agent (propofol) to relax a substance P–induced relaxation in an endogenous GABA-dependent manner was assessed.

Results: GABA levels increased and localized to airway smooth muscle after contractile stimuli in guinea pig upper airways. Acetylcholine-contracted guinea pig tracheal rings exhibited an increase in contracted force upon addition of the GABA_A antagonist gabazine that was subsequently reversed by the addition of the GABA_A agonist muscimol. Propofol dose-dependently relaxed a substance P contraction that was blocked by gabazine.

Conclusion: These studies demonstrate that GABA is endogenously present and increases after contractile stimuli in guinea pig upper airways and that endogenous GABA contributes a tonic prorelaxant component in the maintenance of airway smooth muscle tone.

ASTHMA is a chronic inflammatory disease of the airways that predisposes patients to episodes of severe, acute airway constriction. Despite an increasing worldwide prevalence of asthma,1 new pharmacologic approaches to treat this disease are limited. Although a great deal is known regarding the mechanisms governing airway smooth muscle contraction,2 relatively less is known about mechanisms of airway smooth muscle relaxation. Therefore, greater insights into the mechanisms of endogenous control of airway smooth muscle relaxation are required to identify novel therapeutic strategies.

Interestingly, both volatile and intravenous anesthetics have long been recognized as potent bronchodilators,3–15 yet the exact mechanisms for this effect are incompletely understood and have been attributed to both neural5–7 and direct airway smooth muscle effects.8–10 Despite the well known effect of anesthetics (e.g., propofol) as allosteric potentiators of the action of endogenous γ-aminobutyric acid (GABA) at neuronal GABA_A channels,11 it has been a longstanding belief that any GABAergic contribution to airway tone was largely mediated by GABA_A channels in the brainstem12 or by GABA_B receptors on preganglionic cholinergic nerves in the lung.13,14 Our recent identification of GABA_A channels expressed directly on airway smooth muscle that contribute to relaxation15 raises the novel possibility that a previously unrecognized mechanism of anesthetic mediated airway smooth muscle relaxation could be anesthetics allosterically potentiating the effect of endogenous GABA at airway smooth muscle GABA_A channels. In addition to GABA_A channels, GABA_B receptors and glutamic decarboxylase (the enzyme responsible for GABA synthesis) have also recently been identified in airway epithelial16,17 and smooth muscle cells,18 suggesting that endogenous GABA may have autocrine/paracrine functions in airways. Despite the presence of a complex GABAergic system in the airway, measurements of the endogenous ligand GABA or a tonic effect on airway tone by endogenous GABA has not yet been demonstrated.

Taken together, the expression of airway smooth muscle GABA_A receptors facilitating relaxation and the clinical benefit of anesthetics on hyperreactive airway tone led us to question (1) whether endogenous GABA is present in the airway, (2) whether contractile agonists increase airway GABA release (3) whether liberated GABA tonically modulates airway smooth muscle tone, and (4) whether airway smooth muscle GABA_A channels mediate a component of anesthetic (i.e., propofol)-induced airway smooth muscle relaxation. Defining and
harnessing this novel relaxation pathway may identify new therapeutic options for hyperreactive airway disease.

**Materials and Methods**

*Reagents*

Indomethacin, N-vanillylisononamide (capsaicin analogue), pyrilamine, acetylcholine, γ-aminobutyric acid, o-phenaldialdehyde (OPA), 2-mercaptoethanol (βME), and gabazine were obtained from Sigma (St. Louis, MO). Propofol was obtained from ICN Biomedicals and diluted in dimethyl sulfoxide (DMSO) (Aurora, OH). Tetrodotoxin was obtained from Calbiochem (San Diego, CA).

**Guinea Pig Tracheal Rings**

All animal protocols were approved by the Columbia University Animal Care and Use Committee (New York, New York). Male Hartley guinea pigs (approximately 400 g) were deeply anesthetized with intraperitoneal pentobarbital (100 mg/kg). After opening the chest cavity, the entire trachea was surgically removed and promptly placed in cold (4°C) phosphate-buffered saline (PBS). Each trachea was dissected under a dissecting microscope into closed rings composed of two cartilaginous segments from which mucosa and connective tissue were removed. Epithelium was left intact for high pressure liquid chromatography (HPLC) and immunohistochemistry studies but removed for organ bath experiments. Tissues were placed into cold Krebs-Henseleit (KH) 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) containing 10 μM indomethacin (DMSO vehicle final concentration in organ baths of 0.01%) to block tone due to endogenous release of prostanoids.

