Biased TAS2R Bronchodilators Inhibit Airway Smooth Muscle Growth by Downregulating Phosphorylated Extracellular Signal-regulated Kinase 1/2

Donghwa Kim¹, Soomin Cho², Maria A. Castaño¹, Reynold A. Panettieri³, Jung A. Woo⁴, and Stephen B. Liggett^{1,4}

¹Department of Medicine and ⁴Department of Molecular Pharmacology and Physiology, University of South Florida Morsani College of Medicine, Tampa, Florida; ²Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, California; and ³Rutgers Institute for Translational Medicine and Science, Robert Wood Johnson Medical School, Rutgers, The State University of New Jersey, New Brunswick, New Jersey

Abstract

Bitter taste receptor (TAS2R) agonists dilate airways by receptor-dependent smooth muscle relaxation. Besides their coupling to relaxation, we have found that human airway smooth muscle (HASM) cell TAS2Rs activate (phosphorylate) extracellular signal-related kinase 1/2 (ERK1/2), but the cellular effects are not known. In the present study, we show in HASM cells that TAS2R agonists initially stimulate phosphorylated ERK1/2 (pERK1/2) but by 24 hours cause a marked (50–70%) downregulation of pERK1/2 without a change in total ERK1/2. It was hypothesized that TAS2R agonists suppress cell growth through this pERK1/2 downregulation. Agonist-dependent inhibition of cell proliferation was indeed found in HASM cells derived from normal and asthmatic human lungs, as well as in an immortalized HASM cell line. pERK1/2 downregulation was linked to downregulation of the upstream kinase MEK1/2 (mitogen-activated protein kinase/extracellular signal-regulated kinase). Various structurally

diverse TAS2R agonists evoked a range of inhibition of HASM proliferation, the magnitude of which directly correlated with the downregulation of pERK1/2 ($R^2 = 0.86$). Some TAS2R agonists were as effective as pharmacological inhibitors of Raf1 and MEK1/2 in suppressing growth. siRNA silencing of TAS2Rs (subtypes 10, 14, and 31) ablated the pERK1/2 and growth-inhibitory effects of TAS2R agonists. These phenotypes were attenuated by inhibiting the TAS2R G protein $G_{\alpha i}$ and by knocking down β -arrestin 1/2, indicating a dual pathway, although there may be additional mechanisms involved in this HASM TAS2R multidimensional signaling. Thus, TAS2R agonist structure can be manipulated to maintain the relaxation response and can be biased toward suppression of HASM growth. The latter response is of potential therapeutic benefit in asthma, in which an increase in smooth muscle mass contributes to airway obstruction.

Keywords: asthma; G protein; mitogen-activated protein kinase; bitter taste receptor

β_2 -Adrenergic receptors (β_2 ARs) represent the only currently used target for direct bronchodilators in the treatment of asthma and chronic obstructive pulmonary disease. β -Agonists acting at airway smooth muscle (HASM) cell β_2 ARs relax the airway by coupling to $G_{\alpha s}$, activating adenylyl cyclase, increasing cAMP, and activating protein

kinase A, which leads to relaxation. Recently, another class of G protein-coupled receptor (GPCR) agonists acting as direct bronchodilators and consisting of bitter taste receptor (TAS2R) agonists has been described (1). Three of the 25 TAS2Rs are highly expressed in HASM: subtypes 10, 14, and 31. Each

markedly relaxes HASM contracted by multiple bronchoconstrictive substances such as those acting at receptors for the M_3 -muscarinic, H_1 -histamine, and other receptors on HASM. In contrast to β_2 AR, TAS2R activation does not increase cAMP or activate protein kinase A, but rather couples to $G_{\alpha i}$ (2) and subsequently

(Received in original form June 5, 2018; accepted in final form October 22, 2018)

Supported by National Institutes of Health grants HL045967 (S.B.L.) and HL114471 (S.B.L. and R.A.P.).

Author Contributions: Conception, design, and conduct of experiments; analysis of data; and interpretation of results: D.K., S.C., M.A.C., J.A.W., and S.B.L.; and writing and preparation of the manuscript: D.K., R.A.P., J.A.W., and S.B.L.

Correspondence and requests for reprints should be addressed to Stephen B. Liggett, M.D., Departments of Internal Medicine and Molecular Pharmacology and Physiology, University of South Florida Morsani College of Medicine, 12901 Bruce B. Downs Boulevard, MDC02, Tampa, FL 33612. E-mail: sliggett@health.usf.edu.

This article has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Cell Mol Biol Vol 60, Iss 5, pp 532–540, May 2019

Copyright © 2019 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2018-0189OC on October 26, 2018

Internet address: www.atsjournals.org

phospholipase C, which results in an increase in a restricted intracellular pool of $[Ca^{2+}]_i$, ultimately hyperpolarizing the cell membrane (1).

