Quantitative Single-Cell Analysis of Signaling Pathways Activated Immediately Downstream of Histamine Receptor Subtypes

Jakobus van Unen, Ali Rashidfarrokhi, Eelco Hoogendoorn, Marten Postma, Theodorus W. J. Gadella, Jr., and Joachim Goedhart

Swammerdam Institute for Life Sciences, Section of Molecular Cytology, van Leeuwenhoek Centre for Advanced Microscopy, University of Amsterdam, Amsterdam, The Netherlands

Received March 31, 2016; accepted June 28, 2016

ABSTRACT

Genetically encoded biosensors based on Förster resonance energy transfer (FRET) can visualize responses of individual cells in real time. Here, we evaluated whether FRET-based biosensors provide sufficient contrast and specificity to measure activity of G-protein–coupled receptors. The four histamine receptor subtypes (H1R, H2R, H3R, and H4R) respond to the ligand histamine by activating three canonical heterotrimeric G-protein–mediated signaling pathways with a reported high degree of specificity. Using FRET-based biosensors, we demonstrate that H1R activates Goαi. We also observed that H1R activates Goq, albeit at a 10-fold lower potency. In addition to increasing cAMP levels, most likely via Goαs, we found that the H2R induces Goq-mediated calcium release. The H3R and H4R activated Goi with high specificity and high potency. We demonstrate that a number of FRET sensors provide sufficient contrast to: 1) analyze the specificity of the histamine receptor subtypes for different heterotrimeric G-protein families with single-cell resolution, 2) probe for antagonist specificity, and 3) allow the measurement of single-cell concentration-response curves.

Introduction

The histamine receptor family consists of four known members to date: histamine-1-receptor (H1R), histamine-2-receptor (H2R), histamine-3-receptor (H3R), and the more recently discovered histamine-4-receptor (H4R) (Jablonski et al., 2004). Although the sequence homology is relatively low (e.g., H2R shares approximately 37% identity with the H3R, but less than 20% with the H2R and H3R; Liu et al., 2001; Zhu et al., 2001), all subtypes bind histamine specifically. The histamine receptor family has been implicated in a large number of pathologies (Parsons and Ganellin, 2006; Pino-Ángeles et al., 2012), including cancer (Medina and Rivera, 2010), and is therefore a popular target for therapeutic interventions (Bongers et al., 2010; Seifert et al., 2013).

The H2R is mainly expressed in endothelium, smooth muscle cells, and the central nervous system (CNS) and is best known for its role in various allergic disorders, such as hay fever, urticaria, and allergic rhinitis. The H2R is ubiquitously expressed and its antagonists are widely used for the treatment of gastric ulcers. The H3R binds histamine specifically and is therefore a popular target for therapeutic interventions (Parsons and Ganellin, 2006; Pino-Ángeles et al., 2012), including cancer (Medina and Rivera, 2010), and is therefore a popular target for therapeutic interventions (Bongers et al., 2010; Seifert et al., 2013).

The H2R is mainly expressed in endothelium, smooth muscle cells, and the central nervous system (CNS) and is best known for its role in various allergic disorders, such as hay fever, urticaria, and allergic rhinitis. The H2R is ubiquitously expressed and its antagonists are widely used for the treatment of gastric ulcers. The H3R is predominantly expressed in the CNS, and its antagonists are currently under investigation for the treatment of a wide range of CNS pathologies, including cognitive disorders, sleep disorders, and aberrant homeostasis. The H3R is expressed in leukocytes and mast cells, and is thus possibly involved in inflammatory and immune responses (Thurmond et al., 2008).

Histamine receptors are G-protein–coupled receptors (GPCRs), and the different subtypes couple to distinct heterotrimeric G-protein families. Signaling downstream of the heterotrimeric G-protein complex is often attributed and classified according to the Gα subunit, since it defines the specific downstream signaling events that are activated. GPCRs can signal via four different Gα–protein families: Goαq, Goα12, Goαi, and Goαs (Fig. 1A). Furthermore, the accompanying Gβγ subunit also contributes to relaying the signal (Smrcka, 2008). In addition, signals are transduced via noncanonical pathways that involve β-arrestins (Ostermaier et al., 2014).

The signaling events directly downstream of the GPCR are used in cell-based screens aimed at identifying drugs that target GPCRs. Classically, Ca2+ and cAMP have been the second messengers of choice to detect GPCR activation. Recently, new cell-based screens that measure alternative parameters and enable high-throughput analysis have been reported (Schröder et al., 2010; Inoue et al., 2012; Kroeze et al., 2015). The detection of Ca2+ is performed with Ca2+-sensitive fluorescent probes, enabling real-time analysis. Since Goq-mediated signaling efficiently activates Ca2+ release via
phospholipase C-β (PLCβ), quantification of calcium levels is an important method to screen GPCR activity. To convert the activity of GPCRs that do not increase calcium levels, promiscuous G-proteins can be used (e.g., Gα16) (Thomsen et al., 2005). Although calcium is a very sensitive readout due to magnification of the signal, Ca²⁺ is multiple steps downstream from the G-protein and influenced by cross-talk and signal amplification.

The cAMP levels are used to detect Gαs and Gαi signaling. However, the detection of Gai activity often requires artificial elevation of cAMP levels by forskolin (Sensken et al., 2008). The detection of cAMP in general and Gαi activity in particular has limited temporal resolution. All of the high-content screening methods use population averages, and thus information on cell-to-cell heterogeneity is usually lost. Moreover, since single-cell resolution is not achieved, the strategies for detection of GPCR activation cannot report on spatial information of signaling events. The only exception is detection of Ca²⁺ with calcium-sensitive fluorophores.

