ACS Pharmacology & Translational Science

pubs.acs.org/ptsci

Article

Differential Role of Serines and Threonines in Intracellular Loop 3 and C-Terminal Tail of the Histamine H_4 Receptor in β-Arrestin and G Protein-Coupled Receptor Kinase Interaction, Internalization, and Signaling

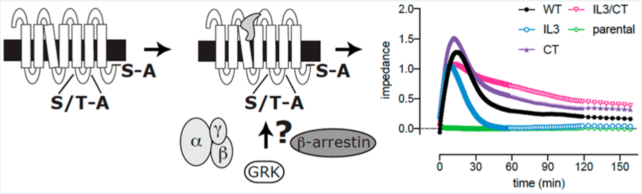
Eléonore W. E. Verweij, Betty Al Araaj, Wimzy R. Prabhata, Rudi Prihandoko, Saskia Nijmeijer, Andrew B. Tobin, Rob Leurs, and Henry F. Vischer*

Cite This: *ACS Pharmacol. Transl. Sci.* 2020, 3, 321−333

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: The histamine H_4 receptor (H_4R) activates $G\alpha_i$-mediated signaling and recruits β-arrestin2 upon stimulation with histamine. β-Arrestins play a regulatory role in G protein-coupled receptor (GPCR) signaling by interacting with phosphorylated serine and threonine residues in the GPCR C-terminal tail and intracellular loop 3, resulting in receptor desensitization and internalization. Using bioluminescence resonance energy transfer (BRET)-based biosensors, we show that G protein-coupled receptor kinases (GRK) 2 and 3 are more quickly recruited to the H_4R than β-arrestin1 and 2 upon agonist stimulation, whereas receptor internalization dynamics toward early endosomes was slower. Alanine-substitution revealed that a serine cluster at the distal end of the H_4R C-terminal tail is essential for the recruitment of β-arrestin1/2, and consequently, receptor internalization and desensitization of G protein-driven extracellular-signal-regulated kinase (ERK)1/2 phosphorylation and label-free cellular impedance. In contrast, alanine substitution of serines and threonines in the intracellular loop 3 of the H_4R did not affect β-arrestin2 recruitment and receptor desensitization, but reduced β-arrestin1 recruitment and internalization. Hence, β-arrestin recruitment to H_4R requires the putative phosphorylated serine cluster in the H_4R C-terminal tail, whereas putative phosphosites in the intracellular loop 3 have different effects on β-arrestin1 versus β-arrestin2. Mutation of these putative phosphosites in either intracellular loop 3 or the C-terminal tail did not affect the histamine-induced recruitment of GRK2 and GRK3 but does change the interaction of H_4R with GRK5 and GRK6, respectively. Identification of H_4R interactions with these proteins is a first step in the understanding how this receptor might be dysregulated in pathophysiological conditions.



KEYWORDS: *histamine, GPCR, β-arrestin, GPCR kinase, internalization, desensitization*

The histamine H_4 receptor (H_4R) is a G protein-coupled receptor (GPCR) that induces chemotaxis and the production of inflammatory cytokines by hematopoietic cells in response to histamine.[1] Currently, H_4R antagonists are being tested in clinical trials to treat histamine-induced itch (JNJ39758979), bronchial allergen challenge (ZPL-3893787), allergic rhinitis (UR-63325), atopic dermatitis (JNJ39758979 and ZPL-3893787), rheumatoid arthritis (Toreforant), asthma (Toreforant and JNJ39758979), and psoriasis (ZPL-3893787 and Toreforant).[2] Interestingly, H_4R-deficient mice are hypersensitive to neuropathic pain, indicating that H_4R-mediated signaling dampens nociception.[3] Indeed, stimulation of H_4R in the central nervous system by intrathecal or intracerebroventricular administration of H_4R agonists attenuates neuropathic pain through inhibition of neuroinflammation and oxidative stress.[4] H_4R receptor expression is reduced in bladder cancer, kidney cancer, breast cancer, gastrointestinal cancer, lung cancer, endometrial cancer, and skin cancer, as compared to healthy tissue.[5] Importantly, preclinical studies in immunodeficient hosts revealed that H_4R agonists display a clear antitumor effect associated with reduced tumor growth and metastatic potential.[5] Hence, understanding the interplay of H_4R signaling and regulatory processes upon agonist stimulation is very relevant for the use of agonists in chronic neuropathic pain and tumor therapy and has so far received limited attention.

The H_4R signals through heterotrimeric $G_{i/o}$ proteins resulting in reduced cAMP production by adenylyl cyclase, increased Ca^{2+} mobilization, activation of extracellular-signal-regulated kinase (ERK)1/2 and Akt, and cytoskeletal changes.[1]

Special Issue: Advances in GPCR Signal Transduction

Received: January 15, 2020
Published: March 16, 2020

ACS Publications

© 2020 American Chemical Society

https://dx.doi.org/10.1021/acsptsci.0c00008

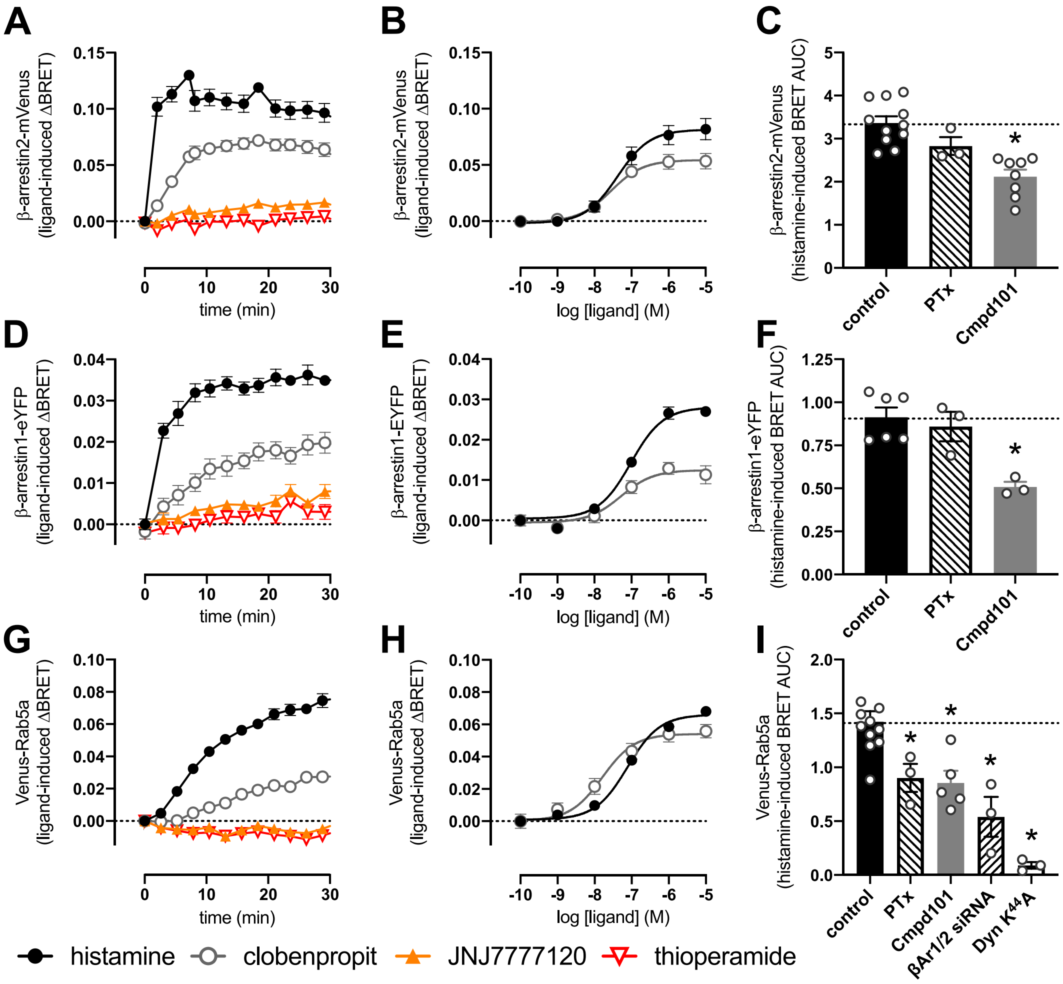


Figure 1. Recruitment of β-arrestin1/2 and H_4R internalization. BRET measurements in HEK293T cells expressing H_4R-Rluc8 in combination with β-arrestin2-mVenus (A and B), β-arrestin1-eYFP (D and E), or Venus-Rab5a (G and H) in real time upon stimulation with 10 μM histamine, JNJ7777120, clobenpropit, or thioperamide (A, D, and G) or after 30 min of incubation with increasing concentrations histamine or clobenpropit (B, E, and H). Data are shown as mean ± SEM from 3 independent experiments performed in triplicate. Ligand-induced BRET changes (ΔBRET) were calculated by subtracting the BRET ratio of vehicle-treated cells. Area under the curve (AUC) of BRET measurements in HEK293T cells expressing H_4R-Rluc8 in combination with β-arrestin2-mVenus (C), β-arrestin1-eYFP (F), or Venus-Rab5a (I) for 30 min in response to 10 μM histamine following pretreatment with vehicle, 100 ng/mL PTx for 16 h, or 3 μM Cmpd101 for 30 min, or cotransfection with β-arrestin1/2 siRNA or dominant-negative dynamin $K^{44}A$ mutant cDNA. AUC of the BRET measurements is shown as mean ± SEM from at least 3 independent experiments performed in triplicate, with scatter plots showing individual AUC values. Statistical differences ($p < 0.05$) compared to control were determined using one-way ANOVA with Dunnett's multiple comparison test and are indicated by an asterisk (*).

Reference antagonist JNJ7777120 can antagonize these histamine-induced cellular responses as well as H_4R-mediated inflammation and pruritus in animal *in vivo* models.[1,2] The H_4R also recruits β-arrestin2 in response to agonist stimulation.[6,7] Binding of β-arrestins to agonist-activated GPCRs is preceded by phosphorylation of GPCR serine and threonine residues in the C-terminal tail (CT) and/or intracellular loop 3 (IL3) by G protein receptor kinases (GRKs) and results in the termination of further G protein coupling by steric hindrance and facilitates clathrin-mediated receptor internalization.[8] Internalized GPCRs are then either recycled back to the cell surface in recycling endosomes or degraded in lysosomes, resulting in a transient or more prolonged downregulation of receptor expression at the cell surface and consequently affecting the responsiveness to agonist stimulation.[9] In addition, receptor-bound β-arrestins can activate mitogen-activated protein kinases, including ERK1/2, p38, and c-Jun, by acting as signaling scaffolds.[8] Differential engagement of GRK subtypes 2, 3, 5 and/or 6 can change the phosphorylation pattern at intracellular GPCR domains and consequently dictate β-arrestin function by modulating the conformation of bound β-arrestin.[10−15] Hence, the dysregulation of GRK subtypes expression levels in tumors[5] but also in the immune system during inflammation[16] can affect receptor phosphorylation, and consequently, the responsiveness of these cells to agonist stimulation. However, it is still unknown which GRK subtypes are potentially involved in the regulation of H_4R activity and how putative phosphorylation sites in IL3 and/or CT affect the recruitment of β-arrestin and the subsequent desensitization of G protein signaling and receptor internalization. Insight in these regulatory processes might contribute to development of H_4R agonists as anticancer and antineuropathic pain drugs with improved therapeutic efficacy by retaining signaling without further downregulation of H_4R levels.