TAS2Rs also stimulate phosphorylation of the extracellular signal-related kinases ERK1 and ERK2 (ERK1/2) in HASM upon short-term exposure to some agonists (2), although the cellular consequences of this signaling have not been ascertained. A prolonged increase in phosphorylated ERK1/2 (pERK1/2) might result in an increase in HASM growth (a known function of ERK1/2) and contribute to an increase in HASM cell mass, which could be detrimental in asthma. Interestingly, pERK1/2 signaling in some cell types displays an early increase, but with chronic stimulation on the order of several hours, it results in a decrease in pERK1/2 below resting concentrations (3–5). For HASM, this might decrease cell growth, which would be an additional therapeutic benefit of TAS2Rs for the treatment of asthma, in which an increase in HASM mass due to cellular hypertrophy and hyperplasia contributes to airflow obstruction.

The functional coupling of GPCRs to multiple diverse signals has been shown to be substantially influenced in some instances by the structure of the agonist (6–8). The current paradigm by which agonists could selectively promote certain events within a GPCR multifunctional output indicates that agonists of different structures stabilize certain specific receptor conformations and thus bias the agonist–receptor pair toward or away from certain functional outcomes (9). Thus, biased agonism is defined functionally. The mechanisms by which agonist structure dictates these differential outcomes include altered receptor–G protein interaction, GPCR kinase phosphorylation of the receptor, and β -arrestin binding to the phosphorylated receptor, as well as differential association with intracellular proteins (10, 11). Regardless of the mechanism, manipulating agonists to tailor the array of physiological responses provides an opportunity to enhance the clinical effectiveness of GPCR agonists. We have extensively defined the coupling of TAS2Rs to the HASM relaxation effect (1, 2, 12–15), which correlates with an increase in a restricted $[Ca^{2+}]_i$ release. We have considered, though, whether agonists acting at these receptors might have other effects

that would act in a favorable manner during treatment of asthma. Of particular interest is the influence of TAS2Rs on HASM cell growth, given that receptor activation stimulates pERK1/2. In the present work, we show that TAS2Rs decrease HASM cell growth, which is dependent on chronic downregulation of pERK1/2 in a biased agonist manner, indicating certain structural constraints that would be necessary to obtain this dual-positive therapeutic effect of TAS2R agonists for the treatment of asthma.

Methods

Cell Culture

Primary HASM cells derived from nonasthmatic (HASM-NA) and asthmatic (HASM-AS) donor lungs (passages 4–6), as well as D9 human telomerase reverse transcriptase immortalized HASM cells (HASM-IM), were grown as previously described (16). Primary cells were maintained in Ham's F-12 media (Thermo

Fisher Scientific) with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1% L-glutamine, 1.7 mM $CaCl_2$, 12 mM NaOH, and 25 mM HEPES. HASM-IM cells were maintained in Dulbecco's modified Eagle's media with 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were treated with vehicle, TAS2R agonist, and inhibitors at the indicated concentrations in media with serum, unless otherwise stated, for the indicated times at 37°C in 95% air and 5% CO_2 and then harvested for various studies.

Cell Proliferation Assays

HASM cells were plated at 1×10^5 cells per well. After treatments, they were trypsinized and counted in quadruplicate using an automated cell counter (Beckman Coulter Life Sciences). For the monolayer expansion assay, adhered HASM cells were grown to 100% confluency, and the plates were scraped in a linear manner across the plate using a pipette tip under vacuum. Occupation of the space by new cells was determined by light microscopy.

Figure 1. Bitter taste receptor (TAS2R) agonist variability decreases human airway smooth muscle (HASM) cell proliferation. Cells from nonasthmatic (HASM-NA) and asthmatic (HASM-AS) lungs at passage 3 were plated at 50% confluency and treated with the TAS2R agonists aloin (ALO), famotidine (FAM), and papaverine (PAP) for 72 hours in media with serum. Studies were performed with two cell lines from nonasthmatic donor lungs and two cell lines from asthmatic donor lungs. Representative experiments are shown in A and B, and mean results from 10 experiments from each cell line ($n = 20$ experiments for HASM-NA and 20 for HASM-AS) are shown in C and D. * $P < 0.01$ versus control. Ctrl = control.

