ORIGINAL RESEARCH

Targeting the γ-Aminobutyric Acid A Receptor α4 Subunit in Airway Smooth Muscle to Alleviate Bronchoconstriction

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Abstract

We previously demonstrated that airway smooth muscle (ASM) cells express γ-aminobutyric acid A receptors ($GABA_AR$s), and that $GABA_AR$ agonists acutely relax ASM. Among the $GABA_AR$ α subunits, human ASM cells express only α4 and α5, providing the opportunity for selective pharmacologic targeting. Novel $GABA_AR$-positive allosteric modulators designed for enhanced α4/α6 subunit selectivity were synthesized using iterative computational analyses (CMD-45 and XHe-III-74). Studies using oocyte heterologous expression systems confirmed that CMD-45 and XHe-III-74 led to significantly greater augmentation of currents induced by a 3% maximal effective concentration (EC_3) of GABA [EC_3]–induced currents in oocytes expressing α4 or α6 subunits (along with β3 and γ2) compared with other α subunits. CMD-45 and XHe-III-74 also led to greater *ex vivo* relaxation of contracted wild-type mouse tracheal rings compared with tracheal rings from $GABA_AR$ α4 subunit (Gabra4) knockout mice. Furthermore, CMD-45 and XHe-III-74 significantly relaxed precontracted human ASM *ex vivo*, and, at a low concentration, both ligands led to a significant leftward shift in albuterol-mediated ASM relaxation. *In vivo*, inhaled XHe-III-74 reduced respiratory system resistance in an asthmatic mouse model. Pretreatment of human ASM cells with CMD-45 and XHe-III-74 inhibited histamine-induced increases in intracellular calcium concentrations *in vitro*, an effect that was lost when calcium was omitted from the extracellular buffer, suggesting that inhibition of calcium influx due to alterations in plasma membrane potential may play a role in the mechanism of ASM relaxation. Selective targeting of the $GABA_AR$ α4 subunit with inhaled ligands may be a novel therapeutic pathway to treat bronchoconstriction, while avoiding sedative central nervous system effects, which are largely mediated by α1–3 subunit–containing $GABA_A$Rs in the brain.

Keywords: XHe-III-74; CMD-45; flexiVent; asthma; $GABA_A$ receptor

Asthma affects hundreds of millions worldwide (1), and its incidence, particularly in urban areas, is growing. In fact, asthma is now the leading cause of emergency room evaluations, hospitalizations, and school absenteeism in New York City (2). Current asthma management strategies include both chronic maintenance therapies (i.e., long-acting β_2-agonists, corticosteroids, and leukotriene modifiers) and acute rescue therapies (i.e., short-acting β_2-agonists) designed to rapidly relax airway smooth muscle (ASM) during acute attacks. However, these therapies inadequately control symptoms for as many as 55% of subjects with asthma, even when care is consistent with current standards (3). Furthermore, the long-acting β_2-agonists are under scrutiny for their safety and efficacy (4–6). As a result, new therapeutic strategies are sorely needed.

We have previously demonstrated that ASM cells express γ-aminobutyric acid A

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Clinical Relevance

Pharmacologic targeting of airway smooth muscle (ASM) cell γ-aminobutyric acid A receptors ($GABA_AR$s) with novel α4 subunit–selective compounds promises to be a new therapeutic strategy in the treatment of bronchoconstriction. These compounds, which can be delivered directly to the airway by aerosol, relax ASM and may avoid sedative central nervous system side effects, which are largely mediated by α1–3 subunit–containing $GABA_A$ receptors.

receptors ($GABA_AR$s), which are ligand-gated chloride channels best known for their role in central nervous system (CNS) inhibitory neurotransmission, and that $GABA_AR$ agonists led to ASM relaxation (7, 8). However, there is legitimate concern that the therapeutic use of $GABA_AR$ modulators to alleviate bronchoconstriction may activate CNS $GABA_AR$s, leading to undesirable CNS side effects. $GABA_AR$s are pentamers made up of a combination of 19 possible subunits (α[1–6], β[1–3], γ[1–3], δ, ε, θ, π, and ρ[1–3]). Most commonly, these receptors contain two α subunits, two β subunits, and one "tertiary" subunit (γ, δ, ε, θ, or π), although other arrangements occur (9, 10). Among the α subunits, human ASM cell $GABA_AR$s contain only α4 or α5 subunits (human ASM cells also express β3, γ2, δ, and θ subunits, thus expressing the necessary complement of subunits to form functional channels) (11). In contrast, the majority of $GABA_AR$s in the brain contain α1, α2, or α3 subunits. In fact, it has been estimated that only 6% of brain $GABA_AR$s contain the α4 subunit (12) (combining with βγ or βδ or other subunits to form the $GABA_AR$ pentamer [10, 12–14]). Furthermore, receptors containing α1, α2, or α3 subunits are generally thought to mediate the phasic, large-amplitude chloride currents that lead to the sedative effects of GABAergic sedatives and anesthetics (15). Therefore, selective activation of $GABA_AR$s with α4 subunits may offer the potential for therapeutic bronchodilation, while avoiding CNS depression.

We hypothesized that positive allosteric modulators of the $GABA_AR$ designed for enhanced selectivity for the α4 subunit will acutely relax ASM. In this article, we demonstrate that two such novel compounds, CMD-45 and XHe-III-74, are positive allosteric modulators of the $GABA_AR$ with enhanced selectivity for receptors containing the α4/α6 subunit, and that these compounds acutely relax ASM from mice and humans. These selective compounds offer the potential to combat bronchoconstriction via a new therapeutic mechanism, while reducing unwanted side effects.

