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Specificity of Olfactory Receptor Interactions with Other G Protein-coupled Receptors*

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Studies on olfactory receptor (OR) pharmacology have been hindered by the poor plasma membrane localization of most ORs in heterologous cells. We previously reported that association with the β_2-adrenergic receptor (β_2-AR) facilitates functional expression of the OR M71 at the plasma membrane of HEK-293 cells. In the present study, we examined the specificity of M71 interactions with other G protein-coupled receptors (GPCRs). M71 was co-expressed in HEK-293 cells with 42 distinct GPCRs, and the vast majority of these receptors had no significant effect on M71 surface expression. However, co-expression with three subtypes of purinergic receptor ($P2Y_1R$, $P2Y_2R$, and $A_{2A}R$) resulted in markedly enhanced plasma membrane localization of M71. Agonist stimulation of M71 co-expressed with $P2Y_1R$ and $P2Y_2R$ activated the mitogen-activated protein kinase pathway via coupling of M71 to $G\alpha_o$. We also examined the ability of β_2-AR, $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$ to interact with and regulate ORs beyond M71. We found that co-expression of β_2-AR or the purinergic receptors enhanced the surface expression for an M71 subfamily member but not for several other ORs from different subfamilies. In addition, through chimeric receptor studies, we determined that the second transmembrane domain of β_2-AR is necessary for β_2-AR facilitation of M71 plasma membrane localization. These studies shed light on the specificity of OR interactions with other GPCRs and the mechanisms governing olfactory receptor trafficking.

Mammalian olfaction begins at the plasma membrane of olfactory sensory neuron (OSN)[2] cilia, where inhaled environmental chemicals bind and activate a subset of G protein-coupled receptors (GPCRs), termed olfactory receptors (ORs). Although ORs were identified over a decade ago and are encoded by the most numerous multigene family in mammals, remarkably few OR-ligand pairs have been characterized (1, 2). Moreover, little is known about the signaling pathways activated by this diverse receptor family. Many ORs can signal through coupling to a specialized G protein, $G\alpha_{olf}$, which results in adenylyl cyclase generation of cAMP and subsequent influx of positive ions through cyclic nucleotide gated channels to cause depolarization. However, considering the enormity of the OR repertoire (more than 1000 ORs in rodents and more than 350 in humans) and the variety of odors detected by mammals, it is probable that these receptors possess diverse signaling mechanisms. Indeed, there are numerous reports about the potential of ORs to signal through a variety of pathways (3). The major obstacle hindering the study of OR pharmacology and signaling has been difficulty expressing functional ORs in heterologous cells, primarily owing to their poor trafficking to the plasma membrane (4).

Some studies performed in heterologous cells have overcome poor cell surface expression by using chimeric ORs or ORs tagged with the N-terminal targeting sequences from proteins such as rhodopsin or the serotonin $5\text{-}HT_3$ receptor (5, 6). One concern with these techniques is that such modifications may alter the true pharmacology of ORs (7). Alternatively, to circumvent improper localization in heterologous cells, adenoviral overexpression and gene targeting strategies in native OSNs have been used to successfully identify OR-ligand pairs and map axon convergence (8–10). Despite the successes achieved by the aforementioned studies, the vast majority of ORs remain orphans, and intracellular retention of ORs continues to impede progress in understanding the pharmacology of these specialized receptors.

The molecular determinants underlying the impaired cell surface localization of ORs in heterologous cells is a topic of intense research interest. There is evidence that OR trafficking is dependent on the C-terminal domains of the receptors (11). Olfactory receptors may contain some type of endoplasmic reticulum retention signal or lack a forward targeting signal. In either of these cases, an accessory protein may be required to facilitate localization at the cell surface. Such an accessory protein may be absent in heterologous cells, leading to nonfunctional ORs trapped inside the cell. Evidence from the chemosensory systems of several species demonstrates the necessity for accessory proteins to properly localize ORs at the plasma membrane. Mutation of the *Caenorhabditis elegans* protein ODR-4, which has been proposed to aid in receptor folding, sorting, or transport, inhibits OR insertion into the plasma membrane (12). *Drosophila* olfaction has been found to depend upon heterodimerization between conventional ORs and an atypical OR named OR83b, which is required for correct local-

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[2] The abbreviations used are: OSN, olfactory sensory neuron; OR, olfactory receptor; GPCR, G protein-coupled receptor; AR, adrenergic receptor; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; KO, knock-out; HA, hemagglutinin; PTX, pertussis toxin; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; ACP, acetophenone; WT, wild type; OE, olfactory epithelium; TMD, transmembrane domain; DAPI, 4′,6′-diamino-2-phenylindole; GFP, green fluorescent protein; ANOVA, analysis of variance.

ization and functionality of fly ORs (13, 14). In mammals, proteins belonging to the receptor transporting protein family help translocate some ORs to the cell surface and enhance responses to odorants in HEK-293 cells (15).

We previously found that association with the β_2-adrenergic receptor (AR) results in enhanced surface expression and functionality of the OR M71 (16). A natural question of interest following this finding was whether GPCRs other than β_2-AR are capable of assembling with M71 to promote its surface expression and conversely whether ORs beyond M71 can undergo heterodimerization with nonolfactory GPCRs to enhance their trafficking to the cell surface. In the present study, we screened 42 distinct nonolfactory GPCRs and identified several purinergic receptor subtypes that also interact with M71 and facilitate plasma membrane localization of this OR. We furthermore found that stimulation of M71 associated with the purinergic subtypes $P2Y_1R$ and $P2Y_2R$ can activate $G\alpha_o$, thereby coupling this OR to a signaling pathway distinct from $G\alpha_{olf}$. We also examined the capacity of ORs besides M71 to interact with other GPCRs and found that an M71 subfamily member, but not ORs from different OR subfamilies, exhibited enhanced cell surface expression upon co-expression with β_2-AR and the purinergic receptors. Finally, using receptor chimeras, we identified the second transmembrane domain of the β_2-AR as a required region for β_2-AR-mediated enhancement of M71 plasma membrane localization.