[Ca²⁺]_i Measurements, Propidium Iodide Staining, and Western Blotting

Intracellular Ca²⁺ was measured in response to exposure of attached HASM cells to TAS2R agonists in 96-well plates loaded with the green fluorescent calcium indicator Fluo-4 (Life Technologies) exactly as described in detail elsewhere (1). Cells

were subjected to staining with propidium iodide and DAPI using reagents from Molecular Probes as described previously (17). For Western blotting, cells were lysed with radioimmunoprecipitation assay buffer, clarified by brief centrifugation, and electrophoresed through 12% SDS-polyacrylamide gels. The antibodies were

from the same sources and were used at the same concentrations as previously described (2).

siRNA Transfections

For β -arrestin 1/2 and TAS2R knockdowns, HASM-IM cells were transfected with 80 nM of siRNAs (Dharmacon) at 24 hours

Figure 2. TAS2R agonists ALO, FAM, and PAP stimulate [Ca²⁺]_i and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in a dose-dependent manner in immortalized human airway smooth muscle cells. For experiments in A, C, and E, cells were loaded with Fluo-4, and [Ca²⁺]_i was measured in triplicate using an automated plate reader. For B, D, and F, cells were serum starved (s/s) for 12 hours, treated with the indicated concentrations of the agonist (in μ M) for 5 minutes, and then prepared for protein immunoblotting as described in the METHODS section. Shown are protein immunoblots for phosphorylated ERK1/2 (pERK1/2), total ERK1/2, and β -actin (loading control). RFU = relative fluorescence units.

and again at 48 hours after plating using Lipofectamine 2000 reagent (Life Technologies) exactly as previously described (2, 16). Although there are no antibodies to TAS2Rs, we have previously shown that this siRNA transfection decreases TAS2R function (1), consistent with protein knockdown.

Drug Treatments

Cells were exposed to the TAS2R agonists alonin (ALO), famotidine (FAM), and papaverine (PAP) at saturating concentrations based on their affinities (18) (200 μ M for ALO, FAM; 100 μ M for PAP) or other concentrations as indicated. The kinase inhibitors GSK2118436 (GSK) and PD98059 were used at concentrations of 20 μ M and 100 μ M, respectively. Pertussis toxin (PTX) was used at a concentration of 100 nM for 24 hours.

Statistical Analysis

Bands from Western blots were quantitated using ImageJ software (NIH). Statistical analysis was performed using Prism software (GraphPad Software), and data are presented as mean $\pm$ SE. For experiments with multiple drugs or conditions, ANOVA with *post hoc t* tests corrected for multiple comparisons was used. *P* values less than 0.05 were considered significant.

Results

TAS2R Signaling by PAP, ALO, and FAM

Our initial screens of TAS2R agonists revealed three known agonists (PAP, ALO, and FAM) that displayed different degrees of growth inhibition of cultured HASM cells derived from nonasthmatic lungs (HASM-NA cells) (Figures 1A and 1C). Cell proliferation was inhibited by as much as approximately 50% by PAP. As we have previously shown, HASM cells derived from lungs of individuals with asthma have a unique phenotype that is maintained in culture (19). We thus studied these HASM-AS cells to confirm that this antiproliferative effect is also observed in this asthmatic model. Figures 1B and 1D show that TAS2R agonists inhibit proliferation in these cells as well, with a rank order of antiproliferation being PAP greater than ALO, with no significant inhibition by FAM. The magnitude of the

inhibition by each drug was not different between HASM-NA and HASM-AS cells. Because HASM-IM cells have characteristics similar to those of primary HASM cell lines (2, 16) and are much more readily amenable to siRNA-based knockdown experiments (2, 16), we subsequently used these cells to investigate the mechanism of TAS2R-mediated growth inhibition. Figure 2 shows the signal transduction in HASM-IM cells evoked by these agonists to the two intracellular pathways that we have previously defined for TAS2Rs in HASM cells (1, 2). All three agonists stimulated $[Ca^{2+}]_i$ in a dose-dependent manner (Figures 2A, 2C, and 2E). The maximal $[Ca^{2+}]_i$ stimulation for the three agonists was less than 20% different, so we considered that for this pathway, these agonists have similar efficacies (and represent “full agonists”). For ERK1/2 phosphorylation, the three agonists evoked marked phosphorylation (pERK1/2 band of each blot in Figures 2B, 2D, and 2F) in a dose-dependent fashion over a 5-minute period of agonist exposure

to HASM-IM cells in serum-free media. Again, the degree of the pERK1/2 signal observed (ratio to total ERK1/2) was similar between the three compounds. The calculated half-maximal effective concentration values for each agonist were consistent with previously published data from our group and others (1, 18).

TAS2R Agonists Downregulate pERK1/2

We next proceeded to ascertain the effects of the three agonists on pERK1/2 after long-term incubation with cells in the presence of 10% serum, which is the condition used for studies of cell growth and the condition relevant to pharmacologic therapy for asthma. Our focus was on the ERK pathway, given its known effect on cell growth (8, 20). Controls for these experiments included PD98059, a mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor that blocks phosphorylation of ERK1/2 by MEK1/2 and inhibits cell growth, and GSK, which inhibits the ERK1/2 pathway through

Figure 3. Long-term treatment of HASM with TAS2R agonists variably downregulates pERK1/2 and cyclin D1. Immortalized HASM cells were maintained in serum-containing media and treated for the indicated times. (A) Representative protein immunoblot depicting the time course of PAP-mediated downregulation of pERK1/2 (see also Figure E1 for mean data from four experiments). (B) Representative protein immunoblot result from 24-hour treatment with the three TAS2R agonists ALO, FAM, and PAP. (C and D) Mean quantitative results from four or five experiments. **P* < 0.01 versus control. GSK = GSK2118436; MEK = mitogen-activated protein kinase kinase; PD = PD98059.