Resonance energy transfer techniques have several unique properties that possibly allow new insights into GPCR signaling and their pharmacology, both in vitro and in vivo (Lohse et al., 2012; Clister et al., 2015; van Unen et al., 2015b). These techniques can provide quantitative data, on/off kinetics with high temporal resolution, can be measured in real time, and allow fast and straightforward analysis of the data (Marullo and Bouvier, 2007; Lohse et al., 2008). Specifically, genetically encoded Förster resonance energy transfer (FRET) sensors allow the assessment of cell-to-cell heterogeneity and the acquisition of multiple responses.

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Histamine receptor signaling. (A) Schematic overview of the canonical heterotrimeric G-protein–mediated signaling pathways activated by the four histamine receptor subtypes. (B) Overview of the different FRET biosensors used in this study to analyze the signaling profiles of the four histamine receptor subtypes. All biosensors are based on a CFP/YFP FRET pair. AC, adenylyl cyclase; DAG, diacylglycerol; mDia, mammalian diaphanous-related formin 1; PKA, protein kinase A; PKC, protein kinase C; RhoGEF, Rho guanine exchange factor; ROCK, Rho-associated coiled-coil-containing protein kinase.
from the same single cell in real time (Lohse et al., 2012). FRET reporters can be developed to measure every step in the GPCR signaling cascade. FRET biosensors are available to measure ligand binding to the GPCR (Stoddart et al., 2015), GPCR activation (Vilardaga et al., 2003), GPCR and G-protein interaction (Hein et al., 2005; Stumpf and Hoffmann, 2016), G-protein activation (Janetopoulos et al., 2001; Adjobo-Hermans et al., 2011), Ca\(^{2+}\) release (Nagai et al., 2004), cAMP production (Klarenbeek et al., 2015), and activation of downstream effectors such as protein kinase C (Verbeek et al., 2008), RhoA (van Unen et al., 2015), and inositol 1,4,5-trisphosphate (Gulyás et al., 2015). The preferred option is to use FRET biosensors that report on the specific activation of one of the heterotrimeric G-protein subfamilies directly stimulated by a GPCR. Since this kind of biosensor is not yet available for all subclasses of G-proteins, we also made use of FRET biosensors that report on G-protein–mediated second-messenger production or activation. With the use of these biosensors, we characterized the canonical G-protein–mediated signaling profiles of the four histamine receptor subtypes. Moreover, we show that these techniques can be used to characterize ligand specificity and calculate potency at these receptors.

Materials and Methods

Construction of Fluorescent Protein Fusions. To obtain N1-xp2A-mCherry, two oligonucleotides encoding for the p2A viral peptide sequence ATNFSLLKQAGDVEENPGP (Kim et al., 2011) were annealed as previously described (Goedhart and Gadella, 2005). Annealing forward 5′-CCGGtggctactaactctgacctgagcagctgcgacgcgctggagacggtggaggagaaccccctgacgctggc-3′ and reverse 5′-CATGgacccaggtccagggtttctctcagcttctgctgctcctgctgctgccttctagta-3′ oligonucleotides yielded the viral peptide xp2A sequence with overhangs (in capitals) on both sides, compatible with AgeI and NcoI restriction sites. The double-stranded linker was ligated into an RSET-mCherry plasmid cut with AgeI and NcoI, resulting in RSET-xp2A-mCherry. This RSET-xp2A-mCherry plasmid was cut with AgeI and BsrGI and ligated into an empty clontech-style N1 vector, resulting in N1-xp2A-mCherry.

It turned out that this xp2A sequence was too short for efficient separation by the viral peptide sequence. To this end, three additional amino acids, GSG, were added to yield GSGATNFSLLKQAGDVEENPGP.

To add the GSG sequence, a PCR was performed on N1-xp2A-mCherry with forward primer 5′-TCCACCGGTGGGATCGGGTGCTACTAACTT-CAGCCTGC-3′ and reverse primer 5′-TCTACAAATGTGGTATGGC-3′. The resulting pcr product was ligated into an empty clontech-style N1 vector using AgeI and BsrGI to create N1-p2A-mCherry.

Human histamine receptors were tagged with fluorescent proteins as described later. N1-H1R-mCherry was obtained by cutting N1-mCherry with NheI and AgeI and ligation with N1-H1R-mTurquoise cut with the same enzymes. N1-H1R-p2A-mCherry was made by cutting N1-p2A-mCherry with AgeI and NotI and ligation with N1-H1R-mCherry cut with the same enzymes.

