Differential Role of Serines and Threonines in Intracellular Loop 3 and C-Terminal Tail of the Histamine H₄ Receptor in β-Arrestin and G Protein-Coupled Receptor Kinase Interaction, Internalization, and Signaling

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ABSTRACT: The histamine H₄ receptor (H₄R) activates Gα-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 H₄R 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 H₄R 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 H₄R did not affect β-arrestin2 recruitment and receptor desensitization, but reduced β-arrestin1 recruitment and internalization. Hence, β-arrestin recruitment to H₄R requires the putative phosphorylated serine cluster in the H₄R 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 H₄R with GRK5 and GRK6, respectively. Identification of H₄R 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 H₄ receptor (H₄R) 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, H₄R 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, H₄R-deficient mice are hypersensitive to neuropathic pain, indicating that H₄R-mediated signaling dampens nociception.³ Indeed, stimulation of H₄R in the central nervous system by intrathecal or intracerebroventricular administration of H₄R agonists attenuates neuropathic pain through inhibition of neuroinflammation and oxidative stress.⁴ H₄R 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 H₄R agonists display a clear antitumor effect associated with reduced tumor growth and metastatic potential.⁶ Hence, understanding the interplay of H₄R 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 H₄R signals through heterotrimeric G₁/o proteins resulting in reduced cAMP production by adenylyl cyclase, increased Ca²⁺ mobilization, activation of extracellular-signal-regulated kinase (ERK)1/2 and Akt, and cytoskeletal changes.¹

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Reference antagonist JNJ7777120 can antagonize these histamine-induced cellular responses as well as H4R-mediated inflammation and pruritus in animal in vivo models. The H4R also recruits β-arrestin2 in response to agonist stimulation. 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. 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. In addition, receptor-bound β-arrestins can activate mitogen-activated protein kinases, including ERK1/2, p38, and c-Jun, by acting as signaling scaffolds. 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.

Hence, the dysregulation of GRK subtypes expression levels in tumors but also in the immune system during inflammation 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 H4R 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 H4R agonists as anticancer and antineuropathic pain drugs with improved therapeutic efficacy by retaining signaling without further downregulation of H4R levels.

In this study, the interactions of H4R with β-arrestin1, β-arrestin2, GRK2, GRK3, GRK5, and GRK6 upon stimulation with the reference ligands histamine, clobenpropit, and 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 H4R-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, 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 (*).

**Figure 1.** Recruitment of β-arrestin1/2 and H4R internalization. BRET measurements in HEK293T cells expressing H4R-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 H4R-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 K44A 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 (*).
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 H4R 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 H4R Internalization. Fusion of Rlu8 to the H4R CT did not affect histamine binding affinity (pKd = 8.4 ± 0.1) and potency (pEC50 = 8.6 ± 0.0) to inhibit forskolin-induced cAMP-responsive element (CRE) reporter gene activity as compared to HA-H4R (pKd = 8.5 ± 0.1 and pEC50 = 8.6 ± 0.1) (Figure S1A–C). However, the expression of H4R-Rluc8 (Bmax = 1.5 ± 0.1 pmol/mg) was 3.2-fold decreased in comparison to HA-H4R (Bmax = 4.9 ± 0.7 pmol/mg) after transient transfection of HEK293T cells.

Stimulation of transiently transfected HEK293T cells with 10 μM histamine rapidly increased BRET between H4R-Rluc8 and both β-arrestin2-mVenus and β-arrestin1-eYFP to maximum steady-state levels within 10 min (Figure 1A,D). Partial H4R agonist clobenpropit induced lower maximum steady-state level within 10 min (Figure 1A,D). However, the expression of H4R-Rluc8 (Bmax = 1.5 ± 0.1 pmol/mg) was 3.2-fold decreased in comparison to HA-H4R (Bmax = 4.9 ± 0.7 pmol/mg) after transient transfection of HEK293T cells.

Pretreatment with the Gaαi/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 H4R 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 H4R-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 to 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 Rlu8 (36 kDa) may differentially affect the efficacy of indolecarboxamide ligands such as JNJ7777120, whereas efficacies of H4R 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 H4R (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 H4R 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 H4R 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 H4R dissociation kinetics as compared to histamine,20 which exceeds the 30 min readout in BRET-based β-arrestin2 recruitment.

Partial H4R agonist clobenpropit induced lower maximum steady-state levels within 10 min (Figure 1A,D). However, the expression of H4R-Rluc8 (Bmax = 1.5 ± 0.1 pmol/mg) was 3.2-fold decreased in comparison to HA-H4R (Bmax = 4.9 ± 0.7 pmol/mg) after transient transfection of HEK293T cells.