Materials and Methods

Reagents, Animals, and Tissues

CMD-45 and XHe-III-74 were designed for enhanced $GABA_AR$ α4 subunit selectivity using iterative computational analyses and synthesized in the laboratory of Dr. James M. Cook (University of Wisconsin–Milwaukee, Milwaukee, WI). All animal studies were approved by the Columbia University (New York, NY) Institutional Animal Care and Use Committee. Male global $GABA_AR$ α4 subunit knockout (KO) mice (Gabra4 KO; gift of Dr. Gregg Homanics [University of Pittsburgh, Pittsburgh, PA) (13) and/or wild-type (WT) C57/Bl6 mice (8–10 weeks old) were used for all mouse studies. WT mice underwent intranasal house dust mite (HDM) antigen sensitization to induce an asthmatic phenotype before *in vivo* respiratory system resistance (R_{RS}) studies.

Human trachea ASM samples were obtained from healthy transplantation donor lungs. Primary human ASM cells used for *in vitro* experiments were obtained from these samples via enzymatic dissociation, as previously described (16). Experiments using these samples were deemed non–human subjects research by Columbia University's Institutional Review Board.

Oocyte Electrophysiological Studies

Xenopus laevis oocytes (Nasco, Fort Atkinson, WI) were coinjected with synthesized rat $GABA_AR$ subunit messenger RNAs (each α subunit [1–6] with β3 and γ2, or α4 with β3 and δ), as described previously (17), to express $GABA_AR$s of prescribed subunit composition. Concentration ranges of CMD-45 or XHe-III-74 were applied for 30 seconds before the addition of a 3% maximal effective concentration (EC_3) of GABA, and voltage-clamp current recordings were performed at a holding potential of −60 mV.

Mouse Tracheal Ring Organ Bath Experiments

Tracheal rings from WT and Gabra4 KO mice were mounted on wire pins in a myograph system (DMT, Ann Arbor, MI), as described previously (18). The rings were contracted with their respective half-maximal effective concentration (EC_{50}) of acetylcholine (ACh) and then exposed to concentration ranges of CMD-45, XHe-III-74, or DMSO vehicle during continuous contraction force recording.

Human ASM Strip Organ Bath Experiments

Human ASM strips were dissected from tracheal samples, the epithelium was removed, and the strips were suspended in glass organ baths. Each strip was contracted with its EC_{50} concentration of ACh. The strips were then exposed to concentration ranges of CMD-45, XHe-III-74, or 0.2% DMSO vehicle during continuous contraction force recording.

In separate studies, human ASM strips were contracted with their EC_{50} concentration of ACh, and increasing concentrations of albuterol were added at 7-minute intervals (half-log increments 100 pM–10 μM). Concurrent with the 500-pM albuterol addition, a single exposure of CMD-45, XHe-III-74 (25 μM), or vehicle (0.2% DMSO) was added.

In Vivo Mouse Respiratory System Resistance Testing

Using a flexiVent (SciReq, Montreal, PQ, Canada) as previously described (18, 19), anesthetized, HDM-sensitized WT mice received nebulized XHe-III-74 (25 μl, 10 mM) or vehicle (25% ethanol in PBS) via tracheostomy 10 minutes before measuring *in vivo* R_{RS} during a nebulized methacholine challenge.

In Vitro Human ASM Cell Calcium Dynamics

Primary human ASM cells were grown to 80% confluence on 96-well plates and loaded with a fluorescent calcium indicator dye (Fura-2 AM; Life Technologies, Grand Island, NY). After a 10-minute pretreatment with concentration ranges of CMD-45, XHe-III-74, or DMSO (0.1%), intracellular

calcium–mediated fluorescence was recorded during exposure to 10 μM histamine using a fluorescent plate reader in the presence and absence of 2 mM external calcium.

Results

Oocyte Electrophysiology Studies

Previous studies have demonstrated that the binding affinity of both XHe-III-74 and CMD-45 is significantly higher for $GABA_A$Rs containing the α4 subunit, followed by those containing α6 (20). In oocytes expressing each α subunit individually (along with β3 and γ2 subunits; α[x]β3γ2), both CMD-45 and XHe-III-74 produced a greater augmentation of GABA [EC_3]-induced currents in oocytes expressing α4 or α6 subunits compared with other α subunits at multiple concentrations (Figures 1 and 2; $P < 0.01$, $n = 3$–4).

Separate studies in oocytes expressing α4 along with the δ subunit (α4β3δ) showed no XHe-III-74–mediated augmentation of GABA ([EC_3])–induced currents, even at the highest concentration of XHe-III-74 tested (10 μM; data not shown; $n = 4$). Similarly, 10 μM CMD-45 showed only a modest augmentation of GABA-induced currents (116 ± 3% of current induced by a GABA EC_3 concentration alone, $P < 0.05$, $n = 4$). CMD-45 did not significantly augment GABA-induced currents at lower concentrations (data not shown). These studies demonstrate the selectivity of CMD-45 and XHe-III-74 for α4/6 subunit–containing receptors, and also demonstrate that they have very little to no activity at receptors containing the α4/δ subunit combination.