regulation of Raf, which is upstream of MEK1/2. The time course of pERK1/2 expression with PAP treatment of HASM cells in serum-containing media is shown in Figure 3A and in Figure E1 in the data supplement, and it indicates an agonist-promoted decrease in pERK1/2 within 6 hours of treatment, without a change in total ERK1/2. In additional studies, we found that 24 hours of exposure to the three TAS2R agonists had different effects on the phosphorylation status of ERK1/2 (Figures 3B and 3C). FAM showed no statistically significant decrease in pERK1/2 with this long-term exposure compared with control (serum-containing media alone), whereas ALO displayed a modest decrease. PAP exposure resulted in the greatest decrease in pERK1/2 (Figures 3B and 3C). These results were consistent with our working hypothesis that TAS2R agonists may affect cell growth through downregulation of pERK1/2 and that certain agonists may be biased toward or away from this pathway compared with other agonists. Cyclin D1, which is a critical protein required for progression of the cell through the G₁ phase of the cell cycle, is regulated by ERK1/2 and participating kinases in the mitogen-activated protein kinase cascade (8, 20). As shown in Figures 3B and 3D, cyclin D1 is decreased by the TAS2R agonists ALO and PAP, but not by FAM, which correlates with the downregulating action of these compounds on pERK1/2 (Figures 3B–3D).

TAS2R Downregulation of ERK1/2 Correlates with Growth Inhibition

To specifically test the potential relationship between inhibition of proliferation and ERK1/2 downregulation by TAS2R agonists, HASM-IM cells were plated at 50% confluence in media with serum and treated with vehicle or the TAS2R agonists, then harvested 24 and 48 hours later, and cell counts were determined in quadruplicate using an automated cell counter. Representative results are shown in Figures 4A–4C, with mean results from multiple experiments at the 48-hour time point shown in Figure 4E. ALO displayed a $27 \pm 4.7\%$ ($P < 0.01$) decrease in cell growth, whereas PAP evoked an even greater decrease ($50 \pm 4.9\%$; $P < 0.01$). In contrast, FAM had no significant effect on cell growth. None of the treatments altered cell viability as determined by propidium iodide staining (Figures 4F and E2). The two

Figure 4. HASM cell proliferation is variably inhibited by TAS2R agonists. Cells in serum-containing media were treated with vehicle or the indicated TAS2R agonist (ALO, FAM, and PAP) or kinase inhibitor (PD and GSK), and cell counts were determined at 0, 24, and 48 hours. PAP displayed the greatest growth inhibition; ALO had a modest effect on growth; and FAM had no effect on growth. (A–D) Representative experiments. (E) Mean results from four to six experiments at the 48-hour time point. (F) Treatment of HASM with the TAS2R agonists ALO, FAM, and PAP for 24 hours did not cause cell death as determined by propidium iodide (PI) staining. Retinoic acid (RA; 50 μ M) and SDS (100 μ M) were positive controls for inducing cell death. Results are representative of five independent experiments. Mean data are shown in Figure E2. * $P < 0.001$ versus vehicle.

known ERK1/2 cascade inhibitors had the expected effects of decreasing cell growth, and in fact the magnitude of the effect was similar to that of PAP (Figures 4D and 4E). As nonconfluent cultured HASM cells proliferate, monolayer coverage into unoccupied regions of the plate is observed. We thus performed additional experiments measuring proliferation in media with serum using this approach to confirm the results from cell counting (Figure 5). Cells were removed in a linear fashion across the plate, and the empty area was monitored

for appearance of new HASM cells. Within about 24 hours, the gap was nearly filled under control conditions, which was blocked by the GSK and PD98059 inhibitors (Figure 5A). Consistent with the proliferation results, PAP markedly inhibited growth; ALO had a modest effect on this phenotype; and FAM had no effect (Figure 5B).

The potential for “off-target” or nonspecific effects of TAS2R agonists on proliferation, particularly the marked effect of PAP, was studied using siRNAs to knock

Figure 5. Antiproliferative effect of TAS2R agonists. Confluent HASM cells were detached and removed in a linear fashion (see dotted lines), and the plate was incubated with serum-containing media and the indicated agents for 24 hours. The surface area occupied by cells as determined by light microscopy from new growth was quantitated. (A) Representative experiment. (B) Mean results from five experiments. * $P < 0.001$. Scale bars: 400 μm .