EXPERIMENTAL PROCEDURES

Receptor Constructs—The FLAG-M71-GFP construct, WT-M71 construct, and α_{1A}-, α_{1B}-, and α_{1D}-AR constructs were generated as previously described (16–18). The rat I7 construct was amplified from rat genomic DNA via PCR using *Pfu* turbo (Stratagene) with a forward primer corresponding to nucleotides 1–25 and a reverse primer corresponding to nucleotides 958–981 (GenBank™ accession number M64386). The hOR17–40 construct was amplified similarly from human genomic DNA with a forward primer corresponding to nucleotides 1–25 and a reverse primer corresponding to nucleotides 921–945 (GenBank™ accession number X80391). The mOR171-4 construct was amplified from mouse genomic DNA with a forward primer corresponding to nucleotides 3–20 and a reverse primer corresponding to nucleotides 915–933 (GenBank™ accession number AY073236). PCR products were inserted into pEGFP-N3 modified to contain a FLAG tag via an XbaI restriction enzyme site in the forward primer and either a KpnI (rat I7, mOR171-4) or BamHI (hOR17–40) restriction enzyme site in the reverse primer. FLAG-M71-GFP was subcloned into the pBK vector to generate a FLAG-tagged M71 construct without the C-terminal GFP. α_{2A}-, α_{2B}-, and α_{2C}-AR constructs were kindly provided by Lee Limbird (Vanderbilt University Medical Center). β_1- and β_2-AR and chimera constructs were kindly provided by Hitoshi Kurose (Kyushu University). The β_3-AR construct was kindly provided by Sheila Collins (CIIT Centers for Health Research). The dopamine D2 receptor construct was kindly provided by David Sibley (National Institutes of Health). Histamine H1–3 receptor constructs were kindly provided by Tim Lovenberg (The R. W. Johnson Pharmaceutical Research Institute). Muscarinic M1–5 acetylcholine receptor constructs were kindly provided by Allan Levey (Emory University School of Medicine). Opioid receptor constructs μ and δ were kindly provided by Ping-Yee Law (University of Minnesota Medical School). The $P2Y_1$ receptor ($P2Y_1R$) construct was kindly provided by Ken Harden (University of North Carolina, Chapel Hill). The dopamine D1 and D5 receptor constructs, melanocortin 3 and 4 receptor constructs, $P2Y_2$ receptor ($P2Y_2R$) construct, adenosine A_1, A_{2A} ($A_{2A}R$), A_{2B}, and A_3 receptor constructs, and trace amine-associated receptor 1 and 3–5 constructs were purchased from the UMR cDNA Resource Center. The serotonin $5HT_{1A}$ receptor construct was kindly provided by John Raymond (Medical University of South Carolina). Metabotropic glutamate receptor constructs, 4b, 7a, and 8 were kindly provided by Jeff Conn (Vanderbilt University School of Medicine). The trace amine-associated receptor 2 construct was kindly provided by Dr. Kenneth Jones (Synaptic Pharmaceutical Corp.).

Cell Culture and Transfection—All of the tissue culture media and related reagents were purchased from Invitrogen. HEK-293 cells were maintained in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) at 37 °C with 5% CO_2. 80–95% confluent cells in 10-cm tissue culture dishes were transfected with 1–3 μg of cDNA mixed with 15 μl of Lipofectamine 2000 in 5 ml of serum-free medium. Following overnight incubation, complete medium was added, and the cells were trypsinized and replated.

For confocal microscopy experiments, a high transfection efficiency was achieved through electroporation using the Nucleofector® following the manufacturer's protocol (Amaxa). Briefly, HEK-293 cells were trypsinized, collected by centrifugation, and resuspended in Nucleofector solution along with 0.7 μg of cDNA/construct. This suspension was then subjected to electroporation in the Nucleofector®, followed by the addition of complete medium and plating of cells directly onto tissue culture treated glass slides (BD Biosciences). The cells were grown for 24 h.

Western Blotting—The samples were resolved by SDS-PAGE on 4–20% Tris-Glycine gels, followed by transfer of protein to nitrocellulose membranes (Bio-Rad). The membranes were incubated in blocking buffer (2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM HEPES, pH 7.4) for 30 min and then incubated with primary antibody for either 1 h at room temperature or overnight at 4 °C. Next, the membranes were washed three times in blocking buffer and incubated with either a horseradish peroxidase-conjugated (HRP) secondary antibody or a fluorescence-conjugated secondary antibody for 30 min at room temperature, followed by three blocking buffer washes. Proteins bound by HRP-conjugated secondaries were visualized via enzyme-linked chemiluminescence using ECL reagent (Pierce). Proteins bound by fluorescence-conjugated secondary antibody were detected using the Odyssey imaging system (Li-Cor).

Surface Luminometer Assay—HEK-293 cells transiently transfected with ORs alone or co-transfected with ORs plus other GPCR subtypes were split into poly D-lysine-coated 35-mm dishes and grown overnight at 37 °C. The cells were washed with phosphate-buffered saline (PBS), fixed with 4%

paraformaldehyde, and washed with PBS again. The cells were then incubated in blocking buffer (2% nonfat milk in PBS, pH 7.4) for 30 min, followed by incubation with HRP-conjugated M2-anti-FLAG antibody (1:600; Sigma) in blocking buffer for 1 h at room temperature. The cells were washed twice with blocking buffer, washed twice with PBS, and then incubated with SuperSignal Pico ECL reagent (Pierce) for 15 s. Luminescence of the entire 35-mm dish was determined using a TD-20/20 luminometer (Turner Designs). The mean values ± S.E. were calculated as percentages of absorbance in arbitrary units and were normalized to total protein in experiments where different cell densities were a factor.

Immunohistochemistry on Nasal Epithelium Slices—Adult female M71-lacZ (19), $P2Y_1R$-knock-out (KO) (20), and $P2Y_2R$-KO (21) transgenic mice were perfused with ice-cold paraformaldehyde, and the olfactory epithelium was dissected. At 1 h post-fixation, the tissue was decalcified at 4 °C in 250 mM EDTA for 1 week. After freezing in optimal cutting temperature compound (Tissue-Tek OCT), the tissue was sectioned at 25 μm using a Leica cryostat, and sections were adhered to Superfrost Plus slides (VWR). The sections were blocked for 3 h in blocking buffer (10% normal donkey serum, 0.1% Triton-X-100 in PBS, pH 7.4) followed by overnight incubation at room temperature with anti-β-galactosidase (1:300; Promega) plus either anti-$P2Y_1R$, $P2Y_2R$, (both 1:25; Zymed Laboratories Inc.), or $A_{2A}R$ (1:25; Chemicon) primary antibodies in PBS plus 2.5% normal donkey serum. After three 10-min washes in wash buffer (PBS plus 0.1% Triton-X-100), the sections were incubated with anti-mouse Alexa-Fluor 488-conjugated and anti-rabbit Alexa-Fluor 546-conjugated secondary antibodies in PBS plus 2.5% normal donkey serum for 1 h. The sections were washed three times for 10 min each in wash buffer and then DAPI-stained, followed by two brief water rinses. The slides were mounted in Vectashield (Vector Labs) and analyzed on a Zeiss LSM 510 laser scanning confocal microscope.

Confocal Microscopy Analysis of Transfected Cells—Nucleofected cells grown on glass slides were rinsed with PBS, fixed in 4% paraformaldehyde, and washed for 5 min three times with PBS. Fixed cells were permeabilized and blocked by incubating in blocking buffer (1× PBS, 2% bovine serum albumin, 0.04% saponin, pH 7.4) for 1 h. Next, the cells were incubated with mouse anti-FLAG antibody (1:1000; Sigma) plus either rat anti-hemagglutinin (HA) antibody (1:1000; Roche), or rabbit anti-$P2Y_2R$ antibody (1:300; Zymed Laboratories Inc.) for 1 h at room temperature. Following three 5-min washes with blocking buffer, the cells were incubated for 30 min with anti-mouse Alexa-Fluor 488-conjugated secondary antibody plus either anti-rat Alexa-Fluor 546-conjugated or anti-rabbit Alexa-Fluor 546-conjugated secondary antibody (1:250; Molecular Probes). The cells were washed in blocking buffer three times for 5 min, DAPI-stained, rinsed twice with water, dehydrated through ethanol, and mounted with Vectashield. A Zeiss LSM 510 laser scanning confocal microscope was used to examine cells.