Mouse Tracheal Ring Organ Bath Studies

Treatment of ACh-contracted WT mouse tracheal rings with CMD-45 and XHe-III-74 led to significant relaxation in *ex vivo* organ bath experiments (Figure 3). Although tracheal rings from Gabra4 KO mice also relaxed in response to CMD-45 and XHe-III-74, this relaxation was significantly less than in WT mice for both CMD-45 and XHe-III-74 at multiple concentrations. This finding further supports the selectivity of these compounds for the $GABA_A$R α4 subunit and the mechanistic role of $GABA_A$R activation in the ASM relaxation. In these experiments, XHe-III-74 was more potent in relaxing murine tracheal rings than CMD-45. This is consistent with the electrophysiological data presented previously here demonstrating larger $GABA_A$R-mediated currents with exposure to XHe-III-74 compared with CMD-45 at equal concentrations (Figure 2). The prorelaxant effects of both compounds were reversible in WT tracheal rings at the highest doses tested (100 μM for CMD-45 and 50 μM for XHe-III-74) after repeated buffer changes. This was demonstrated by showing that XHe-III-74– or CMD-45–treated rings contracted with equal force to 80 mM KCl compared with vehicle-treated rings after these repeated buffer changes (data not shown).

Human ASM

Both CMD-45 and XHe-III-74 led to a significant reduction in contractile force in ACh-contracted human ASM strips at 50 μM ($n = 5$) and 100 μM ($n = 6$) (Figures 4A and 4B $P < 0.05$ for both compounds compared with vehicle control at 50 and 100 μM).

In separate experiments, low concentrations of both CMD-45 and XHe-III-74 (25 μM) potentiated the albuterol-induced relaxation of human ASM contracted with ACh *ex vivo*, leading to a full log decrease in the albuterol EC_{50} (Figure 4C; albuterol EC_{50} was 807.0 nM for DMSO, 69.3 nM for CMD-45 treatment group, and 87.9 nM for the XHe-III-74 treatment group; $n = 4$, $P < 0.01$ for change in albuterol EC_{50} comparing CMD-45 or XHe-III-74 to vehicle [DMSO]).

Mouse *In Vivo* Respiratory System Resistance

XHe-III-74 (25 μl, 10 mM), when administered by inhalation 10 minutes before a bronchoconstrictive challenge, led to a significant reduction in R_{RS}, as measured by the forced oscillation technique (flexiVent) in asthmatic (HDM-sensitized) mice (Figure 5; area under the curve analysis, $P < 0.05$, $n = 3$ for vehicle, 4 for XHe-III-74). This demonstrates the potential for this compound to be therapeutically administered by inhalation to treat bronchospasm.

ASM Calcium Dynamics

In *in vitro* studies using a fluorescent intracellular calcium indicator, removing calcium from the external buffer led to a 29.8% diminution in histamine-mediated increases in intracellular calcium in primary cultures of human ASM cells ($P < 0.001$ comparing 0 [$n = 28$] to 2 mM [$n = 14$] external calcium in DMSO-pretreated and histamine-stimulated cells). In the absence of extracellular calcium, the addition of 100 μM CMD-45 ($n = 17$) or XHe-III-74 ($n = 20$) did not further augment this inhibition of the histamine-induced intracellular calcium response (Figure 6A; P = not significant). However, in the presence of 2 mM external calcium, CMD-45 and XHe-III-74 (25 and 50 μM) inhibited the histamine-mediated increase in intracellular calcium in cultured primary human ASM cells to an extent similar to removing external calcium (25.0% and 30.0% for 50 μM CMD-45 and XHe-III-74, respectively, $P < 0.05$ and $P < 0.001$, respectively; Figures 6B and 6C). This suggests that these compounds lead to ASM relaxation by limiting calcium influx from the extracellular space, likely as a result of altered plasma membrane potential.

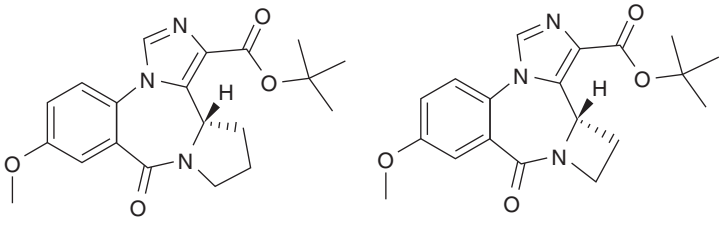

Figure 1. Structure of CMD-45 and XHe-III-74. Both compounds are imidazobenzodiazepine derivatives designed using iterative computational analyses and synthesized for enhanced selectivity for γ-aminobutyric acid A receptors ($GABA_A$Rs) containing α4 or α6 subunits.

