Bitter taste receptors (TAS2Rs) are G-protein-coupled receptors now recognized to be expressed on extraoral cells, including airway smooth muscle (ASM) where they evoke relaxation. TAS2Rs are difficult to express in heterologous systems, with most receptors being trapped intracellularly. We find, however, that co-expression of β2-adrenergic receptors (β2AR) in HEK293T routes TAS2R14 to the cell surface by forming receptor heterodimers. Cell surface TAS2R14 expression was increased by ∼5-fold when β2AR was co-expressed. Heterodimer formation was shown by co-immunoprecipitation with tagged receptors, biomolecular fluorescence complementation, and merged confocal images. The dynamic nature of this interaction was shown by: a gene-dose relationship between transfected β2AR and TAS2R14 expression, enhanced (up to 3-fold) TAS2R14 agonist stimulation of [Ca2+]i, with β2AR co-transfection, ∼53% decrease in [Ca2+]i, signaling with shRNA knockdown of β2AR in H292 cells, and ∼60% loss of [Ca2+]i responsiveness in βAR knock-out mouse ASM. Once expressed on the surface, we detected unidirectional, conformation-dependent, interaction within the heterodimer, with β2AR activation rapidly uncoupling TAS2R14 function (∼65% desensitization). Cross-talk was independent of β2AR internalization and cAMP/PKA, and not accompanied by TAS2R14 internalization. With prolonged β-agonist exposure, TAS2R14 internalized, consistent with slow recycling of naked TAS2R14 in the absence of the heterodimeric milieu. In studies of ASM mechanics, rapid cross-talk was confirmed at the physiologic level, where relaxation from TAS2R14 agonist was decreased by ∼50% with β-agonist co-treatment. Thus the β2AR acts as a double-edged sword: increasing TAS2R14 cell surface expression, but when activated by β-agonist, partially offsetting the expression phenotype by direct receptor:receptor desensitization of TAS2R14 function.

Bitter taste receptors (TAS2R) were initially discovered on taste buds and were thought to have evolved as a mechanism for avoidance of toxic plants (1, 2). However, we found that certain TAS2Rs (subtypes 10, 14, and 31) are expressed on human airway smooth muscle (HASM), and when activated result in marked HASM relaxation and bronchodilation (3, 4). This has brought forth the concept of HASM TAS2Rs being targets for novel agonists in the treatment of asthma and chronic obstructive pulmonary disease (4, 5). TAS2Rs are also expressed on a number of other extraoral cell types, suggesting a previously unrecognized chemo-sensory system that might be exploited for drug development (5, 6). TAS2Rs in taste cells signal by binding to the G-protein gustducin, whose βγ subunit activates phospholipase C, generating inositol 1,4,5-trisphosphate, which activates an endoplasmic reticulum inositol 1,4,5-trisphosphate receptor resulting in an increase in intracellular Ca2+ ([Ca2+]i) (1). There is a divergence in signaling between taste cells and HASM at this juncture. In taste cells, the TAS2R-derived [Ca2+]i, activates a transient receptor potential channel, causing membrane depolarization, release of neurotransmitter, and subsequent activation of the Type III cell, which through sensory nerves communicates to the central nervous system. In HASM, the expressed TAS2Rs act directly to relax the muscle through a non-cAMP dependent mechanism, involving [Ca2+]i modulation (3). Indeed the efficacy of some TAS2R agonists is greater than full β2-adrenergic receptor (β2AR) agonists (4), which are the mainstay of treating bronchospasm in asthma and chronic obstructive pulmonary disease. The relaxation from activation of β2AR expressed on HASM is due to coupling of these receptors to Gαs with generation of cAMP, and a protein kinase A-dependent mechanism of relaxation (7). Given the extensive relaxation evoked from TAS2Rs, and the different mechanisms by which TAS2Rs and β2ARs relax HASM, the idea of using agonists for these receptors singly or in combination has been put forward as a way to optimize therapy (5).

The 25 TAS2Rs have been historically difficult to heterologously express on the cell membrane of model cells (8), which...
has been an impediment for further investigation of their signaling properties. However, in the process of expressing the TAS2R14 subtype with the β2AR, we found an increase in expression in HEK-293T cells. This led to the hypothesis that TAS2R14 and β2AR form a heterodimer in the cytosol, and TAS2R14 cell surface expression is facilitated by the β2AR component. In this report, we show that transfected TAS2R14 is predominately trapped in the cytosol in the absence of co-transfection β2AR, and that β2AR acts as a chaperone to facilitate TAS2R14 membrane insertion and functional coupling. This translocation is due to the formation of TAS2R14:β2AR heterodimers. We show that the heterodimeric unit is stable at the cell surface, and identify a mechanism of unidirectional cross-talk between the two receptors that uncouples TAS2R signaling. Physiologic consequences of the heterodimer and the cross-talk are confirmed in studies of ASM cell mechanics. Taken together, we provide new insight into how TAS2R14 is expressed and regulated by β2AR, and potential interactions between the receptors that may impinge on therapeutic efficacy.