Co-immunoprecipitation—Transfected cells were harvested in 500 μl of ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 1.0% Triton X-100, 5 mM EDTA) and rotated end-over-end at 4 °C for 30 min to solubilize. Unsolubilized membranes were pelleted through centrifugation. 100 μl of the supernatant was reserved to verify construct expression, and 20 μl of 6× sample buffer was added. The remaining supernatant was incubated with 60 μl of anti-FLAG antibody-conjugated agarose beads rotating at 4 °C. Following at least 4 h of incubation, the beads were pelleted and washed five times with 1 ml of lysis buffer. Next, 150 μl of 2× sample buffer was added to elute the proteins. 20 μl of lysate and immunoprecipitated samples were loaded onto gels and analyzed by Western blotting as described above.

ERK Activation Assays—Transfected HEK-293 cells grown in 35-mm dishes were starved in serum-free minimum essential medium overnight. For pertussis toxin (PTX) pretreatment, 10 ng/ml PTX was added to the medium 24 h before the experiment. To stimulate cells, 100 μM acetophenone (Fluka, stock solution prepared in ethanol and diluted to working concentration in water) was added directly to the starvation medium for 2 min at 37 °C. At the end of the stimulation, the medium was removed, and 80 μl of sample buffer was added. The samples were sonicated, heated to 85 °C for 5 min, and centrifuged briefly at 17,000 × *g*. The proteins were resolved by SDS-PAGE, as described above, and extracellular regulated kinase 1/2 (ERK 1/2) was visualized using monoclonal anti-phospho p42/44 and rabbit anti-p42/44 antibodies (1:1000; Cell Signaling) to blot for phosphorylated and total mitogen-activated ERK 1/2, respectively. Fluorescence-conjugated anti-mouse and anti-rabbit secondary signals (1:10,000; Rockland) were detected using the Odyssey imaging system, and band densities were quantified using Odyssey imaging software (Li-Cor).

RESULTS

Enhanced Plasma Membrane Localization of the OR M71 upon Co-expression with the Purinergic Receptors $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$—We have previously reported that association of the OR M71 with the β_2-AR alleviates intracellular retention and yields functional M71 localized at the plasma membrane (16). To determine the specificity of such GPCR-OR interactions and identify whether other GPCRs are similarly capable of enhancing M71 plasma membrane localization, we conducted a screen co-expressing M71 with a multitude of other GPCRs. These co-expressed GPCRs represent families from which at least one receptor subtype is reportedly expressed in the olfactory epithelium (OE) and/or olfactory bulb (16, 22–32) and include the trace amine-associated receptors, a new class of chemosensory receptor in the OE (32). M71 tagged at the N terminus with FLAG and at the C terminus with GFP (FLAG-M71-GFP) was expressed alone and in combination with each of the other GPCRs by transient transfection in HEK-293 cells. Plasma membrane levels of M71 were quantified by detection with an anti-FLAG HRP-conjugated antibody in unpermeabilized cells via a luminometer assay. When expressed alone, only a small amount of M71 was detected at the plasma membrane. Co-expression with the vast majority of receptors examined had no significant effect on M71 surface expression. Strikingly, however, three purinergic receptor subtypes, $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$, significantly increased M71 plasma membrane expression by 4–8-fold, comparable with the previously reported effect of co-expression with β_2-AR (Fig. 1).

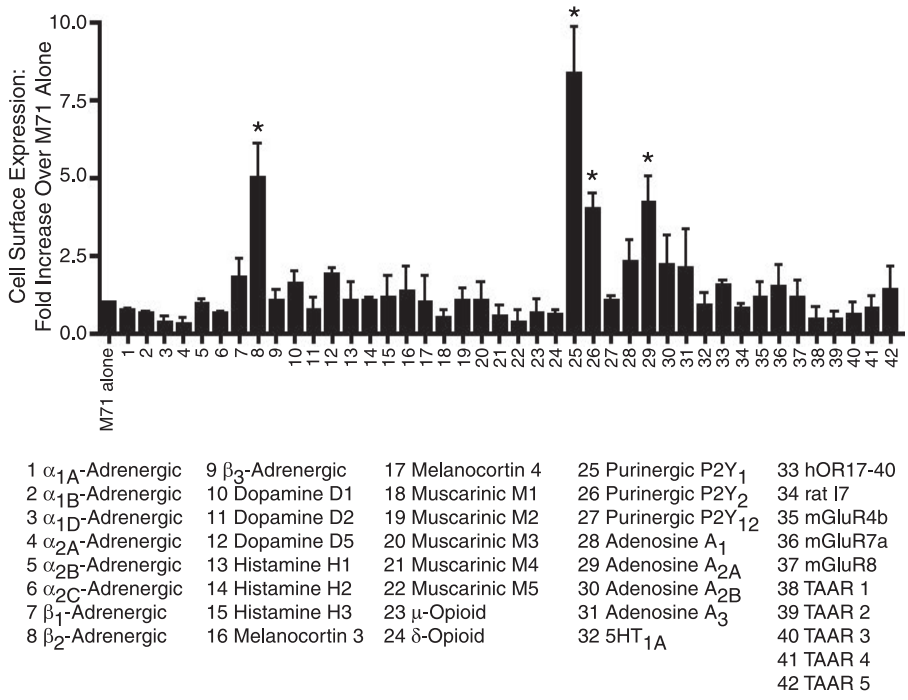

FIGURE 1. **Enhanced M71 plasma membrane localization upon co-expression with β_2-AR, P2Y$_1$R, P2Y$_2$R, and A$_{2A}$R.** FLAG-M71-GFP was expressed alone or co-expressed with 42 other GPCRs in HEK-293 cells. Plasma membrane expression of M71 in unpermeabilized cells was detected via a luminometer assay following incubation with an anti-FLAG HRP-conjugated antibody. The *bars* and *error bars* represent the means ± S.E. from at least three independent experiments and show the fold increase in cell surface expression compared with M71 expressed alone. One-way ANOVA followed by Dunnett's post-hoc test was used to determine statistical significance. The *asterisk* indicates $p < 0.001$.

Expression of P2Y$_1$R, P2Y$_2$R, and A$_{2A}$R in M71-positive Olfactory Sensory Neurons—We performed immunohistochemistry on cryostat sections of olfactory epithelial tissue to elucidate whether P2Y$_1$R, P2Y$_2$R, and A$_{2A}$R are expressed in M71-positive OSNs. To circumvent the lack of an M71-specific antibody, we utilized M71-lacZ transgenic mice for our studies. These mice express the β-galactosidase gene under control of the M71 promoter such that all cells expressing M71 also express β-galactosidase (19). Thus, by labeling sections with an anti-β-galactosidase primary antibody, we identified M71-positive OSNs distributed in the dorso-medial zone of the nasal epithelium, as previously described (19, 33). Using antibodies specific for P2Y$_1$R, P2Y$_2$R, and A$_{2A}$R, we found each of the purinergic receptors to be expressed in olfactory epithelial tissue (Fig. 2). The expression of all three receptors appeared to be ubiquitous throughout the epithelial layer and not restricted to any one population of cells. Both the P2Y$_1$R and P2Y$_2$R showed particularly intense expression on the luminal edge of the olfactory epithelium, where OSN cilia extend and ORs are expressed (Fig. 2, *Field View*). High magnification images showed direct overlap (yellow) of the purinergic receptors expression with M71-positive OSNs (Fig. 2, *Zoom*), and all M71-positive OSNs observed exhibited co-staining with the purinergic receptors. Tissue labeled without purinergic receptor primary antibody exhibited a low level of auto-fluorescence. Control experiments in sections from P2Y$_1$R-KO and P2Y$_2$R-KO mice showed auto-fluorescence levels similar to those of M71-lacZ sections without primary antibody, suggesting that the labeling observed with the purinergic receptor antibodies was specific.