Discussion

In the current study, we demonstrate that CMD-45 and XHe-III-74, novel positive allosteric modulators of the $GABA_A$R,

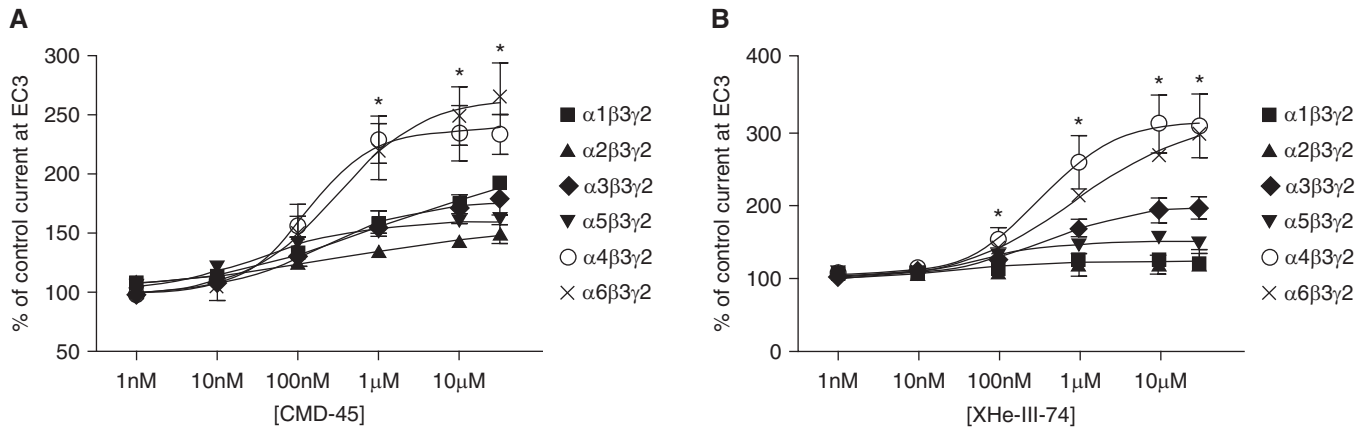

Figure 2. Augmentation of GABA-induced currents in oocytes expressing $GABA_AR$s of specified subunit composition. At multiple concentrations, both CMD-45 and XHe-III-74 led to significantly greater augmentation of $GABA_AR$-mediated currents in oocytes expressing α4 or α6 subunits in combination with β3/γ2 subunits (when each is compared with α1 as a reference in two-way repeated measures ANOVA with Bonferroni post test comparisons). Data are presented as a percent of current induced by a 3% maximal effective concentration (EC_3) of GABA. This demonstrates the subunit selectivity of these novel, positive allosteric modulators of the $GABA_AR$ (*$P < 0.05$ for both α4 and α6 compared with α1 by Bonferroni *post hoc* analysis; $n = 3–4$; mean ± SE).

acutely relax ASM in an α4 subunit–selective manner. Furthermore, in organ bath experiments, these ligands potentiate the relaxation of human ASM induced by albuterol, a β_2-agonist and first line asthma rescue medication. We also demonstrate that XHe-III-74 can be administered by inhalation to treat bronchospasm *in vivo* using a murine asthma model. These studies build upon previous experiments, which demonstrated that human ASM cells express $GABA_AR$s containing α4 or α5 subunits, and that nonselective activation of these receptors leads to acute relaxation *ex vivo* (8). This novel therapeutic approach is exciting, as no new classes of acute bronchodilators have been brought to clinical use in many years, despite a significant clinical need (21). However, given the prominent role that the $GABA_AR$ plays in inhibitory neurotransmission, there is concern that treating bronchospasm with $GABA_AR$ ligands may cause unwanted CNS effects.

There are several possible strategies to attempt to avoid these CNS side effects, including subunit-selective pharmacologic targeting of the $GABA_AR$, aerosolized delivery of ligands directly to the airway followed by peripheral degradation/metabolism, and restricting blood–brain barrier penetration. $GABA_AR$-mediated sedation, as induced by multiple anesthetics, largely results from transient, large-amplitude, hyperpolarizing chloride currents carried by synaptic $GABA_AR$s in the CNS. These $GABA_AR$s generally contain α1, α2, or α3 subunits (15, 22), suggesting that ligands that avoid activity at these subunits may circumvent sedative effects. Extrasynaptic receptors and $GABA_AR$s located outside the CNS more commonly contain α4, α5, or α6 subunits, and mediate tonic, low-amplitude currents (15) (although exceptions likely exist). Human ASM cells, for example, express only $GABA_AR$s containing α4 and α5 (11). Therefore, selective activation of $GABA_AR$s containing α4 subunits offers the potential to treat acute bronchospasm, while minimizing sedative side effects.

Using electrophysiological studies conducted with oocyte heterologous $GABA_AR$ expression systems, we demonstrate that CMD-45 and XHe-III-74 have enhanced functional selectively for receptors containing α4 or α6 over all the other α subunits (expressed with β3 and γ2), and we show that these ligands have very little activity at $GABA_AR$s containing δ subunits (α4β3δ). In additional studies using a $GABA_AR$ α4 subunit KO mouse, we demonstrate that both CMD-45 and XHe-III-74 do, indeed, mediate relaxation of an ACh-induced ASM contraction *ex vivo* in an α4 subunit–selective manner (likely an effect mediated by α4βγ receptors given the ligands inactivity at α4βδ receptors). However, at higher concentrations, both compounds relax ASM from WT and $GABA_AR$ α4 subunit KO mice. This suggests that, at high concentrations, both compounds may be activating $GABA_AR$s containing other α subunits. Mouse ASM may also express $GABA_AR$s containing α6 subunits (human ASM expresses only α4 and α5).