In this study, the interactions of H_4R with β-arrestin1, β-arrestin2, GRK2, GRK3, GRK5, and GRK6 upon stimulation with the reference ligands histamine, clobenpropit, JNJ7777120, and thioperamide were measured by bioluminescence resonance energy transfer (BRET). In addition, the contribution of putative serine and threonine phosphorylation sites in IL3 and CT of H_4R on β-arrestin recruitment, GRK interaction, receptor internalization, and regulation of signal transduction was evaluated by alanine-substitution.

■ RESULTS AND DISCUSSION

BRET-Based Detection of β-Arrestin1/2 Recruitment and H_4R Internalization. Fusion of Rluc8 to the H_4R CT did not affect histamine binding affinity ($pK_D = 8.4 \pm 0.1$) and potency ($pEC_{50} = 8.6 \pm 0.0$) to inhibit forskolin-induced cAMP-responsive element (CRE) reporter gene activity as compared to HA-H_4R ($pK_D = 8.5 \pm 0.1$ and $pEC_{50} = 8.6 \pm 0.1$) (Figure S1A−C). However, the expression of H_4R-Rluc8 ($B_{max} = 1.5 \pm 0.1$ pmol/mg) was 3.2-fold decreased in comparison to HA-H_4R ($B_{max} = 4.9 \pm 0.7$ pmol/mg) after transient transfection of HEK293T cells.

Stimulation of transiently transfected HEK293T cells with 10 μM histamine rapidly increased BRET between H_4R-Rluc8 and both β-arrestin2-mVenus and β-arrestin1-eYFP to maximum steady-state levels within 10 min (Figure 1A,D). Partial H_4R agonist clobenpropit induced lower maximum steady-state level for both β-arrestin1 and 2 recruitment, which are reached with slower kinetics as compared to histamine. Similar partial agonism for clobenpropit in comparison to full agonist histamine has been previously observed in the PathHunter β-galactosidase enzyme-fragment complementation (EFC)-based β-arrestin2 recruitment assay to the H_4R in U2OS cells.[6,17] JNJ7777120 (10 μM) did not induce β-arrestin1 recruitment to H_4R (Figure 1D), whereas only a very minor increase in BRET between H_4R-Rluc8 and β-arrestin2-mVenus was consistently observed in response to 10 μM JNJ7777120 (Figure 1A). However, this minor effect is considerably smaller than its previously observed biased efficacy (~60% of the maximal histamine-induced response) in the EFC-based PathHunter β-arrestin2 recruitment assay.[6,17,18] Considering that full agonist histamine and partial agonist clobenpropit displayed comparable potencies and efficacies between the EFC- and BRET-based β-arrestin2 recruitment assay (Figure 1B; Table 1), the observed efficacy difference of JNJ7777120 between these two assay formats seemed not be related to a possible difference in detection sensitivity. However, it cannot be excluded that fusion of the small β-galactosidase fragment (4 kDa) or the 9-fold larger Rluc8 (36 kDa) may differentially affect the efficacy of indolecarboxamide ligands such as JNJ7777120, whereas efficacies of H_4R ligands from other chemical classes seemed not or less affected by both biosensor configurations. Indeed, thioperamide (10 μM) did not induce β-arrestin1 and 2 recruitment to the H_4R (Figure 1A,D), which corroborates with its lack of efficacy in the EFC-based PathHunter assay.[6,17] Both JNJ7777120 and thioperamide antagonized histamine-induced β-arrestin2 recruitment to H_4R by right-shifting the response curves while also depressing the maximal histamine response at higher concentrations (Figure S2A,B). Both JNJ7777120 and thioperamide have previously been shown to act as competitive surmountable antagonists on the H_4R in various relatively long-term functional readouts;[6,19] hence, the BRET-based β-arrestin2 recruitment assay might be too short in time for these antagonists to re-equilibrate. Indeed, JNJ7777120 displayed considerably slower H_4R dissociation kinetics as compared to histamine,[20] which exceeds the 30 min readout in BRET-based β-arrestin2 recruitment.

Table 1. Potency (pEC_{50}) and Intrinsic Activity of Histamine and Clobenpropit in Wild-Type H_4R-Rluc8-Mediated Responses as Measured in BRET-Based Assays[a]

	histamine	clobenpropit	
BRET assay	pEC_{50}	pEC_{50}	intrinsic activity
β-arrestin1	7.0 ± 0.1	7.3 ± 0.3	0.60 ± 0.1
β-arrestin2	7.3 ± 0.1	7.6 ± 0.0	0.58 ± 0.0
Rab5a	7.1 ± 0.0	7.8 ± 0.1	0.47 ± 0.1
GRK2	6.9 ± 0.1	7.4 ± 0.1	0.37 ± 0.0
GRK3	7.3 ± 0.1	7.6 ± 0.1	0.50 ± 0.0
GRK5	7.9 ± 0.1	8.5 ± 0.4	1.13 ± 0.1

[a] Data are shown as mean ± SEM from at least 3 independent experiments performed in triplicate. The intrinsic activity of clobenpropit was calculated by dividing its maximum response by those of full agonist histamine (intrinsic activity = 1).

In line with their efficacy in β-arrestin recruitment, both histamine and clobenpropit (10 μM) steadily increased BRET between the H_4R-Rluc8 and early endosome marker Venus-Rab5a with significantly slower kinetics than the recruitment of β-arrestin1 and 2, whereas JNJ7777120 and thioperamide were both ineffective to induce H_4R internalization into early endosomes (Figure 1G). This agonist-induced translocation of H_4R to early endosomes corroborated with the internalization kinetics of HA-H_4R in HEK293 upon histamine stimulation as observed with confocal microscopy.[21] The potencies of both histamine and clobenpropit were comparable between recruitment of β-arrestin1 and 2, and receptor internalization into early endosomes (Figure 1B,E,H; Table 1), which is in line with the common paradigm that β-arrestins are involved in internalization by functioning as scaffold for clathrin-mediated internalization in a 1:1 stoichiometry with the receptor.[8]

Pretreatment with the $G\alpha_{i/o}$ protein inhibitor pertussis toxin (PTx; 100 ng mL^{-1}) for 16 h abolished G protein-mediated inhibition of forskolin-induced CRE reporter gene activity in response to histamine (Figure S1C), but this did not affect histamine-stimulated β-arrestin1 and 2 recruitment in the measured 30 min time period (Figure 1C,F). Similarly, PTx treatment did not affect agonist-induced β-arrestin2 recruitment to H_4R in the EFC-based assay.[6] However, receptor internalization was partially (~30%) reduced by PTx (Figure 1I). Pretreatment with Cmpd101 (3 μM) for 30 min partially decreased (35−45%) the BRET change between H_4R-Rluc8 and β-arrestin2-mVenus (Figure 1C), β-arrestin1-eYFP (Figure 1F), and Venus-Rab5a (Figure 1I), during 30 min of stimulation with 10 μM histamine. Hence, these data suggest that phosphorylation by GRK2 and/or GRK3 contributes in part to β-arrestin1/2-mediated H_4R internalization, as previously observed for agonist-activated PAC1, dopamine D2, and μ-opioid receptor.[22−25] Knockdown of β-arrestin1 and 2 decreased the histamine-induced H_4R internalization by 56 ± 13.2% in comparison to control siRNA-treated cells (Figure 1I), indicating that β-arrestins are indeed involved in H_4R internalization. The observed internalization in the presence of β-arrestin-targeting siRNA is most likely due to the only partial knockdown of β-arrestins (Figure S3). Finally, cotransfection of dominant-negative dynamin mutant $K^{44}A$ DNA (2 μg/dish) inhibited 95% of the histamine-induced internalization into early endosomes, indicating that the H_4R internalizes via clathrin-coated pits and/or caveolae.[26]

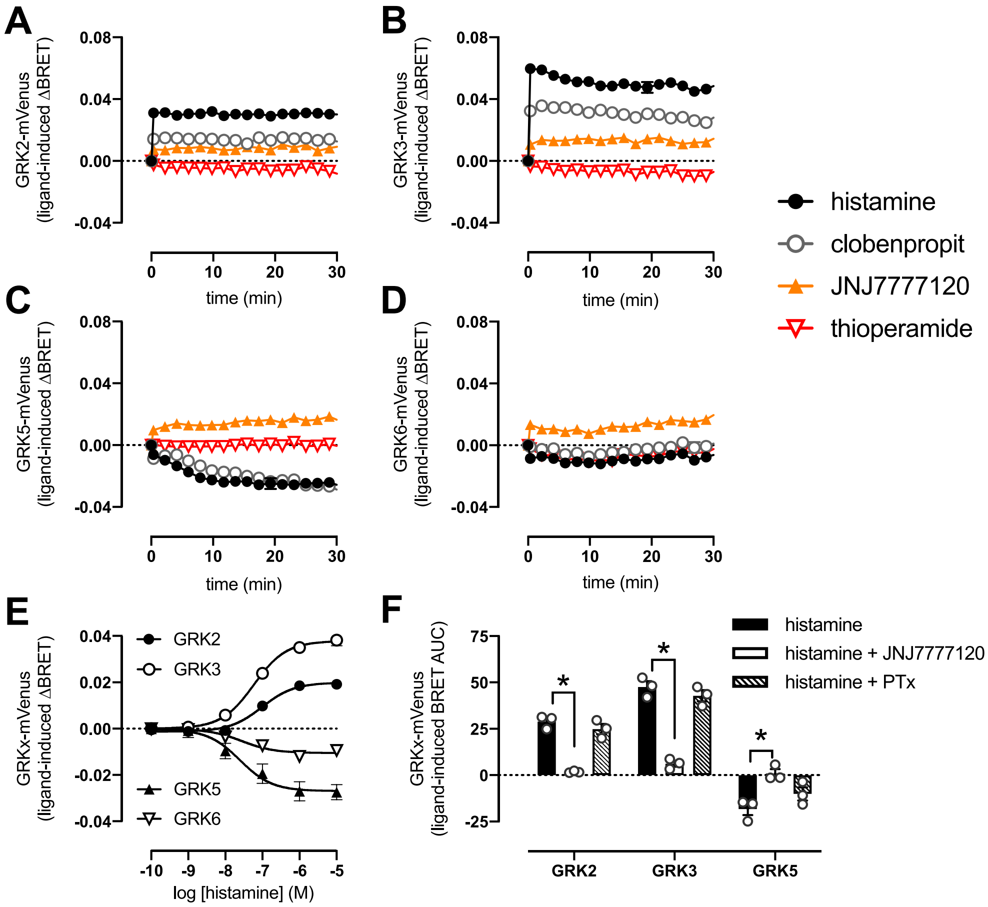


Figure 2. Ligand-induced changes in the interaction between H_4R and GRKs. BRET measurements in HEK293T cells expressing H_4R-Rluc8 in combination with GRK2-mVenus (A, E, and F), GRK3-mVenus (B, E, and F), GRK5-mVenus (C, E, and F), or GRK6-mVenus (D and E) in real time upon stimulation with 10 μM histamine, JNJ7777120, clobenpropit, or thioperamide (A−D) or after 30 min of incubation with increasing concentrations histamine (E). Data are shown as mean ± SEM from 3 independent experiments performed in triplicate. Ligand-induced BRET changes (ΔBRET) were calculated by subtracting BRET ratio of vehicle-treated cells. (F) Area under the curve (AUC) of BRET measurements in HEK293T cells expressing H_4R-Rluc8 in combination with GRK2-mVenus, GRK3-mVenus, or GRK5-mVenus for 20 min in response to 10 μM histamine following pretreatment with vehicle, 50 μM JNJ7777120 for 15 min, or 100 ng/mL PTx for 16 h. AUC is shown as the mean ± SEM of 3 independently performed experiments in triplicate, with scatter plots showing the individual experiments. Statistical differences ($p < 0.05$) compared to control were determined using one-way ANOVA with Dunnett's multiple comparison test and are indicated by an asterisk (*).