Physical Association of M71 with P2Y$_1$R, P2Y$_2$R, and A$_{2A}$R—The observed enhancement of the plasma membrane localization of M71 upon co-expression with P2Y$_1$R, P2Y$_2$R, and A$_{2A}$R receptors, together with confirmation that these purinergic receptors are expressed with M71 in native tissue, suggested that M71 might physically interact with each of these GPCRs. Thus, co-immunoprecipitation studies were performed to determine whether M71 can associate in physical complexes with P2Y$_1$R, P2Y$_2$R, and A$_{2A}$R. FLAG-M71-GFP was expressed together with each of the purinergic receptors, and cell lysates were subjected to immunoprecipitation with anti-FLAG antibody-conjugated agarose beads. Equal levels of expression were observed for M71 transfected alone or co-transfected with the purinergic receptors, and levels of M71 immunoprecipitated were also similar with each of the co-transfected purinergic receptor (data not shown). FLAG-M71-GFP expression was detected as a unique band slightly higher than the 37-kDa protein marker in lysate and immunoprecipitated samples (Fig. 3*A*). Immunoprecipitation of M71 from cells co-expressing HA-P2Y$_1$R yielded a dense immunoreactive band upon blotting with anti-HA antibody (Fig. 3*B*). In addition, both P2Y$_2$R and HA-A$_{2A}$R were also robustly co-immunoprecipitated with M71 (Fig. 3, *C* and *D*). Conversely, a GPCR that does not enhance the cell surface expression of M71, the δ opioid receptor, was not found to co-immunoprecipitate with M71 (Fig. 3*E*). These data demonstrate the ability of M71 to form stable complexes with specific purinergic receptors in a cellular context.

To further verify the cellular localization of M71, we studied transfected HEK-293 cells via confocal microscopy. P2Y$_1$R, P2Y$_2$R, and A$_{2A}$R effectively trafficked to the plasma membrane when expressed alone in HEK cells (data not shown). FLAG-M71, however, exhibited a diffuse staining throughout the entirety of the cytoplasm when expressed alone (Fig. 4*A*). Conversely, upon co-transfection with HA-P2Y$_1$R, P2Y$_2$R, or HA-A$_{2A}$R, a significant amount of M71 localized to the plasma membrane where it co-localized well with the various purinergic receptors (Fig. 4, *B–D*). These data suggest that the purinergic receptors P2Y$_1$R, P2Y$_2$R, and A$_{2A}$R are able to interact in a physical complex with M71 that facilitates localization of the OR to the plasma membrane. Furthermore, the co-localization of M71 and the purinergic receptors at the cell surface indicated by confocal microscopy suggests a persistent association that may potentially have functional consequences.

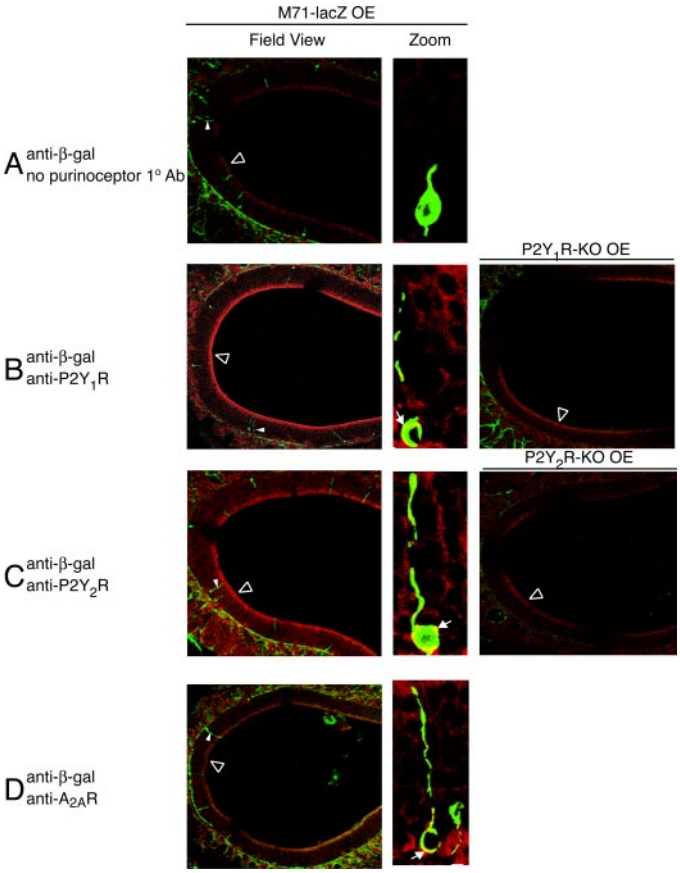

FIGURE 2. **Expression of $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$ in M71-positive olfactory sensory neurons.** Coronal sections (25 μm) of OE from M71-lacZ mice were immunostained with anti-β-galactosidase (*β-gal*) primary antibody followed by Alexa-Fluor 488-conjugated secondary antibody to detect M71-expressing OSNs (*green*). The purinergic receptors were detected by incubation with specific anti-$P2Y_1R$ (*B*), anti-$P2Y_2R$ (*C*), and anti-$A_{2A}R$ (*D*) primary antibodies followed by Alexa-Fluor 546-conjugated secondary antibodies (*red*). To determine background tissue fluorescence, M71-lacZ sections were incubated without purinergic receptor primary antibody (*A*). As a further control, anti-$P2Y_1R$ and anti-$P2Y_2R$ antibodies were incubated with OE sections from $P2Y_1R$-KO and $P2Y_2R$-KO mice (*B* and *C*, *far right panels*). *Small white arrowheads* indicate M71-positive OSNs, *open arrowheads* indicate the luminal edge of the OE, and *white arrows* indicate purinergic receptor staining that overlaps with M71-positive OSNs.

Activation of the MAPK Pathway in Response to Agonist Stimulation of M71 Co-expressed with β_2-AR, $P2Y_1R$, and $P2Y_2R$, but Not $A_{2A}R$—OSNs expressing M71 have been shown to respond to the aromatic ketone acetophenone (ACP) (10). We previously found that ACP stimulation of wild type M71 (WT-M71) expressed in HEK-293 cells did not result in detectable receptor signaling, consistent with the lack of receptor expressed at the plasma membrane, but stimulation of WT-M71 co-expressed with β_2-AR did result in significant cAMP generation (16). These studies demonstrated that when in complex with β_2-AR, heterologously expressed WT-M71 can signal via cAMP generation, as has been reported for many examples of odorant-induced signaling in native OSNs (1, 34). Based on these previous findings, we examined cAMP generation in response to ACP stimulation of WT-M71 co-expressed with $P2Y_1R$, $P2Y_2R$ or $A_{2A}R$. These experiments, however, revealed no evidence of ACP-induced cAMP generation, even with co-transfection of the specialized OSN G-protein, $G\alpha_{olf}$ (data not shown).

