Targeting the restricted α-subunit repertoire of airway smooth muscle GABA$_A$ receptors augments airway smooth muscle relaxation

George Gallos,[1] Peter Yim,[1] Sucie Chang,[1] Yi Zhang,[1] Dingbang Xu,[1] James M. Cook,[2] William T. Gerthoffer,[3] and Charles W. Emala Sr.[1]

[1]*Department of Anesthesiology, College of Physicians and Surgeons of Columbia University, New York, New York;*
[2]*Department of Chemistry, University of Wisconsin, Milwaukee, Wisconsin; and* [3]*Department of Biochemistry and Molecular Pharmacology, University of Southern Alabama, Center for Lung Biology, Mobile, Alabama*

Gallos G, Yim P, Chang S, Zhang Y, Xu D, Cook JM, Gerthoffer WT, Emala CW. Targeting the restricted α-subunit repertoire of airway smooth muscle GABA$_A$ receptors augments airway smooth muscle relaxation. *Am J Physiol Lung Cell Mol Physiol* 302: L248–L256, 2012. First published September 23, 2011; doi:10.1152/ajplung.00131.2011.—The prevalence of asthma has taken on pandemic proportions. Since this disease predisposes patients to severe acute airway constriction, novel mechanisms capable of promoting airway smooth muscle relaxation would be clinically valuable. We have recently demonstrated that activation of endogenous airway smooth muscle GABA$_A$ receptors potentiates β-adrenoceptor-mediated relaxation, and molecular analysis of airway smooth muscle reveals that the α-subunit component of these GABA$_A$ receptors is limited to the α$_4$- and α$_5$-subunits. We questioned whether ligands with selective affinity for these GABA$_A$ receptors could promote relaxation of airway smooth muscle. RT-PCR analysis of GABA$_A$ receptor subunits was performed on RNA isolated by laser capture microdissection from human and guinea pig airway smooth muscle. Membrane potential and chloride-mediated current were measured in response to GABA$_A$ subunit-selective agonists in cultured human airway smooth muscle cells. Functional relaxation of precontracted guinea pig tracheal rings was assessed in the absence and presence of the α$_4$-subunit-selective GABA$_A$ receptor agonists: gaboxadol, taurine, and a novel 8-methoxy imidazobenzodiazepine (CM-D-45). Only messenger RNA encoding the α$_4$- and α$_5$-GABA$_A$ receptor subunits was identified in RNA isolated by laser capture dissection from guinea pig and human airway smooth muscle tissues. Activation of airway smooth muscle GABA$_A$ receptors with agonists selective for these subunits resulted in appropriate membrane potential changes and chloride currents and promoted relaxation of airway smooth muscle. In conclusion, selective subunit targeting of endogenous airway smooth muscle-specific GABA$_A$ receptors may represent a novel therapeutic option for patients in severe bronchospasm.

airway relaxation; CM-D-45; electrophysiology; taurine

ALTHOUGH ASTHMA REMAINS a serious worldwide health challenge (5), new pharmacological approaches to treat this disease are limited. Therapeutic limitations are especially apparent with regard to medications that promote acute airway smooth muscle relaxation, since β-adrenoceptor agonists and anticholinergics remain the only drug classes currently utilized to treat acute airway constriction. Both volatile and intravenous anesthetics (which act on GABA$_A$ receptors) have long been known to dose dependently promote bronchodilation and attenuate bronchoconstriction, respectively. However, it has been a longstanding belief that any GABAergic contribution to airway

tone was largely mediated by neurally mediated mechanisms (2, 17, 26). Our laboratory has demonstrated that airway smooth muscle GABA$_A$ receptor activation can both potentiate β-adrenoceptor agonist-mediated airway smooth muscle relaxation (in vitro) (7) as well as attenuate cholinergic-mediated airway resistance (in vivo) (8).

Although harnessing this novel relaxation pathway may lead to new therapeutic options for the acute treatment of hyperreactive airway disease, there is legitimate concern that systemic and ubiquitous activation of all GABA$_A$ receptors may lead to oversedation and other untoward effects. Although regional administration (i.e., inhaled aerosol) has been used with considerable success with β-adrenoceptor agonists to minimize systemic side effects, an alternative and equally important strategy for minimizing unwanted systemic drug effects involves tissue-specific receptor targeting.

GABA$_A$ receptors exist as pentamers formed by the coassembly of subunits from seven different classes (α$_{1-6}$, β$_{1-3}$, γ$_{1-3}$, δ, π, ε, and ρ$_{1-3}$) with most native GABA$_A$ receptor stoichiometry typically adhering to the combination of two α, two β, and a γ, δ, or π subunit (22). During the last decade, mounting evidence demonstrates not only that subunit composition determines the localization of these receptors, but also that variability in subunit combinations may impart differential pharmacological and kinetic properties. In fact, in the central nervous system two electrophysiologically distinct classes of GABA$_A$ receptors have been identified: the classic synaptic chloride channel with fast kinetics and rapid inactivation, and the slower extrasynaptic channels, which are responsive to lower concentrations of GABA and display slower desensitization properties (9). These GABA$_A$ receptor differences are dictated by differential subunit compositions, with extrasynaptic GABA$_A$ receptors typically requiring the inclusion of either α$_4$, α$_6$ (with δ-subunits) or α$_5$ (with γ-subunits) (14).

We have reported that grossly dissected native airway smooth muscle from both human and guinea pig airways contain mRNA and protein for the GABA$_A$ α$_4$- and α$_5$-subunits, representative of the extrasynaptic phenotype (α$_4$ and α$_5$) (16). However, the possibility for neuronal GABA$_A$ receptors contaminating these airway smooth muscle dissections remains unaddressed. There are pharmacological agonists available with subunit selectivity for the GABA$_A$ channels containing α$_4$- or α$_5$-subunits. Therefore, we hypothesized that direct targeting of the alpha GABA$_A$ receptor subunits restricted to airway smooth muscle cells could elicit membrane potential and chloride conductance changes in vitro and that targeted activation of these restricted GABA$_A$ α-subunits on airway smooth muscle cells would potentiate isoproterenol-

mediated relaxation or induce spontaneous relaxation of airway smooth muscle.

MATERIALS AND METHODS

Reagents. Indomethacin, *N*-vanillynonanamide (capsaicin analog), pyrilamine, acetylcholine, gaboxadol [5,6,7-tetrahydroisoxazolopyridin-3-ol (THIP)], and picrotoxin were obtained from Sigma (St. Louis, MO). Membrane potential dye [fluorescent imaging plate reader (FLIPR) blue reagent] was obtained from Molecular Devices (Sunnyvale, CA). Tetrodotoxin was obtained from Calbiochem (San Diego, CA), and 96-well microfluidic plates were purchased from Fluxion (South San Francisco, CA).

Laser capture microdissection. Since pharmacological targeting of airway smooth muscle GABA_A subunits requires accurate subunit identification, laser capture microdissection (LCMD) was used to select airway smooth muscle cells devoid of surrounding epithelium, nerves, vascular structures, and fibroblasts for RNA isolation prior to RT-PCR analyses to provide greater assurance that our results were not contaminated by other cell types. We sought to confirm interspecies conservation of endogenous GABA_A subunit expression by comparing airway tissue from both guinea pig and human airways. Tracheal rings were embedded in optimal cutting temperature compound followed cryopreservation by using isopentane and/or dry ice. Frozen sections (6 μm) were placed on a single 1-mm PEN-membrane coated slide (PALM Microlaser Technologies) and processed for RNA by use of a LCMD staining kit (Ambion AM1935). Histological confirmation of stained airway smooth muscle guided laser dissection, with only central portions of the airway smooth muscle layer being captured to minimize contamination from other cell types. Airway smooth muscle RNA was isolated by using the Micro scale RNA isolation kit (Ambion AM1931) and underwent RT-PCR using GABA_A subunit specific primers (Table 1) that employed primer designs that flanked large genomic introns to distinguish mRNA from genomic-derived PCR products (15). RNA from guinea pig and human brain served as positive controls, and samples devoid of input cDNA (water blanks) served as negative controls.