Fig. 2. Tagging histamine receptors with fluorescent proteins. (A) Representative confocal images of the localization of the four histamine receptor subtypes. HeLa cells were transiently transfected with a plasmid containing the indicated histamine receptor subtype directly fused to mCherry. (B) Schematic overview of the p2A tagging strategy. To prevent possible FRET between the CFP of a plasma membrane–localized biosensor and the RFP fused to the receptor (left), we introduced a p2A sequence between the receptor and the RFP, leading to separate expression of the RFP and receptor proteins. (C) Confocal image of HeLa cells transfected with the histamine-4-receptor fused to p2A-mCherry, showing the clear cytoplasmic localization of mCherry. Width of the individual images in (A) corresponds to 105 μm, and the width of the image in (C) corresponds to 117 μm.
Fig. 3. G_{aq} signaling by histamine receptors. (A) Activation of the heterotrimeric G-protein G_{aq} by the four histamine receptor subtypes as measured by FRET ratio imaging. (B) HeLa cells transfected with H1R-p2A-RFP and the G_{aq} biosensor were treated with histamine and mepyramine (black). Control cells were transfected with only the G_{aq} biosensor (gray). (C) Cells transfected with H2R-p2A-RFP and the G_{aq} biosensor were treated with histamine and ranitidine (black). In the control condition, cells were transfected only with the G_{aq} biosensor (gray). (D) Cells transfected with H3R-p2A-RFP and the G_{aq} biosensor were treated with histamine and thioperamide (black). In the control condition, cells were transfected with only the G_{aq} biosensor (gray). (E) Cells transfected with H4R-p2A-RFP and the G_{aq} biosensor were treated with histamine and thioperamide (black). In the control condition, cells were only transfected with the G_{aq} biosensor (gray). (F) Box limits indicate the 25th and 75th percentiles as determined by R software (http://www.r-project.org); whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. HeLa cells were stimulated with histamine at t = 32 seconds, and the response was antagonized by the addition of the appropriate antagonist at t = 152 seconds. Time traces show the average ratio change of YFP/CFP fluorescence (± S.E.M.). AC, adenylyl cyclase; DAG, diacylglycerol; mDia, mammalian diaphanous-related formin 1; PKA, protein kinase A; PKC, protein kinase C; ROCK, XXX.
pcDNA3.1-H1R (cDNA.org) was amplified using forward primer 5'-AGGCTTATATAAGCAGACG-3' and reverse primer 5'-ATACC-GTTCCCTCTTCGCACTGGTCGAG-3'. The PCR product was cut with HindIII and AgeI and ligated into an N1-mCherry vector that was cut with the same enzymes. N1-H1R-p2A-mCherry was made by cutting N1-p2A-mCherry with Age1 and BsrGI and ligation with N1-H1R-mCherry cut with the same enzymes.

pDEPH1R-mVenus (a kind gift from Henry Vischer, Vrije Universiteit, Amsterdam, The Netherlands) was amplified with reverse primer ATACCGGTGAGAATCTCGACGACTG and forward primer CAGGTGTCGTGAGGAATTAG, and the product was cut with Age1 and Acc561, resulting in N1-H1R-mTurquoise2. mTurquoise2 was swapped for mCherry and p2A-mCherry by cutting N1-mCherry and N1-p2A-mCherry with Age1 and NotI and ligation with N1-H1R-mTurquoise2 cut with the same enzymes, resulting in N1-H1R-mCherry and N1-H1R-p2A-mCherry. The histamine receptors are available from Addgene.org.

A plasmid encoding YCam3.6 (Ycam, Middlesex, UK) was described previously (van Unen et al., 2015a). 

\[ \text{TEPAC}^{\text{V}} \]

was as previously described (Klarenbeek et al., 2011). The dimerization optimized reporter for activation (DORA) Rhoa sensor was a kind gift from Yi Wu and TaoFei Yin (van Unen et al., 2015a) (University of Connecticut Health Center, Farmington, CT). The FRET biosensors for Gq activation (Adjobo-Hermans et al., 2011) and Gai activation (van Unen et al., 2016) were previously described.

**Drug Treatments.** The different histamine receptors were stimulated at the indicated time points as follows, unless otherwise specified. All substances were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands), except FR900359 and methylhistaprodifen. FR900359 was purchased from the University of Bonn (Bonn, Germany). Methylhistaprodifen (Elz et al., 2000) was a kind gift from Andrea Strasser and Sigurd Elz (Universität Regensburg, Regensburg, Germany). Drugs were dissolved in H2O as 1000 x concentrated stock solutions. Methylhistaprodifen was dissolved in dimethylsulfoxide at 100 and 10 mM and lower concentrations in dimethylsulfoxide/H2O 1:1 (v/v). One microliter of the drug was added to a cell chamber containing 1 ml of liquid followed by rapid mixing by repeated pipetting of the medium. H2R was stimulated with 100 μM histamine and deactivated by 10 μM mepyramine. H3R was stimulated with 100 μM histamine and deactivated by 100 μM ranitidine. The H3R and H4R were stimulated with 100 μM mepyramine and deactivated by 100 μM thioperamide.

For Fig. 5, all receptors were stimulated with 100 μM histamine and 100 μM carbamol. Where indicated, cells were incubated with 100 ng/ml pertussis toxin (PTX) overnight or for 2 hours with the Gqq inhibitor FR900359 (previously known as UBO-QIC) (Schrage et al., 2015) at a concentration of 2 μM.

**Cell Culture and Sample Preparation.** Cell culture, transfection, and live cell microscopy conditions were performed as previously described (van Unen et al., 2015a).