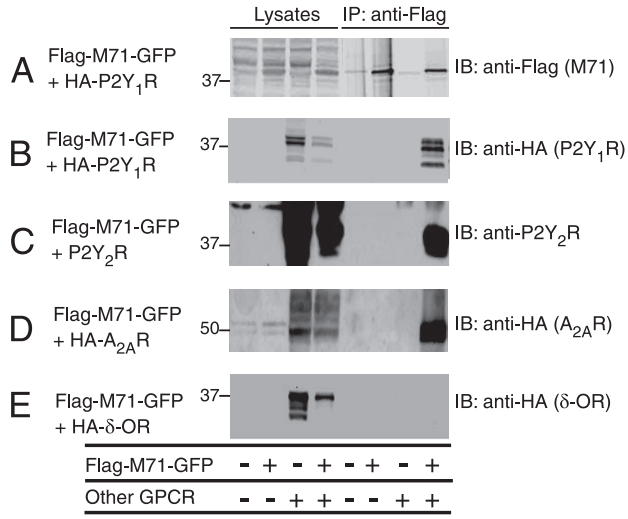

FIGURE 3. **Physical association of M71 with $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$.** HEK-293 cells were transfected with FLAG-M71-GFP alone or in combination with $P2Y_1R$. After harvesting and solubilization, cell lysates were incubated with anti-FLAG antibody-conjugated agarose beads and immunoprecipitated (*IP*). The samples were resolved via SDS-PAGE, and anti-FLAG antibody was used to detect M71 (*A*) and anti-HA antibody was used to detect HA-$P2Y_1R$ (*B*). In additional experiments, FLAG-M71-GFP was co-expressed with $P2Y_2R$, and blots were probed with a specific anti-$P2Y_2R$ antibody (*C*), or FLAG-M71-GFP was co-expressed with either HA-$A_{2A}R$ (*D*) or HA-δ-opioid receptor (*E*), and blots were probed with anti-HA antibody. Each of these experiments was performed at least three times, with similar results. *IB*, immunoblot.

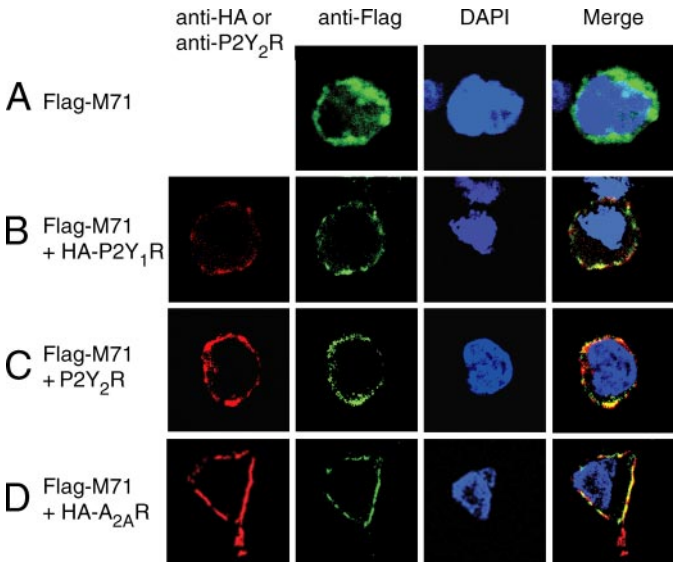

FIGURE 4. **Co-localization of M71 with $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$ at the plasma membrane.** FLAG-M71 was transfected in HEK-293 cells either alone or in combination with HA-$P2Y_1R$, $P2Y_2R$, or HA-$A_{2A}R$. Anti-FLAG primary antibody followed by Alexa-Fluor 488-conjugated secondary (*green*) was used to detect FLAG-M71. $P2Y_2R$ was detected by anti-$P2Y_2R$ antibody, whereas HA-$P2Y_1R$ and HA-$A_{2A}R$ were detected by anti-HA antibody. All three purinergic receptors were visualized using Alexa-Fluor 546-conjugated secondary antibody (*red*). DAPI staining of the nuclei is shown in *blue*. FLAG-M71 expressed alone was localized diffusely throughout cells (*A*). Co-transfection of FLAG-M71 with HA-$P2Y_1R$, $P2Y_2R$, and HA-$A_{2A}R$ resulted in translocation of M71 to the plasma membrane, where it was co-localized with the various purinergic receptors (*B–D*).

In addition to cAMP formation, other signaling pathways that are known to be activated in response to OR stimulation in native OSNs include formation of inositol 1,4,5-bisphosphate and activation of the extracellular regulated kinase/mitogen-

activated protein kinase (ERK/MAPK) pathway (35, 36). ACP stimulation of WT-M71 co-expressed with the various purinergic receptors did not result in detectable accumulation of inositol 1,4,5-bisphosphate (data not shown). However, we did observe small increases in the phosphorylation of ERK1/2 in response to ACP when WT-M71 was co-expressed with the various purinergic receptors or β_2-AR (Fig. 5). Although these ACP-induced increases in phospho-ERK1/2 were not statistically significant, we pursued further studies of this type to see whether the effects could somehow be enhanced.

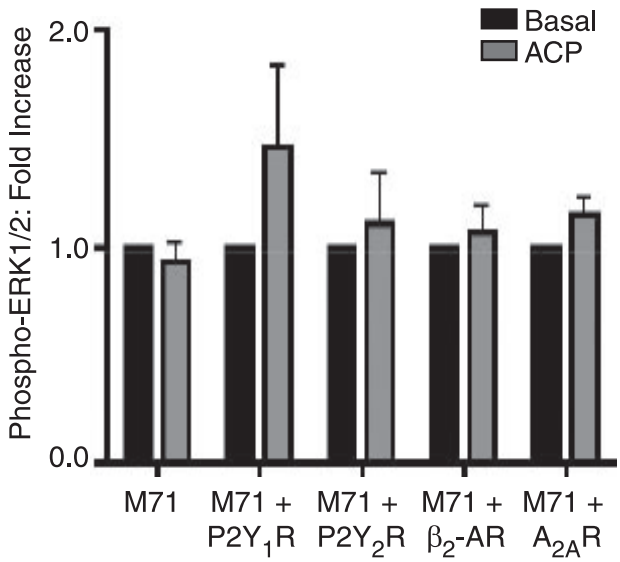

FIGURE 5. **Acetophenone stimulation of M71 co-expressed with $P2Y_1R$, $P2Y_2R$, β_2-AR, or $A_{2A}R$ does not cause significant ERK 1/2 phosphorylation.** WT-M71 was co-transfected with $P2Y_1R$, $P2Y_2R$, β_2-AR, or $A_{2A}R$ in HEK-293 cells and stimulated for 2 min with 100 μM ACP. No significant increase in phosphorylation of ERK 1/2 was found compared with basal levels. *Bars* and *error bars* represent the means ± S.E. from three to six independent experiments. In each experiment, the quantification of phospho-ERK 1/2 immunoreactive bands was normalized to the immunoreactive bands for total ERK 1/2.