Cultured human airway smooth muscle cells. Human immortalized bronchial smooth muscle cell lines prepared as described (10) were grown to confluence in M199 media (GIBCO) containing 10% fetal bovine serum, 0.25 ng/ml epidermal growth factor, 1 ng/ml fibroblast growth factor, ITS supplement (1 mg/ml insulin, 0.55 mg/ml transferrin, 0.67 μg/ml sodium selenium) and antibiotics (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B) in a humidified atmosphere of 5% CO_2-95% air at 37°C. At 24 h prior to study, cells were fed with serum and growth factor-free media.

Membrane potential fluorescent assay. To determine whether activation of GABA_A receptors induce membrane potential changes in cultured human airway smooth muscle cells, the FLIPR in vitro fluorescent dye assay (Molecular Devices) was used as described by Wafford et al. (27). Briefly, human airway smooth muscle cells were grown to 100% confluence in 96-well black-walled plates and were washed with warmed (37°C) low-chloride buffer [consisting of (in mM) 160 sodium-D-gluconate, 4.5 potassium-D-gluconate, 2 CaCl_2, 1 MgCl_2, 10 D-glucose, and 10 HEPES, pH 7.4] four times. A stock solution (100% dye) of FLIPR blue dye was prepared by reconstitution of 1 vial (125 mg) with 100 ml of the low-chloride buffer (assay buffer). A 50% working stock was prepared by further diluting the reconstituted blue dye 1:1 with assay buffer and was used to load cells (90 μl/well) over 20 min at 37°C. All reagents were dissolved in assay buffer. Baseline fluorescence was measured for 3 min prior to the first control additions (assay buffer). Three minutes later, airway smooth muscle cells were exposed to varying concentrations of THIP (0–10 mM) to determine a dose response. The fluorescence produced by membrane potential change following solution additions was quantified after subtracting changes induced by assay buffer alone. For subsequent antagonist assays, the first solution injected was either assay buffer, vehicle control (0.05% DMSO final), or the GABA_A receptor antagonist picrotoxin (200 μM final) followed by THIP (either 0.5 or 1 mM).

Electrophysiology of human airway smooth muscle cells. To corroborate our membrane potentiometric dye findings, we also assessed whether targeted activation of α_4-containing GABA_A receptors induced appropriate electrophysiological changes. On the day of the assay, human airway smooth muscle cells were released from collagen-coated plates with collagenase type IV (Sigma C5138; 500

Table 1. *Human and guinea pig laser capture microdissection PCR primers*

Primer Name	Primer Sequence (5′ to 3′)	RT-PCR Product Size, bp
Primer sequence for human GABA_A subunits		
α_4*	CAA ACC GTA TCA AGT GAA ACC ATC AAA TCA AT GCT TAG TGT GGT CAT GGT GAG GAC AGT TGT TAT	225
α_5*	GCA GAC GGT GGG CAC TGA GAA CA GAT AAG ATC ACG GTC ATT ATG CAG GGA AGG TA	138
β_3	GCT TGC AGA AAA GAC AGC CAA GGC AAA GA TGC CGC CTG AGA CCT CAT TCA TTT CAT T	131
δ	GCC CTG GTG GAG TAC GCC TTT GCT CA GCA GAG AGG GAG AAG AGG ACA ATG GCG TT	131
γ_2*	AGG TCT CCT ATG TCA CAG CGA TGG ATC TCT GAC ACT CAT AGC CGT ACT CTT CAT CTC TCT CT	267/243
Primer sequence for guinea pig GABA_A subunits		
α_4	AGT TTC ATT CTG GAT AAA TAA GGA ATC AGT TCC AGC TA CAT TTG AAT ATT GGT GAA ATA GTT GAC AGC AGC AAA CTC	199
α_5*	TCG GCC CGG TGT CAG ACA CAG AGA TGG AAT AC GGG TGT GGT CAT GTT GTG CGC GAT AGA CTT CTT	197
β_3*	ATG TAC CTC ATC GGC TGC TTC GTC TTC GTG TTC TTG AAC GGT CAT TCT TTG CCT TGG CTG TCT	142
δ	GCT TCA CCA CGG AGC TGA TGA ACT TCA A ATC CAG AAG GAG ACC CAG GAC ATG GC	154
γ_2	TCCAGCCAGAACGTCTTTAGGTATCACCACTG AAAAGATCCATTGCTGTGACATAGGAGACCTT	111

*Primer sequences previously published (19).

units/ml). The cells were resuspended in external buffer solution (in mM: 147 NaCl, 2 MgCl$_2$, 2 CaCl$_2$, 10 HEPES, 10 dextrose; pH 7.2, osmolarity 320 mosM) and rocked at room temperature to allow cells to obtain a spherical shape. Approximately one million cells were centrifuged (300 g, 3 min), washed ×2 in external buffer, and loaded (final volume of 400 μl) into a Fluxion microfluidic plate with each experimental group representing an ordered arrangement of 20 cells in parallel whole cell configuration. Following generation of adequate cellular membrane seals (40–120 mΩ), whole cell recordings were obtained under voltage-clamp conditions (−80 mV) to a dose response to THIP (0.3–300 μM). In separate assays cells were also cotreated simultaneously with 20 μM gabazine (GABA$_A$ receptor antagonist) and 25 μM THIP. In addition, to confirm that THIP-induced currents were due to chloride efflux, in separate experiments the intracellular buffer (in mM: 4 MgCl$_2$, 10 EGTA, 10 HEPES, 130 CsCl, 4 Mg-ATP, 10 CsOH, 7.2 pH, 290 mOsM) was modified by replacing 140 mM CsCl with 140 Cs gluconate to inhibit the magnitude of the outward current at −80 mV.

Guinea pig tracheal rings. All animal protocols were approved by the Columbia University Animal Care and Use Committee. Male Hartley guinea pigs (~400 g) were anesthetized with intraperitoneal pentobarbital (100 mg/kg). Trachea were removed and dissected under a dissecting microscope into closed rings comprised of two cartilaginous segments. Epithelium was removed by gentle abrasion on the tracheal lumen with cotton. Tissues were placed into cold Krebs-Henseleit (KH) buffer (in mM: NaCl 118, KCl 5.6, CaCl$_2$ 0.5, MgSO$_4$ 0.24, NaH$_2$PO$_4$ 1.3, NaHCO$_3$ 25, glucose 5.6, pH 7.4) containing indomethacin 10 μM (DMSO final concentration in organ baths of 0.01%) to block tone due to endogenous release of prostanoids.

Organ baths. Closed guinea pig tracheal rings were suspended in organ baths as previously described (6). Briefly, tissues were hung in a water-jacketed (37°C) 2-ml organ bath (Radnoti Glass Technology, Monrovia, CA) and attached 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, Goleta, CA). KH buffer was continuously bubbled with 95% oxygen and 5% carbon dioxide and tissues were allowed to equilibrate at 1 g isotonic force for 1 h with fresh KH buffer changes every 15 min.

Preliminary contractile challenges. Following equilibration, the capsaicin analog *N*-vanillylnonanamide (10 μM final) was added to the organ baths to first activate and then deplete nonadrenergic, noncholinergic nerves. After *N*-vanillylnonanamide induced force had returned to baseline (~50 min), the tracheal rings were washed and then subjected to two cycles of increasing cumulative concentrations of acetylcholine (0.1 μM to 0.1 mM) to determine the EC$_{50}$ concentrations of acetylcholine required for each individual ring. To avoid bias between treatment groups, tissues were contracted to individually calculated EC$_{50}$ values for acetylcholine and tissues with similar E$_{max}$ values were randomly assigned to treatments within individual experiments. Following extensive KH buffer changes (8–9 times) tissues were allowed to stabilize at isotonic resting tension (~1.0 g). To remove confounding effects of other procontractile pathways, each bath received a complement of antagonists 20 min prior to subsequent contractile challenge. The antagonists included pyrilamine (10 μM; H$_1$ histamine receptor antagonist), and tetrodotoxin (1 μM; blocker of endogenous cholinergic or C-fiber neuronal effects).

