ORIGINAL RESEARCH

Coupling of Airway Smooth Muscle Bitter Taste Receptors to Intracellular Signaling and Relaxation Is via $G_{\alpha i1,2,3}$

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Abstract

Bitter taste receptors (TAS2Rs) are expressed on human airway smooth muscle (HASM) and evoke marked relaxation. Agonist interaction with TAS2Rs activates phospholipase C and increases compartmentalized intracellular Ca^{2+} ($[Ca^{2+}]_i$) via inositol 1,4,5 triphosphate. In taste cells, the G protein gustducin couples TAS2R to phospholipase C; however, we find very low levels of $G_{\alpha gust}$ mRNA or protein in HASM. We hypothesized that another G protein in HASM transmits TAS2R function. TAS2R signaling to $[Ca^{2+}]_i$, extracellular signal–regulated kinase (ERK) 1/2, and physiologic relaxation was sensitive to pertussis toxin, confirming a role for a member of the G_i family. α subunit expression in HASM was $G_{\alpha i2} > G_{\alpha i1} = G_{\alpha i3} > G_{\alpha trans1} \approx G_{\alpha trans2}$, with $G_{\alpha gust}$ and $G_{\alpha o}$ at the limits of detection (>100-fold lower than $G_{\alpha i2}$). Small interfering RNA knockdowns in HASM showed losses of $[Ca^{2+}]_i$ and ERK1/2 signaling when $G_{\alpha i1}$, $G_{\alpha i2}$, or $G_{\alpha i3}$ were reduced. $G_{\alpha trans1}$ and $G_{\alpha trans2}$ knockdowns had no effect on $[Ca^{2+}]_i$ and a minimal, transient effect on ERK1/2 phosphorylation. Furthermore, $G_{\alpha gust}$ and $G_{\alpha o}$ knockdowns did not affect any TAS2R signaling. In overexpression experiments in human embryonic kidney-293T cells, we confirmed an agonist-dependent physical interaction between TAS2R14 and $G_{\alpha i2}$. ASM cells from transgenic mice expressing a peptide inhibitor of $G_{\alpha i2}$ had attenuated relaxation to TAS2R agonist. These data indicate that, unlike in taste cells, TAS2Rs couple to the prevalent G proteins, $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$, with no evidence for functional coupling to $G_{\alpha gust}$. This absence of function for the "canonical" TAS2R G protein in HASM may be due to the very low expression of $G_{\alpha gust}$, indicating that TAS2Rs can optionally couple to several G proteins in a cell type–dependent manner contingent upon G protein expression.

Keywords: bitter taste receptors; G proteins; airway smooth muscle; asthma; chronic obstructive pulmonary disease

Clinical Relevance

Unexpectedly, bitter taste receptors have been localized on airway smooth muscle. These G protein–coupled receptors are activated by a variety of natural and synthetic compounds, resulting in smooth muscle relaxation, thus pointing toward a new class of direct bronchodilators for asthma and chronic obstructive pulmonary disease. A puzzle in the signal transduction cascade for these extraoral receptors is what G protein they use to transmit agonist binding to effector activation. We show here that in airway smooth muscle they do not functionally use the canonical bitter taste receptor G protein $G_{\alpha gust}$ but instead use $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$. These findings now provide for a methodical approach for drug discovery and consideration of whether pathologic processes that modify these $G_{\alpha i}$ proteins could alter therapeutic effectiveness.

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Bitter taste receptors (TAS2Rs) have been traditionally thought to be expressed on taste cells of the tongue, signaling to the brain as part of a system to avoid ingestion of toxic plants (1, 2). These receptors are members of the superfamily of cell surface G protein–coupled receptors (GPCRs). Over the last few years, we and others have demonstrated expression of certain TAS2R subtypes on airway smooth muscle (ASM) of human (3), monkey (4), mouse (3), and guinea pig (5). Activation of TAS2Rs on ASM results in profound relaxation and bronchodilation by a non-cAMP mechanism (3, 6). This action represents a new pathway for development of direct bronchodilators for the treatment of obstructive lung disease. The three highest expressing TAS2R subtypes in human ASM (HASM) are TAS2R10, -14, and -31. Like other GPCRs, TAS2Rs carry out signaling by coupling to G proteins with subsequent activation of an effector enzyme. The TAS2Rs of taste cells (1) have been shown to couple to the G protein, gustducin; agonist activation and coupling results in the dissociation of the α subunit ($G_{\alpha gust}$) and the βγ subunits, with βγ activating phospholipase C (PLC), generating inositol 1,4,5 triphosphate (IP_3). IP_3 binding to its receptor on the endoplasmic reticulum releases intracellular Ca^{2+} ($[Ca^{2+}]_i$), which subsequently activates a transient receptor potential channel, increasing Na^+ influx. This influx depolarizes the cell membrane, causing release of neurotransmitter, which stimulates the adjacent type III taste cell to communicate to the central nervous system.

The pathway diverges in ASM at the IP_3 and/or $[Ca^{2+}]_i$ release steps (3). In HASM cells, membrane potential hyperpolarizes, whereas it depolarizes in taste cells. Indeed, agonists of other GPCRs that increase $[Ca^{2+}]_i$ in HASM, such as acetylcholine, histamine, and leukotrienes, lead to membrane depolarization and smooth muscle contraction, as opposed to the increase in $[Ca^{2+}]_i$ evoked by TAS2R agonists that causes hyperpolarization and relaxation. Studies to date have indicated that the $[Ca^{2+}]_i$ released from TAS2Rs resides in a specialized compartment, which differs from the more global increase in $[Ca^{2+}]_i$ observed with contractile GPCRs (3). Recent data also indicate that IP_3 production is more restricted than previously thought (7), providing additional signaling plasticity by different receptors. Multiwell plate–based fluorescence readers, such as used for this and most TAS2R signaling studies, accurately quantitate $[Ca^{2+}]_i$ even though it may be somewhat localized within the cell.

TAS2Rs have now also been reported to be expressed in a number of other extraoral tissues, including stomach, colon, thyroid, brain, and white blood cells, where pharmacologic activation results in multiple biologic actions (8). The presumption has been with ASM, as well as these other tissues, that TAS2R function occurs by receptor coupling to gustducin. However, the expression of this taste receptor G protein is very low, and indeed difficult to detect at the protein or mRNA levels in HASM and some of these other tissues, compared with taste cells, where it is abundant. Gustducin is a member of the "G_i family" of G protein α subunits, which includes $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o}$, and $G_{\alpha z}$, and the transducins (the visual G proteins), $G_{\alpha trans1}$ and $G_{\alpha trans2}$. Some of these G proteins within this family share a high degree of sequence identity, and, given the very low levels of expression of $G_{\alpha gust}$ in HASM, we have undertaken the task of ascertaining which of the G_i family of G proteins carries out signal transduction of TAS2Rs in HASM. We hypothesized that one or more other members of this family that are abundantly expressed in these cells are responsible for TAS2R functional coupling in HASM.

Materials and Methods

Cell Culture and Transfections

Human embryonic kidney-293T (HEK-293T), primary HASM (passages 3–8; Lonza, Basel, Switzerland), and the D9 human telomerase reverse transcriptase immortalized HASM cells (D9 hTERT) (9) were grown as previously described (9, 10). In some experiments, cells were treated with pertussis toxin (PTX) at the indicated concentrations in media for 24 hours. For Gα subunit knockdowns, HASM cells were transfected with 100 μM of small interfering RNAs (siRNAs) 24 hours and again 48 hours after plating using lipofectamine 2000 (Invitrogene, Carlsbad, CA). The siRNAs are shown in Table E1 in the online supplement. Transfections with cDNAs encoding TAS2R14 and $G_{\alpha i2}$ were performed in HEK-293T (10).