**High Pressure Liquid Chromatography**

To demonstrate that GABA is endogenously present in airways, we used intact guinea pig tracheal ring segments (2 rings per sample) harvested from euthanized guinea pigs. All sample tissues were separately incubated in a 500-μl volume of KH buffer (pH 7.4) on ice for 20 min followed by a 500-μl KH buffer wash. Samples were separated into 3 treatment groups (no treatment, acetylcholine 10 μM and β-alanyl NKA fragment 4–10 for 15 min. Tracheal rings were immediately fixed using 4% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 h at 4°C for GABA immunostaining as previously described. To illustrate the localization patterns of endogenous GABA under basal (resting) and procontractile stimulated conditions, guinea pig tracheal rings were harvested as above and were treated with or without 10 μM β-alanyl NKA fragment 4–10 for 15 min. Tracheal rings were immediately fixed using 4% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 h at 4°C for GABA immunostaining as previously described.

**Immunohistochemistry**

To illustrate the localization patterns of endogenous GABA under basal (resting) and procontractile stimulated conditions, guinea pig tracheal rings were harvested as above and were treated with or without 10 μM β-alanyl NKA fragment 4–10 for 15 min. Tracheal rings were immediately fixed using 4% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 h at 4°C for GABA immunostaining as previously described. Briefly, 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-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, United Kingdom) was used (10% serum in PBS) to block endogenous biotin. Slides were rinsed with PBS and incubated overnight at 4°C in primary antibody against GABA (mouse, MAB316; Chemicon, Temecula, CA) at a concentration of 1:50 in 2% serum in PBS. Tracheal ring sections were also incubated with the same concentration of a mouse isotype IgG antibody (IgGₐ) (as a negative control) or with primary antibody directed against α-smooth muscle actin (mouse, MAB1522; Chemicon, Temecula, CA) at a concentration of 1:10,000 to identify smooth muscle (positive control). After overnight incubation at 4°C, slides were washed with PBS, and primary antibodies were detected using biotinylated anti-mouse antibodies (Vector Laboratories) at a concentration of 1:100. The antigen antibody complex was then visualized by enzymatic reduction of 3,3-diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin and dried, and cover slides were mounted using Polymount (Polysciences, Warrington, PA).
Organ Baths

Closed guinea pig tracheal rings were suspended in organ baths as previously described. Briefly, tissues were attached with silk thread inferiorly to a fixed tissue hook in a water-jacketed (37°C) 2-ml organ bath (Radnoti Glass Technology, Inc., Monrovia, CA) and superiorly to a Grass FT03 force transducer (Grass Telefactor, West Warwick, RI) coupled to a computer via BioPac hardware and Acqknowledge 7.3.3 software (Biopac Systems, Inc., Goleta, CA) for continuous digital recording of muscle force. Tissues were secured such that muscle contraction would align with the vertical plane between the anchoring hook below and transducer above. KH buffer was continuously bubbled with 95% oxygen and 5% carbon dioxide, and tissues were allowed to equilibrate at 1g isotonic force for 1 h with fresh KH buffer changes every 15 min.

Preliminary Contractile Challenges

After equilibration, the capsaicin analog N-vanillyl-norannamide (10 μM final) was added to the organ baths containing guinea pig tracheal rings to first activate and then deplete nonadrenergic, noncholinergic nerves. After N-vanillyl-norannamide-induced force had returned to baseline (50 min), the KH buffer in the organ baths were changed 6 times to wash out added or liberated mediators. Tracheal rings were then subjected to two cycles of contracting cumulative concentrations of acetylcholine (0.1 μM to 1 mM) with 6 buffer changes and resetting of the resting tension between cycles. The resulting concentration response curves were then used to determine the EC50 concentrations of acetylcholine required for each individual ring. Individual tissues have variable sensitivity to contractile agonists (EC50) and variation in the magnitude of contraction (Emax). To avoid bias between treatment groups, tissues were contracted to individually calculated EC40M for acetylcholine and tissues with similar Emax values were randomly assigned to treatments within individual experiments. After preliminary contractile challenges, tissues were subjected to extensive KH buffer changes (8–9 times) and allowed to stabilize at their respective isotonic resting tensions (1.0 g). To remove confounding effects of other procontractile pathways, each airway ring received a complement of antagonists 20 min before subsequent contractile challenge. The antagonists included pyrilamine (10 μM, H1 histamine receptor antagonist), and tetrodotoxin (1 μM; sodium channel blocker negating endogenous neuronal-mediated cholinergic or C-fiber effects).