We further demonstrate that CMD-45 and XHe-III-74 significantly relax human ASM that has been contracted with ACh, a Gq-protein coupled receptor agonist. Interestingly, low concentrations of both CMD-45 and XHe-III-74 were also able to potentiate the albuterol-induced relaxation of human ASM strips. This is potentially of clinical significance, as tolerance to first-line β_2-agonists, such as albuterol, is a clinical limitation, particularly among subjects with severe asthma treated with long-acting β_2-agonists. This is likely a result of β_2-receptor desensitization (23, 24) or "cross-talk" between G protein–coupled receptor pathways. Repeated activation of the β_2-receptor, a Gs protein–coupled receptor, has been shown to lead to activation of components of the procontractile Gq protein–coupled receptor pathway, including phospholipase C-β1 (25).

Using the murine asthma model of HDM sensitization and *in vivo* R_{RS} testing (flexiVent), we demonstrate, for the first time, that a $GABA_AR$ modulator can be delivered by inhalation to alleviate acute bronchospasm. Unlike the *ex vivo* organ bath experimental protocols presented here, the *in vivo* protocol includes exposure to XHe-III-74 before the induction of contraction (primarily because bronchoconstriction induced by methacholine on the flexiVent is transient and spontaneously decreases to near baseline; this makes it difficult to assess prorelaxant effects of compounds after methacholine). This suggests that XHe-III-74 may have the potential to not only alleviate

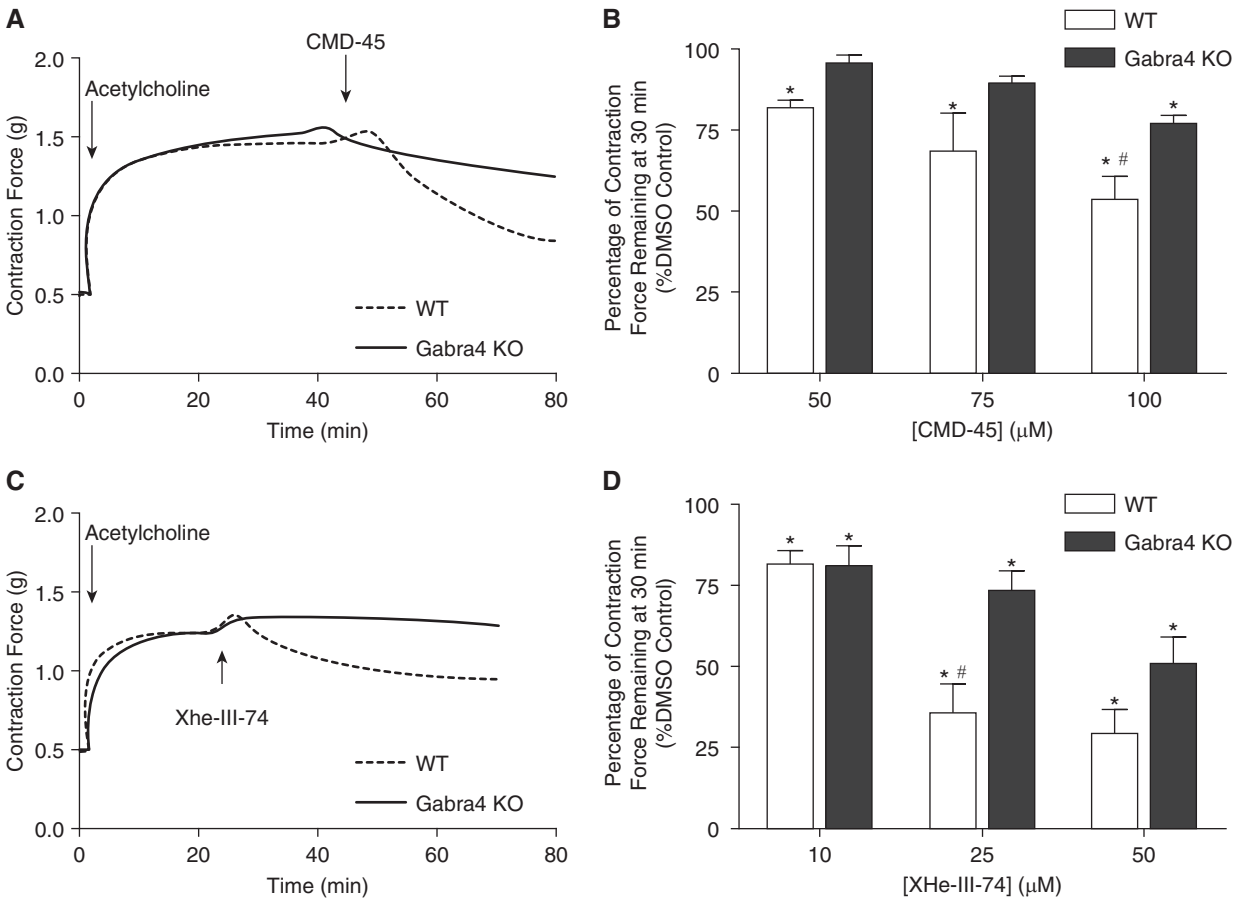

Figure 3. Mouse tracheal ring contraction force in *ex vivo* organ bath preparations. (*A* and *C*) Representative muscle force tracings of acetylcholine (ACh)–contracted murine tracheal rings. (*B*) CMD-45 significantly relaxed precontracted wild-type (WT) murine tracheal rings but not rings from Gabra4 knockout (KO) mice, at 50 μM ($n = 3$) and 75 μM ($n = 3$), consistent with heightened selectivity for the Gabra4 subunit, as demonstrated in Figure 2. (*D*) XHe-III-74 led to significant relaxation of precontracted murine tracheal rings compared with vehicle (0.1% DMSO) in both WT and Gabra4 KO mice at 10 μM ($n = 5$), 25 μM ($n = 5$), and 50 μM ($n = 3$). At 25 μM XHe-III-74, tracheal rings from WT mice relaxed to a greater extent than rings from Gabra4 KO mice, consistent with the heightened Garbra4 selectivity. Contraction force is presented as percent of DMSO vehicle control for WT and Gabra4 KO tracheal rings ($^*P < 0.05$ in comparison to DMSO control, $^{\#}P < 0.05$ in comparison to drug-exposed Gabra4 KO; ANOVA with Bonferroni *post hoc* comparison; mean ± SE).