**Widefield Microscopy.** Ratiometric FRET measurements were performed using a previously described widefield fluorescence microscope (van Unen et al., 2015a). Typical exposure times ranged from 50–200 ms, and camera binning was set to 4 × 4. The 420-nm (slit width 30 nm) excitation light was reflected onto the sample by a 455DCLP dichroic mirror (Omega, Brattleboro, VT), and yellow fluorescent protein (YFP) emission was detected with a BP535/30 filter by rotating the filter wheel. Acquisitions were corrected for background signal and, for FRET ratio imaging, bleedthrough correction, and calculation of the normalized ratio per time point for individual cells. The output of Python was written to Excel (Microsoft, Redmond, WA). Graphs and statistics were conducted using GraphPad version 6.0 for Mac (GraphPad Software, La Jolla, CA; www.graphpad.com). The fit of the concentration-response curves was performed in GraphPad with the following equation:

\[ \text{ratio} = \frac{\text{min}_{\text{ratio}} + (\text{max}_{\text{ratio}} - \text{min}_{\text{ratio}}) \times (1 + 10^{(10 \times (–pEC_{50} - X/n))})}{\text{max}_{\text{ratio}}} \]

where min_{\text{ratio}} and max_{\text{ratio}} represent the experimentally obtained minimal and maximal ratio, respectively; X is the log of the histamine concentration; n represents the Hill coefficient; and pEC_{50} is the \(- \log \) of the concentration (EC_{50}) at which 50% of the maximal effect is observed.

**Confocal Microscopy.** HeLa cells transfected with the indicated constructs were imaged using a Nikon A1 confocal microscope equipped with a 60 x oil immersion objective (Plan Achromat VC, NA 1.4; Nikon Instruments, Melville, NY). The pinhole size was set to 1 Airy unit (< 0.8 μm). Samples were excited with a 561-nm laser line and reflected onto the sample by a 457/514/561 dichroic mirror. Red fluorescent protein (RFP) emission was filtered through a BP595/50 emission filter. Acquisitions were corrected for background signal.

**Results**

**Overview of Histamine Receptor Signaling Pathways and Relevant FRET Sensors.** To study the activation of processes immediately downstream of the four histamine receptor subtypes, we used several FRET biosensors (Fig. 1B). Since the H3R, H2R, and H3R/H4R activate well described, presumably specific classes of G-proteins, we used FRET biosensors that report on these pathways.

The H2R is well known to couple to Gq; therefore, we used an intermolecular FRET biosensor that directly measures the activation (e.g., GDP for GTP exchange) of the heterotrimeric G-protein Gq by monitoring the separation of the Gα subunit and the Gβ subunit (Adjobo-Hermans et al., 2011). Furthermore, we used Ycam (Ycam), a unimolecular FRET sensor based on the Ca^{2+}-binding domains of calmodulin (Nagai et al., 2004), which measures changes in intracellular Ca^{2+} concentration upon Gq-mediated activation of the PLCβ family. More recently, Gqq has been linked to the activation of RhoA via direct interaction with Rho guanine exchange factors (Lutz et al., 2007). To measure the activation of RhoA, we used the DORA RhoA biosensor. This unimolecular FRET sensor measures the GTP loading of RhoA via binding of the Rho-binding domain of PKN1 to the RhoA moiety on the sensor (van Unen et al., 2015a). It should be noted that this sensor might also report on the activity of Gz2/Gz13. The H3R is best known to couple to the Gαi, which is classically assayed by probing the inhibition of forskolin-stimulated cAMP production in cells (Klarenbeek et al., 2011), to measure H2R activation. The H3R and H4R are predominantly linked to the activation of Gαi, which is classically assayed by probing the inhibition of forskolin-stimulated cAMP production in cells (Sensken et al., 2008). To provide a more direct way to measure Gai, we used a recently developed intermolecular FRET biosensor that directly reports on the activation of Gai by monitoring the separation of the Gα subunit and the Gβ subunit (van Unen et al., 2016).
Fig. 4. RhoA signaling by histamine receptors. (A) Activation of the DORA RhoA biosensor by the four histamine receptor subtypes, measured by FRET ratio imaging. (B) Hela cells transfected with H1R-p2A-RFP and the DORA RhoA biosensor were treated with histamine and mepyramine (black). Control cells were transfected with only the DORA RhoA biosensor (gray). (C) Cells transfected with H2R-p2A-RFP and the DORA RhoA biosensor were treated with histamine and ranitidine (black). In the control condition, cells were transfected with only the DORA RhoA biosensor (gray). (D) Cells transfected with H3R-p2A-RFP and the DORA RhoA biosensor were treated with histamine and thioperamide (black). In the control condition, cells were transfected with only the DORA RhoA biosensor (gray). (E) Cells transfected with H4R-p2A-RFP and the DORA RhoA biosensor were treated with histamine and thioperamide (black). In the control condition, cells were transfected with only the DORA RhoA biosensor (gray). The median and average (+) amplitude of the FRET ratio change at t = 100 seconds (F) and the raw YFP/CFP ratio at t = 0 seconds (G) per receptor subtype, quantified from the experiments shown in (B–E). Box limits indicate the
We used these FRET biosensors to measure the signaling responses upon stimulation of the four histamine receptor subtypes to create heterotrimeric G-protein signaling profiles per receptor.

**Tagging of Histamine Receptors with a Fluorescent Protein.** The four human histamine receptor subtype fusions were used in combination with the fluorescent proteins YFP and CFP in HeLa cells for confocal microscopy to examine their localization in living cells. The H1R, H2R, H3R, or H4R was predominantly localized to the plasma membrane (Fig. 2A). Since some of the FRET biosensors used in this study are also localized to the plasma membrane, we anticipated that FRET could occur between fluorescent proteins present in the FRET biosensors and the RFP fused to the C-terminal of the receptor (Fig. 2B, left). To prevent bystander FRET, we used a strategy where the RFP is separated from the receptor protein during translation, and is thus no longer localized to the plasma membrane (Fig. 2B, right). With this strategy, the receptor is essentially untagged, and the RFP can still be used as a reporter for receptor translation. We cloned a previously described p2A sequence (Kim et al., 2011) in between the coding sequences for the receptors and the RFP, resulting in plasmids containing HxR-p2A-RFP (for details, see Materials and Methods). As a result, HeLa cells transfected with these constructs showed cytosolic localization of RFP fluorescence, as shown for H4R-p2A-RFP (Fig. 2C) and the other three histamine receptor subtypes (Supplemental Fig. 1).