In vitro Determination of Dose Responses for Airway Smooth Muscle GABA A Channel Antagonism and Dose Response for Reversal of this GABA A Channel Antagonism by Muscimol

To illustrate that functional antagonism of endogenous GABA at the airway smooth muscle GABA A channel occurs in a dose-dependent fashion, we performed a dose response study using cumulative gabazine concentrations (0–800 μM) administered after an EC50 acetylcholine contraction. Changes in muscle force were analyzed as the percent of change in muscle force from an initial acetylcholine EC50 contraction measured at 15-min intervals after each treatment. Furthermore, to compare relative potency between gabazine and muscimol, we also performed a dose response of muscimol (0–800 μM) after a fixed concentration of gabazine (200 μM) that was given after an acetylcholine EC50 contraction. For these experiments, changes in muscle force mediated by muscimol were analyzed as the percent of change in muscle force from the preceding gabazine (200 μM)-mediated increase in contractile force.

In Vitro Assessment of the Functional Contribution Endogenous GABA Impacts on Resting and Precontracted (Acetylcholine EC50) Airway Smooth Muscle Tone

After equilibration, tracheal rings were randomly assigned to one of four groups: ±gabazine with rings at resting tension (1g) or contracted with an EC50 concentration of acetylcholine, which were allowed to achieve a steady-state plateau of increased force (typically 15 min). To determine the functional effect of endogenous GABA on airway smooth muscle GABA A channels, a single dose of the selective GABA A channel antagonist gabazine (100 μM) was added to the resting or acetylcholine-precontracted tracheal rings. Changes in muscle force were recorded over 15 min. For all groups, changes in muscle force were analyzed as the percent of change in muscle force from an initial baseline or acetylcholine-induced muscle force measured 15 min after treatments. In a separate set of experiments, to confirm that the effect of gabazine was not attributable to nonspecific actions (a non-GABAA channel effect), a single dose of the selective GABA A channel antagonist gabazine (100 μM or 200 μM) was added to acetylcholine-precontracted tracheal rings. After 15 min, treatment groups then received muscimol (200 μM) to reverse the effect of gabazine-induced airway smooth muscle GABA A channel blockade.

In vitro Assessment of an Allosteric GABA A Channel Agent (i.e., propofol) Effect on Airway Smooth Muscle Relaxation after Contraction with Substance P

To illustrate the importance of endogenous GABA acting at GABA A channels to mediate relaxation of airway smooth muscle, we assessed the ability of an allosteric activator of GABA (propofol) to relax airway smooth muscle after a 1 μM substance P contraction. Using the paradigm outlined above, guinea pig rings underwent preliminary contractile challenges with the capsaicin analog and acetylcholine. After extensive washing and a...
resetting of baseline resting tone (1.0 g), tissues were randomly assigned to one of three groups: time control (no treatment), vehicle treatment only (DMSO), and propofol treatment. Guinea pig tracheal rings were then treated with substance P (1 μM), allowed to reach a plateau in muscle force, and then were treated with cumulatively increasing concentrations (0.5, 5, 10, 20, 50, and 100 μM) of either propofol or vehicle (DMSO) equivalents in 6 min increments. In separate experiments substance P-induced contracted guinea pig tracheal rings were pretreated with or without 5 μM gaba- zine 15 min before substance P and with or without cumulatively increasing concentrations (5, 10, 50, 100 μM) of propofol at 6-min increments. For all groups, changes in relaxation were analyzed as a percentage of retained muscle force from the initial substance P contraction and were compared to controls.

**Statistical Analysis**

Each experimental permutation included intrac- experimental controls. Dose response curves were constructed using a sigmoidal dose response analysis function in Prism 4.0 software (GraphPad, San Diego CA), which employs a four-parameter logistic equation according to the Hill model: $Y = minimum + (Emax - minimum)/(1 + 10^{(dose-logEC50)})$, where the minimum represents the initial resting muscle tension. Data were analyzed by one-way ANOVA with Bonferroni posttest comparisons between appropriate groups. Data are presented as mean ± SEM; $P < 0.05$ is considered significant.

**Results**

**Activation of the Neurokinin Receptor 2 Enhances GABA Immunostaining over Airway Smooth Muscle**

Under unstimulated conditions, endogenous GABA was immunohistochemically detectable at the interface between airway smooth muscle and the adjacent epithelium in guinea pig tracheal rings (fig. 1A). Limited staining in small punctate areas within the airway smooth muscle itself was discernable at high magnification (data not shown). In contrast, 15-min exposure to the selective neurokinin receptor 2 agonist, β-ala NKA fragment 4–10 (10 μM) enhanced GABA staining throughout the airway smooth muscle layer (fig. 1B), suggesting that either GABA relocates or demonstrates enhanced release within the airway smooth muscle layer itself. The identity of the airway smooth muscle layer was confirmed using an anti-α-actin antibody (fig. 1C), and a control for primary/secondary antibody nonspecific staining was performed using mouse-specific anti-IgG$_1$ antibody (fig. 1D).