down expression of the three dominant TAS2R subtypes (subtypes 10, 14, 31) in HASM cells that are activated by this agonist. As indicated in Figure 6A, the inhibition of cell proliferation by PAP was not observed with TAS2R10/14/31 knockdowns. Similarly, the downregulation of pERK1/2 by long-term PAP exposure was attenuated by knockdown of the receptors (Figure 6B). These results establish that the PAP effect is indeed due to TAS2R activation by this agonist. We also further explored TAS2R agonist-mediated effects on inhibition of pERK1/2 and the inhibition of growth by performing studies with two additional agonists, diphenhydramine and flufenamic acid (1) (both at 200 μM), and determined the potential relationship between the two outcomes with these five agonists across the range of effects. There was a direct relationship between the downregulating and antiproliferative effects ($R^2 = 0.86$; $P <$

0.001) (Figure 6C). Taken together, the results shown in Figures 4, 6A, and 6C indicate that the tested compounds exert their antiproliferative effect by decreasing pERK1/2 concentrations and that this effect is mediated by agonist activation of TAS2Rs. G_i -coupled GPCRs are known to interact with ERK1/2 via both G_{α} - and β -arrestin-dependent mechanisms, which may be receptor and cell type dependent (21). We thus sought to determine in HASM whether there is a dominant mechanism by which TAS2Rs exert their effect on these pERK1/2 and proliferation phenotypes. Although this exploration is not required to demonstrate biased agonism, it is intriguing to note that both processes (receptor-G protein interaction and receptor- β -arrestin interaction) are, in part, dependent on the receptor conformation established by agonist binding. TAS2Rs in HASM couple to multiple PTX-sensitive members of the G_i

family, primarily $G_{\alpha i1,2,3}$ (2). We thus incubated HASM with the toxin to inactivate these G proteins and ascertained the antiproliferative effects of PAP. The dose of PTX used in these studies is known to fully inactivate $G_{\alpha i}$ (22, 23). PTX treatment impaired the antiproliferative effect of TAS2Rs but did not fully block it (Figure 6D). Knockdown of β -arrestin 1/2 also impaired the effect, but a residual antiproliferative response to PAP was observed (Figure 6E). Of note, these latter studies were performed with approximately 50% knockdown of the β -arrestin 1/2 proteins (Figure E3). However, the fact that partial knockdown caused a partial loss of the agonist-promoted antiproliferative effect implicates a β -arrestin mechanism as well. Studies combining PTX treatment and β -arrestin 1/2 siRNA did not show an additive effect on blocking antiproliferation, but again only modest knockdown of β -arrestin 1/2 was achieved (data not shown). Finally, to gain some insight into the TAS2R-mediated effect on pERK1/2, we examined whether the immediate upstream activating kinase, MEK, was affected by TAS2R agonism. Indeed, pMEK1/2 also underwent downregulation with chronic PAP treatment of HASM (Figure 3A).

Discussion

We previously showed expression of multiple TAS2R subtypes on the cell surface of isolated human and mouse airway smooth muscle cells (1). This was unexpected because the expression of these receptors was believed to be restricted to the tongue. It is now clear that TAS2Rs are expressed on cells of many organs and represent a previously unrecognized chemosensory system in the body (24). The physiological role of TAS2Rs on HASM is not clear, although certain bacteria secrete TAS2R agonists, which may serve to open the airway and improve clearance of bacterial and cellular debris during an infection (25, 26). Nevertheless, as a drug target, TAS2Rs represent an attractive alternative to β_2 ARs for preventing or treating bronchospasm. First, they are highly efficacious. Multiple studies have shown that agonists acting at TAS2Rs on HASM markedly relax the muscle, leading to bronchodilation to a degree greater than or equal to the full β -agonist isoproterenol

Figure 6. Relationship between TAS2R activation, inhibition of HASM proliferation, and pERK1/2 downregulation. (A and B) Knockdown of TAS2Rs 10, 14, and 31 blocks the inhibition of cell growth and the downregulation of pERK1/2 by 24-hour treatment with PAP. (C) Relationship between pERK1/2 downregulation and the HASM proliferative response over 24 hours. $R^2 = 0.86$ with $P < 0.001$ for the linear regression line. $n = 6$ experiments. (D and E) Inhibition of G_i and knockdown of β -arrestin 1/2 in HASM cells attenuates the inhibition of proliferation phenotype by PAP. Results shown are mean values from six or seven quantitating immunoblots from experiments. * $P < 0.01$ versus no-drug control; ** $P < 0.01$ versus the change from no-drug control between the indicated conditions. DPD = diphenhydramine; FFA = flufenamic acid; PTX = pertussis toxin.

(12). In addition, TAS2Rs signal by a different mechanism than β_2 ARs (1) and display bronchodilator activity that is additive with β -agonists when used at low doses (1). Unlike β_2 ARs, TAS2Rs undergo little tachyphylaxis during continuous agonist exposure (15), and TAS2R

expression and function are unaffected by the asthmatic diathesis (13). Although the relaxation properties of TAS2R agonism have been well documented, little is known about other potential signaling pathways. We have recently shown that TAS2Rs on HASM stimulate pERK1/2 (2).