In vitro assessment of two α-subunit-containing GABA$_A$ receptor-selective agonists on β$_2$-adrenoceptor-mediated airway smooth muscle relaxation following an EC$_{50}$ contractile stimulus with acetylcholine. Guinea pig tracheal rings were contracted with an EC$_{50}$ concentration of acetylcholine and allowed to achieve a steady-state plateau of increased force (typically 15 min). Tracheal rings were randomly assigned to one of three groups: isoproterenol-treated controls, isoproterenol dose response in the presence of an α-GABA$_A$-selective agonist (taurine or THIP), or pretreatment with a GABA$_A$ receptor antagonist followed by isoproterenol dose response in the presence of

an α-subunit selective agonist. All groups received cumulatively increasing concentrations of isoproterenol in half-log increments (0.1 nM to 10 μM). To determine the effect of selective GABA$_A$ activation on isoproterenol-mediated relaxation, a single dose of an α$_4$-selective agonist (500 μM THIP or 200 μM taurine) was administered to the study group just prior to a modestly effective concentration of isoproterenol under this regimen (10$^{-8.5 \text{ M}}$). To confirm that the effect of THIP or taurine was not attributable to nonspecific effects elicited by activation of non-GABA$_A$ receptors, the third group received pretreatment with a single dose of the selective GABA$_A$ antagonist gabazine 15 min prior to contractile challenge with exogenous acetylcholine.

In vitro assessment of a novel 8-methoxy imidazobenzodiazepine (13) (CM-D-45; a third α-subunit-containing GABA$_A$ receptor selective agonist) to directly relax airway smooth muscle following either a tetraethylammonium chloride (TEA) or substance P-mediated contraction. To determine the direct relaxant effect of α$_4$-subunit-containing GABA$_A$ receptor activation by use of different contractile agonists, guinea pig tracheal rings were contracted with a single dose (10 mM) of TEA or (1 μM) substance P. Following a plateau in the generated muscle force, a single dose of CM-D-45 (200 μM) or vehicle control (DMSO 0.1%) was added to the organ bath buffer and the percentage of relaxation achieved was assessed over 15 min compared with intraexperimental matched vehicle controls.

Statistical analysis. Each experimental permutation included intraexperimental controls. Where appropriate, we employed repeated measures in a one-way ANOVA using Bonferroni posttest comparisons. In addition, dose-response curves were evaluated by 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: $Y = \text{minimum} + (\text{E}_{max} - \text{minimum})/1 + 10^{\text{dose} - \text{logEC}_{50}}$, where the minimum represents the initial resting muscle tension. In cases where only two experimental groups were being compared a two-tailed Student's *t*-test was employed. Data are presented as means ± SE; $P < 0.05$ in all cases was considered significant.

RESULTS

RT-PCR following laser capture microdissection confirms that airway smooth muscle cells possess a GABA$_A$ receptor α-subunit repertoire that is restricted (α$_4$, α$_5$), is conserved (between human and guinea pig), and collectively mimics the extrasynaptic GABA$_A$ receptor phenotype. Laser capture microdissection allowed for accurate sampling of only those cells displaying airway smooth muscle cell morphology from native tissue. Using gene-specific primers we demonstrate restricted yet abundant expression of mRNA encoding the α$_4$- and α$_5$-subunits (and no expression of mRNA encoding the other GABA$_A$ α-subunits; results not shown). In addition, we also confirmed the presence of complementary subunits (β, γ, δ) required to form a GABA$_A$ receptor of the typical extrasynaptic phenotype (Fig. 1). Since GABA$_A$ receptor α-subunits present in airway smooth muscle cells qualitatively show concordance between human and guinea pig samples, this finding substantiates using guinea pig airway for our functional organ bath studies.

THIP induces an increase in fluorescence indicative of a change in membrane potential that is significantly attenuated by the GABA$_A$ antagonist bicuculline. To establish that targeted activation of airway smooth muscle α$_4$-subunit-containing GABA$_A$ receptors results in membrane potential changes, we loaded human airway smooth muscle cells with FLIPR blue membrane potentiometric dye and exposed the cells to a dose response of THIP (0–10 mM). We found that THIP displayed
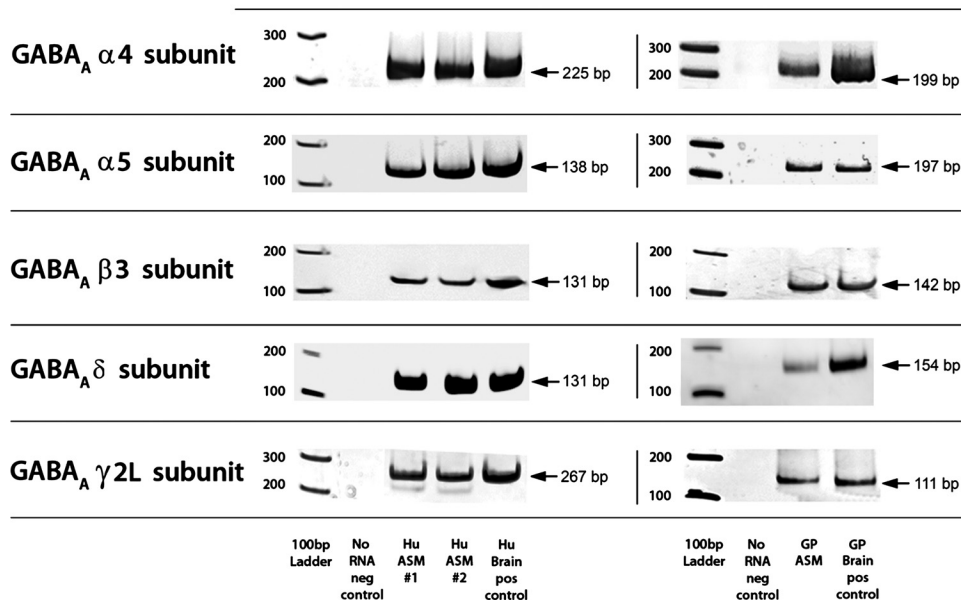
Laser Capture Microdissection RT-PCR

Fig. 1. RT-PCR of GABA$_A$ receptor subunits in airway smooth muscle reveals extrasynaptic phenotype and interspecies conservation. Representative images of RT-PCR products corresponding to specific GABA$_A$ subunits following laser capture microdissection of airway smooth muscle cells harvested from human and guinea pig tracheal airway smooth muscle. bp, Base pairs; ASM, airway smooth muscle; Hu, human; GP, guinea pig; neg, negative; pos, positive. Representative of 2 separate individual human or guinea pig tracheas.

a dose-dependent enhancement of fluorescence (Fig. 2A) with an EC$_{50}$ of 245 μM ($n = 12$; 3 separate cell cultures repeated 4 times per concentration).

To ensure these results were not attributable to any nonspecific fluorescent changes produced by GABA mimetics acting directly with the dye itself, we also performed cell-free assays demonstrating no effect of our drugs on fluorescence in the presence of dye alone (data not shown). In addition, although we considered using other GABA$_A$ antagonists (gabazine and bicuculline) to demonstrate specific blockade of THIP-mediated membrane potential changes, these particular antagonists displayed nonspecific fluorescent changes upon reconstitution in the dye alone and were therefore not used. Since picrotoxin demonstrated no such nonspecific fluorescent changes, it was used to antagonize THIP-mediated membrane potential changes (Fig. 2B). Picrotoxin antagonism of THIP-mediated membrane potential changes persisted even under conditions of high-dose THIP (1 and 0.5 mM), in agreement with its ability to serve as a noncompetitive antagonist at the GABA$_A$ receptor (Fig. 2C).