**Endogenous GABA Eluted from Guinea Pig Tracheal Rings Is Detectable by HPLC, and Increased GABA Levels Are Detected after Stimulation with β-ala NKA Fragment 4–10 or Acetylcholine**

To correlate our immunohistochemical findings, we assessed eluted airway GABA levels using HPLC. GABA has not been previously demonstrated in isolated airway segments. Therefore, representative HPLC controls are first demonstrated in figure 2. A representative chromatogram of KH buffer alone is illustrated in figure 2A. Figure 2B illustrates a representative chromatogram of a 1 pmol standard of GABA in KH buffer. The GABA peak had a retention time of 14.7 min and showed a linear increase in magnitude over a range of GABA standard concentrations (0.25–10 pmol; data not shown). GABA eluted from unstimulated or stimulated guinea pig rings in 200 μl KH buffer at 37°C over a 45-min period is illustrated in figure 2C. Untreated tracheal rings in KH buffer for 45 min eluted measurable amounts of GABA, but the eluted GABA increased in tracheal rings treated for 45 min with 10 μM β-ala NKA fragment 4–10 or acetylcholine. Figure 2D demonstrates the quantitative differences in GABA eluted during 45 min at 37°C in 200 μl KH buffer. The procontractile stimuli acetylcholine and β-ala NKA fragment 4–10 significantly increased eluted GABA ($n = 6$) ($P < 0.01$ compared to control).
Blockade of Endogenous GABA$_A$ Channels Increases Guinea Pig Airway Smooth Muscle Force, and This Effect Is Greater after an Acetylcholine EC$_{50}$ Contraction Compared to Baseline Resting Tension

We next investigated whether antagonism of GABA$_A$ channels in intact guinea pig tracheal rings modulated airway smooth muscle force. Guinea pig tracheal rings suspended in organ baths under optimal resting tension (1.0 g) were treated with 100 $\mu$M gabazine, which resulted in a small increase in muscle force when compared to untreated controls in parallel organ baths (gabazine 4.6 ± 1.35% of baseline tone [n = 8] vs. control -1.91 ± 1.02% of baseline tone [n = 7], respectively; P < 0.01). In contrast, when 100 $\mu$M gabazine was added to the organ baths during a sustained contraction with acetylcholine (EC$_{50}$ concentration), the augmentation of airway smooth muscle force was significantly greater (gabazine 15.67 ± 1.79% increase above acetylcholine EC$_{50}$ plateau force [n = 15] vs. control 4.61 ± 1.35% increase above acetylcholine EC$_{50}$ plateau force [n = 8]; P < 0.001; fig. 3).

Gabazine-mediated Antagonism of Airway Smooth Muscle GABA$_A$ Channels Is Reversed by the Selective GABA$_A$ Channel Agonist Muscimol

A separate set of experiments was performed to confirm that gabazine’s effect occurs selectively at endogenous GABA$_A$ channels. Representative force tracings are shown in figure 4 for 200 $\mu$M gabazine treatment of a sustained acetylcholine contraction (EC$_{50}$) and reversal by 200 $\mu$M muscimol. We used 100 $\mu$M or 200 $\mu$M gabazine during a sustained acetylcholine (EC$_{50}$) contraction and then attempted to reverse both concentrations of gabazine with 200 $\mu$M of muscimol (a specific GABA$_A$ agonist). As before, 100 $\mu$M gabazine during a sustained acetylcholine (EC$_{50}$)-induced contraction resulted in a significant increase in airway smooth muscle force compared to baseline tension with gabazine.
pared to untreated acetylcholine-contracted control tracheal rings in parallel organ baths (gabazine 13.56 ± 3.03% increase above acetylcholine EC₅₀ plateau force [n = 8] vs. control -0.44 ± 1.99% increase above acetylcholine EC₅₀ plateau force [n = 8]; P < 0.05). This 100 μM gabazine-induced increase in muscle force was completely reversed by 200 μM muscimol (-8.54 ± 5.58% increase above acetylcholine EC₅₀ plateau force, n = 8, P < 0.001 compared to 100 μM gabazine alone; fig. 4D). A concentration of 200 μM gabazine during the sustained phase of an EC₅₀ acetylcholine contraction resulted in an even greater increase in airway smooth muscle tension compared to untreated acetylcholine-contracted controls in parallel organ baths or compared to the effect of 100 μM gabazine (200 μM gabazine 29.33 ± 1.30% increase above acetylcholine EC₅₀ plateau force, n = 7, P < 0.001; fig. 4E), and this increase was significantly attenuated by subsequent treatment with 200 μM muscimol (12.35 ± 4.23% increase above acetylcholine EC₅₀ plateau force, n = 7, P < 0.001 compared to 200 μM gabazine alone.