This was observed in serum-starved cells treated for 5 minutes with various TAS2R agonists. Media with serum stimulates pERK1/2 in these cells as well, and the addition of TAS2R agonist for seconds to several minutes in culture leads to an increase in pERK1/2 over that of serum alone (Figure 2). However, we found in the present study that long-term incubation of HASM in serum-containing media with some TAS2R agonists results in an eventual decrease in pERK1/2 below that of basal concentrations, without a loss of total ERK1/2 expression (Figures 3A–3C). Given that a decrease in pERK1/2 would be expected to be antiproliferative, this phenotype was explored with multiple agonists, primarily PAP, ALO, and FAM. In studies in which coupling to the canonical TAS2R signaling pathway (an increase in $[Ca^{2+}]_i$) was examined, all three compounds appear to be full agonists. We have previously shown that this pathway is responsible for TAS2R-mediated airway smooth muscle relaxation (1). Furthermore, in the absence of serum, short-term exposure of HASM to the three agonists phosphorylated ERK1/2 to a similar extent. However, the long-term effects on pERK1/2 and cell growth were quite different between these agonists. This provided the opportunity to establish a cause-and-effect relationship between pERK1/2 downregulation and antiproliferation, as well as to consider the property of biased agonism for this pathway (Figure 7). Indeed, we found a strong positive correlation between the extent of pERK1/2 downregulation and the antiproliferative effect using five structurally diverse TAS2R agonists (Figure 6C). Because these agonists show the same signaling for one pathway (Ca^{2+} signaling) but diverge in efficacy at another pathway (antiproliferation), a biasing by certain agonists is indicated.

Deshpande and colleagues first described an effect on HASM by the TAS2R agonists chloroquine and quinine regarding epidermal growth factor- and platelet-derived growth factor-mediated cell growth (27). In that study, they reported no modulatory effect of these agonists on pERK1/2. However, the cells were studied in the absence of serum and were pretreated with the TAS2R agonists for 15 minutes, then exposed to the growth factor for 30 minutes. Using this approach, these growth factors resulted in phosphorylation

Figure 7. Representation of biased agonism with TAS2R on human airway smooth muscle. The upper equation shows an agonist (A₁; ALO) that binds the receptor and forms an active conformation (A₁R*¹), which leads to one outcome (relaxation). In contrast, the second equation shows another agonist (A₂; PAP) that also leads to the R*¹ state (relaxation) but also stabilizes a second active state (A₂R*²), leading to another outcome (growth inhibition), which is not observed with the A₁ agonist. The A₂ agonist is considered biased toward growth inhibition while maintaining relaxation. Alternatively, the A₁ agonist could be termed an agonist biased away from growth inhibition. Famotidine would be considered an agonist with properties that are intermediate between the extremes of ALO and PAP, in that it evokes a smaller degree of growth inhibition (not depicted).

of ERK1/2, and TAS2R agonist did not alter the extent of the phosphorylation. Deshpande and colleagues went on to implicate other mechanisms for the antimitogenic effects of chloroquine and quinine with long-term exposure. Interestingly, one signal that was modulated by chloroquine and quinine in these studies was a reduction in the growth factor-induced increase in phosphorylated Akt. The PI3K/Akt pathway has been shown to influence Ras-Raf-MEK1/2-ERK1/2 pathways. Activated Akt phosphorylates RAF1, which inhibits its phosphorylation of MEK1/2 and thus the generation of activated ERK1/2 (28, 29). This cross-talk appears to be dose, cell type, and condition dependent (28, 29). In the present work, we studied pERK1/2 and cell growth under quite different conditions from those in the aforementioned studies (25). Our measurement of pERK1/2 was in response to long-term exposure to TAS2R agonists; the stimulus for growth was serum; and we did not use a pretreatment step. Importantly, the effects of TAS2R agonists on ERK1/2 phosphorylation and cell growth of HASM were studied under identical conditions, which provides the best opportunity to relate the signal to cellular consequences. The downregulation

of pERK1/2 by certain TAS2R agonists was unequivocal. The strong correlation between this loss of pERK1/2 and the inhibition of growth further strengthened a causal effect. Additional studies by Deshpande and colleagues also implicate an effect of the TAS2R agonists quinine and chloroquine on HASM autophagy and mitochondrial function during mitogen-induced growth (30). These results indicate, at least for these two agonists, that reduction in smooth muscle mass under these conditions may be due to promotion of proteolytic degradation of cystolic proteins.