Results

Co-expression of β2AR Enhances Cell Membrane TAS2R14 Expression—To begin to address potential TAS2R:β2AR interactions, we attempted to heterologously express the receptors in HEK-293T cells. Our initial approach to transfect these cells with FLAG-TAS2R14 in pcDNA resulted in very little expression in the cytosol or on the cell membrane, as has been documented by others (2, 8). Extension of the short amino terminus with the rat somatostatin receptor 3 amino terminus, and the C terminus with a herpes simplex virus glycoprotein D epitope (a common approach in the TAS2R field, which has been reported to provide for some degree of expression) (2) did not result in consistently detectable expression in our hands. When we added a cleavable leucine-rich N-terminal peptide, termed Lucy (9), to the aforementioned construct (Lucy-Flag-rsst3-TAS2R14-HSV), expression over background was achieved as determined by Western blotting analysis using FLAG or Myc antibodies (Fig. 1, A and B). However, when the above TAS2R14 construct was co-transfected with β2AR in pcDNA, a substantial increase in cell surface expression of TAS2R14 was observed (Fig. 1, A and B). For these studies, cells were transfected with FLAG- or Myc-tagged TAS2R14, in the absence or presence of co-transfection with β2AR, and 48 h later the intact cells were treated with biotin. After purification with avidin immobilized on agarose beads to isolate membrane-bound proteins, the proteins were subjected to SDS-PAGE and immunoblotting with Myc or FLAG antibody. As depicted in Fig. 1, A and B, TAS2R14 cell surface expression was increased when HEK-293T cells were co-transfected with β2AR. In 4 such experiments, the fold-increase of cell surface TAS2R14 when β2AR was co-transfected was determined to be 5.7 ± 0.96-fold ($p < 0.01$ versus TAS2R14-transfected). Confocal imaging of co-transfected cells using the FLAG antibody to identify TAS2R14 (red signal) and concanavalin A to delineate the cell membrane (green signal) confirmed membrane association of the expressed TAS2R14 (yellow signal) (Fig. 1C). Additional confocal imaging studies (Fig. 1D) were concordant with the biotinylation assays. In the absence of β2AR co-transfection, TAS2R14 expression (red signal) was found in ~20% of cells, but rarely at the cell surface. However, when co-transfected with β2AR, most cells were found to express TAS2R14 and its cell surface expression was readily apparent, amounting to 80% of the total (intracellular + cell surface) TAS2R14 expression (Fig. 1D).

β2AR and TAS2R14 Form Heterodimers Facilitating TAS2R14 Expression—These results suggested that the β2AR protein promotes TAS2R14 expression and/or cell surface integration. Biomolecular fluorescence complementation (BiFC) studies were carried out on live HEK-293T cells (Fig. 1E) transfected with the amino terminus of Venus (VN) fused to the carboxyl terminus of β2AR (termed VN-β2AR) and the carboxyl terminus of Venus (VC) fused to carboxyl terminus of TAS2R14 (termed R14-VC). When VN without β2AR was co-transfected with R14-VC there was no fluorescence. Similar findings were observed when β2AR-VN was co-transfected with VC without R14. These results are consistent with the notion that overexpression of the Venus proteins in the absence of fusion to both receptor components is insufficient to result in complementation. Fig. 1E shows a fluorescent signal that includes intracellular and cell surface components only when β2AR-VN and TAS2R14-VC were co-transfected, indicating a close association between the two receptors with reconstitution of the fluorescent chromophore of Venus. Additional studies were performed using co-immunoprecipitation of extracts from cells transfected with FLAG-TAS2R14, Myc-β2AR, or both. As shown in Fig. 2A, immunoprecipitation with anti-FLAG followed by immunoblotting with anti-Myc resulted in a fluorescent signal of the appropriate $M_r$ only when both receptors were transfected together. Similar results were found when extracts were immunoprecipitated with anti-Myc and immunoblotted with anti-FLAG (Fig. 2B). In additional studies, membrane and cytosolic protein fractions were derived from the aforementioned cells, and subjected to co-immunoprecipitation and immunoblotting. TAS2R14-β2AR interactions were found in both compartments (Fig. 2C), consistent with the BiFC results of Fig. 1E. The BiFC and co-immunoprecipitation studies indicate the formation of a TAS2R14:β2AR heterodimer, and together with studies from Fig. 1A, suggests that the β2AR component acts as a chaperone to facilitate TAS2R14 membrane expression. Confocal imaging (Fig. 2D) of HEK-293T cells transfected with FLAG-TAS2R14 showed little cell surface expression (red signals) as was also shown in Fig. 1D. Co-transfection with HA-β2AR showed the expected cell surface expression of this receptor (green signals). When FLAG-TAS2R14 and HA-β2AR were co-transfected, substantial TAS2R14 expression on the cell surface was identified (Fig. 2D). Merged signals from both receptors (yellow) showed association of TAS2R14 and β2AR at the cell surface and within the cell (Fig. 2D).