Fig. 4. After an EC₅₀ acetylcholine contraction, selective γ-aminobutyric acid subtype A channel (GABAₐ) antagonism increases airway smooth muscle force that is reversed by the GABAₐ channel agonist muscimol. Representative tracings in force/time of guinea pig tracheal rings contracted with an EC₅₀ concentration of acetylcholine and treated with (A) 200 μM gabazine followed 15 min later by 200 μM muscimol; (B) 200 μM gabazine; (C) Nothing. Blockade of GABAₐ channels with gabazine results in a sustained increase in muscle force, which is reversed by the selective GABAₐ agonist muscimol. (D) Muscle force in guinea pig tracheal rings contracted to an EC₅₀ with acetylcholine and then subjected to nothing, 100 μM gabazine or 200 μM gabazine followed by 200 μM muscimol. 100 μM gabazine significantly increases muscle force compared to time control (n = 8) (* P < 0.05 compared to untreated time control), and this effect is reversed by 200 μM muscimol (n = 8) (### P < 0.001 compared to 100 μM gabazine). (E) 200 μM gabazine significantly increases muscle force compared to time control (n = 8) (### P < 0.001 compare to untreated time control) and this effect is significantly attenuated by 200 μM muscimol (n = 7) (### P < 0.001 compared to 200 μM gabazine).
Gabazine-mediated GABA<sub>A</sub> Channel Antagonism after an EC<sub>50</sub> Acetylcholine Contraction is Dose-dependent

Additional organ bath experiments were performed to demonstrate cumulatively increasing concentrations of gabazine (from 0 to 800 μM) administered after an EC<sub>50</sub> acetylcholine contraction results in dose-dependent increase in muscle force and exhibits an IC<sub>50</sub> of 163 μM (fig. 5A).

Reversal of Gabazine-mediated GABA<sub>A</sub> Channel Antagonism after an EC<sub>50</sub> Acetylcholine Contraction Also Displays Dose Dependency

Cumulatively increasing concentrations of the GABA<sub>A</sub> channel agonist muscimol (0–800 μM) after a fixed dose of gabazine (200 μM) demonstrates significant reversal of the achieved GABA<sub>A</sub> channel antagonism in a dose-dependent fashion, beginning at a concentration of 100 μM (n = 4); P < 0.05 compared to no treatment (n = 8). Full reversal of the increase in muscle force of 200 μM required a concentration of muscimol greater than 200 μM, demonstrating that gabazine and muscimol are not equally potent under these conditions (fig. 5B).

An Allosteric Agent at GABA<sub>A</sub> Channels Enhances Endogenous GABA Effect of Relaxing Substance P–induced Contraction in Guinea Pig Tracheal Rings

The significant increase in muscle force induced by gabazine during the sustained phase of an acetylcholine contraction suggests that endogenous GABA is present and counterbalances induced contractile muscle force. To illustrate the endogenous presence and contribution of GABA to decreasing muscle force, substance P–induced contractions were treated with an allosteric agent at GABA<sub>A</sub> channels in the absence and presence of a GABA<sub>A</sub> antagonist. Guinea pig tracheal rings were contracted with 1 μM substance P and then subjected to cumulatively increasing concentrations of propofol (5, 10, 20, 50, or 100 μM) or the appropriate concentrations of the vehicle for propofol (DMSO) in the absence or presence of the selective GABA<sub>A</sub> antagonist gabazine (5 μM). Propofol (20–100 μM) had a significant effect on relaxation compared to the spontaneous decay of an untreated time control contraction, with no significant effect of the vehicle (n = 10 in each group); P < 0.05 for 20 μM propofol compared to time control, and P < 0.001 for 50 or 100 μM propofol compared to time control (fig. 6A). To demonstrate that a component of the propofol-induced relaxation was due to propofol allosterically enhancing endogenous GABA effects at GABA<sub>A</sub> channels, substance P–induced contractions were relaxed with propofol (5, 10, 20, 50, 100 μM) in the absence or presence of 5 μM gabazine. Gabazine partially but significantly reversed propofol-induced relaxation, with gabazine alone having no significant effect on the substance P–induced contraction (n = 8 in each group); P < 0.01 for propofol+gabazine compared to propofol alone (fig. 6B).