There is no consensus as to which G-protein(s) mediate OR signaling through the inositol 1,4,5-bisphosphate and MAPK pathways, and it is likely that many if not most ORs are capable of promiscuous G protein coupling (5, 37). Although subsets of OSNs exhibit differential G protein expression, it has been reported that all OSNs express $G\alpha_o$ (38). Interestingly, both $P2Y_1R$ and $P2Y_2R$, as well as β_2-AR, are well known to couple to pertussis toxin-sensitive $G\alpha_{i/o}$, whereas the $A_{2A}R$ receptor has not been reported to couple to $G\alpha_{i/o}$ (39–41).

Given the abundance of $G\alpha_o$ in the olfactory epithelium, we re-examined the capacity of M71 to mediate ACP-induced changes in ERK 1/2 phosphorylation by performing MAPK activation assays in the presence of co-transfected $G\alpha_o$. Under these conditions, we observed ACP stimulation of cells co-expressing WT-M71, $G\alpha_o$, and either $P2Y_1R$ or $P2Y_2R$ resulted in significant increases in ERK 1/2 phosphorylation. ACP stimulation of WT-M71 co-expressed with $G\alpha_o$ and β_2-AR exhibited more modest increases in phospho-ERK 1/2, whereas ACP stimulation of WT-M71 co-expressed with $G\alpha_o$, and $A_{2A}R$ had no effect on ERK 1/2 phosphorylation levels. Pretreatment of cells with PTX, which inactivates $G\alpha_o$, resulted in a marked decrease in ACP-induced ERK 1/2 phosphorylation in cells co-expressing WT-M71 with $P2Y_1R$ or $P2Y_2R$ (Fig. 6). Studies with a specific anti-$G\alpha_o$ antibody revealed that the levels of $G\alpha_o$ expression achieved in these experiments following transfection of HEK-293 cells were roughly comparable with the expression levels of $G\alpha_o$ in native OE tissue (data not shown). Together, these data demonstrate that M71 co-expressed with $P2Y_1R$ or $P2Y_2R$ is functional at the cell surface and capable of coupling to $G\alpha_o$ in an agonist-regulated fashion.

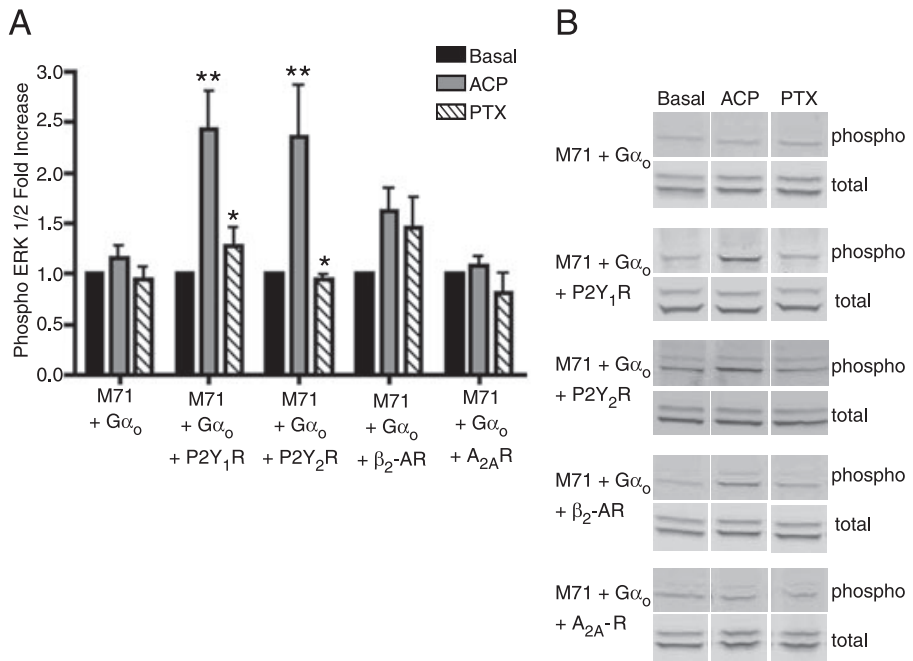

FIGURE 6. **M71 co-expressed with $G\alpha_o$ in addition to $P2Y_1R$, $P2Y_2R$, and β_2-AR exhibits increased phospho-ERK 1/2 signaling when stimulated with acetophenone.** *A*, HEK-293 cells were transfected with WT-M71 plus $G\alpha_o$ or WT-M71 plus $G\alpha_o$ and $P2Y_1R$, $P2Y_2R$, β_2-AR, or $A_{2A}R$. Unstimulated cells were harvested alongside cells exposed to 2-min stimulation with ACP. Some cells were pretreated for 24 h with PTX. ACP stimulation of M71 co-expressed with $P2Y_1R$ and $P2Y_2R$ caused significant increases in ERK 1/2 phosphorylation (n = 12–16; **, $p < 0.01$), which was markedly reduced by PTX pretreatment (n = 3–4; *, $p < 0.05$). A more modest enhancement of ERK 1/2 phosphorylation occurred in ACP-stimulated cells expressing M71 together with $G\alpha_o$ and β_2-AR, whereas no increases in phospho-ERK 1/2 resulted from stimulation of M71 plus $G\alpha_o$ and $A_{2A}R$ (n = 5). The graph represents pooled data analyzed by two-way ANOVA and Bonferroni post-hoc tests. *Bars* and *error bars* represent the means ± S.E. Representative data for each experimental condition are shown in *B*.

Specificity of OR Interactions with Other Receptors—We next assessed whether co-expression with β_2-AR, $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$ might generally result in enhanced plasma membrane localization for many ORs or whether these effects might be specific to particular OR classes. In previous confocal studies, we noted that the β_2-AR did not appear to enhance the surface localization of two ORs that are distantly related to M71: hOR17–40 and rat I7 (16). Similarly, in the current analysis,

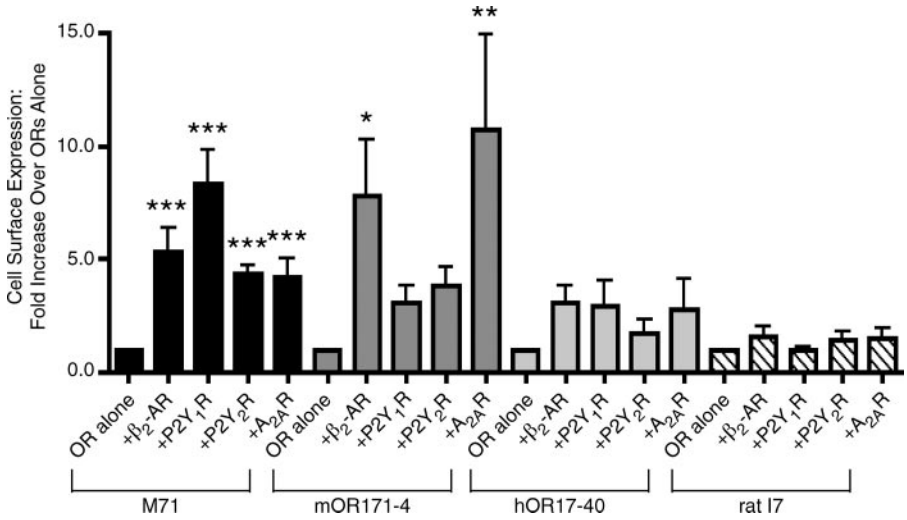

FIGURE 7. **Specificity of OR surface expression enhancement by co-expression with $P2Y_1R$, $P2Y_2R$, β_2-AR, and $A_{2A}R$.** $P2Y_1R$, $P2Y_2R$, β_2-AR, and $A_{2A}R$ were co-expressed with three ORs other than M71: FLAG-mOR171-4-GFP, which shares 67% amino acid identity with M71, FLAG-hOR17–40-GFP (46% identity with M71), and FLAG-rat-I7-GFP (45% identity with M71). The *bars* and *error bars* show the means ± S.E. for fold increases in cell surface expression following co-expression compared with each OR expressed alone. Each data set was analyzed individually by one-way ANOVA and Dunnett's post hoc test. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