Western Blots and Quantitative RT-PCR

To detect G_i α subunit protein expression, SDS-PAGE and Western blots were performed using solubilized whole-cell lysates exactly as previously described (10) using primary antibodies and titers listed in Table E2 and subsequent secondary antibody at a titer of 1:10,000 for chemoluminescence (Thermo Scientific, Waltham, CA) with detection using the ChemiDoc MP (Bio-Rad, Hercules, CA). Bands were quantitated using the provided software or Image-J (National Institutes of Health, Bethesda, MD). Quantitative RT-PCR was performed on HASM RNA using methods exactly as previously described (3). The primers were from Applied Biosystems (Foster City, CA; Table E3). Comparisons were made using the $2^{-\Delta CT}$ method where ΔC_T is the difference in C_T values between the transcript of interest and that of glyceraldehyde 3-phosphate dehydrogenase.

$[Ca^{2+}]_i$, Extracellular Signal–Regulated Kinase 1/2 Measurements

$[Ca^{2+}]_i$ was measured in response to the TAS2R14 agonist, diphenhydramine (DPD), in cells plated in 96-well plates and loaded with the green fluorescent calcium indicator Fluo-4 (Life Technologies, Carlsbad, CA) as previously described (10). Results are shown as either real-time measurements or as graphs showing peak $[Ca^{2+}]_i$ responses from multiple experiments. For extracellular signal–regulated kinase (ERK) 1/2 activation studies, cells were serum starved for 18 hours, treated with vehicle or agonist, and SDS-PAGE and Western blots were performed using antibodies (Table E1) for total ERK1/2 and phospho-ERK1/2 with previously described methods (10, 11).

Receptor-Gα Interaction

cDNA constructs encoding TAS2R14 and $G_{\alpha i2}$ were cotransfected transiently into HEK-293 cells using lipofectamine 2000. After 48 hours, cells were exposed to 500 μM DPD for the indicated time followed by treatment with a membrane-permeant cross-linking reagent (dithiobis (succinimidyl propionate); 1 mM) for 30 minutes in non–amine-containing buffer. Excess cross-linker was quenched in 25 mM Tris (pH 7.4) for 15 minutes at room temperature, and cells were lysed in 0.1% CHAPS buffer, followed by coimmunoprecipitation, as described previously (10).

Magnetic Twisting Cytometry

These studies were performed as previously described (12, 13). Briefly,

Ary-Gly-Asp–coated ferrimagnetic beads were bound to cell surface integrin receptors on ASM cells. The beads are magnetized horizontally and twisted in a vertically aligned magnetic field. Bead displacement measures the increase or decrease in the twist in individual cells, corresponding to contraction and relaxation, respectively.

Genomic and Statistical Analysis

Human G_i family α subunits were aligned using the alignment program COBALT and the identity matrix values were calculated using blastp within BLAST (all programs are from the National Institutes of Health). Results from biochemical studies are presented as mean (±SE) of the indicated number of experiments. Comparisons were performed by paired or unpaired two-sided *t* tests, with significance imparted at *P* less than 0.05. Magnetic twisting cytometry results were analyzed by a nested ANOVA (14).

Results

TAS2R Signaling to $[Ca^{2+}]_i$, ERK1/2, and Relaxation in HASM Is Sensitive to PTX

We first pursued a verification that the intracellular signaling and physiological effects that occur in HASM in response to TAS2R agonist are mediated through the members of the G_i family of G proteins, which (except for $G_{\alpha z}$) are inactivated by PTX. Primary and immortalized HASM were exposed to vehicle or PTX for 24 hours, washed, and loaded with Fluo-4. The $[Ca^{2+}]_i$ response to the TAS2R14 agonist DPD is shown in Figures 1A and 1B. This response in primary HASM is clearly sensitive to PTX treatment, with greater than 90% of the $[Ca^{2+}]_i$ signal inhibited by the toxin. Studies using the immortalized HASM cell line designated D9 hTERT, showed virtually identical results (Figures 1A and 1B). Additional studies were also performed using phosphorylation of ERK1/2 as the signal readout. The early increase in phospho-ERK1/2 from GPCR activation (~5–10 min of agonist exposure) is due to receptor G protein interaction. Responses at the 30-minute time point are β-arrestin dependent and relatively independent of G protein interaction (15, 16). We thus expected that the 5- and 10-minute signals would be blocked by PTX pretreatment if coupling was by one or more $G_{\alpha i}$ subunits. As shown in Figures 1C and 1D, DPD exposure resulted in marked phosphorylation of ERK1/2 in the primary HASM cells, which was inhibited approximately 85% by PTX pretreatment. Similar results were observed in the immortalized HASM (Figures 1E and 1F). Finally, we examined a physiologic response of HASM, using magnetic twisting cytometry. As previously described, TAS2R agonists cause a decrease in twisting force ("relaxation") in HASM (3, 13). Figure 1G shows that the DPD-promoted decrease in twisting force was markedly attenuated by PTX pretreatment. Taken together, these data confirm that the biochemical and physiologic responses to agonist by TAS2Rs in HASM cells are transduced via one or more members of the PTX-sensitive G proteins of the G_i family.

Expression of the G_i Family of α Subunits in HASM

We ascertained both mRNA and protein expression of the eight α subunits. Figure 2A shows the mRNA expression in HASM using quantitative RT-PCR and is expressed as $2^{-\Delta Ct}$, where ΔCt is defined as the difference between Ct values of the given G protein α subunit and glyceraldehyde 3-phosphate dehydrogenase. By far the most abundant mRNAs of this class in HASM are $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$, being more than 5,000-fold greater than $G_{\alpha gust}$. Indeed, $G_{\alpha gust}$ mRNA expression was quite low (C_T values ~30), $G_{\alpha o}$ was typically below the limits of detection, and $G_{\alpha z}$ transcripts were not detected or were very low. $G_{\alpha trans2}$ mRNA levels were reliably detected, but were still several hundred fold less than any of the $G_{\alpha i}$ transcripts, whereas $G_{\alpha trans1}$ transcripts were minimally detected (Figure 2A). Overall, the pattern of the mRNA levels of these $G_{\alpha i}$ subunits was the same between primary HASM cells and the D9 hTERT immortalized HASM line (Figure 2A). For comparison of the protein expression levels, we encountered low expression of some of the α subunits. Thus, in Figure 2B, we show the long exposure times from the images for representative Western blots for $G_{\alpha gust}$, $G_{\alpha o}$, and $G_{\alpha z}$. As indicated, $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ were the most abundantly expressed at the protein level, whereas expression levels of $G_{\alpha gust}$, $G_{\alpha o}$, and $G_{\alpha z}$ were very low. $G_{\alpha trans1}$ and $G_{\alpha trans2}$ were expressed at intermediate levels compared with the others in the group. Figure 2C shows results from multiple experiments, normalized to β-actin, and provides a semiquantitative assessment of $G_{\alpha i}$ family α subunit protein expression in HASM cells. The mRNA and protein data are congruent except for $G_{\alpha trans1}$, where we observe protein expression, but little mRNA expression in either cell line. Taken together, though, the results of these expression studies indicate that $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ are the dominant G_i protein α subunits in HASM, with intermediate levels of $G_{\alpha trans1}$ and $G_{\alpha trans2}$ protein expression. $G_{\alpha gust}$ expression was very low, whereas $G_{\alpha o}$ and $G_{\alpha z}$ were inconsistently detected at the very low level.

siRNA Knockdowns Identify the Primary Transducers of TAS2R Signaling in HASM

We next ascertained the effects of siRNA transfections that decrease specific G_α subunits on the two signaling pathways, $[Ca^{2+}]_i$ and ERK1/2. For these studies, we used the immortalized HASM line (which has the same expression and function phenotypes as the primary HASM cells; *see* Figures 1 and 2), because the former was found to be more readily transfected. Figure 3A shows representative DPD-stimulated $[Ca^{2+}]_i$ tracings from these cells under conditions of scrambled or G_α subunit siRNA transfections. Knockdown of $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ each resulted in an approximately 40–50% decrease in $[Ca^{2+}]_i$ signaling. In contrast, transfections that yielded knockdowns of $G_{\alpha trans1}$, $G_{\alpha trans2}$, $G_{\alpha gust}$, $G_{\alpha o}$, and $G_{\alpha z}$ had no effect on $[Ca^{2+}]_i$ signaling (Figures 3A and 3B). Transfections with siRNAs for all three $G_{\alpha i}$ subunits reduced $[Ca^{2+}]_i$ signaling greater than knockdowns of any single $G_{\alpha i}$ subunit (Figure 3C), but the effect was not additive.