Whole cell recordings of human airway smooth muscle cells demonstrate that specific activation of these restricted GABA$_A$ receptor subtypes generates a chloride current in vitro. Using a microfluidic platform that allowed for 20 human airway smooth muscle cells to simultaneously achieve whole cell configuration in parallel, we demonstrate a robust current upon exposure to 10 μM THIP (Fig. 3A). In addition, utilizing graded additions of THIP (0–300 μM) we demonstrate an EC$_{50}$ of ∼15 μM for THIP (Fig. 3B), a finding in agreement with a mixed population of airway smooth muscle cells demonstrating heterogeneous α$_4$/α$_5$-GABA$_A$ receptor subunit expression. To confirm that our currents were indeed secondary to THIP-mediated activation of GABA$_A$ receptors, we performed two experimental validations. First we demonstrate attenuation of the THIP-induced current (25 μM) under conditions of GABA$_A$ receptor antagonism (gabazine 25 μM). Next, we sought to illustrate that THIP was truly inducing a

chloride current. To achieve this, we demonstrate that following the removal of chloride from our buffer (but replacing it with equiosmotic gluconate) we lose the ability of THIP to induce a current (Fig. 3C).

Two distinct α$_4$-selective GABA$_A$ receptor agonists augment β$_2$-adrenoceptor-mediated airway smooth muscle relaxation following an EC$_{50}$ contractile stimulus with acetylcholine. Selective α-subunit GABA$_A$ receptor activation with THIP significantly potentiated the relaxant effects of isoproterenol after an acetylcholine contraction (Fig. 4A). In guinea pig tracheal rings, cotreatment with THIP and isoproterenol resulted in a significant leftward shift in the isoproterenol relaxation concentration-response curve compared with treatment with isoproterenol alone [EC$_{50}$ = 7.6 nM ($n = 8$) vs. 32.8 nM ($n = 8$); $P < 0.001$]. To prove that the shift in EC$_{50}$ observed was due to selective and specific activation of GABA$_A$ receptors, pretreatment with the selective antagonist gabazine was performed. Pretreatment with gabazine significantly reversed the THIP potentiation of isoproterenol-mediated relaxation [EC$_{50}$ = 11.8 ± 0.6 nM ($n = 8$) vs. EC$_{50}$ = 7.6 ± 0.5 nM ($n = 8$); $P < 0.01$] after an acetylcholine contractile stimulus and significantly returned the concentration-response curve toward baseline [EC$_{50}$ = 11.8 ± 0.6 nM ($n = 8$) vs. 32.8 nM ($n = 8$), respectively; $P > 0.05$]. In addition to a significant shift in the EC$_{50}$ of the isoproterenol concentration-response curve, THIP treatment also resulted in a significant potentiation of relaxation even at a low concentration (10 nM) of isoproterenol (Fig. 4B) [muscle force = 22.9 ± 13.2% ($n = 8$) of initial acetylcholine-induced force for THIP plus isoproterenol vs. 76.5 ± 8.5% for isoproterenol alone ($n = 8$); $P < 0.01$], whereas pretreatment with gabazine reversed this THIP effect [muscle force = 53.1 ± 11.4% ($n = 8$); $P > 0.05$ compared with isoproterenol alone]. In each case n represents total number of individual rings in a treatment group.

We previously published organ bath experiments illustrating the relaxant effect mediated by taurine activation of endogenous glycine receptors on airway smooth muscle and the partial
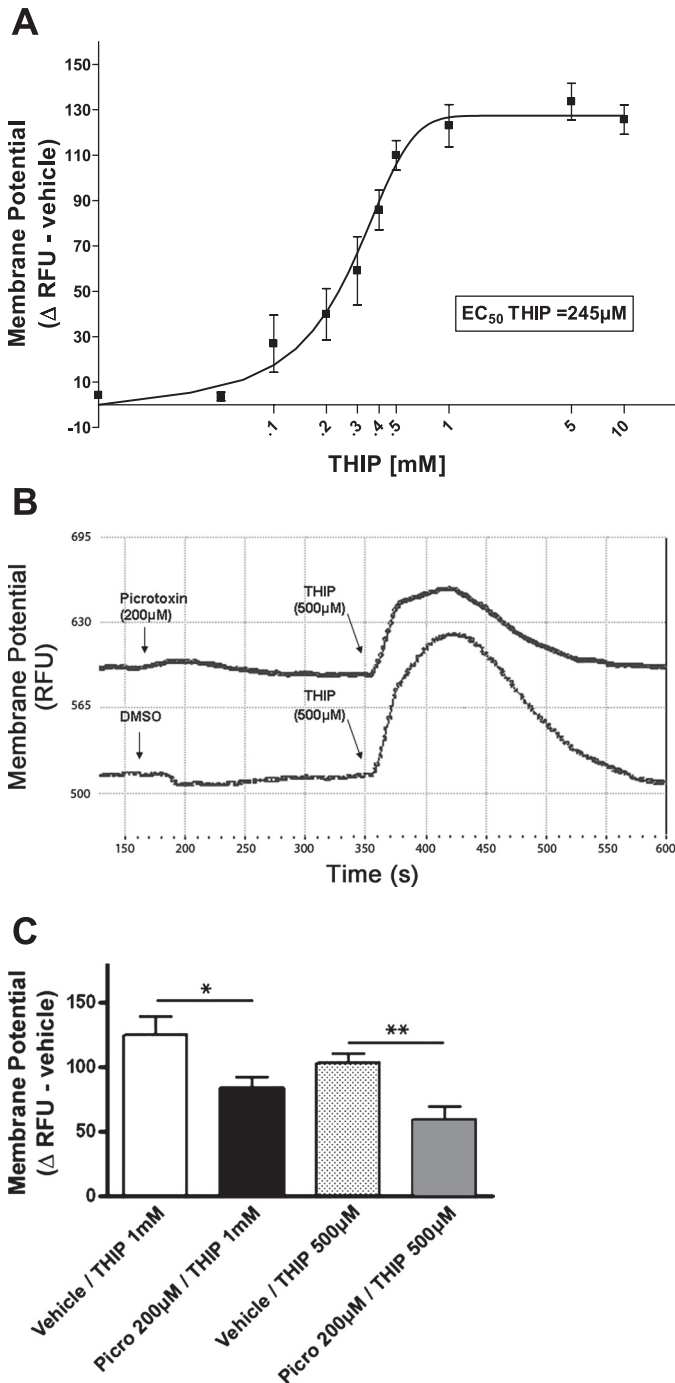
Fig. 2. Membrane potential changes elicited by 5,6,7-tetrahydroisoxazolopyridin-3-ol (THIP) in cultured human airway smooth muscle cells detected by a fluorescent potentiometric dye. *A*: dose-response curve of THIP (from 0 to 10 mM) eliciting a change in relative fluorescence. EC$_{50}$ = 245 μM (*n* = 12). *B*: illustration of attenuation of THIP-induced fluorescent changes under conditions of GABA$_A$ receptor antagonism. Concomitant representative tracings of relative fluorescence unit (RFU) changes over time evoked by addition of THIP. *Bottom tracing*: RFU changes following injection of DMSO vehicle (0.1%; negative control) followed by THIP (500 μM). *Top tracing*: RFU changes following injection of the GABA$_A$ receptor antagonist picrotoxin (200 μM in 0.1% DMSO) and subsequent injection of THIP (500 μM). *C*: picrotoxin (picro)-mediated antagonism of THIP-induced changes in membrane potential at 2 different THIP concentrations (500 μM and 1 mM) (*n* = 12 per group; *$P < 0.05$, **$P < 0.01$ compared with THIP alone).
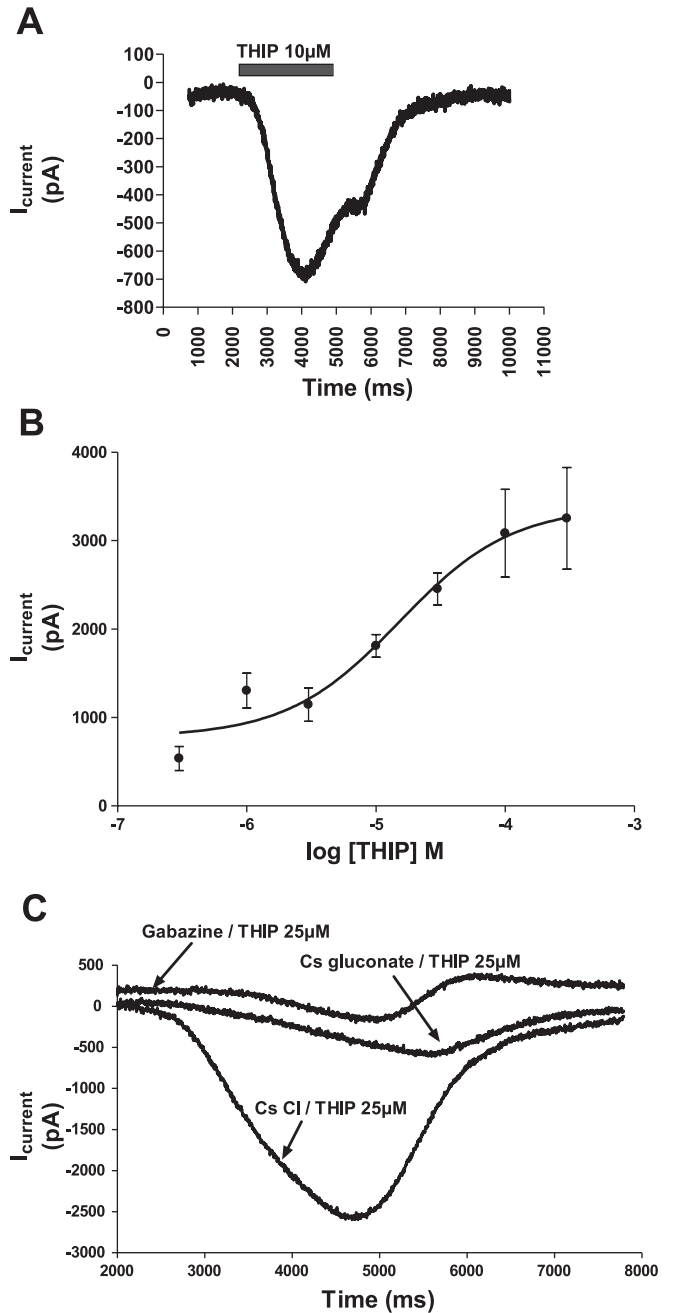