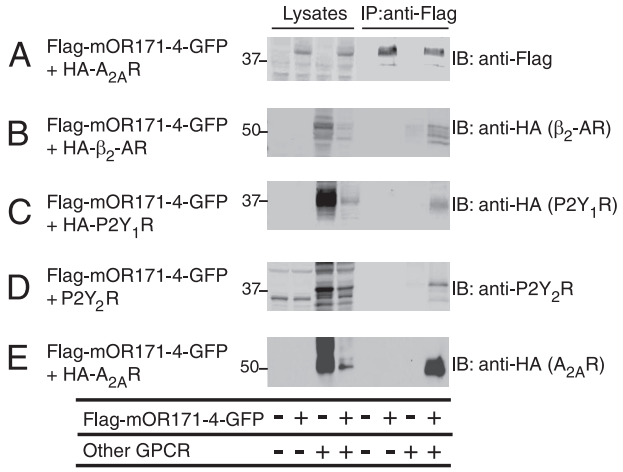

FIGURE 8. **Co-immunoprecipitation of $P2Y_1R$, $P2Y_2R$, β_2-AR and $A_{2A}R$ with mOR171-4.** HEK-293 cells were transfected with FLAG-mOR171-4-GFP alone or FLAG-mOR171-4-GFP plus HA-$A_{2A}R$. The cells were harvested and solubilized, and cell lysates were incubated with anti-FLAG antibody-conjugated agarose beads. Following SDS-PAGE, anti-FLAG antibody was used to detect FLAG-mOR171-4-GFP (*A*). Additional experiments were performed co-expressing FLAG-mOR171-4-GFP with HA-β_2-AR (*B*), HA-$P2Y_1R$ (*C*), $P2Y_2R$ (*D*), and HA-$A_{2A}R$ (*E*). Western blotting using either anti-HA antibody (*B*, *C*, and *E*) or anti-$P2Y_2R$ antibody (*D*) revealed robust co-immunoprecipitation (*IP*) of each receptor with mOR171-4. *IB*, immunoblot.

co-expression with β_2-AR, $P2Y_1R$, $P2Y_2R$, or $A_{2A}R$ did not significantly alter hOR17–40 or rat I7 plasma membrane expression as assessed in luminometer assays (Fig. 7). We also examined the effects of co-expression with the purinergic receptors and β_2-AR on the surface expression of an OR more closely related to M71, mOR171-4, which is a M71 subfamily member that shares ~67% amino acid identity with M71 (42). In luminometer assays of FLAG-mOR171-4-GFP-transfected HEK-293 cells, co-expression with β_2-AR and $A_{2A}R$ significantly elevated levels of the OR at the plasma membrane, whereas co-expression with $P2Y_1R$ and $P2Y_2R$ also modestly enhanced mOR171-4 cell surface expression (Fig. 7). Additionally, co-immunoprecipitation studies demonstrated the ability of mOR171-4 to associate with β_2-AR, $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$ in a cellular context (Fig. 8). These data suggest that β_2-AR, $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$ are not general OR chaperones, but that instead these GPCRs interact specifically with particular classes of ORs, with these interactions facilitating OR plasma membrane localization.

The Second Transmembrane Domain of β_2-AR Is Necessary for β_2-AR-facilitated M71 Plasma Membrane Localization—To identify structural elements that allow specific GPCRs to enhance the cell surface localization of certain ORs, we utilized chimeras that have the transmembrane domains (TMDs) of β_2-AR sequentially replaced with the TMDs of β_1-AR (43). Although the β_1-AR and β_2-AR are closely related, only the β_2-AR significantly increases levels of M71 at the plasma membrane. Chimera 1, in which the N terminus and TMD1 of β_2-AR are replaced by those of β_1-AR, and chimera 3, in which the β_2-AR TMD7 is replaced by that of β_1-AR, both exhibited robust enhancement of M71 surface localization, similar to wild type β_2-AR. Conversely, chimera 2, which contains the TMD2 of β_1-AR, was completely unable to enhance M71 levels at the plasma membrane. In addition, chimera 4, in which both TMD2 and TMD7 of β_2-AR are replaced by those of β_1-AR, was also incapable of localizing M71 to the cell surface (Fig. 9). These data indicate that TMD2 is necessary for β_2-AR-mediated enhancement of M71 plasma membrane expression.

DISCUSSION

The data shown here demonstrate that plasma membrane levels of the OR M71 in HEK-293 cells are significantly enhanced by co-expression with three subtypes of purinergic receptors, $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$. We further found that M71 co-immunoprecipitates as well as co-localizes with each of the purinergic receptors in HEK-293 cells and that $P2Y_1R$, $P2Y_2R$ and $A_{2A}R$ are each present in M71-expressing OSNs *in vivo*. These data suggest that certain non-OR GPCRs can associate with and facilitate the surface expression of M71. These receptor-receptor interactions appear to be highly specific, because the vast majority of the 42 GPCRs that we examined had no significant effect on the localization of M71. Several other examples have been described whereby a GPCR that is retained intracellularly when expressed alone in heterologous cells can be liberated to the plasma membrane upon co-expression and association with another GPCR (17, 44, 45). The most well studied example of this occurrence is the intracellular retention of $GABA_BR1$, which is alleviated by co-expression with $GABA_BR2$ to form a functional heterodimer at the plasma membrane (46–48). Co-expression of $GABA_BR1$ with 35 other