Representative ERK1/2 experiments are shown in Figure 4A. Due to low or nonexistent expressions, $G_{\alpha o}$ and $G_{\alpha z}$ knockdown experiments were not performed. As can be seen, knockdown of any of the $G_{\alpha i}$ subunits caused a decrease in DPD-stimulated phosphorylation of ERK1/2 at the 5- and 10-minute time points (Figure 4B). From these data, we cannot define, in absolute terms, which is the "preferred" $G_{\alpha i}$ subunit for TAS2R signal transduction, because the siRNAs

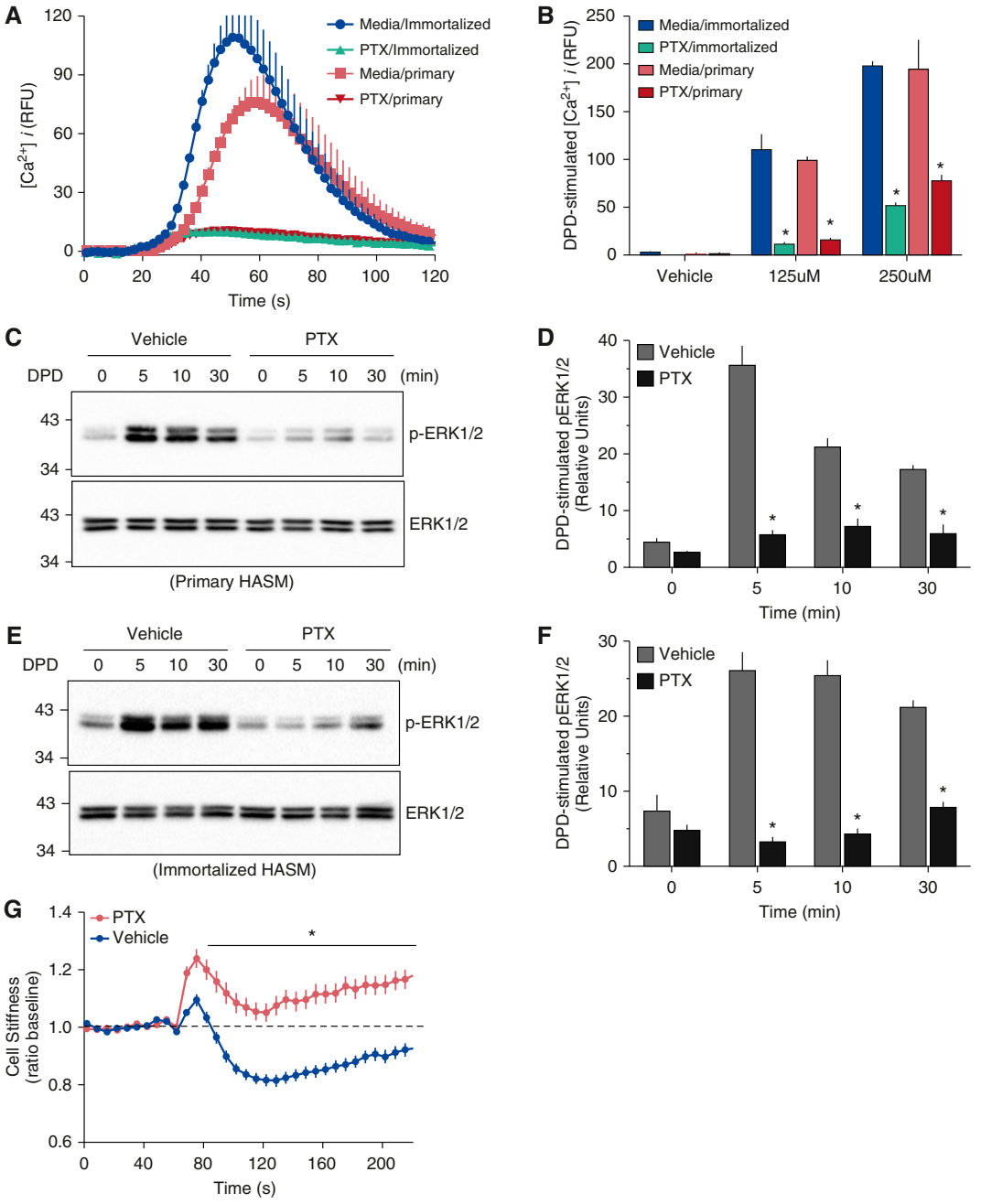

Figure 1. Bitter taste receptor (TAS2R) function in human airway smooth muscle (HASM) is inhibited by pertussis toxin (PTX). Primary HASM cells or D9 immortalized HASM cells were treated with media alone or media with 0.5 μg PTX for 24 hours, and the intracellular Ca^{2+} ($[Ca^{2+}]_i$) response to 250 μM of the TAS2R14 agonist diphenhydramine (DPD) or vehicle was determined. (*A*) Representative tracings in which DPD or vehicle was added at the 19-second time point. (*B*) The mean (±SE) results of the peak $[Ca^{2+}]_i$ from five experiments, $^*P < 0.01$ PTX versus media alone treatment. (*C* and *E*) Representative Western blots of phospho–extracellular signal–regulated kinase (ERK) 1/2 and total ERK1/2 in response to 500 μM DPD from 0 to 30 minutes. (*D* and *F*) Mean (±SE) of the phospho-ERK1/2 responses from four experiments, $^*P < 0.01$ PTX versus media. (*G*) Physiologic response of primary HASM to 250 μM DPD in the absence or presence of PTX pretreatment, as determined by magnetic twisting cytometry. Results are mean (±SE) from 445 to 466 measurements from cells from 4 different culture wells. $^*P < 0.001$ PTX versus vehicle. RFU, relative fluorescent units.

reduced $G_{\alpha i}$ proteins to different levels (Figure 4A). However, it is nevertheless clear that each of these highly expressed subunits contributes to TAS2R coupling to this pathway. For the transducins, we observed a small decrease in phospho-ERK1/2 at the 5-minute, but not at the 10-minute, time point with knockdown of either $G_{\alpha trans1}$ or $G_{\alpha trans2}$ (Figures 4A and 4B). Knockdown of $G_{\alpha gust}$ had no effect on agonist-promoted phosphorylation of ERK1/2.