Discussion

The major finding of this study is that endogenous GABA exists in the guinea pig airway and plays an important role in modulating airway smooth muscle tone by facilitating smooth muscle relaxation via activation of airway smooth muscle GABA<sub>A</sub> channels. To establish the
contribution of endogenous GABA in regulating airway tone, we used both descriptive (HPLC, immunohistochemistry) as well as functional studies (in vitro organ baths), and we demonstrated the importance of GABA by testing its prorelaxant effect against two different contractile stimuli (muscarinic and neurokinin receptor 2 agonists). In addition, to illustrate that the GABA effects were specific to GABAA channels, we performed studies demonstrating a procontractile effect by GABA A antagonism (gabazine), which was reversed by GABA A agonism (muscimol). This is the first demonstration that measurable amounts of endogenous GABA are present in the airway, that GABA release and cellular localization changes in response to procontractile stimuli, and that GABA promotes airway smooth muscle relaxation after smooth muscle contraction.

Many anesthetic agents that facilitate bronchodilation are also positive allosteric agents at GABA A channels, which function to augment the effects of endogenous GABA. Despite these properties, the exact mechanism(s) responsible for anesthetics’ bronchodilatory effect are incompletely understood. It has been a long-standing belief that any potential GABAergic contribution to airway tone was largely mediated by GABA A channels in the brainstem12 or by GABAB receptors on preganglionic cholinergic nerves in the lung.13,14 However, there is emerging evidence that a far more complex airway GABAergic system exists, one which involves the presence of GABA A channels not only on airway nerves, but also on airway epithelium and airway smooth muscle, where they facilitate relaxation.16–18 In addition, since the enzyme responsible for GABA synthesis (glutamic acid decarboxylase) is also present in airway epithelium16,17 and airway smooth muscle,17 it is possible that the bronchodilatory effect of anesthetic agents, which require GABA’s presence to exert their effect, is also attributable to an allosteric effect at airway smooth muscle GABA A channels.

The expression of GABA B receptors on postganglionic parasympathetic nerves terminating in the airways has been known for years,14 and we recently described the expression of GABA B receptors on airway smooth muscle18 and airway epithelial cells17 coupled to Gi signaling pathways. Therefore, in addition to its target on GABA A channels described in the current study, liberated GABA could inhibit parasympathetic nerve release of acetylcholine in the airway but could counteract smooth muscle GABA A channel-mediated relaxation by stimulation of the GABA B receptor, which in turn activates Gi, a signaling cascade classically linked to impairing airway smooth muscle relaxation.

Although we have previously described that GABA is immunohistochemically detectable in the guinea pig airway,18 GABA staining predominated at the interface between the airway smooth muscle and surrounding epithelium in the absence of contractile stimuli, and only

Fig. 6. Relaxation of a substance P-induced contraction is enhanced by the selective positive allosteric effect of propofol on the γ-aminobutyric acid subtype A channel (GABA<sub>A</sub>). (A) Cumulatively increasing concentration response curves expressed as a percent of initial force after a substance P contraction for propofol (squares), dimethyl sulfoxide (DMSO) vehicle (down triangles), or time control (up triangles). Relaxation of guinea pig tracheal rings contracted with 1 μM substance P was significantly enhanced by propofol (≥ 20 μM), *P < 0.05 compared to time control; ***P < 0.001 compared to time control. (B) Cumulatively increasing concentration response curves expressed as a percent of initial force after a substance P contraction for propofol (squares) or with 5 μM gabazine pretreatment (up triangles) before propofol. Partial but significant reversal of propofol-induced relaxation occurred with low-dose gabazine (5 μM) pretreatment (at 50 μM and 100 μM propofol; *P < 0.01 and ***P < 0.001, respectively). Gabazine alone (diamonds) was not different from untreated time controls (down triangles).
sparse staining was noted within the airway smooth muscle layer itself.\textsuperscript{18} This contrasts with the immunohistochemical localization observed for the GABA\textsubscript{\alpha} channel, which was localized throughout the airway smooth muscle layer.\textsuperscript{15} In the current study under unstimulated conditions, GABA immunostaining was again diffusely visible near the smooth muscle layer (between smooth muscle and epithelium and between smooth muscle and cartilage/adventitia). However, GABA immunostaining increased dramatically over the smooth muscle layer after exposure to the procontractile selective neurokinin receptor 2 agonist (\textbeta-aliva NKA fragment 4-10), supporting the possibility that endogenous GABA participates in modulation of airway smooth muscle tone.