BRET-Based Detection of H_4R Interactions with GRKs. Agonist-activated GPCRs are rapidly phosphorylated on serine and threonine residues within their intracellular loops or CT by one or more ubiquitously expressed GRK subtypes 2, 3, 5, or 6 to promote β-arrestin binding and subsequent receptor desensitization and internalization.[11,22,27−30] BRET was used to monitor the interaction of H_4R with these four GRK subtypes in response to stimulation with different ligands. Histamine and clobenpropit (10 μM) increased BRET between H_4R-Rluc8 and GRK2- and GRK3-mVenus with faster kinetics in comparison to β-arrestin1-eYFP and β-arrestin2-mVenus (Figure 2A,B), which suggest that GRK2/3 binding to the receptor precedes recruitment of β-arrestins, as observed for agonist-activated oxytocin and μ-opioid receptor.[22,31] In contrast, histamine and clobenpropit induced a more gradual decrease in BRET between H_4R-Rluc8 and GRK5-mVenus (Figure 2C), suggesting that GRK5 initially colocalizes with the receptor and dissociates upon receptor activation. Indeed, GRK5 contains a lipid-binding motif that targets GRK5 to the cell membrane and in close proximity to unstimulated GPCRs as observed for the β2-adrenergic receptor, bile acid receptor TGR5, and neurokinin-1 receptor.[32−35] Stimulation with substance P decreased the interaction between the neurokinin-1 receptor and GRK5 already before the receptor is internalized,[34] which might involve GRK5 autophosphorylation and interaction with Ca^{2+}-dependent calmodulin following receptor phosphorylation.[36] Also, GRK6 is primarily localized at the cell membrane and activation of the protease-activated receptor 2 by neutrophil elastase or trypsin has been shown to decrease the basal BRET between these proteins.[37] In line with this, histamine and clobenpropit induced only a very minor decrease in BRET between H_4R-Rluc8 and GRK6-mVenus. Clobenpropit acted as partial agonist in modulating the interaction between H_4R and GRK2, 3, and 5, in comparison to full agonist histamine (Figures 2A−E and S4). The potencies of histamine and clobenpropit to recruit GRK2 and GRK3 were comparable to their potencies to induced β-arrestin1/2 recruitment and receptor internalization, whereas ∼8-fold higher potencies were observed for GRK5 (Table 1). Stimulation with 10 μM JNJ7777120 induced a very small increase in BRET between H_4R-Rluc8 and GRK2-, GRK3-, GRK5-, and GRK6-mVenus (Figure 2A−D), while histamine-induced BRET changes between H_4R-Rluc8 and GRK2-, GRK3-, and GRK5-mVenus

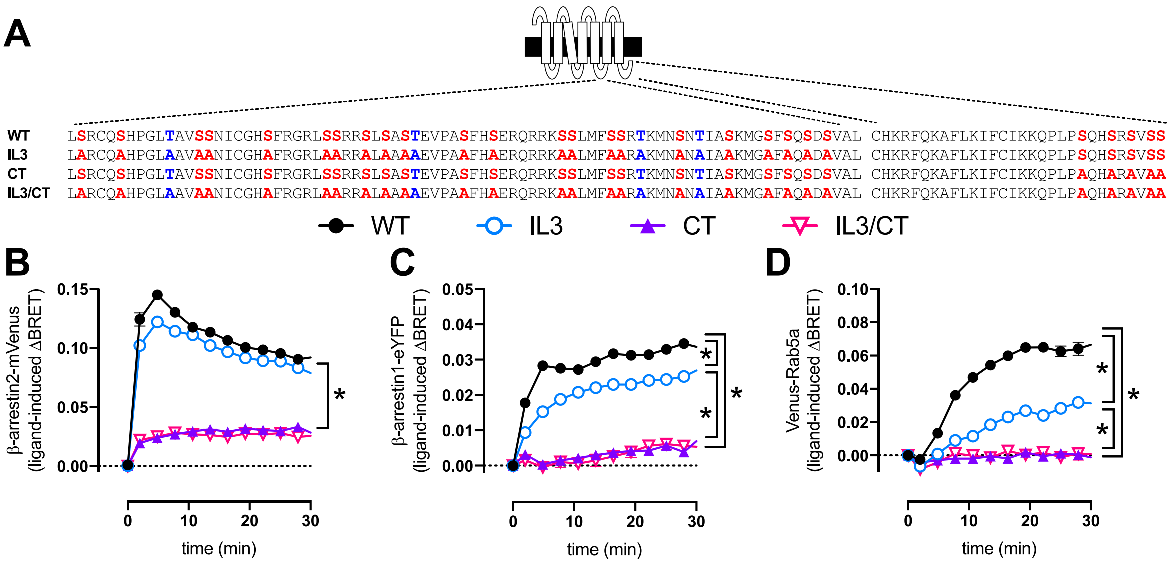


Figure 3. β-arrestin1/2 recruitment and internalization of IL3 and/or CT H_4R mutants. Serine and threonine residues (highlighted in red and blue, respectively) in the IL3 and/or CT were alanine-substituted in the H_4R-IL3, H_4R-CT, and H_4R-IL3/CT mutants (A). BRET measurements in HEK293T cells expressing H_4R-Rluc8 WT or mutants in combination with β-arrestin2-mVenus (B), β-arrestin1-eYFP (C), or Venus-Rab5a (D) in real time upon stimulation with 10 μM histamine. Data are shown as mean ± SEM from 3 independent experiments performed in triplicate. Ligand-induced BRET changes (ΔBRET) were calculated by subtracting BRET ratio of vehicle-treated cells. Statistical differences ($p < 0.05$) between WT and H_4R mutants were determined for the 30 min AUCs of the individual experiments using one-way ANOVA with Tukey's multiple comparison test and are indicated by an asterisk (*).

Table 2. Affinity (pK_D) and Potency (pEC_{50}) of Histamine for WT H_4R and Mutants in Which Serines and Threonines in IL3 and/or CT Are Alanine-Substituted[a]

	Rluc8 pK_D	β-arrestin1 pEC_{50}	β-arrestin2 pEC_{50}	HA-tag pK_D	CRE-luc pEC_{50}	pERK1/2 pEC_{50}	impedance pEC_{50}
WT	8.4 ± 0.1	7.0 ± 0.1	7.3 ± 0.1	8.4 ± 0.0	8.1 ± 0.1	7.5 ± 0.1	8.2 ± 0.2
IL3	8.5 ± 0.0	6.7 ± 0.1	7.2 ± 0.1	8.2 ± 0.1	8.1 ± 0.1	NA	NA
CT	8.5 ± 0.1	ND	7.1 ± 0.1	8.4 ± 0.0	7.9 ± 0.1	NA	NA
IL3/CT	8.2 ± 0.1	ND	7.5 ± 0.3	8.2 ± 0.0	7.5 ± 0.1	NA	NA

[a] Data are shown as mean ± SEM from at least 3 independent experiments that were performed in triplicate. Transiently expressed Rluc8-fused receptors in HEK293T cells were used for β-arrestin1/2 recruitment, whereas stably expressed HA-tagged receptor in HEK293 cells were used in CRE-driven luciferase reporter gene, pERK1/2, impedance assays. NA = not acquired, ND = not detectable.

could be antagonized by 50 μM JNJ7777120 (Figure 2F). These observations corroborate with the very limited effect of JNJ7777120 in BRET-based β-arrestin recruitment assays. As anticipated for an H_4R antagonist, thioperamide had no effect or induced at most a very minor decrease in BRET between H_4R and the GRK subtypes (Figure 2A–D).

Pretreatment with PTx did not affect the histamine-induced GRK2, GRK3, and GRK5 responses (Figure 2F), indicating that these processes are independent of $G\alpha_i$ protein activation as previously reported for GRK2 recruitment to the β_2-adrenergic and dopamine D_2 receptors.[24,38]

Serine and Threonine Residues in IL3 and CT Play Differential Roles in β-arrestin1/2 Recruitment, H_4R Internalization, and GRK Interactions. Crystal structures of active β-arrestin1 and visual arrestin in complex with a V_2 vasopressin receptor CT phosphopeptide and phosphorylated rhodopsin, respectively, revealed an ionic bonding network between a cluster of three negatively charged groups within the receptor CT (i.e., phosphorylated S^{357}, T^{360}, and S^{363} in V_2 vasopressin receptor, and phosphorylated T^{336} and S^{338} in combination with E^{341} in rhodopsin) with three positively charged pockets on the surface of the (β-)arrestin(1) N-domain.[39,40] Alanine-substitution of these serine and threonine residues impaired V_2 vasopressin receptor and rhodopsin to recruit β-arrestin1 and arrestin, respectively.[40] Moreover, agonist-induced phosphorylation of serine and threonine residues in IL3 has been observed by mass spectrometry for several GPCRs,[10,41−44] and found to be required for a stable interaction of β-arrestin2 with the M_1 muscarinic acetylcholine receptor.[45]

The H_4R harbors a cluster of 5 serine residues (i.e., ^{385}SRSVSS^{390}) at the distal end of its CT and 26 serine/threonine residues in its relatively long IL3 (Figure 3A). To investigate the contribution of these putative phosphosites in the interaction of H_4R with β-arrestins, we substituted alanine for all serine/threonine residues in IL3 and/or CT. The generated mutants H_4R-IL3-Rluc8, H_4R-CT-Rluc8, and H_4R-IL3/CT-Rluc8 displayed comparable binding affinities for [^{3}H]histamine (Figure S5A–D; Table 2) and did not affect the expression levels of β-arrestin2-mVenus, β-arrestin1-eYFP, Venus-Rab5a, GRK2-mVenus, GRK3-mVenus, GRK5-mVenus, and GRK6-mVenus, as compared to wildtype (WT) H_4R-Rluc8 (Figure S5G–M). The H_4R-CT-Rluc8 (B_{max} = 1.6 ± 0.1 pmol/mg) was expressed at comparable levels as WT H_4R-Rluc8 (B_{max} = 1.5 ± 0.1 pmol/mg) in transiently transfected HEK293T cells, whereas H_4R-IL3-Rluc8 (B_{max} = 0.3 ± 0.1 pmol/mg) and H_4R-IL3/CT-Rluc8 (B_{max} = 0.4 ± 0.0 pmol/mg) were expressed at 3.75- and 5-fold lower levels,