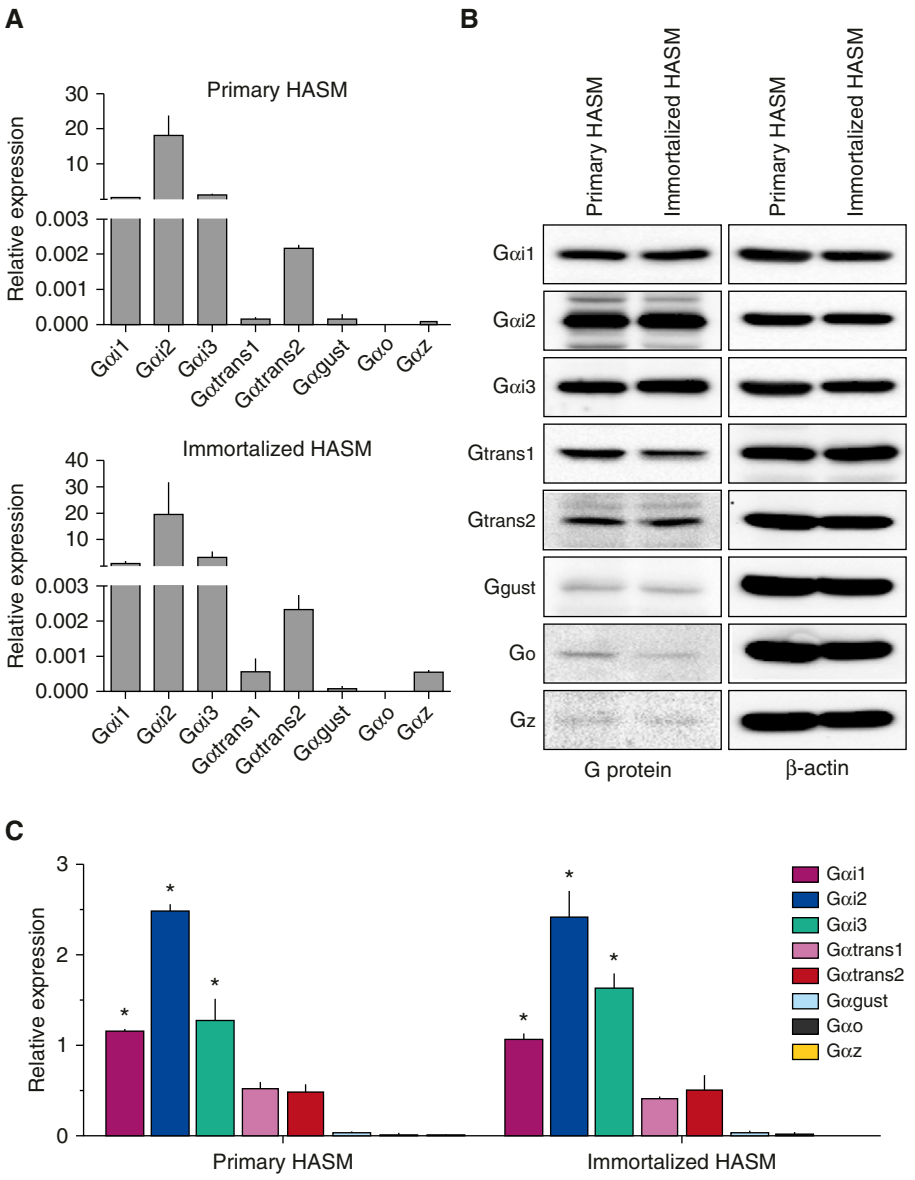

Figure 2. Expression of G_{α} subunits in HASM. (*A*) Mean mRNA expression as determined by quantitative RT-PCR (three experiments). (*B*) Representative Western blots for the eight G_{α} subunits and β-actin (the exposures for $G_{\alpha gust}$, $G_{\alpha o}$, and $G_{\alpha z}$ were greater than the other subunits). (*C*) Mean (±SE) of protein expression presented as the ratio of the α subunit to β-actin from three to four experiments (*$P < 0.001$ compared with all other subunits).

$G_{\alpha i2}$ Directly Couples to TAS2R14 in an Agonist-Dependent Manner

As indicated in the above studies, each of the $G_{\alpha i}$ subunits appears to be capable of transducing TAS2R signaling to $[Ca^{2+}]_i$ or ERK1/2. Given that $G_{\alpha i2}$ has roughly 68% amino acid identity with the canonical TAS2R G protein, gustducin (Table 1), we performed experiments to confirm that $G_{\alpha i2}$ can indeed directly interact with TAS2R14 (the receptor for DPD) in an agonist-dependent manner. HEK-293T cells were transfected with $G_{\alpha i2}$ and FLAG-tagged TAS2R14. After 48 hours, the cells were exposed to vehicle or the agonist, DPD, and, 3 minutes later, the cells were treated with the cross-linking agent, dithiobis (succinimidyl propionate). The cross-linking step was used to ensure that any physical coupling between G protein and receptor was not lost during the subsequent coimmunoprecepitation steps. The cells were then solubilized, immunoprecipitated with FLAG antibody, and the precipitate subjected to SDS-PAGE and Western blotting with anti-$G_{\alpha i2}$ antibody. As shown in Figure 5, the coimmunoprecipitation signal was increased when cells were treated with the agonist, consistent with agonist-promoted physical coupling of the receptor to this G protein α subunit.

Signaling and Physiologic Effects of Overexpressing $G_{\alpha i2}$ and Its Peptide Inhibitor on TAS2R14 Function in ASM

The above results lead us to explore TAS2R signaling with previously constructed transgenic mouse ASM cell lines overexpressing $G_{\alpha i2}$ ($G_{\alpha i2}$OE) or expressing a $G_{\alpha i2}$ inhibitory peptide ($G_{\alpha i2}$IP). ASM cells derived from nontransgenic (NTG) littermates were used as controls. In transgenic $G_{\alpha i2}$IP ASM, DPD-mediated $[Ca^{2+}]_i$ signaling was significantly attenuated by over 50% compared with NTG cells (Figures 6A and 6B). These results are consistent with the knockdown studies shown in Figure 3B. In the transgenic $G_{\alpha i2}$OE ASM, DPD-mediated $[Ca^{2+}]_i$ signaling was enhanced by roughly twofold. The results of TAS2R-mediated phosphorylation of ERK1/2 in these cells are shown in Figures 6C and 6D, and revealed a decrease in this signal in $G_{\alpha i2}$IP cells and a modest increase in the $G_{\alpha i2}$OE cells. To ascertain the physiologic consequences of this altered functional coupling to $G_{\alpha i2}$, we used magnetic twisting cytometry (Figure 6E). Cells were twisted (contracted) with endothelin-1 or 5-hydroxytryptamine. NTG mouse ASM displayed approximately 35% decrease in cell stiffness (relaxation) upon TAS2R14 activation. In contrast, cells expressing the $G_{\alpha i2}$IP showed little or no change in TAS2R-mediated stiffness, consistent with depressed coupling between the receptor and native $G_{\alpha i}$. However, the $G_{\alpha i2}$OE mouse ASM showed a decrease in stiffness with TAS2R14 activation that was not different than NTG.

Discussion

The typical therapeutic agents for treating asthma and chronic obstructive pulmonary disease are corticosteroids to reduce inflammation, and direct and indirect bronchodilators for treating airway obstruction. Indirect bronchodilators (17, 18) are antagonists for receptors (such as the M_3-muscarinic) that contract ASM, which are activated by local increases