Downregulation of pERK1/2 by various stimuli has been reported (3–5) and appears to be cell type dependent. For example, insulin treatment of 3T3-L1 preadipocytes causes an early increase in pERK1/2, but by 3 hours pERK1/2 begins to decrease, and by 24 hours, it is reduced more than 75% (4). In contrast, differentiated 3T3-L1 adipocytes fail to display this downregulation of pERK1/2 (4). Even some growth factors, such as epidermal growth factor, evoke an early increase and then a later downregulation of pERK1/2 (5). Long-term exposure of fibroblasts to the GPCR agonists thrombin and methacholine has also been reported to initially stimulate and then downregulate pERK1/2 (3). For HASM, we have demonstrated that the downregulation of pERK1/2 and the inhibition of growth by TAS2R agonists require the initial engagement of the receptor to the phosphorylation of ERK1/2, which is similar to the studies mentioned above. The mechanism of this coupling for TAS2Rs in HASM appears to be bimodal, requiring receptor-G_i interaction and receptor-β-arrestin interaction. This is similar to what has been described with agonists for the GPCR parathyroid hormone type 1 and angiotensin II type 1a receptors (21, 31). In the present work with HASM cells, we found that both PTX and β-arrestin siRNAs decreased cell proliferation to some extent in the absence of TAS2R agonist. Nevertheless, TAS2R agonist further decreased HASM cell proliferation under PTX or β-arrestin knockdown conditions, which implicates both a G_{αi}-mediated component and a β-arrestin-mediated component. However, because baseline proliferation

was altered, it is difficult to quantitate the relative contribution of each of these pathways to TAS2R-mediated inhibition of proliferation. However, both mechanisms of GPCR signaling are in fact dependent on the conformation of the receptor in the agonist-bound state and thus have potential to be influenced by the structure of the agonist. Thus, our findings of different degrees of pERK1/2 downregulation and inhibition of proliferation by agonists with nontrivial differences in structure are consistent with a biasing effect by certain agonists toward this outcome. The composition of the scaffolding complex that apparently includes the TAS2R β-arrestin, the upstream mediator of ERK1/2 phosphorylation MEK, and G_{αi} (or a second messenger from G_{αi} activation) remains unclear, but similar complexes with other receptors have been described (32, 33).

Conclusions

HASM TAS2R agonists represent a new class of bronchodilators, and these receptors appear to display multifunctional signaling. In HASM cells, TAS2Rs not only relax the muscle but also stimulate pERK1/2 upon brief agonist activation. With prolonged activation, they downregulate pERK1/2, which leads to a significant decrease in cell proliferation. The pERK1/2 downregulation is dependent on the structure of the agonist, indicating that TAS2Rs can be stabilized in a conformation that biases signaling toward this clinically favorable response. It has been estimated that there are over 10,000 compounds that act as TAS2R agonists that are found in plants or are synthetic agents used for other purposes. This extensive structural diversity may provide for other forms of biasing of the TAS2R response in HASM, giving rise to additional therapeutic benefits in the treatment of obstructive lung disease. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Lisa Steward for manuscript preparation and the National Disease Research Interchange for providing the primary HASM cell lines.