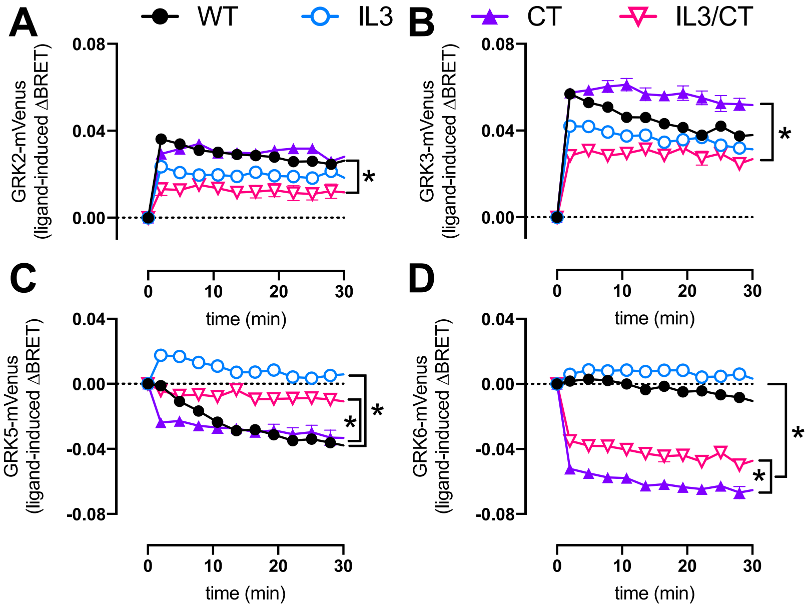


Figure 4. Interaction of IL3 and/or CT H_4R mutants with GRKs. BRET measurements in HEK293T cells expressing H_4R-Rluc8 WT or mutants in combination with GRK2-mVenus (A), GRK3-mVenus (B), GRK5-mVenus (C), or GRK6-mVenus (D) in real time upon stimulation with 10 μM histamine. Data are shown as mean ± SEM from at least 3 independent experiments performed in triplicate. Ligand-induced BRET changes (ΔBRET) were calculated by subtracting BRET ratio of vehicle-treated cells. Statistical differences ($p < 0.05$) between WT and H_4R mutants were determined for the 30 min AUCs of the individual experiments using one-way ANOVA with Tukey's multiple comparison test and are indicated by an asterisk (*).

respectively (Figure S5A–D). Alanine substitution of the CT serine cluster significantly reduced histamine-induced recruitment of β-arrestin2, whereas removal of all the serine/threonine residues in the IL3 had no significant effect (Figures 3B and S5E). This indicated that serine/threonine phosphorylation within the IL3 does not contribute to β-arrestin2 recruitment, whereas serines (and potentially phospho-serines) within the CT do contribute to β-arrestin2 recruitment. In contrast, serine/threonine residues within both the IL3 and CT contribute to β-arrestin1 recruitment, although the largest contribution is made by CT serines (Figures 3C and S5F). Consistent with a role for β-arrestin in receptor internalization, as suggested by siRNA knockdown of β-arrestin1/2 (vide supra), we observed the partial disruption of β-arrestin1 recruitment resulting from the removal of serine/threonine within the IL3, correlated with a partial disruption of internalization into early endosomes (Figure 3D). The CT serine-mutated receptor (where β-arrestin1/2 recruitment was abolished) showed no significant receptor internalization (Figure 3D).

Alanine substitution of all serine/threonine residues in either IL3 or CT did not affect GRK2 and GRK3 recruitment as compared to WT H_4R-Rluc8 (Figure 4A,B), whereas concurrent substitution of these putative phosphosites in IL3 and CT seemed to partially reduce the interaction with GRK2 and GRK3. These findings corroborate with the identification of basic residues in IL3 of the α_{2A}-adrenergic receptor as key drivers for both GRK2 binding and activation.[46] Indeed, the IL3 of H_4R harbors multiple arginine and lysine residues that might engage in such interaction (Figure 3A). In contrast, however, alanine substitution of all phosphosites in the proximal ^{354}TSST^{357} and/or more distal ^{370}TREHPSTANT^{379} clusters within the μ-opioid receptor CT reduced the maximal DAMGO-induced GRK2 and GRK3 recruitment in a β-galactosidase EFC-based assay.[22] Surprisingly, however, mutation of the ^{354}TSST^{357} cluster did not affect GRK2-Venus recruitment to μ-opioid receptor-Rluc8 in a BRET-based assay. H_4R-IL3-Rluc8 and H_4R-IL/CT-Rluc8 did not display a histamine-induced decrease in BRET with GRK5-mVenus as compared to WT H_4R-Rluc8 and H_4R-CT-Rluc8 (Figure 4C), suggesting that serines/threonines in IL3 may be involved in the interaction of H_4R with GRK5. Cross-linking and hydrogen−deuterium exchange mass spectrometry experiments have previously suggested that GRK5 interacts with IL3 of the β_2-adrenergic receptor, but these experiments did not reveal whether phosphosites were involved in this interaction.[47] Interestingly, alanine substitution of the distal serine cluster in the CT of H_4R (i.e., H_4R-CT-Rluc8 and H_4R-IL3/CT-Rluc8) reduced the BRET with GRK6-mVenus upon stimulation with histamine, whereas no histamine-induced response was observed for H_4R-Rluc8 and H_4R-IL3-Rluc8 (Figure 4D), suggesting that putative phosphoserines in the CT might hamper the basal interaction with GRK6. Hierarchical and sequential phosphorylation of multiple phosphor-acceptor sites by kinases has been observed for the CXCR4 chemokine receptor and μ-opioid receptor, whereas GRK2, 5, and 6 were shown to compete for angiotensin II receptor type 1 phosphorylation.[29,48,49]

Serine Cluster in CT Limits Duration of Signaling. We then evaluated the effect of removing all putative phosphosites in IL3 and/or CT on histamine-induced H_4R signaling. To this end, we generated clonal HEK293 cell lines that stably express HA-tagged WT H_4R, H_4R-IL3, or H_4R-IL3/CT at comparable levels on their cell surface, whereas cell surface expression of H_4R-CT was 2-fold lower as determined by enzyme-linked immunosorbent assay (ELISA, Figure 5A). Histamine inhibited forskolin-induced cAMP-driven CRE reporter gene activity with comparable potency in these HEK293 cells expressing WT H_4R, H_4R-IL3, H_4R-CT, or H_4R-IL3/CT, which is in agreement with the comparable binding affinity of

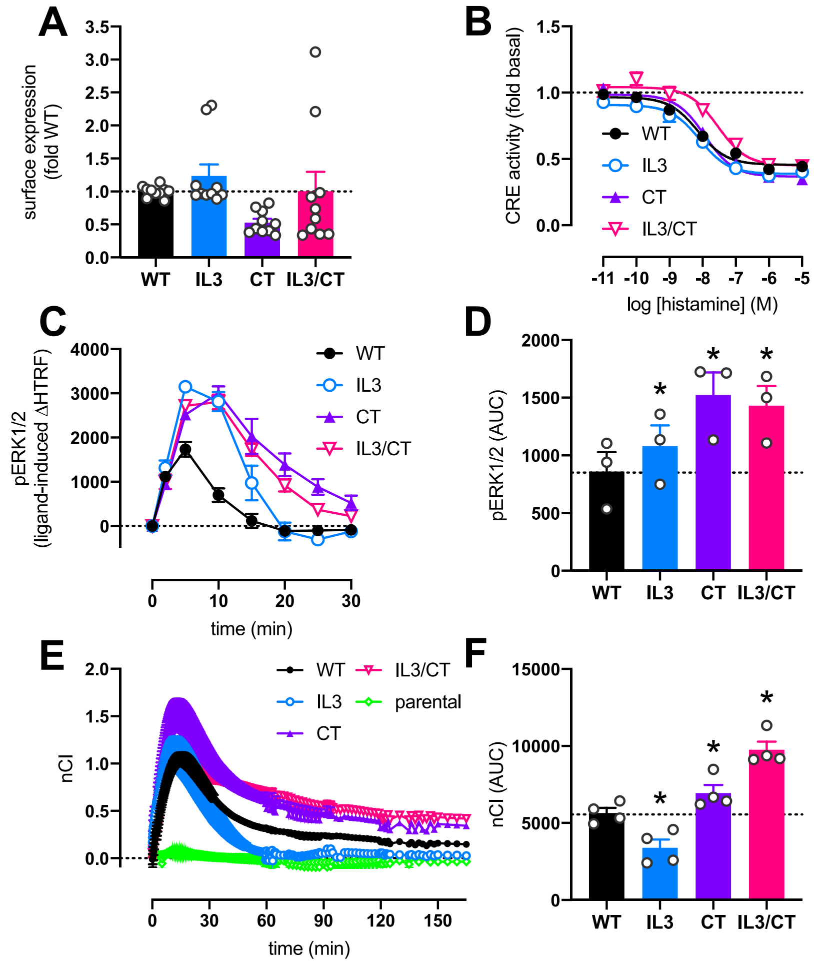


Figure 5. Signaling by IL3 and/or CT H_4R mutants. HA-tagged H_4R WT, H_4R-IL3, H_4R-CT, and H_4R-IL3/CT were stably expressed in HEK293 cells. (A) Expression of HA-tagged H_4R WT and mutants was measured at the cell surface using ELISA. Data is presented as fold over WT expression and shown as mean ± SEM from 4 independent experiments, with scatter plots showing individual data. (B) Inhibition of forskolin-induced CRE-driven reporter gene activity in response to stimulation with histamine for 6 h. Data are shown as mean ± SEM from 3 independent experiments in triplicate and expressed as fold over basal (C) Histamine-induced (100 μM) ERK1/2 phosphorylation was measured using an HTRF-based detection kit at indicated time points. Data points are presented as histamine-induced HTRF changes (ΔHTRF) by subtracting the HTRF ratio of vehicle-treated cells and expressed as mean ± SEM from 3 independent experiments performed in duplicate. (D) Duration of histamine-induced changes in ERK1/2 phosphorylation by HA-H_4R WT and mutants was quantified as the AUC after conversion of the data to percentages of their corresponding maximum responses. Grouped AUC is shown as mean ± SEM from 3 independent experiments, with scatter plots showing the AUC from individual experiments. (E) Data points are presented as histamine-induced (10 μM) cellular impedance changes (nCI) by subtracting the cellular impedance that is observed directly before stimulation for each sample and expressed as mean ± SEM from 4 independent experiments performed in duplicate. (F) Duration of histamine-induced changes in cellular impedance by HA-H_4R WT and mutants was quantified as the AUC after conversion of the data to percentages of their corresponding maximum responses. Grouped AUC is shown as mean ± SEM from 4 independent experiments, with scatter plots showing the AUC from individual experiments. Statistical differences ($p < 0.05$) compared to HA-H_4R WT were determined using two-way ANOVA with Dunnett's multiple comparison test and are indicated by an asterisk (*).