Heterologously Expressed TAS2R14 Signal in Response to TAS2R Agonists—Functional signaling of TAS2R14 was ascertained by fluorescent microscopy and by a plate-based fluorescent assay of Fluo-4-loaded cells. Cells were transfected with the chimeric G-protein Gα16/G44 and pcDNA, and TAS2R14 +
In the imaging studies, \( \beta_2\text{AR} \) co-transfected cells showed no response to vehicle, nor did pcDNA-transfected (control) cells show a \([\text{Ca}^{2+}]_i\) response (Fig. 3A) to multiple TAS2R agonists. However, increases in \([\text{Ca}^{2+}]_i\) were observed for quinine, which activates subtypes 10, 14, and 31, and the TAS2R14 agonists diphenhydramine (DPD) and flufenamic acid (FFA). In contrast, bitter taste receptor agonists for TAS2R31 (saccharin) and TAS2R10 (strychnine) caused no increase in \([\text{Ca}^{2+}]_i\). Taken together, these data indicate the expected agonist specificity for TAS2R14 (Fig. 3B) (2). In the plate-based studies, co-transfected cells showed the expected dose-responses (2) to DPD (Fig. 3C) and quinine (data not shown). Furthermore, cells transfected with TAS2R14, \( \beta_2\text{AR} \), and \( \alpha_{16}/G44 \) showed a significant increase in \([\text{Ca}^{2+}]_i\) response to DPD and FFA (Fig. 4, A and B) compared with the TAS2R14 + G16/G44 cells, consistent with the increase in cell surface TAS2R14 expression evoked by \( \beta_2\text{AR} \) as observe by the biotinylation assay (Fig. 1, A and B), and the confocal imaging (Figs. 1D and 2D). In these experiments, expression of \( \beta_2\text{AR} \) compared with control pcDNA did not decrease TAS2R14 mRNA levels as determined by quantitative PCR (4.1 ± 0.12 versus 3.5 ± 0.08 units, respectively, \( n = 4 \), \( p > 0.05 \)).
We further confirmed the dynamic nature of $\beta_2$AR in controlling TAS2R14 expression using two approaches. First, we decreased $\beta_2$AR expression using small hairpin RNA (shRNA) targeting $\beta_2$AR in H292 cells, a mucoepithelial cell line that endogenously expresses $\beta_2$AR and TAS2R14. $\beta_2$AR-shRNA stable transfection caused an 81 ± 2.2% ($n = 4$) decrease in $\beta_2$AR protein expression (data not shown) compared with scrambled control shRNA. In $[Ca^{2+}]_i$ assays, this decrease in $\beta_2$AR was associated with a 46 ± 5.6% decrease in DPD-stimulated $[Ca^{2+}]_i$ compared with scrambled shRNA ($n = 4$, $p < 0.01$, Fig. 4C). These results are consistent with the effects observed when TAS2R14 and $\beta_2$AR are co-transfected in HEK-293T cells, and also indicated that the phenotype is present in cells that endogenously express both receptors. Given our
interest in TAS2Rs on ASM as novel targets for treating bronchospasm, we also examined extracellular calcium ([Ca^{2+}]_{i}) responses from cultured ASM cells derived from trachea of WT mice or mice with deleted β_{1}AR and β_{2}AR genes (βAR-KO) (10), with the expectation that TAS2R expression (and thus the cellular response to agonist) would be reduced in the βAR-KO ASM cells because β_{2}AR was not encoded and thus not available to facilitate TAS2R14 expression at the cell surface. Indeed, the βAR-KO mouse ASM cells displayed a 62.9% decrease (n = 3, p < 0.01) in the [Ca^{2+}]_{i} response to DPD compared with the WT ASM cells (Fig. 4D). The quinine response was also significantly less in βAR-KO versus the WT mouse ASM cells (Fig. 4E).

**Functional Consequences of the TAS2R14:β_{2}AR HeterodimERIC Complex**—Taken together, these data show, in several cell types using multiple approaches, that the enhancement of TAS2R14 cellular signaling by β_{2}AR is due to increased TAS2R14 cell surface expression. We next addressed the potential interactions of the two receptors once the heterodimer is inserted into the membrane at the cell surface.

HASM cells were treated with agonist for one receptor for 5 min or 1 h, then the other receptor was activated and function of the second receptor ([Ca^{2+}]_{i} or cAMP) quantitated. The 5-min time point is adequate for agonist engagement of the receptors, which tests whether a conformational change in one receptor influences the conformation/function of the other receptor, a concept that we refer to as “direct receptor:receptor cross-talk.” This time period is also adequate for generation of the immediate second messengers and potential subsequent feedback, which we addressed as a potential mechanism as indicated below. The 1-h pre-exposure is a time period where significant intracellular trafficking and interactions with other proteins may occur, which are distal events after immediate receptor activation, and thus includes other types of regulation as opposed to direct receptor-receptor interaction. To test the effect of activation of β_{2}AR on TAS2R14 function, cells were treated with isoproterenol (ISO) for the indicated times followed by DPD or vehicle challenge and immediate measurement of stimulated [Ca^{2+}]_{i} (Fig. 5A). Under these conditions, a
64 ± 7.6% decrease in DPD-stimulated [Ca^{2+}]_i, was observed after ISO exposure of 5 min, and a 78 ± 6.9% decrease (n = 4, p < 0.05 versus 5 min) with the 1 h treatment with ISO (Fig. 5A). ISO exposure for 1 h did not decrease TAS2R14 mRNA compared with vehicle control (3.5 ± 0.29 versus 3.5 ± 0.52 units, respectively, p > 0.05). The loss of TAS2R14 function by β₂AR exposure was not replicated by exposing cells to forskolin, which also increases intracellular cAMP (Fig. 5B), indicating that the desensitization of TAS2R14 by β₂AR is not due to cAMP, or cAMP-promoted PKA activation, with negative feedback to TAS2R14. In contrast, engagement of β₂AR with the neutral antagonist propranolol, or the β₂AR-specific inverse agonist ICI118551, had no effect on TAS2R14-stimulated [Ca^{2+}]_i (Fig. 5C). The relatively rapid loss of TAS2R14 function, the independence from cAMP, and the lack of an effect with neutral or inverse agonists, suggested that there is a direct receptor to receptor interaction imposed on TAS2R when β₂AR conformation is stabilized by an agonist, leading to a less favorable conformation for TAS2R14 to couple to G-protein.