**Analysis of Gqα Signaling by Four Histamine Receptor Subtypes.** To study which of the histamine receptor subtypes is capable of activating the heterotrimeric G-protein Gqα, we performed live cell measurements on HeLa cells transfected with the Gqα biosensor (Fig. 3A) and cotransfected with H1R-p2A-RFP, H2R-p2A-RFP, H3R-p2A-RFP, or H4R-p2A-RFP. YFP and CFP fluorescence was monitored over time, and cells were stimulated with the indicated amount of agonist and antagonist. Activation of the H1R was achieved by stimulating the cells at the indicated time points with histamine, and the response was antagonized by the addition of the H1R-specific antagonist mepyramine (Leurs et al., 1995). A fast drop in YFP/CFP FRET ratio (10–30%) was observed after addition of histamine, indicating a fast activation of the receptor and subsequent separation of Gqα and G12/13 by these receptors. No change in YFP/CFP FRET ratio was observed in cells transfected with H2R or H3R after stimulation with histamine and subsequent addition of the H2R-specific antagonist ranitidine (Leurs et al., 1995) or the H3R/H4R-specific antagonist thioperamide (Leurs et al., 1995), indicating no activation or deactivation of Gqα by H2R, H3R, or H4R (Fig. 3, C–E, black traces). Cells in control conditions (no GPCR coexpression) were transfected with the Gqα biosensor and did not show any change in FRET ratio upon addition of the relevant agonists and antagonists (Fig. 3, B–E, gray traces). The amplitude of the FRET ratio change at t = 100 seconds, was quantified from single cells per histamine receptor subtype (Fig. 3F). The basal FRET ratio of the biosensors at the start of every experiment (t = 0) was used to evaluate basal activity. We did not observe large differences in FRET ratio between the receptor subtypes at the start of the experiment (Fig. 3G).

From these results, we conclude that only the H1R effectively couples to the heterotrimeric G-protein Gqα, and this biosensor provides high selectivity and sensitivity to readout H1R activation.

**Analysis of RhoA Signaling by Four Histamine Receptor Subtypes.** To study the activation of the small GTPase RhoA, we performed live cell measurements on HeLa cells transfected with the DORA RhoA biosensor (Fig. 4A) and cotransfected with H1R-p2A-RFP, H2R-p2A-RFP, H3R-p2A-RFP, or H4R-p2A-RFP. Activation of the H1R resulted in a fast increase in YFP/CFP FRET ratio (30–60%), indicating a fast activation of the receptor and subsequent exchange of GDP for GTP on the RhoA biosensor. The signal rapidly returned to baseline after addition of mepymazine (Fig. 4B, black trace). Activation of the H2R resulted in a small reversible change in YFP/CFP FRET ratio, whereas no change in YFP/CFP FRET ratio was observed after activation of the H3R (Fig. 3, D and E, black traces). Cells in the control condition that were transfected with the DORA RhoA biosensor showed a minor reversible change in FRET ratio (<5%) upon addition of histamine (Fig. 4, B–E, gray traces). We observed this small response previously (van Unen et al., 2015a), and it can most likely be attributed to the activation of the endogenous guanine exchange factor trio (van Rijssel and van Buul, 2012) by endogenous H2R receptors. We repeated this experiment in human embryonic kidney 293 (HEK293) cells, which do not contain endogenous H2R receptors, and found similar results for the activation of RhoA by ectopically expressed H2R, but no change in YFP/CFP FRET ratio for the control condition (Supplemental Fig. 2).

The amplitude of the FRET ratio change at t = 100 seconds (Fig. 4F) and the start ratio (Fig. 4G) were quantified from single cells per histamine receptor to allow comparison between the receptor subtypes.

From these results, we conclude that the H1R effectively signals to the small GTPase RhoA. The small effects of the H2R and H3R on the DORA RhoA biosensor that were observed are possibly mediated by a minor activation of endogenous Go12/13 by these receptors.