[^{3}H]histamine for WT and these H_4R mutants (Figure 5B; Table 2). In addition, the three H_4R mutants inhibited the forskolin-induced CRE activity to the same extent as WT H_4R, indicating that G_i protein signaling upon 6 h of stimulation with histamine was not affected by alanine substitution of serines and threonines in the IL3 and/or CT (Figure 5B).

To evaluate whether these mutations affect the duration of G protein-mediated H_4R signaling, the phosphorylation of ERK1/2 and whole-cell impedance responses were measured over time in response to histamine stimulation. Histamine induced a transient increase in ERK1/2 phosphorylation in WT H_4R-expressing cells that peaked after 5 min and decreased to basal levels in the subsequent 15 min (Figure 5C), as previously reported using Western blot analysis.[6,50] Histamine had a 4-fold lower potency to stimulate pERK1/2 as compared to its effect on cAMP-driven reporter gene activity (Figure S6A; Table 2) and could be fully antagonized by the specific H_4R inverse agonist VUF10558 (Figure S6B). H_4R signaling to pEKR1/2 is G_i protein dependent as revealed by the inhibition of both basal and histamine-induced pERK1/2 upon PTx pretreatment (Figure S6B), confirming previously reported observations.[50]

Histamine induced an approximately 2-fold higher maximal pERK1/2 levels in cells expressing the mutants H_4R-IL3, H_4R-CT, or H_4R-IL3/CT, as compared to WT H_4R (Figure 5C). Also the duration of the histamine-induced pERK1/2 activation was significantly sustained in the case of the H_4R-CT and H_4R-IL3/CT mutants, as determined by analysis of their AUC (Figure 4D).

In a whole-cell analysis of H_4R response, histamine was seen to transiently increased cellular impedance to a peak response within approximately 15 min, followed by a sustained phase that returns to near-basal levels within 2–3 h (Figure 5E). The potency of histamine in this global impedance response was comparable to the CRE-reporter gene assay and 4-fold higher than ERK1/2 activation by the WT H_4R (Figure S6C,D; Table 2), whereas no histamine-induced change in cellular impedance was observed in parental HEK293 cells (Figure 5E). H_4R-CT induced a higher impedance response than did WT H_4R upon stimulation with histamine, whereas a comparable maximal impedance response was observed in cells expressing H_4R-IL3 or H_4R-IL3/CT (Figure 5E). Alanine substitution of the CT serine cluster resulted in a slower decrease of the maximal peak response to basal over time as compared with WT H_4R (Figure 5E), which was significantly further delayed by concomitant mutation of serines and threonines in IL3 (Figure 5F). These data corroborate with the prolonged histamine-induced ERK1/2 phosphorylation by H_4R-CT and H_4R-IL3/CT as compared to WT H_4R. Surprisingly, however, the histamine-induced impedance response returned much faster to basal levels, without a second descending shoulder phase (Figure 5E), in cells expressing H_4R-IL3 as compared to those expressing WT H_4R (Figure 5F).

Hence, the serine cluster in the H_4R CT is important for the recruitment of β-arrestin1 and 2. Mutation of this cluster impaired H_4R internalization, while enhancing and elongating ERK1/2 phosphorylation and cellular impedance responses, suggesting that bound β-arrestin mediates H_4R internalization and receptor desensitization. Similarly, decreased internalization in combination with enhanced ERK1/2 phosphorylation was observed for agonist-activated parathyroid hormone receptor 1,[51] free fatty acid receptor FFA4,[12,52] and β2-adrenergic receptor[53] upon reducing their ability to interact with β-arrestins by mutation of putative phosphorylation sites in their CT or decreasing β-arrestin levels using siRNA. In contrast, removal of putative phosphosites in IL3 of the H_4R had no significant effect on the recruitment of β-arrestin2, while partially reducing β-arrestin1 recruitment and receptor internalization. Although maximal ERK1/2 phosphorylation was slightly increased as compared to WT H_4R, which might be in part related to the somewhat higher cell surface expression of H_4R-IL3, the duration of ERK1/2 was not significantly affected, whereas the duration of cellular impedance was even decreased. These data suggest that putative phosphosite-mediated interactions between IL3 and β-arrestins are not involved in desensitization of G protein signaling, while interaction of this domain with β-arrestin1 is important to mediate receptor internalization. Although, β-arrestin2 recruitment to H_4R-IL3 was not affected, we cannot exclude the possibility that β-arrestin2 is not fully engaged to the receptor core domain and consequently unable to support internalization. Likewise, alanine substitution of 15 serines in IL3 of the M_3 muscarinic acetylcholine receptor (M_3R) reduced β-arrestin2 recruitment and receptor internalization upon agonist stimulation but did not affect M_3R desensitization.[54] Phosphorylation of an N- and C-terminal clusters serines/threonines in IL3 of M_2 muscarinic acetylcholine receptor (M_2R) was required for β-arrestin-mediated internalization, whereas only the C-terminal cluster was involved in receptor desensitization.[55,56] Similarly, phosphorylation of at least two out of four adjacent serines in the IL3 of the α_{2A} adrenergic receptor is required for receptor desensitization.[57] The diversity in length of IL3 (80 ± 57 amino acids; mean ± SD) in combination with lack of sequence conservation among aminergic GPCRs (i.e., histamine, muscarinic acetylcholine; adrenergic, dopamine, and serotonin receptors) might explain the differences in the role of putative phosphosites in these regulatory processes. Moreover, other class A GPCRs (i.e., protein, peptide, lipid, and nucleotide receptors) have shorter IL3s (9.6 ± 16 amino acids; mean ± SD). Mutation of three putative phosphosites in the relatively short IL3 of the nucleotide receptor P2Y1 did not affect ADP-induced β-arrestin2 recruitment and internalization,[58] whereas mutation of two phosphosites in the short IL3 of somatostatin receptor 5 reduced both β-arrestin2 recruitment and receptor internalization upon somatostatin stimulation.[59] The very short IL3 loop of rhodopsin interacts in the crystal structure with the β-strand VI and the back loop of active visual arrestin, which form a groove together with the C-loop, the loop between β-strands VII and VIII, the 160-loop, and the loop between β-strand VI and helix I.[60] This groove is structurally conserved in both β-arrestin1 and 2 and might indeed accommodate part of a longer IL3. However, how putative phosphosites in the long H_4R IL3 affect interaction and subsequent conformation of β-arrestins remains to be investigated, as well as how this interaction translates into the differential effect on β-arrestin1/2 recruitment. Indeed, differential conformations and effects of receptor bound β-arrestin1/2 have been recently described.[61]

Signaling of histamine H_1, H_2, and H_3 receptor subtypes (H_1R, H_2R, and H_3R) is regulated by GRK2, while GRK3 was also reported to be involved in desensitization of H_2R and H_3R.[62−64] GRK5 and GRK6 do not affect H_1R and H_2R signaling[62,64] and have to the best of our knowledge not been experimentally assessed for the H_3R. However, considering the variation in length, sequence, and number of putative phosphosites for both IL3 and CT within histamine receptor subfamily, regulation of receptor signaling, and trafficking by GRKs is likely to occur via distinct phosphorylation profiles.

In conclusion, our findings highlight that putative phosphosites in IL3 and CT differently affect the interaction with GRKs and β-arrestins, and consequently, H_4R desensitization and internalization in transfected HEK293(T) cells. It remains to be addressed in future research which of these 31 putative phosphorylation sites contributes (individually or in combination) to the differential interactions with GRKs and β-arrestins. However, considering that H_4R expression is downregulated in multiple tumors in comparison to healthy tissues[5] while GRK subtypes are differently up- or downregulated in tumors,[65] identification of which putative phosphosites are actually phosphorylated in diseased cells in relation to GRK expression levels might be assessed first. In combination with CRISPR/Cas9 genomic editing to individually deplete GRK subtypes, these phosphorylation profiles might provide valuable information to evaluate their regulatory effects on H_4R desensitization and internalization in cancers and guide future site-directed mutagenesis studies. Various H_4R agonists have been shown to attenuate tumor growth and neuropathic pain,[4,5] and identification of biased ligands that induce H_4R signaling without further receptor downregulation might potentially increase their therapeutic efficacy.[17]

■ METHODS

Materials. Poly-L-lysine, PTx, forskolin, and histamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other

H_4R ligands were synthesized in the Medicinal Chemistry Department of the Vrije Universiteit Amsterdam (Amsterdam, The Netherlands).[17] Cmpd101 was obtained from Tocris Bioscience (Bristol, UK). Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salt solution (HBSS), BCA protein assay kit, On-target plus β-arrestin1, β-arrestin2, and control siRNA were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals were of analytical grade and purchased from standard commercial suppliers.

DNA Constructs. HA-H_4R, H_4R-Rluc8, β-arrestin2-mVenus, and β-arrestin1-eYFP constructs in pcDEF3 expression plasmid have been previously described.[7,66,67] Alanine substitution of all serine and threonine residues in IL3 and/or CT were introduced by DNA synthesis followed by subcloning using internal *Eco*RI and *Ppu*MI restriction sites or by PCR-based site-directed mutagenesis, respectively. Mutant receptors were either HA-tagged at their N-terminus or fused with Rluc8 at their C-tail. DNA encoding bovine GRK2 and GRK3 and human GRK5 and GRK6 were kindly provided by Dr. S. Cotecchia (Laussane, Switzerland)[68] and genetically fused with mVenus in pcDEF3, as previously described.[7] All generated constructs were verified by DNA sequencing. The CRE-driven luciferase reporter gene plasmid pTLNC-21CRE[69] and DNA encoding for Venus-Rab5a[70] and dominant-negative dynamin $K^{44}A$[26] were kindly provided by Dr. W. Born (Denver, CO, USA), Dr. N. Lambert (Augusta, GA, USA), and Dr. C van Koppen (Essen, Germany), respectively.

Cell Culture and Transfection. HEK293 and HEK293T cells (ATCC; Manassas, VA, USA) were cultured in DMEM supplemented with 10% fetal bovine serum (Bodinco; Alkmaar, The Netherlands), 50 IU mL^{-1} penicillin, and 50 mg mL^{-1} streptomycin (GE healthcare; Uppsala, Sweden) at 37 °C with 5% CO_2. Cells were transfected with indicated amounts of DNA plasmids per 10 cm dish using 25 kDa linear polyethylenimine (Polysciences; Warrington, PA, USA), as previously described.[7] Total DNA amounts were kept equal by adding an empty pcDEF3 plasmid. HEK293T cells were transfected with β-arrestin1 and 2 siRNA (1:1) or scrambled siRNA using Lipofectamine 2000 (Invitrogen; Paisley, UK), as previously described.[67] Monoclonal stable HEK293 cell lines expressing WT or mutant HA-H_4R were selected and maintained in the presence of 400 ng μL^{-1} G418 (Biovision; San Fransico, CA, USA).