Table 1. Potency (pEC50) and Intrinsic Activity of Histamine and Clobenpropit in Wild-Type H4R-Rluc8-Mediated Responses as Measured in BRET-Based Assays

| BRET assay | histamine | clobenpropit |
|------------|-----------|--------------|
| β-arrestin1| 7.0 ± 0.1 | 7.3 ± 0.3    |
| β-arrestin2| 7.3 ± 0.1 | 7.6 ± 0.0    |
| Rab5a      | 7.1 ± 0.0 | 7.8 ± 0.1    |
| GRK2       | 6.9 ± 0.1 | 7.4 ± 0.1    |
| GRK3       | 7.3 ± 0.1 | 7.6 ± 0.1    |
| GRK5       | 7.9 ± 0.1 | 8.5 ± 0.4    |

*pEC50* and intrinsic activity

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).
BRET-Based Detection of H₄R 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. BRET was used to monitor the interaction of H₄R with these four GRK subtypes in response to stimulation with different ligands. Histamine and clobenpropit (10 μM) increased BRET between H₄R-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. In contrast, histamine and clobenpropit induced a more gradual decrease in BRET between H₄R-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. Stimulation with substance P decreased the interaction between the neurokinin-1 receptor and GRK5 already before the receptor is internalized, which might involve GRK5 autophosphorylation and interaction with Ca²⁺-dependent calmodulin following receptor phosphorylation. 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. In line with this, histamine and clobenpropit induced only a very minor decrease in BRET between H₄R-Rluc8 and GRK6-mVenus. Clobenpropit acted as partial agonist in modulating the interaction between H₄R and GRK2, 3, and 5, in comparison to full agonist histamine (Figures 2A–D 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 H₄R-Rluc8 and GRK2-, GRK3-, GRK5-, and GRK6-mVenus (Figure 2A–D), while histamine-induced BRET changes between H₄R-Rluc8 and GRK2-, GRK3-, and GRK5-mVenus were observed.

Figure 2. Ligand-induced changes in the interaction between H₄R and GRKs. BRET measurements in HEK293T cells expressing H₄R-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 H₄R-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 (*).
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 H4R antagonist, thioperamide had no effect or induced at most a very minor decrease in BRET between H4R 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 Gαi protein activation as previously reported for GRK2 recruitment to the β2-adrenergic and dopamine D2 receptors.24,38

Serine and Threonine Residues in IL3 and CT Play Differential Roles in β-arrestin1/2 Recruitment, H4R Internalization, and GRK Interactions. Crystal structures of active β-arrestin1 and visual arrestin in complex with a V2 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 S357, T360, and S363 in V2 vasopressin receptor, and phosphorylated T338 and S338 in combination with E41 in rhodopsin) with three positively charged pockets on the surface of the (β-)arrestin1 N-domain.39,40 Alanine-substitution of these serine and threonine residues impaired V2 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,10,11,41 and found to be required for a stable interaction of β-arrestin2 with the M1 muscarinic acetylcholine receptor.42,43

The H4R harbors a cluster of 5 serine residues (i.e., 385SRSVSS390) 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 H4R with β-arrestins, we substituted alanine for all serine/threonine residues in IL3 and/or CT. The generated mutants H4R-IL3-Rluc8, H4R-CT-Rluc8, and H4R-IL3/CT-Rluc8 displayed comparable binding affinities for [3H]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) H4R-Rluc8 (Figure S5G–M). The H4R-CT-Rluc8 (Bmax = 1.6 ± 0.1 pmol/mg) was expressed at comparable levels as WT H4R-Rluc8 (Bmax = 1.5 ± 0.1 pmol/mg) in transiently transfected HEK293T cells, whereas H4R-IL3-Rluc8 (Bmax = 0.3 ± 0.1 pmol/mg) and H4R-IL3/CT-Rluc8 (Bmax = 0.4 ± 0.0 pmol/mg) were expressed at 3.75- and 5-fold lower levels,

### Table 2. Affinity (pKp) and Potency (pEC50) of Histamine for WT H4R and Mutants in Which Serines and Threonines in IL3 and/or CT Are Alanine-Substituted

| Mutant | Rluc8 pKp | β-arrestin1 pEC50 | β-arrestin2 pEC50 | HA-tag pKp | CRE-luc pEC50 | pERK1/2 pEC50 | Impedance pEC50 |
|--------|-----------|------------------|------------------|------------|-------------|-------------|---------------|
| 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            |