Interestingly, β₂AR internalization from the cell surface to the interior is also underway after 5 min of agonist exposure (11, 12). We considered, then, that within the context of the heterodimer, TAS2R14 may co-internalize with β₂AR, thereby resulting in an overall decreased cellular response to TAS2R14 agonist. However, whereas loss of β₂AR cell surface expression was clearly apparent after 5 min of ISO exposure, TAS2R14

**FIGURE 4.** β₂AR expression dynamically regulates TAS2R14 functional expression. HEK-293T cells were transfected with TAS2R14 + G₁₁₆/G44, without or with β₂AR (A and B). The [Ca^{2+}]_i response to the TAS2R14 agonists DPD and FFA are increased when β₂AR is co-transfected, consistent with the increased expression of TAS2R14 (Fig. 1, A and B). In 4 experiments, the [Ca^{2+}]_i stimulation was increased by 2.4 ± 0.11 and 2.1 ± 0.12, respectively, when β₂AR was co-expressed (p < 0.01 versus absence of β₂AR). In C, H292 cells, which endogenously express TAS2R14 and β₂AR were transfected with β₂AR shRNA (or sh-control) and treated with vehicle or the TAS2R14 agonist DPD. Knockdown of β₂AR by β₂AR shRNA resulted in decreased TAS2R14-mediated [Ca^{2+}]_i signaling. In D and E, β₂AR knock-out mouse (10) ASM cells (which express no detectable β₂AR) were challenged with TAS2R agonists and revealed >50% reduction in TAS2R-stimulated [Ca^{2+}]_i. Results are from 4 representative experiments.
cell surface expression was not changed (Fig. 6). In quantitative imaging studies, there was a readily detectable increase in intracellular β₂-AR after 5 min of treatment with agonist, and no statistically significant parallel increase in intracellular TAS2R14 at this time point (Fig. 7A). After a 1-h exposure to ISO, both β₂-AR and TAS2R14 intracellular expressions were increased, with β₂-AR > TAS2R14 (Figs. 6 and 7A). These imaging studies were confirmed using the cell surface biotinylation assay. In these assays we found β₂-AR cell surface loss was rapid and amounted to > 25%, whereas TAS2R14 cell surface loss was minimally detected at 5 min. However, by 1-h ISO exposure, TAS2R14 cell surface expression was clearly decreased and approached > 50% loss (Fig. 7B). So, at least with brief β-agonist exposure, the evidence does not support co-internalization. However, by 1-h ISO exposure, there was detectable loss of cell surface expression of TAS2R14, measured as a gain of intracellular expression (Figs. 6 and 7A), or loss of cell surface immunoreactivity in the biotinylation assays (Fig. 7B). This suggested that internalization of TAS2R14 during prolonged β-agonist exposure could be the basis for the further loss of cellular responsiveness to TAS2R14 agonist at this more prolonged time point. To further explore this, we blocked receptor internalization with the dynamin inhibitor dynasore. As shown in Fig. 7C, dynamin inhibition only partially rescued TAS2R14 desensitization by β-agonist after a 1-h exposure, indicating two processes at play: an early event that may be uncoupling of TAS2R14 to its G-protein due to interactions within the heterodimer, and a later internalization of TAS2R14 such that cell surface expression is reduced.

We next addressed the reverse scenario, where TAS2R14 conformation is altered by pretreatment with its agonist, and then β₂-AR function ascertained by measuring the cAMP response to ISO. With 5 min pre-treatment with FFA, ISO-stimulated cAMP was depressed (Fig. 8). However, this decrease was also observed with forskolin-stimulated cAMP (which increases cAMP by direct activation of adenylyl cyclase). With vehicle treatment, the ratio of the ISO response to the forskolin response was 1.3 ± 0.20, and with 5 min of treatment with DPD, the ratio was 1.5 ± 0.15 (p > 0.05). With the 1-h treatment with DPD, both ISO- and forskolin-stimulated cAMP levels were further depressed, and when corrected for the decreased forskolin response the ISO response was not impaired compared with vehicle treatment (Fig. 8). We thus conclude that there is no direct receptor:receptor cross-talk between activated TAS2R2 and β₂-AR, but rather a heterologous regulation distal to the receptor.

**Physiological Consequences of the β₂-AR-TAS2R14 Interaction**—Given the loss of TAS2R2 function evoked by activation of β₂-AR as measured by [Ca²⁺], we tested the relevance to ASM relaxation by studying single-cell mechanics with cultured HASM cells using magnetic twisting cytometry (3, 13, 14). Here, ferrimagnetic beads are attached to cell surface integrin receptors and perturbed magnetic fields serve to quantify increased or decreased cell stiffness (analogous to contraction and relaxation, respectively). Both β₂-ARs and TAS2Rs relax HASM. Because they do so by independent mechanisms, if there was no interaction we would expect an additive degree of relaxation when both receptors are activated, or, at least an...
equivalent degree (if at the maximal possible response). However, if β2AR interact with TAS2Rs to decrease TAS2R function, then β-agonist would depress TAS2R14 agonist-mediated relaxation. The results of these physiologic studies are shown in Fig. 9A, and indeed show an impairment of TAS2R14 function when β2AR is activated by ISO. Relaxation to FFA was impaired by ~50%, which is remarkably similar in degree to the loss of TAS2R14-stimulated \([Ca^{2+}]_i\) observed in the cell-based studies (Fig. 5A). We also utilized this physiologic readout to ascertain if there was concordance with the \([Ca^{2+}]_i\) results (Fig. 5C) observed when the β2AR is engaged by the neutral antagonist propranolol or the inverse agonist ICI118551. As shown in Fig. 9B, neither agent altered HASM relaxation by the TAS2R14 agonist FFA, consistent with the \([Ca^{2+}]_i\) studies. Taken together, these results further strengthen the notion that the agonist-bound conformation of the β2AR is required to promote TAS2R14 dysfunction.