Radioligand Binding. Cell homogenates were prepared 2 days after transfection of HEK293T cells with 1 μg of HA-H_4R or 0.5 μg of WT or mutant H_4R-Rluc8 DNA, or from HEK293 cells that stably express WT or mutant HA-H_4R, as previously described.[17] Binding of $[^3H]$histamine (PerkinElmer; Waltham, MA, USA) to these homogenates was measured in 50 mM Tris-HCl buffer (pH7.4) for 2 h at 25 °C, as previously described.[17] Nonspecific binding was determined in the presence of 50 μM JNJ7777120.

BRET Assays. HEK293T cells were transfected with 0.5 μg of WT or mutant H_4R-Rluc8 in combination with 4 μg of β-arrestin1-eYFP, β-arrestin2-mVenus, or GRK2/3/5/6-mVenus DNA, or 2 μg of Venus-Rab5a DNA. At 24 h post-transfection, cells were transferred to poly-L-lysine-coated white 96-well plates (Greiner Bio-one; Frickenhausen, Germany). At 48 h post-transfection, baseline BRET was measured using 5 μM coelenterazine-H (Promega; Madison, WI, USA) in HBSS in a Mithras LB940 multilabel plate reader (Berthold Technologies; Bad Wildbad, Germany) followed by stimulation with H_4R ligands at 37 °C, as previously described.[67] The BRET ratio was calculated by dividing acceptor light emission at 540 nm by Rluc8 light emission at 480 nm.

CRE Reporter Gene Assay. HEK293T cells transiently transfected with 1 μg of HA-H_4R or H_4R-Rluc8, and HEK293 cells stably expressing WT or mutant HA-H_4R were cotransfected with 2.5 μg of pTLNC-21CRE plasmid. At 24 h post-transfection, cells were transferred to poly-L-lysine-coated white 96-well plates. At 48 h post-transfection, cells were stimulated with histamine in serum-free DMEM supplemented with 1 μM forskolin for 6 h, and reporter gene activity was measured using luciferase assay reagent containing D-luciferin (Promega) in a Mithras LB940 multilabel plate reader, as previously described.[17]

Western Blot. Transfected cells were cultured in 6-well plates. At 48 h post-transfection, cells were lysed in RIPA buffer supplemented with 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and 1× cOmplete protease inhibitor cocktail (Roche Diagnostics; Mannheim, Germany) for 20 min on ice, sonicated for 5 s, and centrifuged at 20 800*g* for 10 min at 4 °C. SDS-PAGE and immunoblot analysis using 1:1000 anti-β-arrestin1/2 clone D24H9 or 1:1000 anti-STAT3 clone 79D7 primary antibodies (Cell Signaling Technology; Danvers, MA, USA), and 1:5000 horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories; Hercules, CA, USA) were performed as previously described,[67] except that samples were incubated at 95 °C for 5 min before being subjected to SDS-PAGE.

ELISA. Expression of WT and mutant HA-H_4R on the surface of intact HEK293 cells was detected by ELISA using 1:800 anti-HA high-affinity clone 3F10 primary antibody (Sigma-Aldrich) and 1:5000 horseradish peroxidase-conjugated goat anti-rat secondary antibody (Thermo Fisher Scientific), as previously described.[67] Peroxidase activity was measured using 3,3′5,5′-tetramethylbenzidine liquid substrate system (Abcam; Cambridge, UK) on a Victor3 1420 multilabel plate reader (PerkinElmer) at 450 nm.

ERK1/2 Phosphorylation Assay. Histamine-induced ERK1/2 phosphorylation was measured in HEK293 cells that stably express WT or mutant HA-H_4R using a homogeneous time-resolved fluorescence (HTRF) phospho-ERK (Thr202/Tyr204) kit (Cisbio; Codolet, France), according to manufacturer's instructions. HTRF ratios were detected at 620 and 665 nm in PHERAstar FS (BMG Labtech; Ortenberg, Germany) upon excitation at 337 nm.

Cellular Impedance. Histamine-induced changes in morphology of HEK293 cells that stably express WT or mutant HA-H_4R were measured as impedance of electron flow in a poly-L-lysine-coated E-plate VIEW 96 PET using the xCELLigence RTCA-SP system (ACEA Biosciences; San Diego, CA, USA). The first baseline was measured using 45 μL of cell culture medium per well. Next, 5 × 10^4 cells were added per well in 50 μL of culture medium and equilibrated at 24 °C for 30 min before inserting the E-plate into the xCELLigence system to monitor impedance at 37 °C and 5% CO_2. After 18 h, 5 μL of prewarmed vehicle or histamine in DMEM was added to the cells, and impedance was continuously recorded at 15 s intervals. The impedance signal was converted to cell index (CI) and normalized to the CI values directly before ligand addition for each well using the RTCA software 1.2.1, followed by subtraction of the vehicle control at each time point.

Data Analysis. GraphPad Prism 8 was used for analysis of pharmacological data by nonlinear regression and statistics. Statistical difference was accepted if $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.0c00008.

> Pharmacological characterization of H_4R-Rluc8 fusion protein and H_4R mutants, JNJ7777120, and thioperamide anatagonize histamine-induced β-arrestin2 recruitment to H_4R, β-arrestin1/2 expression upon siRNA transfection, concentration response curves of clobenpropit on BRET between H_4R and GRKs, expression of β-arrestin2-mVenus, β-arrestin1-eYFP, GRKx-mVenus, and Venus-Rab5a in cells coexpressing WT H_4R-Rluc8 or mutants, and concentration response curves of histamine on H_4R-mediated ERK1/2 activation and cellular impedance (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Henry F. Vischer − *Division of Medicinal Chemistry, Amsterdam Institute for Molecules, Medicines and Systems, Faculty of Science, Vrije Universiteit Amsterdam, 1081 HZ Amsterdam, The Netherlands;* orcid.org/0000-0002-0184-6337; Email: H.F.Vischer@vu.nl

Authors

Eléonore W. E. Verweij − *Division of Medicinal Chemistry, Amsterdam Institute for Molecules, Medicines and Systems, Faculty of Science, Vrije Universiteit Amsterdam, 1081 HZ Amsterdam, The Netherlands*

Betty Al Araaj − *Division of Medicinal Chemistry, Amsterdam Institute for Molecules, Medicines and Systems, Faculty of Science, Vrije Universiteit Amsterdam, 1081 HZ Amsterdam, The Netherlands*

Wimzy R. Prabhata − *Division of Medicinal Chemistry, Amsterdam Institute for Molecules, Medicines and Systems, Faculty of Science, Vrije Universiteit Amsterdam, 1081 HZ Amsterdam, The Netherlands*

Rudi Prihandoko − *Centre for Translational Pharmacology, Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom*

Saskia Nijmeijer − *Division of Medicinal Chemistry, Amsterdam Institute for Molecules, Medicines and Systems, Faculty of Science, Vrije Universiteit Amsterdam, 1081 HZ Amsterdam, The Netherlands;* orcid.org/0000-0001-8037-4659

Andrew B. Tobin − *Centre for Translational Pharmacology, Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom;* orcid.org/0000-0002-1807-3123

Rob Leurs − *Division of Medicinal Chemistry, Amsterdam Institute for Molecules, Medicines and Systems, Faculty of Science, Vrije Universiteit Amsterdam, 1081 HZ Amsterdam, The Netherlands;* orcid.org/0000-0003-1354-2848

Complete contact information is available at: https://pubs.acs.org/10.1021/acsptsci.0c00008

Author Contributions

E.W.E.V., S.N., A.B.T., R.L., and H.F.V. participated in research design. E.W.E.V., B.A.A., W.R.P., R.P. S.N., and H.F.V. conducted experiments and performed data analysis. E.W.E.V., A.B.T., R.L., and H.F.V. contributed to writing of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by The Netherlands Organisation for Scientific Research (NWO) ECHO project 711.013.014 (R.L. and H.F.V).

■ ABBREVIATIONS

AUC, area under the curve; BRET, bioluminescence resonance energy transfer; CI, cell index; CRE, cAMP-responsive element; CT, C-tail; EFC, enzyme-fragment complementation; ERK1/2, extracellular-signal-regulated kinase 1/2; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; H_4R, histamine H_4 receptor; HTRF, homogeneous time-resolved fluorescence; IL3, intracellular loop 3; PTx, pertussis toxin; SD, standard deviation; SEM, standard error of the mean; WT, wildtype

■ REFERENCES

(1) Panula, P., Chazot, P. L., Cowart, M., Gutzmer, R., Leurs, R., Liu, W. L. S., Stark, H., Thurmond, R. L., and Haas, H. L. (2015) International Union of Basic and Clinical Pharmacology. XCVIII. Histamine Receptors. *Pharmacol. Rev. 67*, 601−655.

(2) Thurmond, R. L., Venable, J., Savall, B., La, D., Snook, S., Dunford, P. J., and Edwards, J. P. (2017) Clinical Development of Histamine H_4 Receptor Antagonists. *Handb. Exp. Pharmacol. 241*, 301−320.

(3) Sanna, M. D., Mello, T., Masini, E., and Galeotti, N. (2018) Activation of ERK/CREB pathway in noradrenergic neurons contributes to hypernociceptive phenotype in H4 receptor knockout mice after nerve injury. *Neuropharmacology 128*, 340−350.

(4) Sanna, M. D., Lucarini, L., Durante, M., Ghelardini, C., Masini, E., and Galeotti, N. (2017) Histamine H_4 receptor agonist-induced relief from painful peripheral neuropathy is mediated by inhibition of spinal neuroinflammation and oxidative stress. *Br. J. Pharmacol. 174*, 28−40.

(5) Nicoud, M. B., Formoso, K., and Medina, V. A. (2019) Pathophysiological Role of Histamine H_4 Receptor in Cancer: Therapeutic Implications. *Front. Pharmacol. 10*, 556.

(6) Rosethorne, E. M., and Charlton, S. J. (2011) Agonist-Biased Signaling at the Histamine H_4 Receptor: JNJ7777120 Recruits β-Arrestin without Activating G Proteins. *Mol. Pharmacol. 79*, 749−757.

(7) Nijmeijer, S., Engelhardt, H., Schultes, S., van de Stolpe, A. C., Lusink, V., de Graaf, C., Wijtmans, M., Haaksma, E. E. J., de Esch, I. J. P., Stachurski, K., Vischer, H. F., and Leurs, R. (2013) Design and pharmacological characterization of VUF14480, a covalent partial agonist that interacts with cysteine 98(3.36) of the human histamine H_4 receptor. *Br. J. Pharmacol. 170*, 89−100.

(8) Peterson, Y. K., and Luttrell, L. M. (2017) The Diverse Roles of Arrestin Scaffolds in G Protein-Coupled Receptor Signaling. *Pharmacol. Rev. 69*, 256−297.

(9) Shenoy, S. K., and Lefkowitz, R. J. (2011) β-arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol. Sci. 32*, 521−533.

(10) Butcher, A. J., Prihandoko, R., Kong, K. C., McWilliams, P., Edwards, J. M., Bottrill, A., Mistry, S., and Tobin, A. B. (2011) Differential G-protein-coupled receptor phosphorylation provides evidence for a signaling bar code. *J. Biol. Chem. 286*, 11506−11518.