acute bronchoconstriction, but also to protect against bronchoconstriction. Furthermore, the ability to deliver XHe-III-74 directly to the airway, combined with its α subunit selectivity, promises to limit the degree of systemic drug absorption and unwanted side effects.

In vitro, CMD-45 and XHe-III-74 inhibit the increase in ASM intracellular calcium concentrations ($[Ca^{2+}]_i$) induced by the procontractile Gq protein–coupled receptor agonist histamine by 25–30%, but these compounds provided no further inhibition in the absence of extracellular calcium. This finding offers potential insights into the mechanism of relaxation induced by these compounds. ASM tone is regulated by $[Ca^{2+}]_i$ changes and oscillations that result from sarcoplasmic reticulum release, as well as external Ca^{2+} entry through voltage-sensitive and -insensitive pathways (26). Complex signaling pathways mediate this regulation and ultimately dictate the phosphorylation state and Ca^{2+} sensitivity of the contractile proteins, actin and myosin. As the $GABA_AR$ is a chloride channel, the effects of $GABA_AR$ modulators on ASM tone are likely mediated by membrane potential. Unlike in mature neurons, activating a $GABA_AR$ is expected to depolarize membrane potential in a resting ASM cell due to its higher internal chloride concentration. However, during contraction, the membrane potential of ASM cells increases from a resting potential of approximately −50 to −60 mV to a potential of approximately −20 mV, crossing over the predicted chloride equilibrium potential such that opening of a chloride channel would now favor chloride influx and relative hyperpolarization. Under these conditions, there may be an inhibition of voltage-sensitive Ca^{2+} entry pathways (i.e., entry via voltage-gated calcium channels [26, 27], the Na^+/Ca^{2+} exchanger [28, 29], and/or members of the transient potential receptor family of channels [30]). This is consistent with the *in vitro* data presented here showing that CMD-45 and XHe-III-74 led to a 25–30% decrease in the rise of ASM cell $[Ca^{2+}]_i$ after exposure to histamine. This is equivalent to the proportion of the histamine-induced $[Ca^{2+}]_i$ increase during contraction that is thought to result from extracellular Ca^{2+} entry, as demonstrated here and elsewhere (31). Furthermore, exposure to high concentrations of these compounds