Although HPLC detection and quantification of amino acid neurotransmitters has been performed extensively in neuronal tissues,\textsuperscript{19} it has never been applied to airway tissues. Recent studies highlight the methodologic pitfalls involved in GABA analysis by HPLC,\textsuperscript{20} which were taken into account in the current study. Using a validated approach, we were able to identify discrete, symmetrical, and reproducible GABA chromatograms derived from buffers into which intact airway tissues eluted GABA. In agreement with our immunohistochemical findings, we demonstrate that 10 \mu M acetylcholine or \beta-aliva NKA fragment 4-10 increased eluted GABA levels. It is possible that physical manipulation or trauma to our dissected airway tissues induces release of GABA. To minimize this effect, dissected tracheal rings were washed extensively until wash buffer concentrations of GABA were undetectable, and then the tracheal rings were incubated undisturbed in a small volume of buffer in the absence or presence of a contractile agonist such that initial buffer concentrations of GABA were undetectable, and GABA that subsequently eluted over 45 min was quantitated. We measured GABA that had permeated into the incubation buffer; therefore, our method does not directly assess the changes in GABA levels that occur within the interstitium of the airway smooth muscle layer. This is an important consideration because we likely are underestimating the magnitude of change in endogenous airway GABA levels that airway smooth muscle GABA\textsubscript{\alpha} channels are actually exposed to. The current study does not allow us to determine the cellular source of GABA. Taken together, our immunohistochemical and HPLC findings demonstrate that airway GABA levels increase after procontractile stimuli and raise the intriguing possibility that liberated GABA acting at airway smooth muscle GABA\textsubscript{\alpha} channels may contribute to relaxation, thus functioning to counterregulate the procontractile stimuli that induced its release.

The cellular source of GABA liberated into the airway was not determined in the present study, but at least 3 structures in the airway appear to be candidate sources. The enzyme that synthesizes GABA (glutamic acid decarboxylase) was recently identified by us in airway smooth muscle (unpublished results, March 2008) and airway epithelial cells.\textsuperscript{16,17} In addition, different types of nerve fibers (parasympathetic, nonadrenergic/noncholinergic) terminally branch in the airway and may contain GABA. As such, identifying the cellular source of GABA in the airway is an active topic of investigation in our laboratory. The current study provides evidence that the contractile agonists acetylcholine and \beta-alina NKA increase GABA release. There is evidence in the central nervous system\textsuperscript{22} and spinal cord\textsuperscript{23} that these agonists induce the release of GABA from neuronal cell types.

To determine if endogenous GABA enhances relaxation via activation of airway smooth muscle GABA\textsubscript{\alpha} channels, we conducted in vitro functional organ bath studies using intact guinea pig airway smooth muscle. In these studies, epithelial or neuronal contributions were eliminated by denuding the epithelium and by pretreatment with capsaicin and tetrodotoxin, respectively.

We initially examined the functional consequence of eliminating the ability of endogenous GABA to exert its prorelaxant effect via the airway smooth muscle GABA\textsubscript{\alpha} channel. We used the GABA\textsubscript{\alpha} channel antagonist gabazine because it is water-soluble (obviating the need for vehicle controls), is a competitive inhibitor of the GABA\textsubscript{\alpha} channel (unlike picrotoxin, thereby allowing for a reversal of its effect), is not known to have nonspecific effects at targets other than the GABA\textsubscript{\alpha} channel (like bicuculline methiodide),\textsuperscript{24} and has been successfully used by us in previous organ bath studies.\textsuperscript{25} Gabazine increased smooth muscle force and had a greater effect under contracted conditions, which correlates with conditions under which we demonstrated enhanced GABA release. The ability of the GABA\textsubscript{\alpha} agonist muscimol to reverse gabazine’s effect further supported the specificity of gabazine’s effect at the GABA\textsubscript{\alpha} channel. Another and perhaps more important consideration that may account for a larger effect of the GABA\textsubscript{\alpha} antagonist under contracted conditions is the status of the membrane potential under baseline versus acetylcholine-contracted conditions. Opening of the GABA\textsubscript{\alpha} channel in smooth muscle at resting membrane potential (approximately –45 mV) may actually allow efflux of intracellular chloride, favoring depolarization and contraction.\textsuperscript{26} However, after an acetylcholine contraction in airway smooth muscle, the chloride reversal potential is exceeded,\textsuperscript{27} allowing for chloride entry and membrane hyperpolarization to occur (establishing a milieu that favors smooth muscle relaxation).\textsuperscript{28} However, the current study did not correlate resting membrane potential with resting muscle force.