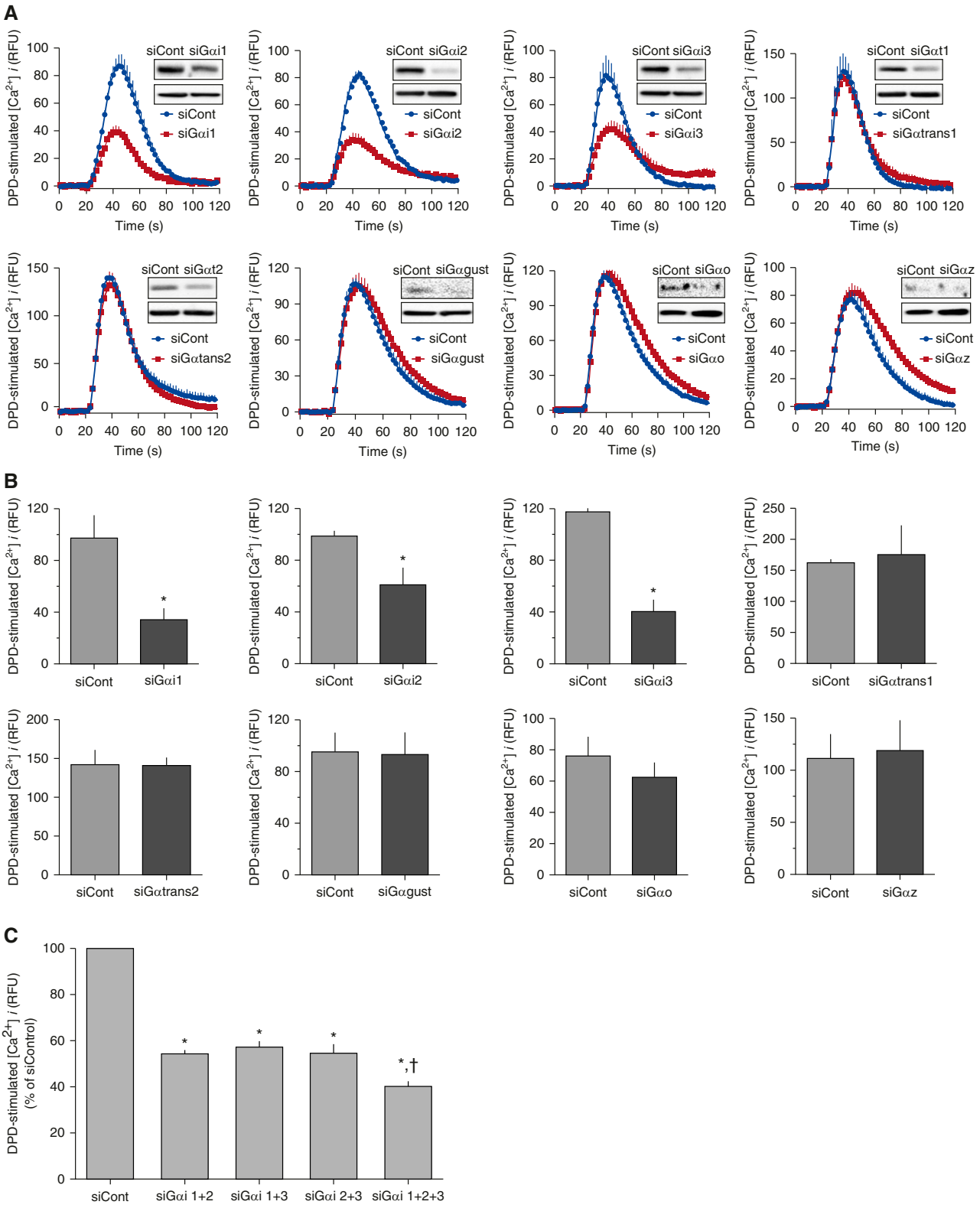

Figure 3. Selective knockdowns of certain G_{α} subunits in HASM attenuate TAS2R signaling to $[Ca^{2+}]_i$. (*A*) Representative $[Ca^{2+}]_i$ tracings in response to 250 μM DPD after transfection of immortalized HASM cells with control (scrambled) or α subunit–specific small interfering RNA (siRNA), with the *inset* showing a representative Western blot indicating the degree of α subunit knockdown. (*B*) Mean (±SE) of the peak $[Ca^{2+}]_i$ response to 250 μM DPD from four experiments. $^*P < 0.01$ versus control siRNA. (*C*) Peak $[Ca^{2+}]_i$ DPD responses to various combinations of $G_{\alpha i1}$, $G_{\alpha i2}$, or $G_{\alpha i3}$ knockdowns. $^*P < 0.01$ versus control siRNA, $^{\dagger}P < 0.05$ versus all other combinations ($n = 5$–6).

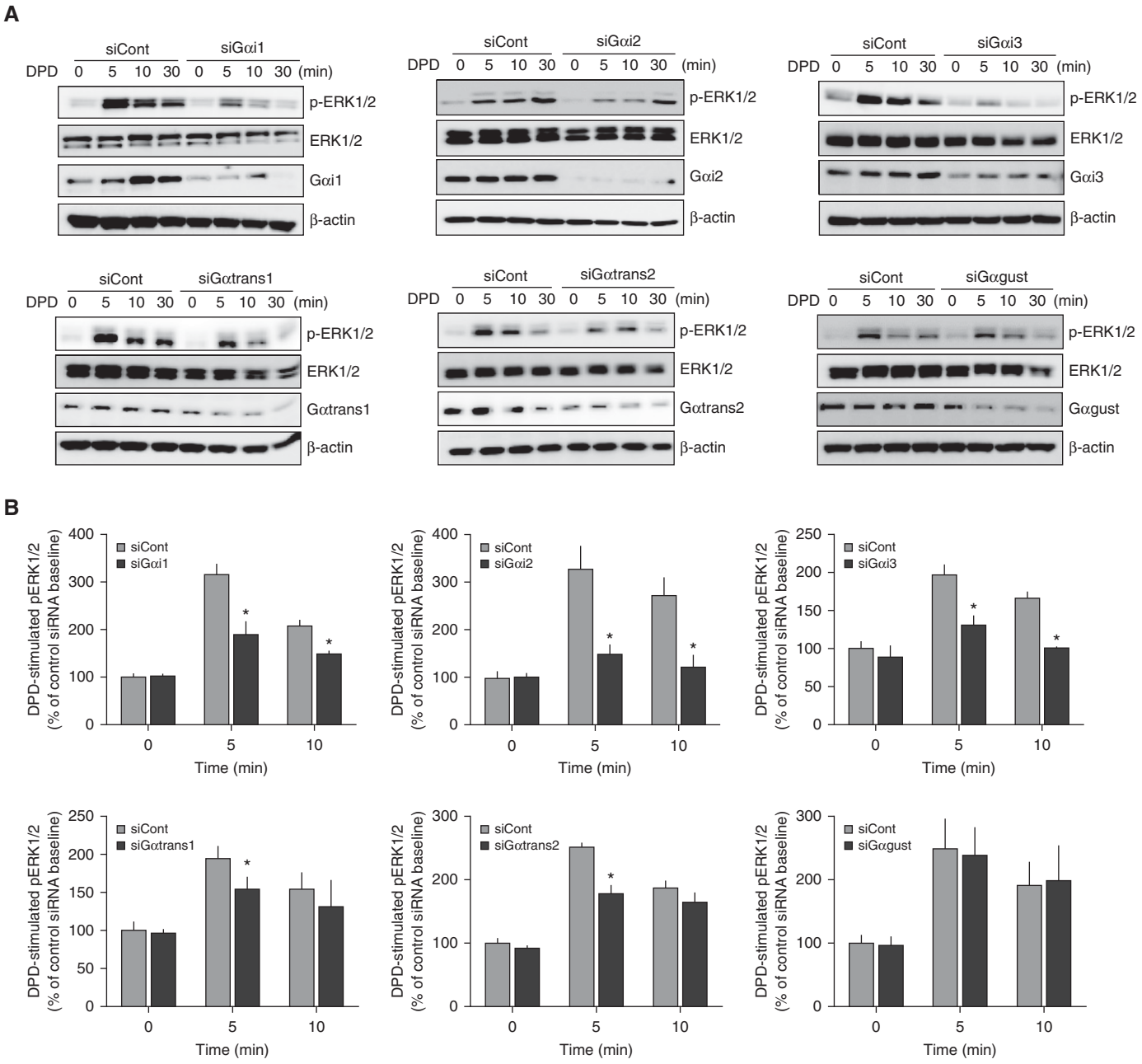

Figure 4. Selective knockdowns of certain G_α subunits in HASM attenuates TAS2R signaling to ERK1/2 phosphorylation. (*A*) Representative Western blots from immortalized HASM cells showing phosphorylated ERK1/2 (pERK1/2), total ERK1/2, the G_α subunit expression, and β-actin, under control or siRNA knockdown conditions. Cells were treated with 500 μM DPD for the indicated times. (*B*) Mean (±SE) results of pERK1/2 expression normalized to the group mean baseline ($t = 0$) condition from four experiments. $^*P < 0.05$ versus control siRNA.

in bronchospastic mediators (such as acetylcholine). Their effectiveness is dependent on the relative activity of a given pathway. In contrast, direct bronchodilators relax ASM regardless of the constrictive signal. Currently, only one class of direct bronchodilators, β-agonists, acting at β_2-adrenergic receptors (β_2AR), are available for clinical use. β-agonists are used for acute relief of airway obstruction and as controller therapy for prevention. The treatment response to β-agonists, however, is associated with significant interindividual variability and a number of unfavorable outcomes (19–25).