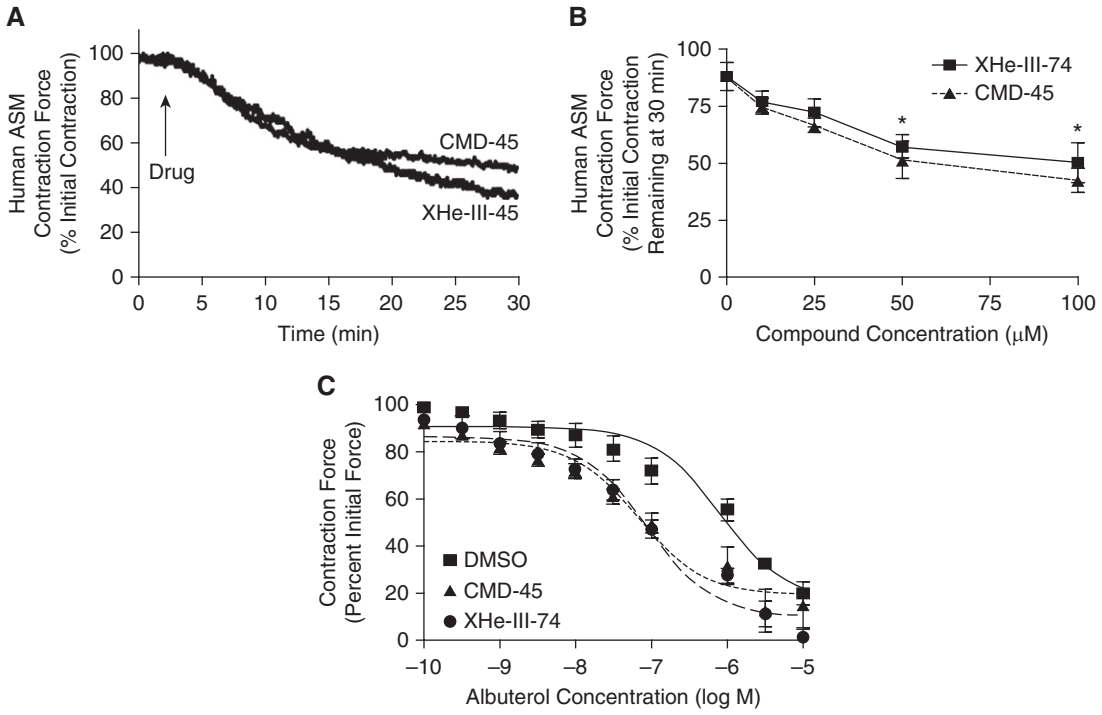

Figure 4. Human tracheal airway smooth muscle (ASM) strips in *ex vivo* organ bath preparations. (*A*) Representative muscle force tracings of the 100 μM CMD-45– and XHe-III-74–induced direct relaxation of ACh [EC_{50}]–precontracted human tracheal ASM strips. (*B*) CMD-45 or XHe-III-74 (50 and 100 μM) induced significant relaxation of ACh-precontracted tracheal ASM strips compared with vehicle (0.2% DMSO) controls at 30 minutes (*n* for 0, 10, 25, 50, and 100 μM, respectively: CMD-45, 6, 6, 4, 5, and 6; XHe-III-74, 6, 4, 3, 5, and 6. *$P < 0.05$ for both CMD-45 and XHe-III-74 compared with vehicle control, ANOVA with Bonferroni *post hoc* comparison). (*C*) CMD-45 (*short dashed line*) and XHe-III-74 (*long dashed line*) at low dose (25 μM) both induced significant leftward shifts in the dose–response curve for albuterol (β_2-adrenoceptor–selective agonist)-mediated human ASM relaxation compared with DMSO control (*solid line*). The EC_{50} concentration of albuterol with coadministration of CMD-45 was 69.3 nM, and with coadministration of XHe-III-74 was 87.9 nM compared with 807.0 nM for DMSO ($n = 4$ per group; $P < 0.01$ for leftward shift in EC_{50} for both ligands compared with vehicle; mean ± SE).

(100 μM) in the absence of extracellular calcium does not further inhibit ASM intracellular calcium increases in response to histamine, further supporting the likelihood that the mechanism of action of these compounds is the inhibition of calcium influx.

As mentioned previously, human ASM cells also express $GABA_A$Rs containing α5 subunits, and we have previously published a report demonstrating ASM relaxation with α5 subunit–selective ligands *ex vivo* (31). However, targeting the α4 subunit may offer advantages over α5 for two reasons. First, in recent mouse studies an α5 subunit–selective ligand produced memory impairments (32), and postanesthesia cognitive impairment was associated with up-regulation of the α5 subunit in the brain (33). Second, the expression of $GABA_A$Rs containing α2 and α5 has been demonstrated on human airway epithelium cells, and activation of these receptors was demonstrated to stimulate mucus production (34). Thus, targeting the α4 subunit may allow for ASM relaxation in the absence of these potential side effects.

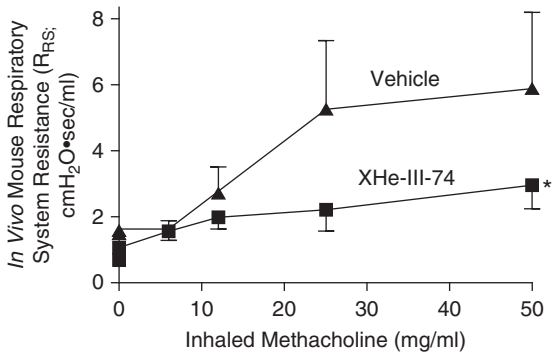

Figure 5. *In vivo* mouse respiratory system resistance (R_{RS}) testing. Inhalation of XHe-III-74 10 minutes before a bronchoconstrictive challenge (methacholine) significantly reduced R_{RS} in house dust mite antigen–sensitized WT mice (asthma model) compared with inhaled vehicle control (*$P < 0.05$ for area under the curve analysis; $n = 3$ for vehicle control, 4 for XHe-III-74; mean ± SE).

Pharmacokinetic studies are underway in mice to determine lung, brain, and liver tissue concentrations of XHe-III-74 after administration by nebulization to determine the extent of systemic absorption. Experiments are also planned to further characterize the potential CNS side effect profile of the drug, which we believe will be limited, given its route of delivery and $GABA_A$R subunit selectivity. However, a modest number of synaptic α4βγ2 receptors does exist in the brain (10), and these receptors may mediate clinical effects.