Fig. 3. Electrophysiological characterization of human airway smooth muscle cells following exposure to THIP. *A*: compiled and smoothed tracings (pA/ms) of evoked current from human airway smooth muscle cells following exposure to 10 μM THIP (representative of 11/12 arrays of whole cell configurations with mean current of −670.4 ± 27.6 pA). Each array consisted of 20 human airway smooth muscle cells simultaneously held in parallel whole cell configuration on a microfluidic Fluxion automated patch-clamp platform with a minimal seal resistance >300 MΩ. *B*: dose-response curve of THIP (0 to 300 μM) eliciting a current from human airway smooth muscle cells held in whole cell configuration and voltage clamped at −80 mV. EC$_{50}$ = 15 μM. Data are obtained from 4 separate experiments. *C*: gabazine or chloride free buffer attenuation of THIP induced whole cell currents. Representative tracings depicting THIP-induced currents over time for human airway smooth muscle cells held in voltage clamp at −80 mV under control conditions (CsCl/THIP 25 μM), selective GABA$_A$ receptor antagonism (gabazine/THIP 25 μM), and removal of chloride from the buffer (Cs gluconate/THIP 25 μM). I$_{current}$, ionic current.

reversal of this effect by strychnine (29). We now present a separate subset of these experiments, directed at determining the component of taurine's effect at the GABA$_A$ receptor. Utilizing the same paradigm outlined with THIP above, we found that taurine (200 μM) potentiated isoproterenol-mediated relaxation of acetylcholine precontracted airway smooth muscle (Fig. 5). Cotreatment with taurine and isoproterenol resulted in a significant leftward shift in the isoproterenol relaxation concentration-response curve compared with treatment with isoproterenol alone [EC$_{50}$ = 2.4 nM (n = 7) vs. 15.8 nM (n = 6), respectively; $P < 0.01$]. To demonstrate that α$_4$-containing GABA$_A$ receptor activation is an important component of taurine's effect, the GABA$_A$ selective antagonist gabazine (200 μM) was used in this experimental permutation
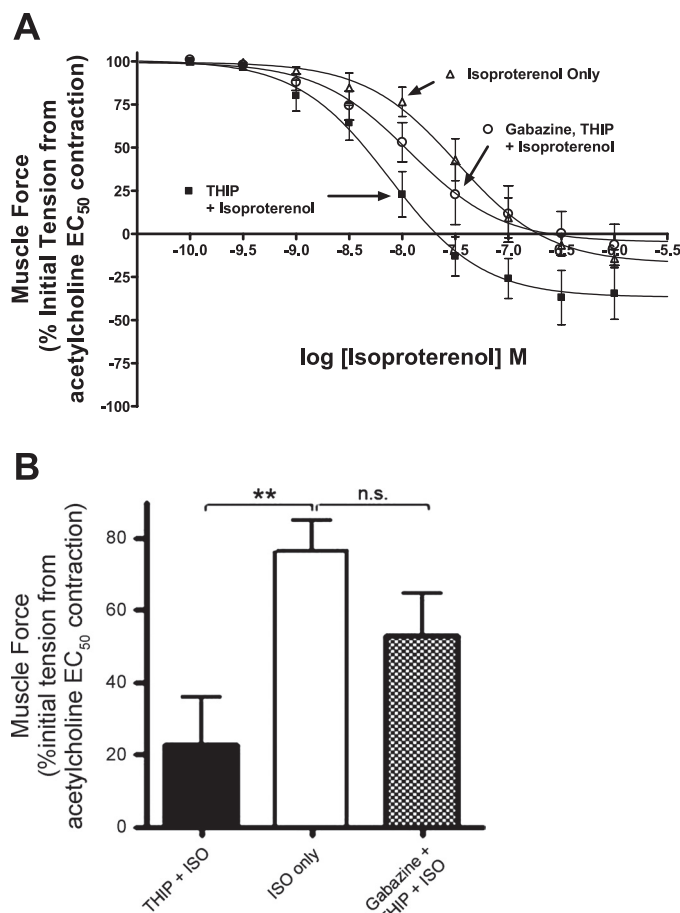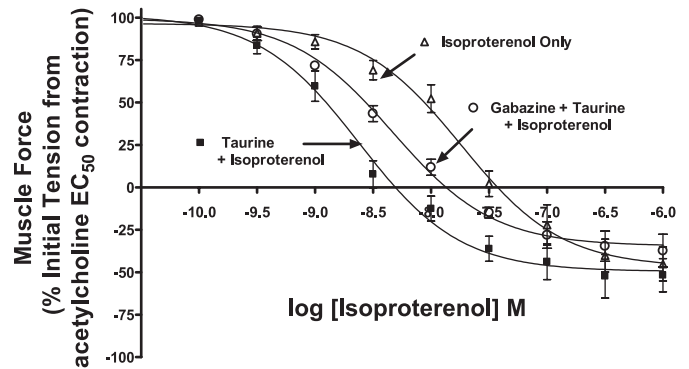

Fig. 5. Taurine activation of α$_4$-containing GABA$_A$ receptors potentiates β$_2$-adrenoceptor-mediated airway smooth muscle relaxation following an EC$_{50}$ contractile stimulus with acetylcholine. Compiled isoproterenol concentration-response curves comparing treatment with isoproterenol only [EC$_{50}$ = 15.8 nM (n = 6); △] to isoproterenol after a single concentration of 200 μM taurine [EC$_{50}$ = 2.4 nM (n = 7); ■] and isoproterenol with a single concentration of 200 μM taurine subsequent to 200 μM gabazine pretreatment [EC$_{50}$ = 4.7 nM (n = 7); ○]. Taurine treatment results in a severalfold reduction in the EC$_{50}$ for isoproterenol response, and this prorelaxant effect is significantly but partially reversed ($P < 0.05$, respectively) by GABA$_A$ receptor antagonist pretreatment.