"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."
IL3 of H4R harbors multiple arginine and lysine residues that might engage in such interaction (Figure 3A). In contrast, serine/threonine residues within both the IL3 and CT did not affect GRK2 and GRK3 recruitment as compared to WT H4R-Rluc8 (Figure 4A,B), whereas cell surface expression of H4R-IL3/CT-Rluc8) reduced the BRET with GRK6-mVenus upon stimulation with histamine, whereas no histamine-induced interaction of H4R with GRK5. Cross-linking and hydrogen–deuterium exchange mass spectrometry experiments have previously suggested that GRK5 interacts with IL3 of the β2-adrenergic receptor, but these experiments did not reveal whether phosphosites were involved in this interaction. Interestingly, alanine substitution of the distal serine cluster in the CT of H4R (i.e., H4R-CT-Rluc8 and H4R-IL3/CT-Rluc8) reduced the BRET with GRK6-mVenus upon stimulation with histamine, whereas no histamine-induced response was observed for H4R-Rluc8 and H4R-IL3-Rluc8 (Figure 4C), 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 H4R signaling. To this end, we generated clonal HEK293 cell lines that stably express HA-tagged WT H4R, H4R-IL3, or H4R-IL3/CT at comparable levels on their cell surface, whereas cell surface expression of H4R-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 H4R, H4R-IL3, H4R-CT, or H4R-IL3/CT, which is in agreement with the comparable binding affinity of

respectively (Figure SSA–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 SSE). 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 S5E). 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 H4R-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 α3-adrenergic receptor as key drivers for both GRK2 binding and activation.46 Indeed, the IL3 of H4R 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 354TSST357 and/or more distal T376REHPSTANT379 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 356TSST357 cluster did not affect GRK2-Venus recruitment to μ-opioid receptor-Rluc8 in a BRET-based assay. H4R-IL3-Rluc8 and H4R-IL/CT-Rluc8 did not display a histamine-induced decrease in BRET with GRK5-mVenus as compared to WT H4R-Rluc8 and H4R-CT-Rluc8 (Figure 4C), suggesting that serines/threonines in IL3 may be involved in the interaction of H4R with GRK5. Cross-linking and hydrogen–deuterium exchange mass spectrometry experiments have previously suggested that GRK5 interacts with IL3 of the β2-adrenergic receptor, but these experiments did not reveal whether phosphosites were involved in this interaction. Interestingly, alanine substitution of the distal serine cluster in the CT of H4R (i.e., H4R-CT-Rluc8 and H4R-IL3/CT-Rluc8) reduced the BRET with GRK6-mVenus upon stimulation with histamine, whereas no histamine-induced response was observed for H4R-Rluc8 and H4R-IL3-Rluc8 (Figure 4C), suggesting that putative phosphoserines in the CT might hamper the basal interaction with GRK6.

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Figure 4. Interaction of IL3 and/or CT H4R mutants with GRKs. BRET measurements in HEK293T cells expressing H4R-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 H4R 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 (*).
[\textsuperscript{3}H]histamine for WT and these H\textsubscript{4}R mutants (Figure 5B; Table 2). In addition, the three H\textsubscript{4}R mutants inhibited the 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. 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\textsubscript{4}R 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 performed in duplicate. (F) Duration of histamine-induced changes in cellular impedance by HA-H\textsubscript{4}R 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\textsubscript{4}R WT were determined using two-way ANOVA with Dunnett’s multiple comparison test and are indicated by an asterisk (*).

Figure 5. Signaling by IL3 and/or CT H\textsubscript{4}R mutants. HA-tagged H\textsubscript{4}R WT, H\textsubscript{4}R-IL3, H\textsubscript{4}R-CT, and H\textsubscript{4}R-IL3/CT were stably expressed in HEK293 cells. (A) Expression of HA-tagged H\textsubscript{4}R 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 4 independent experiments with scatter plots showing individual data. (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\textsubscript{4}R 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, with scatter plots showing the AUC from individual experiments. Statistical differences (p < 0.05) compared to HA-H\textsubscript{4}R WT were determined using two-way ANOVA with Dunnett’s multiple comparison test and are indicated by an asterisk (*).
In a whole-cell analysis of H3R response, histamine was seen to transiently increase 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 H3R (Figure S6C,D; Table 2), whereas no histamine-induced change in cellular impedance was observed in parental HEK293 cells (Figure 5E). H3R-CT induced a higher impedance response than did WT H3R upon stimulation with histamine, whereas a comparable maximal impedance response was observed in cells expressing H3R-IL3 or H3R-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 H3R (Figure 5F), which was significantly further delayed by concomitant mutation of serines and threonines in IL3 (Figure SF). These data corroborate with the prolonged histamine-induced ERK1/2 phosphorylation by H3R-CT and H3R-IL3/CT as compared to WT H3R. Surprisingly, however, the histamine-induced impedance response returned much faster to basal levels, without a second descending shoulder phase (Figure 5E), in cells expressing H3R-IL3 as compared to those expressing WT H3R (Figure 5F).