We have undertaken a systematic approach to identify and characterize HASM G protein–coupled receptors to find other pathways that act to constrict or dilate airways, so as to consider additional receptors for therapeutic development (26). Unexpectedly, we found expression of multiple TAS2Rs on HASM, with 6 of the 25 human subtypes expressed at levels greater than the β_2AR (3). Agonists for these TAS2Rs evoked marked relaxation of HASM in *ex vivo* studies, single-cell measurements,

Table 1. Homology Matrix of Amino Acid Identities Shown as Percent Identity for the Human $G_{\alpha i}$ Family of G Proteins

	$G_{\alpha gust}$	$G_{\alpha trans1}$	$G_{\alpha trans2}$	$G_{\alpha i1}$	$G_{\alpha i2}$	$G_{\alpha i3}$	$G_{\alpha o}$	$G_{\alpha z}$	Accession Number
$G_{\alpha gust}$	100	78	80	68	68	68	62	58	A8MTJ3.2
$G_{\alpha trans1}$	—	100	82	68	66	65	63	54	P11488.5
$G_{\alpha trans2}$	—	—	100	69	70	69	61	57	NP_005263.1
$G_{\alpha i1}$	—	—	—	100	88	94	71	67	NP_002060.4
$G_{\alpha i2}$	—	—	—	—	100	86	68	66	CAG33064.1
$G_{\alpha i3}$	—	—	—	—	—	100	70	67	AAM12621.1
$G_{\alpha o}$	—	—	—	—	—	—	100	62	AAH30027.2
$G_{\alpha z}$	—	—	—	—	—	—	—	100	CAG30381.1

and an *in vivo* murine model of asthma (3). These findings have led to considering TAS2R agonists as therapy for obstructive lung diseases, either as primary agents or in addition to β-agonists (17, 27). Ongoing studies have used high-throughput screening and medicinal chemistry to identify agonists with high affinity and selectivity (2). Subsequently, TAS2Rs have been identified on cell types in other organs, indicating a previously unrecognized chemosensory system in the body that has a broad range of physiologic and pathologic implications, and also represents new avenues for drug development (8). Of concern in understanding TAS2R signaling in extraoral tissues is the very low expression of $G_{\alpha gust}$ found in most of these cell types. Although it is now recognized that GPCRs can couple to more than one G protein, this is typically in the setting of abundantly expressed G proteins (28, 29). In terms of non–taste cell TAS2Rs, such as those on HASM, the presumed canonical G protein is at the limit of detection by quantitative RT-PCR and Western blots, suggesting that the main transducer of TAS2R signaling in HASM is not $G_{\alpha gust}$. Because of the degree of amino acid identity (Table 1), and the sensitivity of the biochemical and physiological responses of TAS2R activation in HASM to PTX, we hypothesized that one or more members of the G_i family, which are abundantly expressed in HASM, carries out signal transduction from receptor to effector in this cell type.

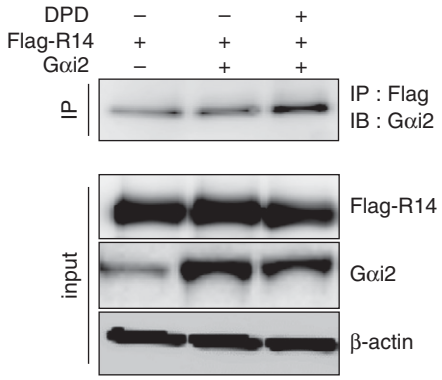

Figure 5. TAS2R14 physically couples to $G_{\alpha i2}$. Human embryonic kidney-293T cells were transfected with cDNA constructs to express FLAG-TAS2R14 and $G_{\alpha i2}$, exposed to the agonist DPD or vehicle for 3 minutes, and then the cells were treated with the cross-linking agent dithiobis (succinimidyl propionate). Solubilized lysates were immunoprecipitated with FLAG-antibody and the precipitates immunoblotted for $G_{\alpha i2}$. An increase in the coimmunoprecipitation signal was observed with TAS2R14 agonist exposure. Results are representative of three experiments. IB, immunoblot; IP, immunoprecipitation.

Here, we show that, in ASM, the $G_{\alpha i1,2,3}$ subfamily clearly carries out TAS2R signal transduction, with no measurable contribution from $G_{\alpha gust}$. This is most likely due to the fact that these $G_{\alpha i}$ subunits have the capacity to couple to TAS2Rs (as we show here), and that the low expression of $G_{\alpha gust}$, which we also know couples to TAS2Rs, in essence minimizes any potential functional relevance of this G protein in this particular cell type. The transducins, which have even greater amino acid identity with gustducin than $G_{\alpha i1,2,3}$ (Table 1), also appear to play a minor role, at least in signaling to ERK1/2. The lower expression levels of the transducins may also be the basis for its minor role in TAS2R signaling in HASM, as we also know that these G proteins can couple to TAS2Rs (30). The fact that the ERK1/2 pathway, but not the $[Ca^{2+}]_i$ pathway, was affected by transducin knockdowns is a phenomenon observed in other multidimensional signaling systems. It may be related to different levels of signaling amplification that are present between two pathways, such that there are spare receptor G protein units for one pathway versus another (31). This also illustrates the advantages of the knockdown strategy we used here as compared with simply overexpressing a given α subunit, which could lead to either promiscuous coupling or saturation. Indeed, we did not observe an increase in ASM relaxation to TAS2R agonist in the mouse ASM $G_{\alpha i2}$OE cells (Figure 6E), probably because of the abundance of this α subunit in ASM cells in the native state.

We have previously shown that TAS2R signaling is sensitive to gallein (which sequesters the $G_{\beta\gamma}$ subunit) and PLC inhibitors (3). Taken together with the current data, it is clear that $G_{\beta\gamma}$ released from a heterodimer composed of $G_{\alpha i1}$, $G_{\alpha i2}$, or $G_{\alpha i3}$ α subunits carries out the majority of TAS2R signaling in HASM, and, indeed, multiple $G_{\beta\gamma}$ subunit combinations found in G_i heterodimers have been shown to stimulate PLC (32, 33). It is recognized that G protein α subunits are dynamically regulated by multiple pathologic events, and we can now place the relevance of such changes into the context of TAS2R agonist treatment of obstructive airways disease. $G_{\alpha i2}$ (and/or $G_{\alpha i1}$ and $G_{\alpha i3}$) is increased in various animal or cell models of bronchial hyperresponsiveness, airway inflammation, and asthma (11, 34–38). This increase has also been shown to decrease β-agonist bronchodilator responsiveness, because β_2ARs also couple to $G_{\alpha i}$, and thus attenuate receptor-activated adenylyl cyclase (11). However, this increase in $G_{\alpha i}$ would not be expected to affect TAS2R function, and may even enhance it, given that this subfamily is responsible for the ASM relaxation response to TAS2R activation. Chronic activation of some G_i-coupled receptors has been shown to down-regulate cellular levels of $G_{\alpha i}$ protein. For example, with the α_{2A}AR, stably transfected and natively expressing cells display approximately 40% loss of $G_{\alpha i2}$ after 24 hours of exposure to high (saturating) concentrations of receptor agonist (39, 40). There are many G_i-coupled receptors expressed on HASM (26), and it is conceivable that prolonged exposure to certain locally produced substances in pathologic states, such as asthma or chronic obstructive pulmonary disease, might result in $G_{\alpha i}$ down-regulation. Although we are not aware of such decreases, this particular scenario might lead to a reduction of the effectiveness of TAS2R agonists in relaxing ASM. Even a complete loss of one of these