Specificity of the β2AR-TAS2R14 Interaction—We next tested whether the closely related β1AR subtype formed a complex with TAS2R14, and, whether β1AR activation altered TAS2R14 function. For the co-immunoprecipitation experiments, two phylogenetically distant GPCRs (15), the neuropeptide receptor NMU2R and the chemokine receptor CXCR6, were also studied. HEK-293T cells were transfected with FLAG-tagged TAS2R14 in the absence or presence of HA-tagged β2AR, β1AR, NMU2R, or CXCR6, immunoprecipitated with HA antibody, and immunoblotted with anti-FLAG antibodies (Fig. 10). As shown, β1AR, as well as the previously shown β2AR, formed complexes with TAS2R14 (top panel). In contrast, there was no signal for NMU2R and a minimally detected signal for CXCR6. The other panels are controls for the immunoprecipitation (second panel) and the inputs to the immunoprecipitation reaction (bottom two panels). These results suggested, then, that β1AR activation might also desensitize TAS2R14, as was seen with β2AR. Because human ASM do not express β1AR and β2AR, we utilized the H1299 cell line that expresses β1AR, β2AR, and TAS2R14 (as determined by quantitative PCR, data not shown). Cells were treated with carrier (control) and ISO with pretreatment with vehicle, the β1AR antagonist betaxolol, or the β2AR antagonist ICI118551. The latter two conditions isolate β2AR and β1AR activation, respectively. After 5 min of agonist exposure, cells were treated with DPD and \([Ca^{2+}]_i\) and immediately recorded (Fig. 11). There was a small (17%) but statistically significant desensitization of TAS2R14 function under the conditions of β1AR activation. When β2AR was selectively activated, there was a greater decrease in TAS2R14 function, amounting to ~50% (see Fig. 11).

**Discussion**

Here we show an interaction between TAS2R14 and β2AR on two levels. First, β2AR form heterodimers with TAS2R14 in the
cytosol, which promotes expression of TAS2R14 on the cell membrane. This phenomenon was observed in heterologously expressing cells where receptors were transfected singly or together, and the expression phenotype was also found in cells that endogenously express both receptors where \( \beta_2 \)AR expression was depressed by shRNA, and in ASM cells from gene-ablated mice lacking \( \beta_1 \)AR and \( \beta_2 \)AR expression. The data were highly consistent using these multiple approaches and cell types. The observation that even intracellular TAS2R14 expression is increased when \( \beta_2 \)AR are co-expressed suggests that the heterodimer stabilizes an otherwise unstable and readily degraded TAS2R14. Our results have some similarities to what has been reported with the mouse odorant receptor (OR) M71 (16). This receptor could not be readily expressed by heterologous transfection, remaining trapped in the cytosol. Co-transfection of \( \beta_2 \)AR was found to promote OR M71 expression via the formation of heterodimers. Although TAS2Rs and ORs are both chemoreceptors for exogenous substances involved in bitter taste and smell perception, respectively, they are not in the same family within the GPCR superfamily as indicated by the GRAFS classification system (15). ORs are part of the large rhodopsin family, whereas TAS2Rs are in the Frizzled family, one of four non-rhodopsin families. This suggests that this chaperone phenotype of \( \beta_2 \)AR is unlikely to be due to a highly specific amino acid sequence, given the difference in primary sequence between the two families. Nevertheless, a general region of the \( \beta_2 \)AR is likely involved. This notion is consistent with our finding that TAS2R14 also forms complexes with the related \( \beta_2 \)AR (which has a 72% amino acid identity in the transmembrane domains with \( \beta_2 \)AR), but not NMU2R (26% identity) or CXCR6 (19% identity). Of note, TAS2Rs have small extracellular N termini and intracellular C termini, as well as the loops that interconnect the transmembrane domains. Thus these compact
receptors may require heterodimer formation with the dissimilar \( \beta_2 \)AR (longer N and C termini and third intracellular loop) to gain cell surface expression. Unlike the marked divergence in protein sequences and primary coupling pathways between TAS2R14 and \( \beta_2 \)AR, heterodimer formation between very closely related GPCR subtypes has also been reported (17, 18), including the facilitation of cell surface expression of a cytosol-locked receptor (17). For example, the \( \alpha_1D \)AR forms a heterodimer with the \( \alpha_1D \)AR, which results in cell surface expression of the latter (17). In this instance, specific amino acid residues may be responsible for the interaction of such closely related receptors.