Fig. 4. THIP augmentation of β$_2$-adrenoceptor-mediated guinea pig airway smooth muscle relaxation following an EC$_{50}$ contractile stimulus with acetylcholine. A: compiled isoproterenol concentration-response curves comparing treatment with isoproterenol only (EC$_{50}$ = 32.8 nM; n = 8; △) to isoproterenol after a single concentration of 500 μM THIP (EC$_{50}$ = 7.6 nM; n = 8; ■) and isoproterenol with a single concentration of 500 μM THIP subsequent to 500 μM gabazine pretreatment (EC$_{50}$ = 11.8 nM; n = 8; ○). Selective GABA$_A$ receptor subunit activation results in a significant severalfold reduction in the EC$_{50}$ for isoproterenol response; this THIP-mediated prorelaxant effect is eliminated by GABA$_A$ receptor antagonist pretreatment. B: THIP treatment also resulted in a significant potentiation of relaxation even at a low concentration (10 nM) of isoproterenol (ISO) [muscle force = 22.9 ± 13.2% (n = 8) of initial acetylcholine-induced force for THIP plus isoproterenol vs. 76.5 ± 8.5% for isoproterenol alone (n = 8); $P < 0.01$], whereas pretreatment with gabazine reversed this THIP effect [muscle force = 53.1 ± 11.4% (n = 8); $P > 0.05$ compared with isoproterenol alone]. **$P < 0.01$, n.s. = not significant.

to block taurine's effect. As seen with our THIP studies, pretreatment with gabazine significantly reversed the potentiation of isoproterenol-mediated relaxation provided by taurine [EC$_{50}$ = 4.7 nM (n = 7) vs. 2.4 nM (n = 7); $P < 0.05$]. However, in agreement with a partial role of GABA$_A$ receptor activation in taurine-mediated relaxation, gabazine did not completely reverse the relaxation afforded by taurine treatment [EC$_{50}$ = 4.7 nM (n = 7) vs. 15.8 nM (n = 6); $P < 0.05$]. Each n represents the total number of individual rings in a treatment group.

Selective activation of α$_4$-subunit-containing GABA$_A$ receptors using a novel α$_4$-subunit ligand induces spontaneous relaxation of airway smooth muscle precontracted with either TEA or substance P. To further demonstrate that selective activation of α$_4$-subunit-containing airway smooth muscle GABA$_A$ receptors can spontaneously induce relaxation, we also tested a third compound known to also be a selective agonist for α$_4$-subunit-containing GABA$_A$ receptors: CM-D-45 (13). Following a plateau in the force generated by TEA (10 mM), addition of 200 μM CM-D-45 resulted in direct relaxation compared with matched DMSO (vehicle)-treated controls (Fig. 6A). Treatment of TEA-precontracted airway smooth muscle with CM-D-45 resulted in a significant decrease in initial muscle force (reported as % gram tension remaining from pretreatment levels) over 15 min (54.3% ± 14.3; n = 14) compared with DMSO 0.1%-treated tissues (99.9% ± 9.5; n = 13; $P < 0.0001$). Similar results were achieved following challenge with a neurokinin receptor-mediated airway smooth muscle contraction using substance P. As before, CM-D-45 treatment induced a significant direct relaxation of precontracted airway smooth muscle (61.3% ± 5.3; n = 10) compared with matched vehicle controls (89.52% ± 1.9; n = 8; $P < 0.001$), illustrating that this effect is not limited to a specific contractile agent. Each n represents the total number of individual rings in a treatment group.

DISCUSSION

The major findings of this study are that human airway smooth muscle possesses GABA$_A$ receptors with a restricted
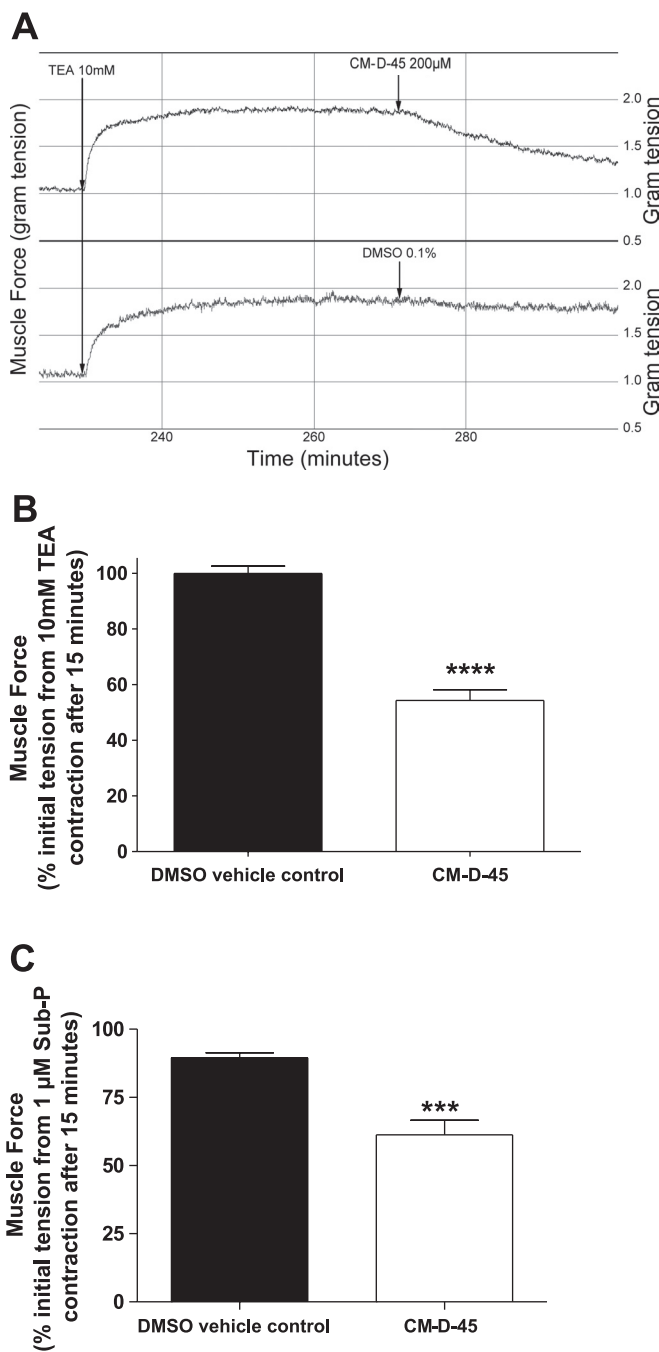
Fig. 6. CM-D-45 activation of α$_4$-containing airway smooth muscle GABA$_A$ receptors induces direct relaxation of TEA and substance P-induced contractions. Representative tracings (in muscle force/time) illustrating direct relaxation achieved by CM-D-45 following a contraction achieved by 10 mM TEA (*top tracing*) compared with 0.1% DMSO vehicle control (*bottom tracing*). CM-D-45 induces a significant degree of spontaneous relaxation following a 10 mM TEA-mediated contraction compared with treatment with 0.1% DMSO vehicle control at 15 min following drug addition. Muscle force = 54.3 ± 14.3% ($n = 13$) of initial TEA-induced force following CM-D-45 treatment vs. 99.1 ± 9.5% for vehicle control alone ($n = 13$); ****$P < 0.0001$. CM-D-45 also induces significant spontaneous relaxation following a 1 μM substance P-mediated contraction compared with treatment with 0.1% DMSO vehicle control at 15 min following drug addition. Muscle force = 61.3 ± 16.9% ($n = 10$) of initial TEA-induced force following CM-D-45 treatment vs. 89.5 ± 5.3% for vehicle control alone ($n = 10$); ***$P < 0.001$.