**Analysis of Calcium Signaling by Four Histamine Receptor Subtypes.** To investigate changes in intracellular Ca2+ concentration upon stimulation of the four histamine receptor subtypes, we performed live cell measurements on HEK293 cells transfected with the Ycam biosensor (Fig. 5A) and cotransfected with H1R-p2A-RFP, H2R-p2A-RFP, H3R-p2A-RFP, or H4R-p2A-RFP. HEK293 cells were used in this experiment because endogenous H1 receptors in HeLa cells interfere with the measurements of intracellular Ca2+. Carbachol was added at the indicated time points to stimulate...
Fig. 5. Calcium signaling by histamine receptors. (A) Activation of the Ycam calcium biosensor by the four histamine receptor subtypes, measured by FRET ratio. (B) HEK293 cells transfected with H1R-p2A-RFP and the Ycam biosensor were treated with histamine and carbachol (black). Control cells transfected with only the Ycam biosensor were treated with histamine and carbachol (gray). This control condition is the same for all receptor subtypes in this experiment. (C) Cells transfected with H2R-p2A-RFP and the Ycam biosensor were treated with histamine and carbachol (black). (D) Cells transfected with H3R-p2A-RFP and the Ycam biosensor were treated with histamine and carbachol (black). (E) Cells transfected with H4R-p2A-RFP and the Ycam were treated with histamine and carbachol (black). The median and average (+) amplitude of the FRET ratio change at t = 100 seconds (F) and the raw YFP/CFP ratio at t = 0 seconds (G) per receptor subtype, quantified from the experiments shown in (B–E). Box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. HEK293 cells were stimulated with histamine at t = 32 seconds and stimulated with carbachol at t = 152 seconds. Time traces show the average ratio change of YFP/CFP fluorescence (± S.E.M.). AC, adenylyl cyclase; DAG, diacylglycerol; mDia, mammalian diaphanous-related formin 1; PKA, protein kinase A; PKC, protein kinase C; ROCK, Rho-associated coiled-coil-containing protein kinase.
endogenous M₁/M₃ receptors as a positive endpoint control for intracellular Ca²⁺ release (Zhu et al., 1998). Stimulation of the H₁R resulted in a fast transient increase in YFP/CFP FRET ratio (300–400%), which is indicative of a rise in intracellular calcium. The signal decreased and stabilized again at an elevated ratio compared with baseline (Fig. 5B, black trace). Stimulation with carbachol did not further change the YFP/CFP ratio, suggesting depletion of intracellular Ca²⁺ stores upon histamine stimulation or desensitization of Gq signaling. Interestingly, we observed a fast transient increase in YFP/CFP FRET ratio (200–300%) upon stimulation of the H₂R (Fig. 5C, black trace). Subsequent stimulation with carbachol resulted in a similar fast transient increase in YFP/CFP FRET ratio (200–300%). This indicates that activation of the H₂R causes release of intracellular Ca²⁺. Preincubation with the specific Gq inhibitor FR900359 (Schrage et al., 2015) resulted in a complete abrogation of intracellular Ca²⁺ release downstream of H₂R, either directly or indirectly. Gq-mediated Ca²⁺ release is a process that involves multiple steps that amplify the response (Berridge et al., 2000). This may explain why, after H₂R activation, the response of the Gq biosensor remains under the threshold of detection, but still leads to robust calcium release.

Activation of the H₃R or H₄R did not result in a change of YFP/CFP FRET ratio (Fig. 5, D and E, black traces). In control cells transfected with Ycam, we did not observe a change in YFP/CFP FRET ratio upon stimulation with histamine, but stimulation with carbachol resulted in a transient increase in YFP/CFP FRET ratio (250–350%) (Fig. 5, B–E, gray traces).

The amplitude of the FRET ratio change at t = 100 seconds was quantified from single cells per histamine receptor subtype (Fig. 5F). We did not observe large differences in basal FRET ratio between the receptor subtypes (Fig. 5G).

From this, we conclude that activation of the H₂R and, surprisingly, the H₃R leads to release of intracellular Ca²⁺, providing evidence for Gq coupling at both of these receptors.

**Analysis of cAMP Signaling by Four Histamine Receptor Subtypes.** To assess the production of cAMP upon stimulation of the four histamine receptor subtypes, we performed live cell measurements on HeLa cells transfected with a previously published Gα₁ biosensor (van Unen et al., 2016) (Fig. 7A) and cotransfected with H₁R-p2A-RFP, H₂R-p2A-RFP, H₃R-p2A-RFP, or H₄R-p2A-RFP. Stimulation of the H₁R resulted in fast reversible change in YFP/CFP FRET ratio (10–20%). Overnight preincubation of cells with PTX completely abrogated this response, further strengthening the evidence for activation of Gα₁ by H₁R (Supplemental Fig. 5). Stimulation of the H₂R did not result in a change in YFP/CFP FRET ratio. Stimulation of control cells transfected with only the Gα₁ biosensor did not result in a change in YFP/CFP FRET ratio (Fig. 7, B–E, gray traces). The amplitude of the FRET ratio change at t = 100 seconds was quantified from single cells per histamine receptor subtype, showing clear activation of the Gα₁ biosensor by subtypes H₁R, H₂R, and H₃R (Fig. 7F). We did not observe large differences in basal FRET ratio between the receptor subtypes (Fig. 7G).

These results led us to conclude that the H₁R, H₂R, and H₃R can robustly couple to and activate the heterotrimeric G-protein Gα₁.

**Single-Cell Analysis of Pharmacological Parameters with FRET-Based Biosensors**

Based on the systematic interrogation with FRET biosensors that measure the activation of different G-protein families in this study, we propose a revision of G-protein selectivity at the four histamine receptor subtypes (summarized in Fig. 8A).