References

- Deshpande DA, Wang WCH, McIlmoyle EL, Robinett KS, Schillinger RM, An SS, *et al.* Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction. *Nat Med* 2010;16:1299–1304.
- Kim D, Woo JA, Geffken E, An SS, Liggett SB. Coupling of airway smooth muscle bitter taste receptors to intracellular signaling and relaxation is via $G_{\alpha 1,2,3}$. *Am J Respir Cell Mol Biol* 2017;56:762–771.
- Kahan C, Seuwen K, Meloche S, Pouyssegur J. Coordinate, biphasic activation of p44 mitogen-activated protein kinase and S6 kinase by growth factors in hamster fibroblasts: evidence for thrombin-induced signals different from phosphoinositide turnover and adenylylcyclase inhibition. *J Biol Chem* 1992;267:13369–13375.
- Shen N, Yu X, Pan FY, Gao X, Xue B, Li CJ. An early response transcription factor, Egr-1, enhances insulin resistance in type 2 diabetes with chronic hyperinsulinism. *J Biol Chem* 2011;286:14508–14515.
- Zubilewicz A, Hecquet C, Jeanny J, Soubrane G, Courtois Y, Mascarelli F. Proliferation of CECs requires dual signaling through both MAPK/ERK and PI 3-K/Akt pathways. *Invest Ophthalmol Vis Sci* 2001;42:488–496.
- Swift SM, Schwarb MR, Muhlbachler KA, Liggett SB. Pleiotropic β -agonist-promoted receptor conformations and signals independent of intrinsic activity. *Am J Respir Cell Mol Biol* 2007;36:236–243.
- Eason MG, Jacinto MT, Liggett SB. Contribution of ligand structure to activation of alpha 2-adrenergic receptor subtype coupling to Gs. *Mol Pharmacol* 1994;45:696–702.
- Eishingdrelo H, Kongsamut S. Minireview: Targeting GPCR activated ERK pathways for drug discovery. *Curr Chem Genomics Transl Med* 2013;7:9–15.
- Wisler JW, Xiao K, Thomsen AR, Lefkowitz RJ. Recent developments in biased agonism. *Curr Opin Cell Biol* 2014;27:18–24.
- Smith JS, Lefkowitz RJ, Rajagopal S. Biased signalling: from simple switches to allosteric microprocessors. *Nat Rev Drug Discov* 2018;17:243–260.
- Whalen EJ, Rajagopal S, Lefkowitz RJ. Therapeutic potential of β -arrestin- and G protein-biased agonists. *Trends Mol Med* 2011;17:126–139.
- 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.
- Robinett KS, Koziol-White CJ, Akoluk A, An SS, Panettieri RA Jr, Liggett SB. Bitter taste receptor function in asthmatic and nonasthmatic human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2014;50:678–683.
- An SS, Wang WC, Koziol-White CJ, Ahn K, Lee DY, Kurten RC, *et al.* TAS2R activation promotes airway smooth muscle relaxation despite β_2 -adrenergic receptor tachyphylaxis. *Am J Physiol Lung Cell Mol Physiol* 2012;303:L304–L311.
- 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.
- Kim D, Cho S, Woo JA, Liggett SB. A CREB-mediated increase in miRNA let-7f during prolonged β -agonist exposure: a novel mechanism of β_2 -adrenergic receptor down-regulation in airway smooth muscle. *FASEB J* 2018;32:3680–3688.
- Woo JA, Zhao X, Khan H, Penn C, Wang X, Joly-Amado A, *et al.* Slingshot-cofilin activation mediates mitochondrial and synaptic dysfunction via A β ligation to β 1-integrin conformers. *Cell Death Differ* 2015;22:921–934.
- Meyerhof W, Batram C, Kuhn C, Brockhoff A, Chudoba E, Bufer B, *et al.* The molecular receptive ranges of human TAS2R bitter taste receptors. *Chem Senses* 2010;35:157–170.
- An SS, Mitzner W, Tang WY, Ahn K, Yoon AR, Huang J, *et al.* An inflammation-independent contraction mechanophenotype of airway smooth muscle in asthma. *J Allergy Clin Immunol* 2016;138:294–297.e4.
- Sun Y, Liu WZ, Liu T, Feng X, Yang N, Zhou HF. Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. *J Recept Signal Transduct Res* 2015;35:600–604.
- Gesty-Palmer D, Chen M, Reiter E, Ahn S, Nelson CD, Wang S, *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.
- Spangrude GJ, Sacchi F, Hill HR, Van Epps DE, Daynes RA. Inhibition of lymphocyte and neutrophil chemotaxis by pertussis toxin. *J Immunol* 1985;135:4135–4143.
- Goldman DW, Chang FH, Gifford LA, Goetzl EJ, Bourne HR. Pertussis toxin inhibition of chemotactic factor-induced calcium mobilization and function in human polymorphonuclear leukocytes. *J Exp Med* 1985;162:145–156.
- An SS, Liggett SB. Taste and smell GPCRs in the lung: evidence for a previously unrecognized widespread chemosensory system. *Cell Signal* 2018;41:82–88.
- Brockhoff A, Behrens M, Massarotti A, Appendino G, Meyerhof W. Broad tuning of the human bitter taste receptor hTAS2R46 to various sesquiterpene lactones, clerodane and labdane diterpenoids, strychnine, and denatonium. *J Agric Food Chem* 2007;55:6236–6243.
- Sbarbati A, Tizzano M, Merigo F, Benati D, Nicolato E, Boschi F, *et al.* Acyl homoserine lactones induce early response in the airway. *Anat Rec (Hoboken)* 2009;292:439–448.
- Sharma P, Panebra A, Pera T, Tiegs BC, Hershfeld A, Kenyon LC, *et al.* Antimitogenic effect of bitter taste receptor agonists on airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2016;310:L365–L376.
- Moelling K, Schad K, Bosse M, Zimmermann S, Schweneker M. Regulation of Raf-Akt cross-talk. *J Biol Chem* 2002;277:31099–31106.
- Zhou J, Du T, Li B, Rong Y, Verkhatsky A, Peng L. Crosstalk between MAPK/ERK and PI3K/AKT signal pathways during brain ischemia/reperfusion. *ASN Neuro* 2015;7:1759091415602463.
- Pan S, Sharma P, Shah SD, Deshpande DA. Bitter taste receptor agonists alter mitochondrial function and induce autophagy in airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2017;313:L154–L165.
- Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, *et al.* Independent β -arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci USA* 2003;100:10782–10787.
- Shenoy SK, Lefkowitz RJ. Angiotensin II-stimulated signaling through G proteins and β -arrestin. *Sci STKE* 2005;2005:cm14.
- Carmona-Rosas G, Alcántara-Hernández R, Hernández-Espinosa DA. Dissecting the signaling features of the multi-protein complex GPCR/ β -arrestin/ERK1/2. *Eur J Cell Biol* 2018;97:349–358.