(yet conserved) α-subunit phenotype that can be pharmacologically targeted by selective agonists to generate electrophysiological changes and facilitate relaxation of precontracted airway smooth muscle. Our mRNA profiling of RNA isolated from airway smooth muscle using laser capture microdissection validates the use of guinea pig airway smooth muscle as a surrogate for human tissue since the GABA$_A$ α-subunit repertoire is shared between these two species. Variability in GABA$_A$ receptor subunit composition not only is known to alter agonist binding but also can affect the responsiveness of a given GABA$_A$ receptor to allosteric activation from a wide variety of agents including steroids, ethanol, and zinc (21, 24, 28). The expression of only two subtypes of GABA$_A$ α-subunits is an important finding since these subunits play a key role in determining GABA$_A$ receptor drug selectivity. Specifically, the α-subunit, for which there are six isoforms (α$_{1-6}$), plays an important role in ligand, agonist, and allosteric binding. For example, the GABA binding site has been mapped to the interface between both the α- and β-subunits in the pentamer (18). Additionally, the particular α-subunit isoform present has been shown to have dramatic effects on the allosterism achievable by classic benzodiazepines, with the α$_4$- and α$_6$-subunits demonstrating low affinity for this drug class and consequently a marked insensitivity to typical benzodiazepine-mediated effects (11, 23). Behavioral responses also show subunit isoform specificity, with α$_1$ mediating sedative effects (20), α$_{2/3}$ mediating anxiolytic effects (4), and α$_5$ mediating effects on memory (3). The impact of α-subunit isoforms also seems to influence the responsiveness of GABA$_A$ receptors to neurosteroid-mediated allosteric potentiation, with α$_1$ and α$_3$ demonstrating a heightened responsiveness to this steroid class (19).

Given the potential for differential drug responses afforded by particular GABA$_A$ receptor α-subunit isoforms, it is particularly fortuitous that the repertoire of these isoforms on human airway smooth muscle is restricted to only the α$_4$- and α$_5$-subunits. This is particularly true for the α$_4$-subunit, for which there are several commercially available agonists showing enhanced activation of GABA$_A$ receptors containing this alpha isoform. For example, gaboxadol (THIP) displays selectivity for α$_4$-containing GABA$_A$ receptors (1), especially if composed of both α$_4$- and δ-subunits (25). Interestingly in a heterologous single-cell overexpression system, THIP has been shown to demonstrate preferential activation of distinct GABA$_A$ receptor α-subunit combinations in a dose-dependent manner with activation of α$_4$/δ receptors occurring at low concentrations (EC$_{20}$ 1–2 μM) followed by activation of α$_5$/γ receptors occurring at increased concentrations (EC$_{20}$ 10–20 μM). We first sought to demonstrate that THIP could induce a dose-dependent change in membrane potential through the use of a membrane potentiometric dye (FLIPR blue). Our results indicate that THIP dose dependently changes membrane potential, with fluorescent changes yielding an EC$_{50}$ of 245 μM. There are several reasons why this value may be higher than THIP's effective concentration in previous studies: First, extrapolating results from an overexpression model of a single subunit conformation in an oocyte cell is not a reasonable comparison because our human airway smooth muscle cells express a mixture of different GABA$_A$ subunit combinations. Given this inherent heterogeneity, we expect THIP to display a wider dose-dependent effect than may be expected in a pure

single-construct expression model. In addition, there is an inherent insensitivity in using this membrane potentiometric dye. For example, we have previously demonstrated that a significant degree of membrane potential change is required to elicit respective fluorescence changes in human airway smooth muscle cells and occurs at a ratio of 15 mV:100 relative fluorescence units (29). Although the possibility exists that high dose THIP may be activating other targets than GABA$_A$ receptors, we demonstrate reversal of the THIP effect with the selective GABA$_A$ receptor blocker picrotoxin. Nevertheless, given these limitations we performed automated whole cell patch clamp of human airway smooth muscle cells to complement our FLIPR studies. Using an electrophysiological based platform, we demonstrate an EC$_{50}$ of 15 μM following the THIP dose response and illustrate significant reversal of THIP-induced currents under conditions of GABA$_A$ receptor blockade with gabazine. In addition, we demonstrate that THIP specifically is inducing a chloride current since replacement of chloride with gluconate eliminates the current observed during addition of THIP. These findings establish that THIP can be used to pharmacologically target human airway smooth muscle GABA$_A$ receptors and elicit electrophysiological changes consistent with GABA$_A$ receptor activation.

To demonstrate functional relevance, we next sought to demonstrate that treatment of THIP could also lead to enhanced airway smooth muscle relaxation. Using a guinea pig airway smooth muscle in vitro organ bath model we demonstrate that THIP treatment can potentiate isoproterenol relaxation of precontracted airway smooth muscle and that this effect is reversed under pretreatment with a GABA$_A$ receptor antagonist. However, given the close pharmacological overlap with this agonist and its effects achievable at α$_4$- and α$_5$-containing GABA$_A$ receptors and the inherent heterogeneity of subunit expression that likely exists in airway smooth muscle tissue, we purposely included other agents to more selectively target the α$_4$ population of GABA receptors in an attempt to determine whether more selective targeting of this subtype would be sufficient to achieve functional relaxation.

Given the ability of taurine to act as an agonist at α$_4$-subunit-containing GABA$_A$ receptors in neuronal cells (12), we questioned whether this agent would reproduce the relaxation achieved with THIP. We found that taurine also potentiated isoproterenol-mediated airway smooth muscle relaxation; however, we noted that although gabazine antagonism did significantly attenuate taurine's effect on relaxation the reversal was partial. This demonstration of partial reversal is consistent with taurine having a non-GABA$_A$ receptor effect. Since taurine is also a ligand at glycine receptors, this finding propelled us to the novel finding that glycine receptors also are expressed and contribute to taurine-mediated relaxation (29). Therefore, whereas taurine effects on relaxation were reversible under pretreatment with the GABA$_A$ receptor antagonist gabazine, its lack of specificity for only α$_4$-containing GABA$_A$ receptors prompted us to employ a third agonist with higher specificity in our functional studies.

Using a synthetic derivative (an 8-methoxy imidazobenzodiazepine) with high specificity to α$_4$/α$_6$-containing GABA$_A$ receptors (CM-D-45), we expanded our functional studies to determine whether activation of α$_4$-containing GABA receptors would induce spontaneous relaxation of precontracted airway smooth muscle. Given the absence of α$_6$-subunit ex-

pression in airway smooth muscle, this drug holds the highest possibility for truly targeting solely α$_4$-subunit-containing GABA$_A$ receptors in our tissue among the different agonists we tested. We conducted a similar experimental design for these organ bath studies but omitted treatment with isoproterenol. We found that CM-D-45 directly relaxed airway smooth muscle precontracted with either TEA or substance P, suggesting that targeting α$_4$-containing GABA$_A$ receptors may elicit spontaneous relaxation on its own against both a pure hyperpolarizing agent (TEA) or following a G-coupled contractile agonist (substance P).