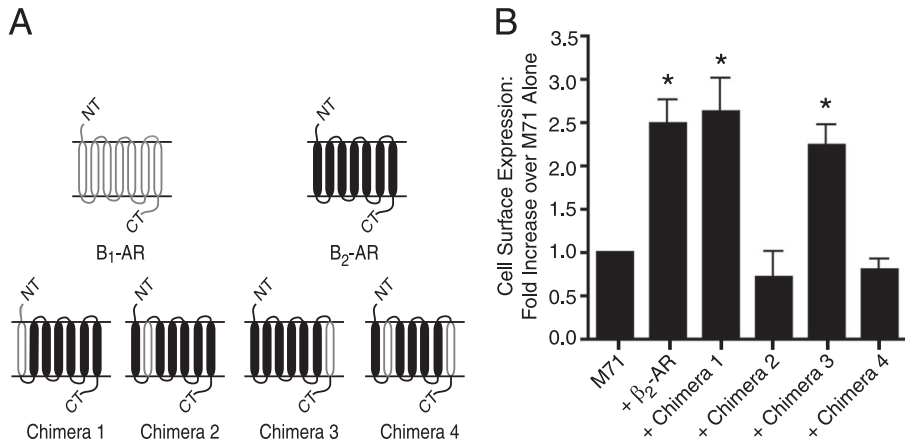

FIGURE 9. **β_1/β_2-AR chimera effects on M71 plasma membrane localization.** *A*, HEK-293 cells were transfected with M71 plus wild type β_2-AR or chimeras in which various TMDs of the β_2-AR were replaced with those of the β_1-AR. The chimera junctions occurred at the following amino acid positions in the human β_1-AR and β_2-AR sequences: chimera 1, $\beta_1$1–84/β_2 60–413; chimera 2, $\beta_2$1–71/β_1 97–131/$\beta_2$107–413; chimera 3, β_2 1–295/β_1 347–381/β_2 331–413; and chimera 4, β_2 1–71/β_1 97–131β_2 107–295/β_1 347–381/β_2 331–413. *CT*, C terminus; *NT*, N terminus. *B*, plasma membrane levels of M71 were quantified through surface luminometer assays, and *bars* and *error bars* represent means and standard errors from three independent experiments, analyzed by one-way ANOVA, using Dunnett's post-hoc analysis. *, $p < 0.01$.

GPCRs, however, does not affect $GABA_BR1$ surface trafficking, exemplifying the specificity of this interaction (49).

Interactions between receptors can potentially serve as the basis for receptor-receptor cross-talk. With respect to OR interactions with non-OR GPCRs, it is interesting to note that OR signaling and olfaction in general are known to be modulated by various hormones and neurotransmitters. For example, adrenaline strongly enhances odorant contrast in newt olfactory receptor cells (29), and dopamine has been demonstrated to suppress odorant-induced Ca^{2+} signaling in mouse OSNs and depress overall OSN excitability (26). Most relevant to this study, purinergic nucleotides have been found to reduce odor responsiveness in cultured mouse OSNs (25). In addition, the expression of the purinergic receptor subtypes $P2Y_1R$ and $P2Y_2R$ has previously been characterized in olfactory epithelium (25, 50) consistent with our findings in the current study. Thus, the present data, taken together with previous findings, suggest that purinergic receptors *in vivo* may associate with certain ORs, such as M71, to promote OR surface expression and regulate OR functionality. This model for the regulation of mammalian ORs by receptor heterodimerization bears similarity to recent findings in the field of *Drosophila* olfaction, where typical ORs have been found to require heterodimerization with an atypical OR, OR83b, to achieve proper localization and activity (13, 14).

The association of the OR M71 with $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$, whether by direct physical dimerization or via interactions in a multi-protein complex, offers a novel mechanism by which nucleotides may modulate olfaction. Direct associations between ORs and other GPCRs might also potentially alter receptor conformation in a way that results in new pharmacological properties, as has been established for heterodimers between taste receptors (51, 52). In the case of ORs, differential interacting partners could create altered affinities for odorants or contribute to the ability of ORs to be activated by multiple odorants (53).

In addition to potential effects on receptor pharmacology, OR associations with other GPCRs may also influence OR signaling pathways. In our studies, we observed weak activation of the MAPK pathway in response to agonist stimulation of M71 co-expressed with $P2Y_1R$, $P2Y_2R$, and β_2-AR. Strikingly, however, agonist stimulation of M71 co-expressed with exogenous $G\alpha_o$ in addition to $P2Y_1R$, $P2Y_2R$, or β_2-AR resulted in much more significant ACP-induced phosphorylation of ERK 1/2. Notably, $P2Y_1R$, $P2Y_2R$, and β_2-AR have all been demonstrated to signal via $G\alpha_{i/o}$ (39–41). $A_{2A}R$, however, is not known to couple to $G\alpha_{i/o}$, and thus M71 interacting with $A_{2A}R$ may signal through an alternate pathway that does not result in phosphorylation of ERK 1/2. OR signaling through $G\alpha_o$ has not been previously reported, but a number of studies do suggest an ability of ORs to couple to G proteins besides $G\alpha_{olf}$, for example $G\alpha_s$ and $G\alpha_{15/16}$ (37). In addition, $G\alpha_o$ has been strongly implicated in olfactory signaling. Goa-1, the *C. elegans* orthologue of mammalian $G\alpha_o$, has been shown to modulate olfactory habituation (54), and $G\alpha_o$ knock-out mice exhibit dramatically impaired olfaction (54, 55). We propose that association with other GPCRs, such as $P2Y_1R$, $P2Y_2R$, and β_2-AR, imparts to M71 the ability to initiate signaling through coupling to $G\alpha_o$. Further studies may clarify how the downstream effects of OR signaling through $G\alpha_o$ differ from those that occur by OR signaling through $G\alpha_{olf}$.

Using receptor chimeras, we found that replacing the second TMD of the β_2-AR with TMD2 of β_1-AR abolishes β_2-AR-mediated enhancement of M71 at the plasma membrane. Protein alignments, however, did not reveal any obvious motif similarities in TMD2 among β_2-AR, $P2Y_1R$, $P2Y_2R$ and $A_{2A}R$ that were not found in other GPCRs, suggesting that the structural elements that mediate interaction with M71 may vary from receptor to receptor. Indeed, the TMDs implicated in GPCR dimerization appear to be highly receptor-dependent. TMD6 of β_2-AR has been shown to constitute a necessary interface for receptor homodimerization, whereas this domain was determined to be of limited importance for dopamine D1 receptor dimerization (56, 57). Oligomerization of the yeast α-factor receptor was reported to be mediated by the N terminus, TMD1 and TMD2, and two independent groups identified TMD4 as the interface of dopamine D2 receptor homodimers (58–60). CCR5 receptor dimerization appears to depend on residues in TMD1 and TMD4, whereas oligomerization of the $A_{2A}R$ has been demonstrated to involve the fifth TMD (61, 62). Most recently, oligomerization of the cholecystokinin receptor was shown to be most influenced by TMD7 (63). In summary, the necessity of TMD2 for β_2-AR mediated enhancement of M71 surface localization adds to the growing consensus that the mechanisms of GPCR dimerization are based on unique

structural complexities distinct to particular interacting partners.

Our results indicate that not all ORs share the propensity to associate with non-OR GPCRs such as β_2-AR, $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$. We found that an OR with 67% identity to M71 does associate with β_2-AR and the purinergic receptors, whereas two ORs with 46% or less identity to M71 do not. ORs with greater than 60% identity are thought to be activated by similar types of odorants and are therefore classified into the same subfamily (42). We speculate that non-OR GPCRs such as β_2-AR, $P2Y_1R$, $P2Y_2R$, and $A_{2A}R$ may interact with specific subfamilies of ORs, but not all ORs, to facilitate cell surface expression and modulate responsiveness to odorants. Furthermore, such OR interactions with other receptors may act in concert with OR associations with accessory proteins (15) to control OR trafficking. Considering the enormous size of the OR family, a number of distinct mechanisms are likely to contribute to the regulation of OR plasma membrane localization and functionality.

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