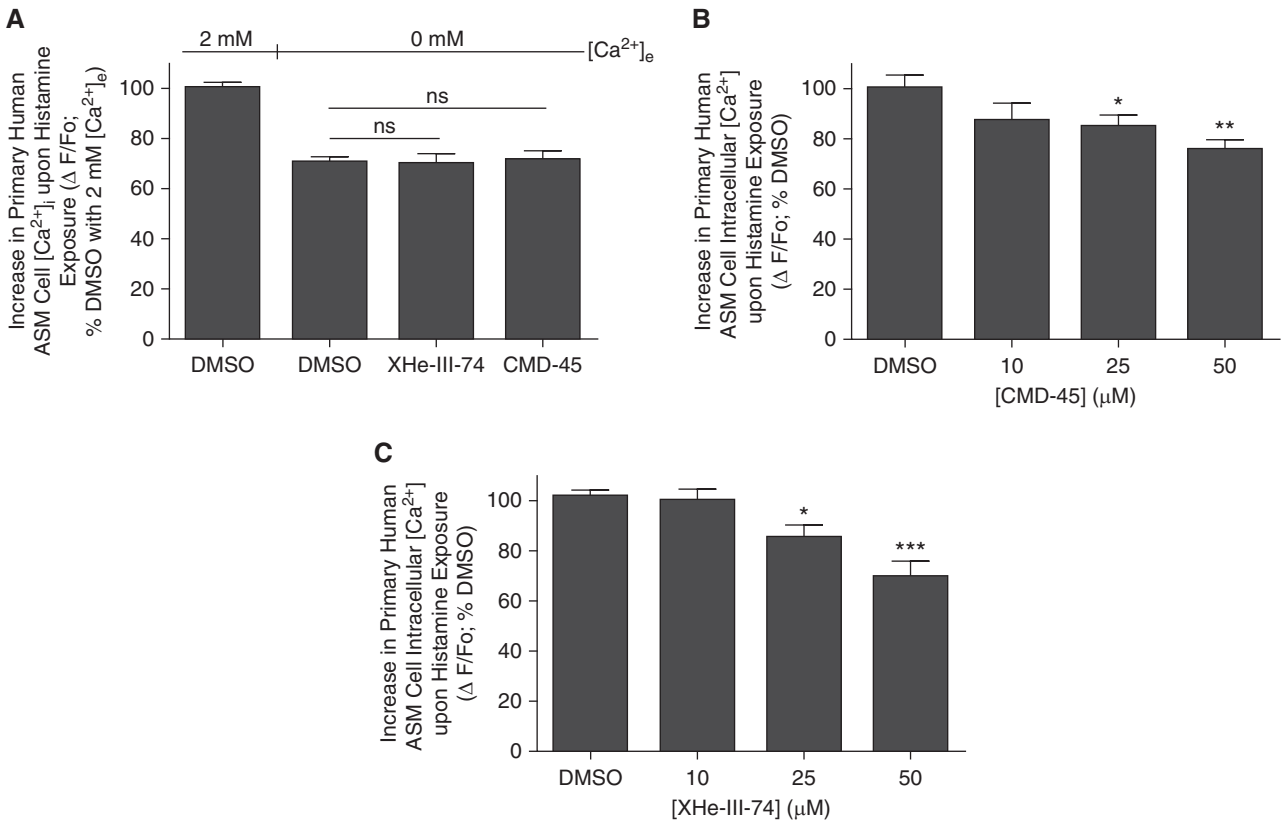

Figure 6. *In vitro* primary human ASM cells calcium dynamics. (*A*) The increase in intracellular calcium concentrations ($[Ca^{2+}]_i$) in primary human ASM upon exposure to 10 μM histamine is inhibited 29% ($P < 0.05$; $n = 28$) by the removal of extracellular calcium ($[Ca^{2+}]_e$; 0 mM). The addition of 100 μM XHe-III-74 ($n = 20$) or CMD-45 ($n = 17$) does not further inhibit histamine-induced increases in $[Ca^{2+}]_i$ ($P > 0.05$). (*B* and *C*) In contrast, in the presence of 2 mM external calcium, pretreatment with 25 or 50 μM CDM-45 or XHe-III-74 significantly inhibited subsequent histamine-induced increases in peak $[Ca^{2+}]_i$ (n [DMSO, 10, 25, and 50 μM]: [*B*] (CMD-45): 12, 9, 11, 9; [*C*] (XHe-III-74): 16, 10, 14, 14; human ASM cell lines established from three donor patients; data are presented as percent of average of simultaneously tested vehicle control wells; n = total plate wells; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA with Bonferroni *post hoc* comparison; mean ± SE). ΔF/Fo, peak change in fluorescence/baseline fluorescence; ns, not significant.

Furthermore, extrasynaptic receptors containing α4 subunits are present in the thalamus and dentate gyrus of mice, and selective activation of these receptors by the $GABA_AR$ ligand, gaboxadol, led to sedation in a previous study (13). However, this effect was thought to be mediated by α4βδ receptors, and, as we demonstrate here, CMD-45 and XHe-III-74 have very little activity at this receptor. Finally, $GABA_ARs$ containing α6 are also expressed in the CNS at modest levels, mainly in the cerebellum (10), although little is known about the consequences of activating these receptors. This may be important, as CMD-45 and XHe-III-74 demonstrate significant modulation of α6β3γ2 $GABA_ARs$ as well.

In an attempt to avoid these potential pitfalls, efforts are underway to develop derivatives of XHe-III-74 that will not enter the CNS. The aim of these efforts is to design and synthesize functional XHe-III-74 derivatives that are either degraded in the periphery after inhalational administration or that are incapable of penetrating the blood–brain barrier. Ultimately, these efforts may prove most effective in limiting all CNS side effects.

In conclusion, we demonstrate that two novel, positive allosteric modulators of the $GABA_AR$, CMD-45 and XHe-III-74, acutely relax mouse and human ASM in an α4 subunit–selective manner. Of great clinical interest, both compounds also augment β_2-agonist–induced human ASM relaxation. Furthermore, the subunit selectivity of these novel agents, along with the ability to administer them directly to ASM by inhalation, offers the potential to limit CNS side effects. The mechanism of this relaxation appears to involve alterations in ASM Ca^{2+} handling, as the compounds limit $[Ca^{2+}]_i$ increases in response to a contractile agonist *in vitro*. Although further pharmacokinetics and pharmacodynamics studies are needed, α subunit–selective targeting of the $GABA_AR$ may allow for the development of much-needed novel therapeutics working via an alternative mechanistic pathway to combat bronchoconstrictive diseases. ■

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