(11) Nobles, K. N., Xiao, K., Ahn, S., Shukla, A. K., Lam, C. M., Rajagopal, S., Strachan, R. T., Huang, T.-Y., Bressler, E. A., Hara, M. R., Shenoy, S. K., Gygi, S. P., and Lefkowitz, R. J. (2011) Distinct phosphorylation sites on the β_2-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Sci. Signaling 4*, No. ra51.

(12) Prihandoko, R., Alvarez-Curto, E., Hudson, B. D., Butcher, A. J., Ulven, T., Miller, A. M., Tobin, A. B., and Milligan, G. (2016) Distinct Phosphorylation Clusters Determine the Signaling Outcome of Free Fatty Acid Receptor 4/G Protein-Coupled Receptor 120. *Mol. Pharmacol. 89*, 505−520.

(13) Yang, Z., Yang, F., Zhang, D., Liu, Z., Lin, A., Liu, C., Xiao, P., Yu, X., and Sun, J.-P. (2017) Phosphorylation of G Protein-Coupled Receptors: From the Barcode Hypothesis to the Flute Model. *Mol. Pharmacol. 92*, 201−210.

(14) Mayer, D., Damberger, F. F., Samarasimhareddy, M., Feldmueller, M., Vuckovic, Z., Flock, T., Bauer, B., Mutt, E., Zosel, F., Allain, F. H. T., Standfuss, J., Schertler, G. F. X., Deupi, X., Sommer, M. E., Hurevich, M., Friedler, A., and Veprintsev, D. B. (2019) Distinct G protein-coupled receptor phosphorylation motifs modulate arrestin affinity and activation and global conformation. *Nat. Commun. 10*, 1261.

(15) Sente, A., Peer, R., Srivastava, A., Baidya, M., Lesk, A. M., Balaji, S., Shukla, A. K., Babu, M. M., and Flock, T. (2018) Molecular mechanism of modulating arrestin conformation by GPCR phosphorylation. *Nat. Struct. Mol. Biol. 25*, 538−545.

(16) Lombardi, M. S., Kavelaars, A., Cobelens, P. M., Schmidt, R. E., Schedlowski, M., and Heijnen, C. J. (2001) Adjuvant arthritis induces down-regulation of G protein-coupled receptor kinases in the immune system. *J. Immunol. 166*, 1635−1640.

(17) Nijmeijer, S., Vischer, H. F., Rosethorne, E. M., Charlton, S. J., and Leurs, R. (2012) Analysis of Multiple Histamine H_4 Receptor Compound Classes Uncovers $G\alpha_i$ Protein- and β-Arrestin2-Biased Ligands. *Mol. Pharmacol. 82*, 1174−1182.

(18) Nijmeijer, S., Vischer, H. F., Sirci, F., Schultes, S., Engelhardt, H., de Graaf, C., Rosethorne, E. M., Charlton, S. J., and Leurs, R. (2013) Detailed analysis of biased histamine H_4 receptor signalling by JNJ 7777120 analogues. *Br. J. Pharmacol. 170*, 78−88.

(19) Lim, H. D., van Rijn, R. M., Ling, P., Bakker, R. A., Thurmond, R. L., and Leurs, R. (2005) Evaluation of histamine H_1-, H_2-, and H_3-receptor ligands at the human histamine H_4 receptor: identification of 4-methylhistamine as the first potent and selective H_4 receptor agonist. *J. Pharmacol. Exp. Ther. 314*, 1310−1321.

(20) Smits, R. A., Lim, H. D., van der Meer, T., Kuhne, S., Bessembinder, K., Zuiderveld, O. P., Wijtmans, M., de Esch, I. J. P., and Leurs, R. (2012) Ligand based design of novel histamine H_4 receptor antagonists; fragment optimization and analysis of binding kinetics. *Bioorg. Med. Chem. Lett. 22*, 461−467.

(21) Nguyen, T., Shapiro, D. A., George, S. R., Setola, V., Lee, D. K., Cheng, R., Rauser, L., Lee, S. P., Lynch, K. R., Roth, B. L., and O'Dowd, B. F. (2001) Discovery of a novel member of the histamine receptor family. *Mol. Pharmacol. 59*, 427−433.

(22) Miess, E., Gondin, A. B., Yousuf, A., Steinborn, R., Mösslein, N., Yang, Y., Göldner, M., Ruland, J. G., Bünemann, M., Krasel, C., Christie, M. J., Halls, M. L., Schulz, S., and Canals, M. (2018) Multisite phosphorylation is required for sustained interaction with GRKs and arrestins during rapid -opioid receptor desensitization. *Sci. Signaling 11*, No. eaas9609.

(23) Lowe, J. D., Sanderson, H. S., Cooke, A. E., Ostovar, M., Tsisanova, E., Withey, S. L., Chavkin, C., Husbands, S. M., Kelly, E., Henderson, G., and Bailey, C. P. (2015) Role of G Protein-Coupled Receptor Kinases 2 and 3 in μ-Opioid Receptor Desensitization and Internalization. *Mol. Pharmacol. 88*, 347−356.

(24) Pack, T. F., Orlen, M. I., Ray, C., Peterson, S. M., and Caron, M. G. (2018) The dopamine D2 receptor can directly recruit and activate GRK2 without G protein activation. *J. Biol. Chem. 293*, 6161−6171.

(25) Shintani, Y., Hayata-Takano, A., Moriguchi, K., Nakazawa, T., Ago, Y., Kasai, A., Seiriki, K., Shintani, N., and Hashimoto, H. (2018) β-Arrestin1 and 2 differentially regulate PACAP-induced PAC1 receptor signaling and trafficking. *PLoS One 13*, No. e0196946.

(26) Vögler, O., Bogatkewitsch, G. S., Wriske, C., Krummenerl, P., Jakobs, K. H., and van Koppen, C. J. (1998) Receptor subtype-specific regulation of muscarinic acetylcholine receptor sequestration by dynamin. Distinct sequestration of m2 receptors. *J. Biol. Chem. 273*, 12155−12160.

(27) Busillo, J. M., Armando, S., Sengupta, R., Meucci, O., Bouvier, M., and Benovic, J. L. (2010) Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling. *J. Biol. Chem. 285*, 7805−7817.

(28) Doll, C., Pöll, F., Peuker, K., Loktev, A., Glück, L., and Schulz, S. (2012) Deciphering μ-opioid receptor phosphorylation and dephosphorylation in HEK293 cells. *Br. J. Pharmacol. 167*, 1259−1270.

(29) Just, S., Illing, S., Trester-Zedlitz, M., Lau, E. K., Kotowski, S. J., Miess, E., Mann, A., Doll, C., Trinidad, J. C., Burlingame, A. L., von Zastrow, M., and Schulz, S. (2013) Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. *Mol. Pharmacol. 83*, 633−639.

(30) Luo, J., Busillo, J. M., Stumm, R., and Benovic, J. L. (2017) G Protein-Coupled Receptor Kinase 3 and Protein Kinase C Phosphorylate the Distal C-Terminal Tail of the Chemokine Receptor CXCR4 and Mediate Recruitment of β-Arrestin. *Mol. Pharmacol. 91*, 554−566.

(31) Hasbi, A., Devost, D., Laporte, S. A., and Zingg, H. H. (2004) Real-time detection of interactions between the human oxytocin receptor and G protein-coupled receptor kinase-2. *Mol. Endocrinol. 18*, 1277−1286.

(32) Thiyagarajan, M. M., Stracquatanio, R. P., Pronin, A. N., Evanko, D. S., Benovic, J. L., and Wedegaertner, P. B. (2004) A predicted amphipathic helix mediates plasma membrane localization of GRK5. *J. Biol. Chem. 279*, 17989−17995.

(33) Tran, T. M., Jorgensen, R., and Clark, R. B. (2007) Phosphorylation of the β_2-adrenergic receptor in plasma membranes by intrinsic GRK5. *Biochemistry 46*, 14438−14449.

(34) Jorgensen, R., Holliday, N. D., Hansen, J. L., Vrecl, M., Heding, A., Schwartz, T. W., and Elling, C. E. (2008) Characterization of G-protein coupled receptor kinase interaction with the neurokinin-1 receptor using bioluminescence resonance energy transfer. *Mol. Pharmacol. 73*, 349−358.

(35) Jensen, D. D., Godfrey, C. B., Niklas, C., Canals, M., Kocan, M., Poole, D. P., Murphy, J. E., Alemi, F., Cottrell, G. S., Korbmacher, C., Lambert, N. A., Bunnett, N. W., and Corvera, C. U. (2013) The bile acid receptor TGR5 does not interact with β-arrestins or traffic to endosomes but transmits sustained signals from plasma membrane rafts. *J. Biol. Chem. 288*, 22942−22960.

(36) Komolov, K. E., Bhardwaj, A., and Benovic, J. L. (2015) Atomic structure of GRK5 reveals distinct structural features novel for G protein-coupled receptor kinases. *J. Biol. Chem. 290*, 20629−20647.

(37) Zhao, P., Lieu, T. M., Barlow, N., Sostegni, S., Haerteis, S., Korbmacher, C., Liedtke, W., Jimenez-Vargas, N. N., Vanner, S. J., and Bunnett, N. W. (2015) Neutrophil elastase activates protease-activated receptor-2 (PAR2) and Transient Receptor Potential Vanilloid 4 (TRPV4) to cause inflammation and pain. *J. Biol. Chem. 290*, 13875−13887.

(38) Vasudevan, N. T., Mohan, M. L., Gupta, M. K., Martelli, E. E., Hussain, A. K., Qin, Y., Chandrasekharan, U. M., Young, D., Feldman, A. M., Sen, S., Dorn, G. W., DiCorleto, P. E., and Prasad, S. V. N. (2013) $G_{\beta\gamma}$-independent recruitment of G-protein coupled receptor kinase 2 drives tumor necrosis factor α-induced cardiac β-adrenergic receptor dysfunction. *Circulation 128*, 377−387.

(39) Shukla, A. K., Manglik, A., Kruse, A. C., Xiao, K., Reis, R. I., Tseng, W.-C., Staus, D. P., Hilger, D., Uysal, S., Huang, L.-Y., Paduch, M., Tripathi-Shukla, P., Koide, A., Koide, S., Weis, W. I., Kossiakoff, A. A., Kobilka, B. K., and Lefkowitz, R. J. (2013) Structure of active β-arrestin-1 bound to a G-protein-coupled receptor phosphopeptide. *Nature 497*, 137−141.

(40) Zhou, X. E., He, Y., de Waal, P. W., Gao, X., Kang, Y., Van Eps, N., Yin, Y., Pal, K., Goswami, D., White, T. A., Barty, A., Latorraca, N. R., Chapman, H. N., Hubbell, W. L., Dror, R. O., Stevens, R. C., Cherezov, V., Gurevich, V. V., Griffin, P. R., Ernst, O. P., Melcher, K., and Xu, H. E. (2017) Identification of Phosphorylation Codes for Arrestin Recruitment by G Protein-Coupled Receptors. *Cell 170*, 457−469.