Interestingly, for transfected TAS2R14, there is some level of expression in HEK-293T cells in the absence of co-transfecting \( \beta_2 \)AR. However, this cell line does express endogenous \( \beta_2 \)AR, which may act to provide some degree of expression of the transfected TAS2R14. Similarly, we do not obtain complete elimination of \( \beta_2 \)AR in the shRNA studies, which may explain detectable TAS2R14 signals in this setting. However, in the \( \beta_2 \)AR-KO mouse ASM cells, there is no detectable \( \beta_2 \)AR, yet we repeatedly detect a low TAS2R14 signal. This may suggest that a small portion of intracellular TAS2R14 is ultimately inserted into the membrane in the absence of the \( \beta_2 \)AR chaperone effect, or, that another GPCR can also perform this function. In the context of the potential use of TAS2R agonists as bronchodilators, these expression data collectively indicate

**FIGURE 9.** Physiologic consequences of TAS2R: \( \beta_2 \)AR cross-talk in airway smooth muscle. HASM cells were studied with magnetic twisting cytometry, which measures cell stiffness, where a decrease in stiffness in response to an agonist is the correlate to airway relaxation. In A, cells were treated with 10 \( \mu \)M ISO, 100 \( \mu \)M FFA, or ISO + FFA. All treatment conditions resulted in a decrease in cell stiffness compared with baseline (\( p < 0.001 \)). However, maximal relaxation was not additive when both agonists were used. The response to the combination was less than when cells were treated with FFA alone. *, response less than FFA alone, \( p < 0.001 \). Data shown are results from 104 to 390 individual cell measurements from 3 independent experiments. In B, cells were exposed to FFA in the absence or presence of the neutral \( \beta_2 \)AR antagonist propranolol (10 \( \mu \)M) or the \( \beta_1 \)AR inverse agonist ICI118551 (10 \( \mu \)M). The FFA-promoted relaxation response was unaffected by either agent. Results are from 231–272 individual cell measurements from 3 independent experiments.

**FIGURE 10.** TAS2R14 co-immunoprecipitation studies with other transfected GPCRs. FLAG-TAS2R14 was transfected into HEK293T cells alone or with one of the following HA-tagged GPCRs: \( \beta_1 \)AR, NMU2R, or CXCR6. Lysates were immunoprecipitated with HA and immunoblotted with FLAG. The upper section shows co-immunoprecipitation of \( \beta_1 \)AR and \( \beta_2 \)AR with TAS2R14. No (or very little) co-immunoprecipitation was observed with HA-NMU2R or HA-CXCR6 transfection. Results are representative of 4 independent experiments.

**FIGURE 11.** Effects of \( \beta_1 \)AR or \( \beta_2 \)AR activation on TAS2R14 signaling to [Ca\(^{2+}\)]. H1299 cells were pretreated with carrier, the \( \beta_2 \)AR antagonist ICI118551 (10 \( \mu \)M), or the \( \beta_1 \)AR antagonist betaxolol (10 \( \mu \)M) for 10 min, and then cells were treated for 5 min with 10 \( \mu \)M ISO. (The latter two conditions selectively activate \( \beta_2 \)AR and \( \beta_1 \)AR, respectively.) Desensitization of TAS2R14 signaling was observed with activation of either \( \beta_2 \)AR subtype, and was greater when \( \beta_2 \)AR was activated compared with \( \beta_1 \)AR. *, \( p < 0.01 \) versus carrier; #, \( p < 0.01 \) + ICI118551 versus + betaxolol. Results are from 4 experiments.
that measures to maintain β₂AR expression would be beneficial in attaining higher TAS2R14 expression so as to obtain maximal airway relaxation.

However, β₂AR acts as a double-edged sword once TAS2R14 is expressed in the cell membrane. The TAS2R14:β₂AR heterodimer remains intact at rest, and activation of the β₂AR component leads to rapid uncoupling of TAS2R14 to its signal transduction pathway. This effect is not due to generation of the β₂AR second messenger cAMP, or co-internalization of TAS2R14, but appears to be due to induction of an unfavorable conformation of the TAS2R14 transmitted from the agonist-bound conformation of the β₂AR. With prolonged β₂-agonist exposure, the uncoupling process remains in play, and internalization of TAS2R14 is observed, which further contributes to a loss of cellular responsiveness. Although most β₂AR stabilize a similar set of conformations, there is evidence from cell-based and biophysical studies, of atypical conformations, which are nevertheless, coupled to Gₐ/cAMP when bound to certain agonists (19, 20). Thus from a therapeutic standpoint, specific β₂AR agonist/TAS2R14 agonist combinations would need to be tested to ascertain potential interactions of the two receptors.

The combined effects of expression modulation and coupling efficiency that β₂AR has on TAS2R14 indicate a mechanism by which TAS2R14 functional responses can be regulated in a given cell. Although it has been suggested that proteins such as Ric8b (a putative GEF) may increase OR and TAS2R expression, the effect appears to be small (9). We propose that β₂AR expression represents a major mechanism of stabilizing TAS2R14 in the cell and insertion into the membrane, via formation of a heterodimer. This mechanism is not restricted to overexpressing cells, but is clearly shown to be at play in endogenously expressing cells, and, to have physiological relevance. The consequences of activated (i.e. agonist occupied) β₂AR on quenching TAS2R14 function has a mitigating effect on β₂AR-mediated enhanced expression of TAS2R14. The ultimate impact of this cross-talk is dependent on the number of “spare receptors,” and, the read-out that is measured. In our studies with ASM cells, which express relatively low levels of TAS2R14 that cause a significant physiologic response, we have considered that there are few spare receptors and a high degree of amplification from receptor to ASM relaxation (3, 4). Thus the impact of the β-agonist-induced uncoupling of TAS2R14 is readily observed, amounting to ~50% loss of function.