Hence, the serine cluster in the H3R CT is important for the recruitment of β-arrestin1 and 2. Mutation of this cluster impaired H3R internalization, while enhancing and elongating ERK1/2 phosphorylation and cellular impedance responses, suggesting that bound β-arrestin mediates H3R internalization and receptor desensitization. Similarly, decreased internalization in combination with enhanced ERK1/2 phosphorylation was observed for agonist-activated parathyroid hormone receptor 1,33 free fatty acid receptor FFA4,32,55 and β2-adrenergic receptor55 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 H3R 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 H3R, which might be in part related to the somewhat higher cell surface expression of H3R-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 H3R-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 M3 muscarinic acetylcholine receptor (M3R) reduced β-arrestin2 recruitment and receptor internalization upon agonist stimulation but did not affect M3R desensitization.54 Phosphorylation of an N- and C-terminal clusters serines/threonines in IL3 of M3 muscarinic acetylcholine receptor (M3R) 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 α2A 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 1.60 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 H3R 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 H1, H2, and H3 receptor subtypes (H1R, H2R, and H3R) is regulated by GRK2, while GRK3 was also reported to be involved in desensitization of H1R and H2R.62–64 GRK5 and GRK6 do not affect H1R and H2R signaling64 and have to the best of our knowledge not been experimentally assessed for the H3R. 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, H3R 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 H3R expression is downregulated in multiple tumors in comparison to healthy tissues while GRK subtypes are differently up- or down-regulated 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 H3R desensitization and internalization in cancers and guide future site-directed mutagenesis studies. Various H3R agonists have been shown to attenuate tumor growth and neuropathic pain,4,5 and identification of biased ligands that induce H3R 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

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H₂R ligands were synthesized in the Medicinal Chemistry Department of the Vrije Universiteit Amsterdam (Amsterdam, The Netherlands). Compound 101 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-arrestin 1, β-arrestin 2, 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-H₂R, H₂R-Rluc8, β-arrestin 2-mVenus, and β-arrestin 1-eYFP constructs in pcDEF3 expression plasmid have been previously described.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 EcoRI and PpuMI restriction sites or by PCR-based site-directed mutagenesis, respectively. Mutant receptors were either HA-tagged at their N-terminus or by PCR-based site-directed mutagenesis, respectively. All generated constructs were verified by DNA sequencing. The CRE-driven luciferase reporter gene plasmid pTLNC-21CRE and DNA encoding for Venus-Rab5a 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 μg mL⁻¹ streptomycin (GE healthcare; Uppsala, Sweden) at 37 °C with 5% CO₂. 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.67 Total DNA amounts were kept equal by pretransfection, baseline BRET was measured using 5 μg of WT or mutant HA-H₂R DNA, or from HEK293 cells transfected 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 β-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 Na₃VO₄, and 1× Complete protease inhibitor cocktail (Roche Diagnostics; Mannheim, Germany) for 20 min on ice, sonicated for 5 s, and centrifuged at 20 800g 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 7D9 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, except that samples were incubated at 95 °C for 5 min before being subjected to SDS-PAGE.

**ELISA.** Expression of WT and mutant HA-H₂R 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-H₂R 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-H₂R 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 × 10⁴ 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% CO₂. 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 H₄R-Rluc8 fusion protein and H₄R mutants, JNJ7777120, and thioperamide antagonize histamine-induced β-arrestin2 recruitment to H₄R, β-arrestin1/2 expression upon siRNA transfection, concentration response curves of clobenpropit on BRET between H₁R and GRKs, expression of β-arrestin2-mVenus, β-arrestin1-eYFP, GRKx-mVenus, and Venus-Rab5a in cells coexpressing WT H₄R-Rluc8 or mutants, and concentration response curves of histamine on H₂R-mediated ERK1/2 activation and cellular impedance (PDF)

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**Notes**
The authors declare no competing financial interest.

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**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; H₂R, histamine H₂ receptor; H₄R, histamine H₄ receptor; HTRF, homogeneous time-resolved fluorescence; IL3, intracellular loop 3; PTx, pertussis toxin; SD, standard deviation; SEM, standard error of the mean; WT, wildtype

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