(41) Butcher, A. J., Bradley, S. J., Prihandoko, R., Brooke, S. M., Mogg, A., Bourgognon, J.-M., Macedo-Hatch, T., Edwards, J. M., Bottrill, A. R., Challiss, R. A. J., Broad, L. M., Felder, C. C., and Tobin, A. B. (2016) An antibody biosensor establishes the activation of the M_1 muscarinic acetylcholine receptor during learning and memory. *J. Biol. Chem. 291*, 8862−8875.

(42) Hinz, L., Ahles, A., Ruprecht, B., Küster, B., and Engelhardt, S. (2017) Two serines in the distal C-terminus of the human β_1-adrenoceptor determine β-arrestin2 recruitment. *PLoS One 12*, No. e0176450.

(43) Trester-Zedlitz, M., Burlingame, A., Kobilka, B., and von Zastrow, M. (2005) Mass spectrometric analysis of agonist effects on posttranslational modifications of the β-2 adrenoceptor in mammalian cells. *Biochemistry 44*, 6133−6143.

(44) Hayashi, K., Gotou, M., Matsui, T., Imahashi, K., Nishimoto, T., and Kobayashi, H. (2017) Identification of phosphorylation sites on β_1-adrenergic receptor in the mouse heart. *Biochem. Biophys. Res. Commun. 488*, 362−367.

(45) Jung, S.-R., Kushmerick, C., Seo, J. B., Koh, D.-S., and Hille, B. (2017) Muscarinic receptor regulates extracellular signal regulated kinase by two modes of arrestin binding. *Proc. Natl. Acad. Sci. U. S. A. 114*, E5579−E5588.

(46) Pao, C. S., and Benovic, J. L. (2005) Structure/Function Analysis of α_{2A}-Adrenergic Receptor Interaction with G Protein-coupled Receptor Kinase 2. *J. Biol. Chem. 280*, 11052−11058.

(47) Komolov, K. E., Du, Y., Duc, N. M., Betz, R. M., Rodrigues, J. P. G. L. M., Leib, R. D., Patra, D., Skiniotis, G., Adams, C. M., Dror, R. O., Chung, K. Y., Kobilka, B. K., and Benovic, J. L. (2017) Structural and Functional Analysis of a β_2-Adrenergic Receptor Complex with GRK5. *Cell 169*, 407−421.

(48) Mueller, W., Schütz, D., Nagel, F., Schulz, S., and Stumm, R. (2013) Hierarchical Organization of Multi-Site Phosphorylation at the CXCR4 C Terminus. *PLoS One 8*, No. e64975.

(49) Heitzler, D., Durand, G., Gallay, N., Rizk, A., Ahn, S., Kim, J., Violin, J. D., Dupuy, L., Gauthier, C., Piketty, V., Crépieux, P., Poupon, A., Clément, F., Fages, F., Lefkowitz, R. J., and Reiter, E. (2012) Competing G protein-coupled receptor kinases balance G protein and β-arrestin signaling. *Mol. Syst. Biol. 8*, 590.

(50) Morse, K. L., Behan, J., Laz, T. M., West, R. E., Greenfeder, S. A., Anthes, J. C., Umland, S., Wan, Y., Hipkin, R. W., Gonsiorek, W., Shin, N., Gustafson, E. L., Qiao, X., Wang, S., Hedrick, J. A., Greene, J., Bayne, M., and Monsma, F. J. (2001) Cloning and characterization of a novel human histamine receptor. *J. Pharmacol. Exp. Ther. 296*, 1058−1066.

(51) Zindel, D., Engel, S., Bottrill, A. R., Pin, J.-P., Prezeau, L., Tobin, A. B., Bünemann, M., Krasel, C., and Butcher, A. J. (2016) Identification of key phosphorylation sites in PTH1R that determine arrestin3 binding and fine-tune receptor signaling. *Biochem. J. 473*, 4173−4192.

(52) Alvarez-Curto, E., Inoue, A., Jenkins, L., Raihan, S. Z., Prihandoko, R., Tobin, A. B., and Milligan, G. (2016) Targeted elimination of G proteins and arrestins defines their specific contributions to both intensity and duration of G protein-coupled receptor signaling. *J. Biol. Chem. 291*, 27147−27159.

(53) O'hayre, M., Eichel, K., Avino, S., Zhao, X., Steffen, D. J., Feng, X., Kawakami, K., Aoki, J., Messer, K., Sunahara, R., Inoue, A., von Zastrow, M., and Gutkind, J. S. (2017) Genetic evidence that β-arrestins are dispensable for the initiation of β_2-adrenergic receptor signaling to ERK. *Sci. Signaling 10*, eaal3395.

(54) Poulin, B., Butcher, A., McWilliams, P., Bourgognon, J.-M., Pawlak, R., Kong, K. C., Bottrill, A., Mistry, S., Wess, J., Rosethorne, E. M., Charlton, S. J., and Tobin, A. B. (2010) The M_3-muscarinic receptor regulates learning and memory in a receptor phosphorylation/arrestin-dependent manner. *Proc. Natl. Acad. Sci. U. S. A. 107*, 9440−9445.

(55) Lee, K. B., Ptasienski, J. A., Pals-Rylaarsdam, R., Gurevich, V. V., and Hosey, M. M. (2000) Arrestin binding to the M_2 muscarinic acetylcholine receptor is precluded by an inhibitory element in the third intracellular loop of the receptor. *J. Biol. Chem. 275*, 9284−9289.

(56) Pals-Rylaarsdam, R., and Hosey, M. M. (1997) Two homologous phosphorylation domains differentially contribute to desensitization and internalization of the m_2 muscarinic acetylcholine receptor. *J. Biol. Chem. 272*, 14152−14158.

(57) Eason, M. G., Moreira, S. P., and Liggett, S. B. (1995) Four consecutive serines in the third intracellular loop are the sites for beta-adrenergic receptor kinase-mediated phosphorylation and desensitization of the α_{2A}-adrenergic receptor. *J. Biol. Chem. 270*, 4681−4688.

(58) Reiner, S., Ziegler, N., Leon, C., Lorenz, K., von Hayn, K., Gachet, C., Lohse, M. J., and Hoffmann, C. (2009) β-Arrestin-2 interaction and internalization of the human P2Y1 receptor are dependent on C-terminal phosphorylation sites. *Mol. Pharmacol. 76*, 1162−1171.

(59) Peverelli, E., Mantovani, G., Calebiro, D., Doni, A., Bondioni, S., Lania, A., Beck-Peccoz, P., and Spada, A. (2008) The third intracellular loop of the human somatostatin receptor 5 is crucial for arrestin binding and receptor internalization after somatostatin stimulation. *Mol. Endocrinol. 22*, 676−688.

(60) Kang, Y., Zhou, X. E., Gao, X., He, Y., Liu, W., Ishchenko, A., Barty, A., White, T. A., Yefanov, O., Han, G. W., Xu, Q., de Waal, P. W., Ke, J., Tan, M. H. E., Zhang, C., Moeller, A., West, G. M., Pascal, B. D., Van Eps, N., Caro, L. N., Vishnivetskiy, S. A., Lee, R. J., Suino-Powell, K. M., Gu, X., Pal, K., Ma, J., Zhi, X., Boutet, S., Williams, G. J., Messerschmidt, M., Gati, C., Zatsepin, N. A., Wang, D., James, D., Basu, S., Roy-Chowdhury, S., Conrad, C. E., Coe, J., Liu, H., Lisova, S., Kupitz, C., Grotjohann, I., Fromme, R., Jiang, Y., Tan, M., Yang, H., Li, J., Wang, M., Zheng, Z., Li, D., Howe, N., Zhao, Y., Standfuss, J., Diederichs, K., Dong, Y., Potter, C. S., Carragher, B., Caffrey, M., Jiang, H., Chapman, H. N., Spence, J. C. H., Fromme, P., Weierstall, U., Ernst, O. P., Katritch, V., Gurevich, V. V., Griffin, P. R., Hubbell, W. L., Stevens, R. C., Cherezov, V., Melcher, K., and Xu, H. E. (2015) Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. *Nature 523*, 561−567.

(61) Ghosh, E., Dwivedi, H., Baidya, M., Srivastava, A., Kumari, P., Stepniewski, T., Kim, H. R., Lee, M.-H., van Gastel, J., Chaturvedi, M., Roy, D., Pandey, S., Maharana, J., Guixà-González, R., Luttrell, L. M., Chung, K. Y., Dutta, S., Selent, J., and Shukla, A. K. (2019) Conformational Sensors and Domain Swapping Reveal Structural and Functional Differences between β-Arrestin Isoforms. *Cell Rep. 28*, 3287−3299.

(62) Willets, J. M., Taylor, A. H., Shaw, H., Konje, J. C., and Challiss, R. A. J. (2008) Selective regulation of H_1 histamine receptor signaling by G protein-coupled receptor kinase 2 in uterine smooth muscle cells. *Mol. Endocrinol. 22*, 1893−1907.

(63) Osorio-Espinoza, A., Escamilla-Sánchez, J., Aquino-Jarquin, G., and Arias-Montaño, J.-A. (2014) Homologous desensitization of human histamine H_3 receptors expressed in CHO-K1 cells. *Neuropharmacology 77*, 387−397.

(64) Rodriguez Pena, M. S., Timmerman, H., and Leurs, R. (2000) Modulation of histamine H_2 receptor signalling by G-protein-coupled receptor kinase 2 and 3. *Br. J. Pharmacol. 131*, 1707−1715.

(65) Sun, W.-Y., Wu, J.-J., Peng, W.-T., Sun, J.-C., and Wei, W. (2018) The role of G protein-coupled receptor kinases in the pathology of malignant tumors. *Acta Pharmacol. Sin. 39*, 1699−1705.

(66) van Rijn, R. M., Chazot, P. L., Shenton, F. C., Sansuk, K., Bakker, R. A., and Leurs, R. (2006) Oligomerization of recombinant and endogenously expressed human histamine H_4 receptors. *Mol. Pharmacol. 70*, 604−615.

(67) de Munnik, S. M., Kooistra, A. J., van Offenbeek, J., Nijmeijer, S., de Graaf, C., Smit, M. J., Leurs, R., and Vischer, H. F. (2015) The viral G protein-coupled receptor ORF74 hijacks β-arrestins for

endocytic trafficking in response to human chemokines. *PLoS One 10*, No. e0124486.

(68) Diviani, D., Lattion, A. L., Larbi, N., Kunapuli, P., Pronin, A., Benovic, J. L., and Cotecchia, S. (1996) Effect of different G protein-coupled receptor kinases on phosphorylation and desensitization of the α_{1B}-adrenergic receptor. *J. Biol. Chem. 271*, 5049−5058.

(69) Flühmann, B., Zimmermann, U., Muff, R., Bilbe, G., Fischer, J. A., and Born, W. (1998) Parathyroid hormone responses of cyclic AMP-, serum- and phorbol ester-responsive reporter genes in osteoblast-like UMR-106 cells. *Mol. Cell. Endocrinol. 139*, 89−98.

(70) Lan, T.-H., Kuravi, S., and Lambert, N. A. (2011) Internalization dissociates β_2-adrenergic receptors. *PLoS One 6*, No. e17361.