In summary, we have delineated a mechanism whereby β₂AR promotes cell surface expression of the predominately intracellular TAS2R14. This function is due to formation of a β₂AR: TAS2R14 heterodimer. Once inserted in the membrane, the TAS2R14 is functional, coupling to the canonical pathway, an increase in 

**Experimental Procedures**

**Cell Culture**—HEK-293T were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Primary ASM cells were derived as previously described (21, 22) or obtained from Lonza (lot number 7F3984), maintained in SmBM media plus the SmGM–2 SingleQuot with growth factors (Lonza), and utilized at passages 3–8. H292 and H1299 cells were grown in RPMI 1640 in 10% FBS. HEK-293T, H292, and H1299 cell lines were obtained from ATCC. Cells were maintained at 37 °C, in a 95% air, 5% CO₂ incubator, including periods when drugs were added to the cells. For the 96-well plate-based [Ca²⁺], studies, cells were seeded at 40,000–80,000 cells/well the night before assays, and studied the next day at confluence.

**cDNA Constructs and Transfections**—The TAS2R14 cDNAs were subcloned into a pcDNA3.1 ( HindIII-XhoI) construct by PCR amplification, which included (5′ to 3′) the in-frame coding sequences for the Lucifer peptide (MRPQILLLLALLTLGLA) (9), as well as the following: the FLAG tag, the extracellular N-terminal portion (first 45 amino acids of the coding sequence) of the rat somatostatin-3 receptor, TAS2R14, and a 12-amino acid epitope of HSV as described (2). pBiFC-VN155 and pBiFC-VC155 were obtained from Addgene. The β₂AR-VN and TAS2R14-VC constructs were subcloned (EcoRI-XhoI) into pBiFC-VN155 and pBiFC-VC155, respectively. For co-IP studies, HEK-293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen) and used for studies 36–48 h later. In other studies, cells were also transfected with Ga16/G44 to facilitate TAS2R14 signaling to [Ca²⁺], or Gₐ-gust (GNAT3), the canonical G-protein for TAS2R8. For confocal imaging studies, HEK-293T cells were transiently transfected and then passaged to coverslips or chamber slides. For the BiFC studies, HEK-293T cells were transiently transfected with the indicated plasmids onto the Lab-TekII chamber slides (Fisher). For shRNA transfections, the following β₂AR shRNA sequences were individually cloned into pLKO.1-puro: 5′-CCGGCCTCTCTAAATTTGAGATGGTACTCGAGTAGCCTATCAAAATTGGAGGTTTTT-T3′; 5′-CCGGCCCTCAAGACGTTAGGCATCATCTCGAGATTGATGCCTAACGTCTTGAGGTTTT-3′; 5′-CCGGGCGCATTCAACTGCTATGCCAATCTCGAGATTGATGCCTAACGTCTTGAGGTTTTT-3′; 5′-CCGGGCCATCAACTGCTATGCCAATCTCGAGATTGATGCCTAACGTCTTGAGGTTTTT-3′ (Sigma TRCN0000008084, TRCN-0000008085, and TRCN0000008086, respectively). The control shRNA sequence was 5′-CCGGCAACAAAGTGAAGAGCA-CCAAACTCGAGTTTGTCTTCTTCTCTTGTGTTTTT-3′ (Sigma SHC002). H292 were transfected with the three β₂AR constructs or the control constructs using Lipofectamine 2000 with puromycin (7 μg/ml) selection to generate stable cell lines. TAS2R14, β₂AR, and β-actin mRNA were determined by quantitative PCR using primers, conditions, and analysis exactly as previously described (3).

**Intracellular Calcium Measurements**—Measurements of [Ca²⁺], mobilization in the aforementioned cell lines were determined as previously described (23, 24). Briefly, cells seeded in 96-well plates were loaded with the Ca²⁺-sensitive fluorescence indicator Fluo-4 (Life Technologies) and probene-
acid in Hanks’ balanced salt solution, containing 1.3 mM CaCl₂, 0.5 mM MgCl₂·6H₂O, 0.4 mM MgSO₄·7H₂O, 5.3 mM KCl, 0.4 mM KH₂PO₄, 4.2 mM NaHCO₃, 137.9 mM NaCl, 0.3 mM Na₂HPO₄, 5.5 mM D-glucose, and 20 mM HEPES. Loading consisted of a 30-min incubation in a 5% CO₂ atmosphere at 37 °C followed by a 30-min incubation at 25 °C at ambient atmosphere. The plate was read in a FlexStation3 plate reader (Molecular Devices), with excitation of 485 nm, emission of 525 nm, and a cut-off value of 515 nm. [Ca²⁺]r-stimulating agents or vehicle controls were robotically added at the 19-s point, and the fluorescent signal was measured every 1.52 s up to 120 s. In experiments with transiently transfected cells, [Ca²⁺]r-measurements were made 36–48 h after transfection.

**cAMP Measurements**—cAMP was measured using a fluorescent competitive immunoassay (Molecular Devices) as previously described (12). Cells were cultured in 48-well plates and washed once with pre-warmed Krebs-Ringer bicarbonate buffer (10 mM glucose, 15 mM NaHCO₃, pH 7.4). The cells were treated at 37 °C with 100 μM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine for 30 min, and then exposed to forskolin (10 μM) or forskolin (10 μM) in the presence of 100 μM 1-ascorbic acid for 30 min. Cells were lysed by the addition of a hypotonic buffer, and cAMP was measured on the FlexStation3, with concentrations determined using a standard curve.