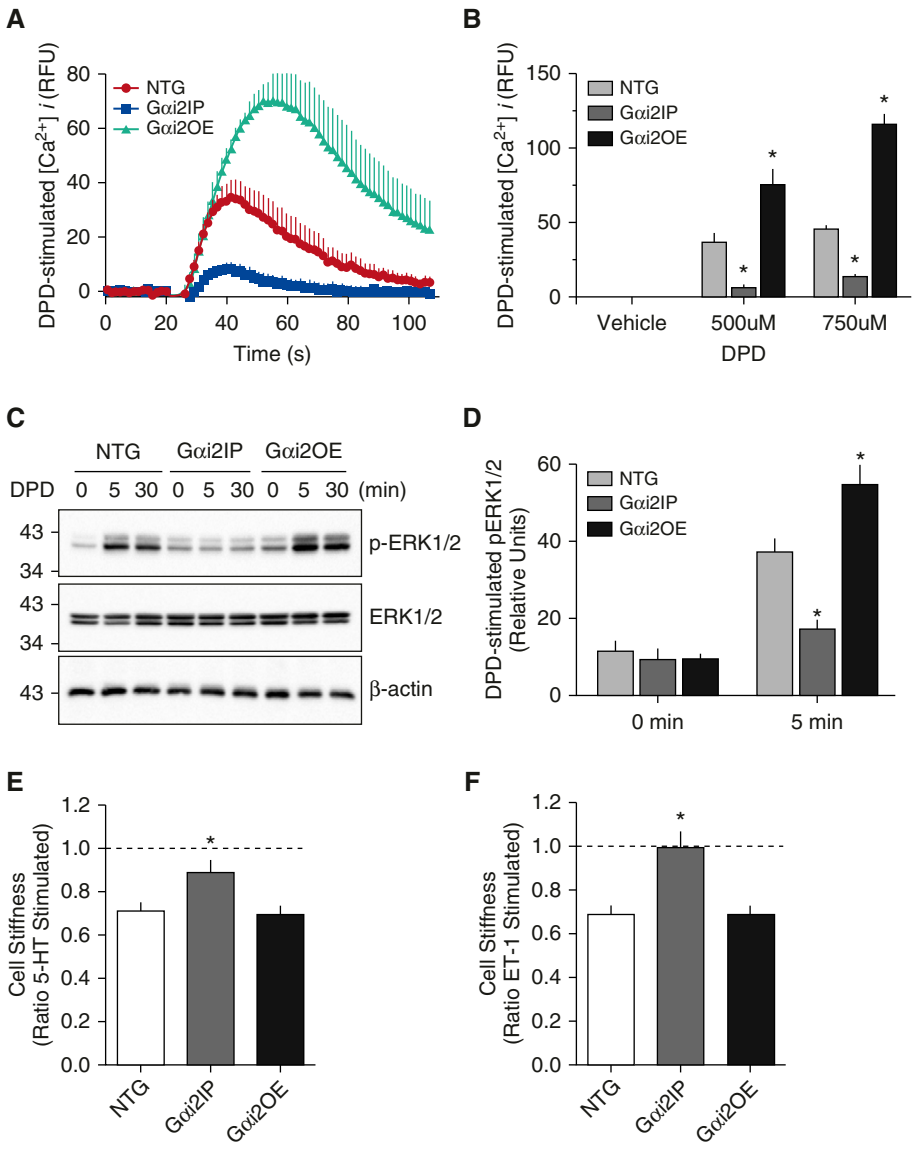

Figure 6. Biochemical and physiologic signaling in ASM cells from transgenic mice overexpressing $G_{\alpha i2}$ ($G_{\alpha i2}$OE) or a $G_{\alpha i2}$ peptide inhibitor ($G_{\alpha i2}$IP). (*A*) Representative $[Ca^{2+}]_i$ tracings from mouse ASM $G_{\alpha i2}$OE or $G_{\alpha i2}$IP cells in response to 500 μM DPD. (*B*) Mean (±SE) results of peak $[Ca^{2+}]_i$ response from four experiments. *$P < 0.01$ versus nontransgenic (NTG). (*C*) Representative results of the pERK1/2 response from the three cell lines. (*D*) Mean (±SE) of the pERK1/2 response from four experiments, *$P < 0.05$ versus NTG. (*E* and *F*) Physiologic consequences of inhibiting and overexpressing $G_{\alpha i2}$, as determined using magnetic twisting cytometry. Cells were first contracted with 10 μM of endothelin-1 (ET-1) or 5-hydroxytryptamine (5-HT), as indicated. Results are from 178–293 measurements. *$P < 0.005$ versus NTG.

subunits, however, would not be expected to result in total loss of TAS2R function, because $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ all appear to be capable of transmitting TAS2R activation in HASM.

In the taste field, $G_{\alpha gust}$ has been considered the primary G protein for TAS2Rs; however, there is evidence within several studies that suggest alternative coupling. $G_{\alpha gust}$ knockout mice retain some sensitivity to bitter tastants, suggesting that one or more additional G proteins in taste cells may provide for coupling (41). Given the high degree of homology between gustducin and transducin (Table 1), a transgenic mouse was developed overexpressing $G_{\alpha trans1}$ on the $G_{\alpha gust}$ null background. Bitter taste responsiveness in this mouse was partially, but not fully, restored, which suggested that $G_{\alpha trans1}$ can substitute for $G_{\alpha gust}$, but expression levels or heterogeneity of transgene expression may have limited rescue (30). In other studies, where single-cell recordings were undertaken, in some cells there was actually no difference in TAS2R signaling by some agonists between $G_{\alpha gust}(-)$ and $G_{\alpha gust}(+)$ cells, again pointing to alternative G proteins that can functionally couple to TAS2Rs (42). Taken together with the current work, where $G_{\alpha gust}$ expression in HASM is very low, it may be the case that any of several G_i family α subunits will couple to TAS2Rs, and that the principal TAS2R-activated G protein in a given cell type is based primarily on expression levels. As indicated in Table 1 and the amino acid sequence alignment in Figure E1, one can readily group these eight G proteins into several subfamilies. $G_{\alpha trans1}$ and $G_{\alpha trans2}$ show the greatest homology to $G_{\alpha gust}$ (78 and 80%, respectively). $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ are highly similar to each other, and are 68% identical to $G_{\alpha gust}$. Given our results, and the locations of the differences in amino acid sequences between this group and $G_{\alpha gust}$ (43) (Figure E1), this degree of homology appears to be adequate for TAS2R coupling. Although $G_{\alpha o}$ has some degree of sequence identity to $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$, the identity to $G_{\alpha gust}$ is 62%. A similar comparison is apparent for $G_{\alpha z}$, with identity to $G_{\alpha gust}$ being 58%. It thus seems less likely that $G_{\alpha o}$ and $G_{\alpha z}$ participate in TAS2R signaling in HASM due to this lower amino acid similarity to $G_{\alpha gust}$, the low protein expression of these subunits, and the PTX insensitivity, which excludes $G_{\alpha z}$. Regardless, our goal for this study was not to determine the "affinities" of the eight α subunits for coupling to TAS2Rs, but rather to ascertain which of these α subunits are pertinent for TAS2R signaling in HASM. We show that, in this cell type, unlike that of the taste cell, $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ are the principal G proteins to which TAS2Rs couple to intracellular signaling and relaxation. ■