Finally, we tested whether FRET-based biosensors can be used to determine important pharmacological parameters, including antagonist specificity and concentration-response curves. To demonstrate the application of a FRET sensor for the rapid testing of multiple antagonists, we transfected HeLa cells with the TEPACVV biosensor and cotransfected with H₂R-p2A-RFP. Cells were sequentially stimulated with histamine, mepyramine, thioperamide, and ranitidine at the indicated time points (Fig. 8B). Histamine addition resulted in an expected
Fig. 6. cAMP signaling by histamine receptors. (A) Production of cAMP by the four histamine receptor subtypes visualized by the \( \text{EPAC}^{\text{CFP}} \) biosensor and measured by FRET ratio. (B) Hela cells transfected with H1R-p2A-RFP and the \( \text{EPAC}^{\text{CFP}} \) biosensor were treated with histamine and mepyramine (black). Control cells were transfected with only the \( \text{EPAC}^{\text{CFP}} \) biosensor (gray). (C) Cells transfected with H2R-p2A-RFP and the \( \text{EPAC}^{\text{CFP}} \) biosensor were treated with histamine and ranitidine (black). Control cells were transfected with only the \( \text{EPAC}^{\text{CFP}} \) biosensor (gray). (D) Cells transfected with H3R-p2A-RFP and the \( \text{EPAC}^{\text{CFP}} \) biosensor were treated with histamine and thioperamide (black). In the control condition, cells were transfected with only the \( \text{EPAC}^{\text{CFP}} \) biosensor (gray). (E) Cells transfected with H4R-p2A-RFP and the \( \text{EPAC}^{\text{CFP}} \) biosensor were treated with histamine and thioperamide (black). In the control condition, cells were transfected with only the \( \text{EPAC}^{\text{CFP}} \) biosensor (gray). (F) The median and average (+) amplitude of the FRET ratio change at t = 100 seconds (F) and the raw YFP/CFP ratio at t = 0 seconds (G) per receptor subtype, quantified from the experiments shown in (B–E). Box limits
fast drop in YFP/CFP ratio (40–60%), and only upon addition of ranitidine did the ratio partly return to baseline levels, showing the specific inhibition of the H2R by ranitidine. To explore the possibility of using FRET sensors for single-cell concentration-response curves, we transfected HeLa cells with H1R-p2A-RFP and the Goq biosensor, the Go1i biosensor, or the DORA RhoA biosensor. Titration of increasing amounts of histamine resulted in concentration-response curves with pEC50 values of 6.05 [95% confidence interval (CI), 6.21–7.88] for Goq activation (Fig. 8C), 5.07 (95% CI, 5.63–4.50) for RhoA activation (Fig. 8D), and 5.05 (95% CI, 5.78–4.33) for Go1i activation (Fig. 8E).

Next, we evaluated the effect of a potent synthetic H1R ligand, methylhistaprodifen (Elz et al., 2000), on Goq versus Go1i activation. The concentration-response curves yielded pEC50 values of 7.50 (95% CI, 7.61–7.27) for Goq activation (Fig. 8C) and 5.30 (95% CI, 6.53–4.07) for Go1i activation (Fig. 8E). The extent of Go1i activation by methylhistaprodifen was attenuated relative to histamine.

To assess the potency of the H1R and H2R for the activation of Go1i, we transfected HeLa cells with the Go1i biosensor and the H2R-p2A-RFP or H1R-p2A-RFP. We found pEC50 values of 7.71 (95% CI, 7.94–7.48) and 8.09 (95% CI, 8.28–7.91) for the H2R and H1R, respectively (Fig. 8F). When we titrated increasing histamine concentrations in cells transfected with H2R-p2A-RFP and the 1EPACV biosensor, we observed a transient full response on 1EPACV even at the lowest concentrations used, which rendered the data unsuitable for concentration-response curve analysis (for raw YFP/CFP ratio traces, see Supplemental Fig. 6). From these data, we conclude that FRET biosensors can be used to assess antagonist specificity at receptors, and that they can be used to obtain single-cell concentration-response curves.

**Discussion**

Using several biosensors based on FRET, we have characterized the canonical G-protein–coupled signaling profiles of the four histamine receptors. Our results provide evidence that, besides the well known activation of Goq, the H2R can also couple efficiently to Go1i proteins, in agreement with previously published results (Murayama et al., 1990; Seifert et al., 1994). We also found a small increase in cAMP production following H1R activation, which provides evidence toward Gαs coupling via H1R. Activation of the H2R greatly increased the production of cAMP, which was described previously, but surprisingly we also found a Goq-mediated increase in Ca2+ upon stimulation of this receptor. The H3R and H4R seem to couple exclusively to Go1i proteins, which is in good agreement with the literature. The absence of Ca2+ release by H3R and H4R indicates that Gβγ-mediated activation of PLC, which is strongly cell-type dependent, is not effective under our conditions (Khan et al., 2013). Moreover, the experiments presented in this paper show that FRET biosensors can be used to examine antagonist specificity and potency of GPCR ligands. It must be noted that we did not assess the specific activation of Ga12/Gα13 proteins by histamine receptors, as robust and specific FRET sensors for this G-protein family do not exist yet. Still, we expect that Ga12/Gα13 activity can be picked up by the DORA RhoA sensor, and it can be separated from a Goq response by using the specific Goq inhibitor FR900359. The H2R-mediated activation of RhoA has been described in detail before and is specifically induced by Goq (van Unen et al., 2015a), but we cannot exclude the possibility that the small responses on the DORA RhoA biosensor after H2R and H3R activation are partly mediated by Ga12/Gα13 activation. Previously, we determined that, under similar experimental conditions, HeLa cells have 710 fmol/mg binding sites for H2R (Adjobo-Hermans et al., 2011), which corresponds roughly to 680,000 receptors per cell at a 25% transfection efficiency (assuming a cell volume of 2 pl and a protein concentration of 0.2 mg/ml). We did not notice differences in fluorescence levels between the four different isoforms in this study, either when directly tagged or in the case of a cotranslated mCherry. We note that the fluorescence intensity of the cotranslated mCherry can be used as a measure for receptor level since the 2A peptide produces two proteins in a 1:1 stoichiometry.