**Receptor and [Ca²⁺]r Imaging**—To localize epitope-tagged β₂AR, TAS2R14, and cellular calcium, confocal microscopy was employed as previously described (12) with an Olympus FV1000 MPE multiphoton laser-scanning microscope. After transfection, HEK-293T cells were transfected onto 12-mm coverslips coated with poly-L-lysine in 6-well plates and studied using the EVOS Cell Imaging System (Thermo Fisher) with excitation at 488 nm and a 515–540 nm emission filter.

**Co-immunoprecipitation and Western Blots**—These studies were carried out using methods as previously described (18, 22) with modifications. Transfected cells were washed in ice-cold PBS and lysed in 1 ml of TNEN (100 mM Tris-Cl, 75 mM NaCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 0.3% Triton X-100 with proteinase inhibitor mixture) buffer. Cell debris was removed by centrifugation at 14,000 rpm for 25 min at 4 °C. Total cell lysate (400 μg) was pre-cleared by incubation with protein A/G (2:1)-agarose beads (Gibco BRL) for 30 min, centrifuged to remove the beads, and incubated with primary antibody overnight at 4 °C. Protein A/G beads were then added, and the suspension was incubated at 4 °C for 2 h with constant rotation. The beads were washed four times with TNEN, and proteins were released by addition of 2× Laemmli sample buffer. Equivalent amounts of eluted proteins were subjected to SDS-PAGE, transferred to PVDF (Millipore) membranes, and immunoblots were performed with the indicated antibodies. Bands were visualized using chemiluminescence (Thermo Scientific, 1:1000 secondary antibody titer) and detection by the ChemiDoc MP imaging system (Bio-Rad). Bands were quantitated using the provided software or Image-J (National Institutes of Health). For certain experiments, a membrane protein extraction system (MemPER, Thermo Scientific) was utilized to separate membrane and cytosolic cell fractions. Briefly, transfected HEK-293T were detached and washed by centrifugation, and 0.75 ml of permeabilization buffer added to the pellet. Cells were incubated for 20 min at 4 °C with constant rotation. Permeabilized cells were centrifuged for 15 min at 16,000 rpm, and the supernatant (cytosolic fraction) was removed. The pellet (membrane fraction) was solubilized with 0.5 ml of the included solubilization buffer for 30 min at 4 °C, and was clarified by centrifugation. For standard immunoblotting 8–10 μg of each fraction was used, whereas 200–250 μg was used for immunoprecipitation. For these and other studies, protein concentrations were determined by the method of Bradford (25). For the biotinylation studies, a cell surface protein isolation system (Pierce, Thermo Scientific) was utilized. Briefly, cells in a 10-cm culture dish were washed with PBS and then 10 ml of biotin solution consisting of 12 mg of sulfo-NHS-SS-Biotin in 50 ml of cold PBS was added to the dish, which was then rocked on an orbital shaker for 1 h at 4 °C. The reaction was stopped by addition of 500 μl of quenching solution. Cells were detached by scraping, washed twice by centrifugation, and resuspension in Tris-buffered saline, and then solubilized in 1 ml of RIPA buffer. Cell debris was removed by centrifugation at 14,000 rpm. To the supernatant, 60 μl of NeutrAvidin-agarose beads were added and incubated overnight at 4 °C. The beads were washed four times with lysis buffer and the proteins were released by addition of 2× Laemmli sample buffer. The catalogue numbers and sources for the antibodies were: anti-FLAG antibody (F7425, Sigma), anti-HA antibody (11-583-816001, Roche), anti-β-actin (A1978, Sigma), anti-Myc (SC4, Santa Cruz), anti-Na-K-ATPase (3010, Cell Signaling), anti-GAPDH (2118, Cell Signaling), anti-mouse conjugate FITC (AP127F, Sigma), anti-rabbit conjugate Alexa 594 (A11012, Thermo Fisher), anti-α-agarose bead (A2095, Sigma, for IP), and anti-FLAG M2 magnetic beads (M8823, Sigma, for IP).

**Magnetic Twisting Cytometry**—The physiological consequences of the TAS2R14-β₂AR interaction were ascertained by examining single cell mechanics of HASM using magnetic twisting cytometry (3, 13, 14, 24). These experiments were performed exactly as previously described (3, 13, 26). Briefly, RGD-coated ferrimagnetic microbeads were attached to cell surface integrin receptors, magnetized horizontally, and then twisted in a vertically aligned magnetic field. Lateral bead displacement measures smooth muscle “contraction” and “relaxation,” in response to the application of bronchoreactive drugs to the
media, which correlates with airway constriction and dilation in the ex vivo and in vivo settings (3, 14).

Statistical Analyses—The data from the biochemical studies were analyzed by two-sided, paired or unpaired t-tests (as appropriate). Multiple comparisons were performed by two-way analysis of variance with post hoc t-tests adjusted by Bonferroni’s method. For the magnetic twisting cytometry studies, nested design analysis was used as previously described (27), which controls for random effects of repeated measurements of multiple cells from the same flask. For all studies, significance was imparted when \( p < 0.05 \). Analysis was performed using SAS V9.2 (SAS Institute Inc.) and Prism (GraphPad). Results are shown as mean ± S.E.

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