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References

1. Meyerhof W. Elucidation of mammalian bitter taste. *Rev Physiol Biochem Pharmacol* 2005;154:37–72.
2. Meyerhof W, Batram C, Kuhn C, Brockhoff A, Chudoba E, Bufe B, Appendino G, Behrens M. The molecular receptive ranges of human TAS2R bitter taste receptors. *Chem Senses* 2010;35:157–170.
3. Deshpande DA, Wang WC, McIlmoyle EL, Robinett KS, Schillinger RM, An SS, Sham JS, Liggett SB. Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction. *Nat Med* 2010;16:1299–1304.
4. Robinett KS, Deshpande DA, Malone MM, Liggett SB. Agonist-promoted homologous desensitization of human airway smooth muscle bitter taste receptors. *Am J Respir Cell Mol Biol* 2011;45: 1069–1074.
5. Pulkkinen V, Manson ML, Säfholm J, Adner M, Dahlén SE. The bitter taste receptor (TAS2R) agonists denatonium and chloroquine display distinct patterns of relaxation of the guinea pig trachea. *Am J Physiol Lung Cell Mol Physiol* 2012;303:L956–L966.
6. Deshpande DA, Robinett KS, Wang WC, Sham JS, An SS, Liggett SB. Bronchodilator activity of bitter tastants in human tissue. *Nat Med* 2011;17:776–778.
7. Dickinson GD, Ellefsen KL, Dawson SP, Pearson JE, Parker I. Hindered cytoplasmic diffusion of inositol trisphosphate restricts its cellular range of action. *Sci Signal* 2016;9:ra108.
8. Clark AA, Liggett SB, Munger SD. Extraoral bitter taste receptors as mediators of off-target drug effects. *FASEB J* 2012;26:4827–4831.
9. 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* 2006;291: L523–L534.
10. Kim D, Pauer SH, Yong HM, An SS, Liggett SB. β2-adrenergic receptors chaperone trapped bitter taste receptor 14 to the cell surface as a heterodimer and exert unidirectional desensitization of taste receptor function. *J Biol Chem* 2016;291:17616–17628.
11. McGraw DW, Elwing JM, Fogel KM, Wang WC, Glinka CB, Mihlbachler KA, Rothenberg ME, Liggett SB. Crosstalk between Gi and Gq/Gs pathways in airway smooth muscle regulates bronchial contractility and relaxation. *J Clin Invest* 2007;117:1391–1398.
12. An SS, Laudadio RE, Lai J, Rogers RA, Fredberg JJ. Stiffness changes in cultured airway smooth muscle cells. *Am J Physiol Cell Physiol* 2002;283:C792–C801.
13. Camoretti-Mercado B, Pauer SH, Yong HM, Smith DC, Deshpande DA, An SS, Liggett SB. Pleiotropic effects of bitter taste receptors on $[Ca^{2+}]_i$ mobilization, hyperpolarization, and relaxation of human airway smooth muscle cells. *PLoS One* 2015;10:e0131582.
14. Krzywinski M, Altman N, Blainey P. Points of significance: nested designs: for studies with hierarchical noise sources, use a nested analysis of variance approach. *Nat Methods* 2014;11:977–978.
15. Gesty-Palmer D, Chen M, Reiter E, Ahn S, Nelson CD, Wang S, Eckhardt AE, Cowan CL, Spurney RF, Luttrell LM, *et al*. Distinct β-arrestin– and G protein–dependent pathways for parathyroid hormone receptor–stimulated ERK1/2 activation. *J Biol Chem* 2006; 281:10856–10864.
16. Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O, Lefkowitz RJ. β-arrestin–dependent, G protein–independent ERK1/2 activation by the β2 adrenergic receptor. *J Biol Chem* 2006;281:1261–1273.
17. Liggett SB. Bitter taste receptors on airway smooth muscle as targets for novel bronchodilators. *Expert Opin Ther Targets* 2013;17:721–731.
18. Liggett SB. Bitter taste receptors in the wrong place: novel airway smooth muscle targets for treating asthma. *Trans Am Clin Climatol Assoc* 2014;125:64–74; discussion 74–75.
19. Beasley R, Pearce N, Crane J, Burgess C. β-agonists: what is the evidence that their use increases the risk of asthma morbidity and mortality? *J Allergy Clin Immunol* 1999;104:S18–S30.
20. Cheung D, Timmers MC, Zwinderman AH, Bel EH, Dijkman JH, Sterk PJ. Long-term effects of a long-acting β_2-adrenoceptor agonist, salmeterol, on airway hyperresponsiveness in patients with mild asthma. *N Engl J Med* 1992;327:1198–1203.
21. Drazen JM, Silverman EK, Lee TH. Heterogeneity of therapeutic responses in asthma. *Br Med Bull* 2000;56:1054–1070.
22. Kraan J, Koëter GH, vd Mark TW, Sluiter HJ, de Vries K. Changes in bronchial hyperreactivity induced by 4 weeks of treatment with antiasthmatic drugs in patients with allergic asthma: a comparison between budesonide and terbutaline. *J Allergy Clin Immunol* 1985; 76:628–636.
23. Lipworth BJ. Airway subsensitivity with long-acting β2-agonists: is there cause for concern? *Drug Saf* 1997;16:295–308.
24. Salpeter SR, Wall AJ, Buckley NS. Long-acting β-agonists with and without inhaled corticosteroids and catastrophic asthma events. *Am J Med* 2010;123:322–8.e2.
25. Sears MR, Taylor DR. The β2-agonist controversy: observations, explanations and relationship to asthma epidemiology. *Drug Saf* 1994;11:259–283.
26. Einstein R, Jordan H, Zhou W, Brenner M, Moses EG, Liggett SB. Alternative splicing of the G protein–coupled receptor superfamily in human airway smooth muscle diversifies the complement of receptors. *Proc Natl Acad Sci USA* 2008;105:5230–5235.
27. Pera T, Penn RB. Bronchoprotection and bronchorelaxation in asthma: new targets, and new ways to target the old ones. *Pharmacol Ther* 2016;164:82–96.
28. Eason MG, Kurose H, Holt BD, Raymond JR, Liggett SB. Simultaneous coupling of α2-adrenergic receptors to two G-proteins with opposing effects: subtype-selective coupling of α2C10, α2C4, and α2C2 adrenergic receptors to Gi and Gs. *J Biol Chem* 1992;267: 15795–15801.
29. Eason MG, Liggett SB. Identification of a Gs coupling domain in the amino terminus of the third intracellular loop of the α2A-adrenergic receptor: evidence for distinct structural determinants that confer Gs versus Gi coupling. *J Biol Chem* 1995;270:24753–24760.
30. He W, Danilova V, Zou S, Hellekant G, Max M, Margolskee RF, Damak S. Partial rescue of taste responses of α-gustducin null mice by transgenic expression of α-transducin. *Chem Senses* 2002;27:719–727.
31. Costa-Neto CM, Parreiras-E-Silva LT, Bouvier M. A pluridimensional view of biased agonism. *Mol Pharmacol* 2016;90:587–595.
32. Boyer JL, Graber SG, Waldo GL, Harden TK, Garrison JC. Selective activation of phospholipase C by recombinant G-protein α- and β γ-subunits. *J Biol Chem* 1994;269:2814–2819.
33. Blank JL, Brattain KA, Exton JH. Activation of cytosolic phosphoinositide phospholipase C by G-protein β γ subunits. *J Biol Chem* 1992;267:23069–23075.
34. Hakonarson H, Herrick DJ, Grunstein MM. Mechanism of impaired β-adrenoceptor responsiveness in atopic sensitized airway smooth muscle. *Am J Physiol* 1995;269:L645–L652.
35. Hakonarson H, Herrick DJ, Serrano PG, Grunstein MM. Mechanism of cytokine-induced modulation of β-adrenoceptor responsiveness in airway smooth muscle. *J Clin Invest* 1996;97:2593–2600.
36. Hakonarson H, Maskeri N, Carter C, Hodinka RL, Campbell D, Grunstein MM. Mechanism of rhinovirus-induced changes in airway smooth muscle responsiveness. *J Clin Invest* 1998;102:1732–1741.
37. Grunstein MM, Hakonarson H, Maskeri N, Chuang S. Autocrine cytokine signaling mediates effects of rhinovirus on airway responsiveness. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L1146–L1153.
38. Hotta K, Emala CW, Hirshman CA. TNF-α upregulates $G_{i\alpha}$ and $G_{q\alpha}$ protein expression and function in human airway smooth muscle cells. *Am J Physiol* 1999;276:L405–L411.
39. Gasic S, Green A. G_i down-regulation and heterologous desensitization in adipocytes after treatment with the α2-agonist UK 14304. *Biochem Pharmacol* 1995;49:785–790.
40. Jewell-Motz EA, Donnelly ET, Eason MG, Liggett SB. Agonist-mediated downregulation of $G_{\alpha i}$ via the α_2-adrenergic receptor is targeted by receptor-G_i interaction and is independent of receptor signaling and regulation. *Biochemistry* 1998;37:15720–15725.
41. Wong GT, Gannon KS, Margolskee RF. Transduction of bitter and sweet taste by gustducin. *Nature* 1996;381:796–800.
42. Caicedo A, Pereira E, Margolskee RF, Roper SD. Role of the G-protein subunit α-gustducin in taste cell responses to bitter stimuli. *J Neurosci* 2003;23:9947–9952.
43. Conklin BR, Bourne HR. Structural elements of G α subunits that interact with G β γ, receptors, and effectors. *Cell* 1993;73:631–641.