In summary, we present the following evidence: *1*) through highly selective sampling of airway smooth muscle, there is a restricted and conserved repertoire of α$_4$ and α$_5$-GABA$_A$ α-subunits in airway smooth muscle, *2*) agonists pharmacologically targeting this repertoire produce characteristic electrophysiological changes indicative of GABA$_A$ receptor activation, *3*) these selective agonists can augment isoproterenol-mediated relaxation, and *4*) GABA$_A$ α$_4$-receptor activation can directly and spontaneously relax precontracted airway smooth muscle from a variety of procontractile agents. As such, these studies hold promise and potential for improving the armamentarium of pharmacological agents available to treat acute airway bronchoconstriction.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. **Chandra D, Jia F, Liang J, Peng Z, Suryanarayanan A, Werner DF, Spigelman I, Houser CR, Olsen RW, Harrison NL, Homanics GE.** GABA$_A$ receptor alpha 4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. *Proc Natl Acad Sci USA* 103: 15230–15235, 2006.
2. **Chapman RW, Danko G, Rizzo C, Egan RW, Mauser PJ, Kreutner W.** Prejunctional GABA-B inhibition of cholinergic, neurally-mediated airway contractions in guinea-pigs. *Pulm Pharmacol* 4: 218–224, 1991.
3. **Collinson N, Kuenzi FM, Jarolimek W, Maubach KA, Cothliff R, Sur C, Smith A, Otu FM, Howell O, Atack JR, McKernan RM, Seabrook GR, Dawson GR, Whiting PJ, Rosahl TW.** Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the alpha 5 subunit of the GABA$_A$ receptor. *J Neurosci* 22: 5572–5580, 2002.
4. **Dias R, Sheppard WF, Fradley RL, Garrett EM, Stanley JL, Tye SJ, Goodacre S, Lincoln RJ, Cook SM, Conley R, Hallett D, Humphries AC, Thompson SA, Wafford KA, Street LJ, Castro JL, Whiting PJ, Rosahl TW, Atack JR, McKernan RM, Dawson GR, Reynolds DS.** Evidence for a significant role of alpha 3-containing GABA$_A$ receptors in mediating the anxiolytic effects of benzodiazepines. *J Neurosci* 25: 10682–10688, 2005.
5. **Eder W, Ege MJ, von Mutius E.** The asthma epidemic. *N Engl J Med* 355: 2226–2235, 2006.
6. **Gallos G, Gleason NR, Virag L, Zhang Y, Mizuta K, Whittington RA, Emala CW.** Endogenous gamma-aminobutyric acid modulates tonic guinea pig airway tone and propofol-induced airway smooth muscle relaxation. *Anesthesiology* 110: 748–758, 2009.
7. **Gallos G, Gleason NR, Zhang Y, Pak SW, Sonett JR, Yang J, Emala CW.** Activation of endogenous GABA$_A$ channels on airway smooth muscle potentiates isoproterenol-mediated relaxation. *Am J Physiol Lung Cell Mol Physiol* 295: L1040–L1047, 2008.
8. **Gleason NR, Gallos G, Zhang Y, Emala CW.** The GABA$_A$ agonist muscimol attenuates induced airway constriction in guinea pigs in vivo. *J Appl Physiol* 106: 1257–1263, 2009.

9. **Glykys J, Mody I.** Activation of GABA$_A$ receptors: views from outside the synaptic cleft. *Neuron* 56: 763–770, 2007.
10. **Gosens R, Stelmack GL, Dueck G, McNeill KD, Yamasaki A, Gerthoffer WT, Unruh H, Gounni AS, Zaagsma J, Halayko AJ.** Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 291: L523–L534, 2006.
11. **Hevers W, Luddens H.** The diversity of GABA$_A$ receptors. Pharmacological and electrophysiological properties of GABA$_A$ channel subtypes. *Mol Neurobiol* 18: 35–86, 1998.
12. **Jia F, Yue M, Chandra D, Keramidas A, Goldstein PA, Homanics GE, Harrison NL.** Taurine is a potent activator of extrasynaptic GABA(A) receptors in the thalamus. *J Neurosci* 28: 106–115, 2008.
13. **Li XY, Ma CR, He XH, Yu JM, Han DM, Zhang CC, Atack JR, Cook JM.** Studies in search of diazepam-insensitive subtype selective agents for GABA(A)/Bz receptors. *Med Chem Res* 11: 504–537, 2002.
14. **McKernan RM, Whiting PJ.** Which GABAA-receptor subtypes really occur in the brain? *Trends Neurosci* 19: 139–143, 1996.
15. **Mizuta K, Osawa Y, Mizuta F, Xu D, Emala CW.** Functional expression of GABA$_B$ receptors in airway epithelium. *Am J Respir Cell Mol Biol* 39: 296–304, 2008.
16. **Mizuta K, Xu D, Pan Y, Comas G, Sonett JR, Zhang Y, Panettieri RA Jr, Yang J, Emala CW Sr.** GABA$_A$ receptors are expressed and facilitate relaxation in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 294: L1206–L1216, 2008.
17. **Moore CT, Wilson CG, Mayer CA, Acquah SS, Massari VJ, Haxhiu MA.** A GABAergic inhibitory microcircuit controlling cholinergic outflow to the airways. *J Appl Physiol* 96: 260–270, 2004.
18. **Olsen RW, Sieghart W.** GABA A receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology* 56: 141–148, 2009.
19. **Rahman M, Lindblad C, Johansson IM, Backstrom T, Wang MD.** Neurosteroid modulation of recombinant rat alpha5beta2gamma2L and alpha1beta2gamma2L GABA(A) receptors in Xenopus oocyte. *Eur J Pharmacol* 547: 37–44, 2006.
20. **Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Mohler H.** Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. *Nature* 401: 796–800, 1999.
21. **Shingai R, Sutherland ML, Barnard EA.** Effects of subunit types of the cloned GABA$_A$ receptor on the response to a neurosteroid. *Eur J Pharmacol* 206: 77–80, 1991.
22. **Sieghart W, Sperk G.** Subunit composition, distribution and function of GABA(A) receptor subtypes. *Curr Top Med Chem* 2: 795–816, 2002.
23. **Slany A, Zezula J, Fuchs K, Sieghart W.** Allosteric modulation of [^{3}H]flunitrazepam binding to recombinant GABA$_A$ receptors. *Eur J Pharmacol* 291: 99–105, 1995.
24. **Smart TG, Moss SJ, Xie X, Huganir RL.** GABA$_A$ receptors are differentially sensitive to zinc: dependence on subunit composition. *Br J Pharmacol* 103: 1837–1839, 1991.
25. **Storustovu SI, Ebert B.** Pharmacological characterization of agonists at delta-containing GABA$_A$ receptors: functional selectivity for extrasynaptic receptors is dependent on the absence of gamma2. *J Pharmacol Exp Ther* 316: 1351–1359, 2006.
26. **Tohda Y, Ohkawa K, Kubo H, Muraki M, Fukuoka M, Nakajima S.** Role of GABA receptors in the bronchial response: studies in sensitized guinea-pigs. *Clin Exp Allergy* 28: 772–777, 1998.
27. **Wafford KA, van Niel MB, Ma QP, Horridge E, Herd MB, Peden DR, Belelli D, Lambert JJ.** Novel compounds selectively enhance delta subunit containing GABA A receptors and increase tonic currents in thalamus. *Neuropharmacology* 56: 182–189, 2009.
28. **Wafford KA, Whiting PJ.** Ethanol potentiation of GABA$_A$ receptors requires phosphorylation of the alternatively spliced variant of the gamma 2 subunit. *FEBS Lett* 313: 113–117, 1992.
29. **Yim PD, Gallos G, Xu D, Zhang Y, Emala CW.** Novel expression of a functional glycine receptor chloride channel that attenuates contraction in airway smooth muscle. *FASEB J* 25: 1706–1717, 2011.