Under tonic exposure to high concentrations of GABA, classic neuronal GABA\textsubscript{\alpha} channels desensitize (as is seen within the synaptic cleft after vesicular release of GABA).\textsuperscript{29} However, there is emerging evidence that subunit composition plays an important role in determining...
the threshold for GABA<sub>A</sub> channel desensitization. Indeed, two electrophysiologically distinct classes of GABA<sub>A</sub> channels have been identified in neurons; the classic synaptic chloride channels with fast kinetics and rapid inactivation (collectively termed “phasic”), and the slower extrasynaptic channels that are responsive to lower concentrations of GABA and display slower desensitization properties (collectively termed “tonic”).

Interestingly, tonic GABA<sub>A</sub> channels require the inclusion of either an α4, α5, or α6 subunit with a δ subunit, and we have previously demonstrated that airway smooth muscle GABA<sub>A</sub> channels express the δ subunit in addition to having α subunit expression limited to α4 and α5 subunits. This finding indicates that the repertoire of subunits required in neurons to form tonic GABA<sub>A</sub> channels are also expressed in airway smooth muscle.

To illustrate the functional role of endogenous GABA in promoting relaxation of contracted tissues, anesthetic with positive allosteric effects at the GABA<sub>A</sub> channel (i.e., propofol) was used. Positive allosterism enhances the native effect of a ligand at its target channel/receptor (and by itself cannot activate the target channel/receptor); therefore, it provides a unique opportunity to demonstrate a ligand’s endogenous effect. We demonstrate a dose-dependent improvement in relaxation of a substance P contraction by propofol, which was partially reversed by low-dose GABA<sub>A</sub> channel antagonism. This partial reversal may indicate that propofol relaxation of airway smooth muscle may involve additional mechanisms or that insufficient concentrations of gabazine were employed to demonstrate complete reversal of propofol. However, we were limited in the concentrations of gabazine that could be used in these propofol studies because, as illustrated above, higher concentrations of gabazine increase muscle force by blocking relaxation induced by endogenous GABA.

This latter set of experiments adds yet another possible explanation for propofol’s preferential protection from reflex-induced bronchoconstriction during intubation, which has traditionally been attributed to its action on airway efferent parasympathetic cholinergic nerves and less convincingly to postjunctional modulation of L-type calcium channels or inositol phosphate signaling in airway smooth muscle itself. However, these previous studies have illustrated these smooth muscle effects only at concentrations of propofol (> 100 μM) above those typically achieved clinically. Several studies in airway tissues from humans and animal models have shown that propofol can attenuate contractile responses from acetylcholine, histamine, and endothelin, but only at concentrations of 100–300 μM. These previous studies that only demonstrated a propofol effect at high concentrations have largely focused on cellular signaling pathways involved in the initiation and maintenance of contraction (L-type calcium channels, intracellular calcium changes, or inositol phosphate synthesis). Conversely, in the current study, lower concentrations of propofol (20 μM) facilitated airway smooth muscle relaxation likely due to different signaling pathways regulating relaxation as opposed to those regulating contraction.

Although the measurement of plasma propofol concentrations during the administration of clinically relevant doses of propofol is complex, induction doses of propofol (2–3 mg/kg IV) typically result in peak plasma concentrations of 60–80 μM while maintenance infusions of propofol reportedly achieve approximately 30 μM concentrations. Although the concentration in individual tissue compartments is unknown, high tissue uptake of propofol by the lung (30% of bolus dose) has been reported. Of note, the prorelaxant effect we demonstrate with propofol occurs at a concentration (20 μM) that is comparable to levels typically achieved during propofol used for either induction or maintenance of anesthesia.

In summary, the findings of (1) endogenous airway GABA levels existing at measurable levels in the airway, (2) GABA levels increasing after procontractile stimuli, (3) selective blockade of endogenous GABA activation of airway smooth muscle GABA<sub>A</sub> channels resulting in increased muscle force, (4) GABA<sub>A</sub> channel antagonism is greater under contracted versus resting muscle tone, and (5) positive allosteric agonism with propofol enhances endogenous GABA-mediated relaxation of precontracted tissue provide evidence of a prorelaxant GABAergic system existing in airway smooth muscle that contributes to the modulation of contractile force.

References
20. Rea K, Cremers TI, Westerink BH: HPLC conditions are critical for the detection of GABA by microdialysis. J Neurochem 2005; 94:672–9