Although not explored in this work, FRET biosensors are also very well suited to report spatial signaling information, which can be used to distinguish signaling at the plasma membrane (G-protein activation) from signaling in the cytosol (cAMP production/Ca2+ release) or other subcellular locations (Pilić and Schultz, 2008). Specifically, the Go1 biosensors measure Gαi activation in a more relevant cellular state than the classic biochemical assays that require forskolin-induced cAMP production. On the other hand, population-based methods allow for a higher throughput.

Furthermore, FRET biosensors can be multiplexed, meaning that multiple signaling readouts can be measured at the same time in the same single cell (Pilić and Schultz, 2008). A related approach is to combine information on the FRET biosensor readouts with spatial and temporal information on cell shape or cell behavior (van Unen et al., 2015a). The combination of FRET biosensors and microfluidics approaches (Martins et al., 2012; Sackmann et al., 2014) can be used to solve more detailed questions around GPCRs by delivering more precisely defined stimulations to cells (for example, repeated stimuli or gradients).
Fig. 7. Gαi signaling by histamine receptors. (A) Activation of the heterotrimeric G-protein Gαi by the four histamine receptor subtypes, as measured by FRET ratio. (B) Hela cells transfected with H1R-p2A-RFP and the Gαi biosensor were treated with histamine and mepyramine (black). Control cells were transfected with only the Gαi biosensor (gray). In the control condition, cells were transfected with only the Gαi biosensor (gray). (C) Cells transfected with H2R-p2A-RFP and the Gαi biosensor were treated with histamine and ranitidine (black). In the control condition, cells were transfected with only the Gαi biosensor (gray). (D) Cells transfected with H3R-p2A-RFP and the Gαi biosensor were treated with histamine and thioperamide (black). In the control condition, cells were transfected with only the Gαi biosensor (gray). (E) Cells transfected with H4R-p2A-RFP and the Gαi biosensor were treated with histamine and thioperamide (black). In the control condition, cells were transfected with only the Gαi biosensor (gray). The median and average (+) amplitude of the FRET ratio change at t = 100 seconds (F) and the raw YFP/CFP ratio at t = 0 seconds (G) per receptor subtype, quantified from the experiments shown in (B–E). Box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. HeLa cells were stimulated with histamine at t = 32 seconds, and the response was antagonized by the addition of the appropriate antagonist at t = 152 seconds. Time traces show the average ratio change of YFP/CFP fluorescence (± S.E.M.). AC, adenylyl cyclase; DAG, diacylglycerol; mDia, mammalian diaphanous-related formin 1; PKA, protein kinase A; PKC, protein kinase C; ROCK, Rho-associated coiled-coil-containing protein kinase.
We demonstrate that the contrast of several existing FRET biosensors is sufficient to use them for real-time single-cell analysis. Whether these FRET-based sensors provide sufficient sensitivity for high-throughput cell-based screening remains to be investigated.

A general limitation of (FRET-based) biosensors is the buffering or amplification of signals by the overexpressed biosensors. This is especially relevant at longer timescales when the signals are part of feedback or feedforward loops. This can be prevented by limiting the expression levels and measuring for short time periods.

A specific limitation of the $G_{\alpha q}$ and $G_{\alpha i}$ FRET biosensors is that they depend on overexpression of the heterotrimer, which possibly affects the natural coupling preference of the GPCR for certain classes of G-proteins. Future studies using gene-editing techniques such as CRISPR-Cas9 (Lackner et al., 2015) can overcome this limitation by tagging the endogenous subunits with fluorescent proteins instead.

A small percentage of the HEK293 control cells (5%) in the calcium release experiment show a response on the Ycam biosensor after the first stimulation with histamine. This possibly represents a $G_{\alpha q}$-mediated response in cells that have a high expression of endogenous H2R. There is conflicting evidence about endogenously expressed histamine receptors in HEK293 cells (Iwata et al., 2005; Atwood et al., 2011). A possible explanation could be that this $G_{\alpha q}$-mediated response is due to the recently found heterodimerization or cross-talk of possible endogenous H1R and the ectopically expressed H2R (Alonso et al., 2013), which could also provide an alternative explanation for the results of the TEPACVV biosensor presented in Supplemental Fig. 4A.

The different additions of the concentration-response curves in our experiments are cumulatively added to cells. Because of the repeated stimulation regimen, desensitization effects or receptor internalization events could take place during our experiments. However, our obtained pEC50 values for histamine...
at the H2R (6.05), H3R (7.71), and H4R (8.09) compare well to previously published pEC50 values obtained in GTPase assays in SF9 insect cells (Seifert et al., 2013).

The concentration-response curves obtained with methylhistadipriden show a preference of Goq over Goi when compared with the natural ligand histamine, suggesting ligand-biased signaling. Whether such a preference is conveyed to downstream signaling and a different physiologic outcome will be an interesting future direction.

Internal calcium release, as measured by the Yeam FRET biosensor, leads to a fast transient calcium spike in receptor overexpression conditions when stimulated with saturating agonist concentration. The measured responses after endogenous receptor stimulation (carbachol in our experiments) can differ vastly from oscillatory behavior in various frequencies to a single or multiple sparsely distributed spikes. Because of the extreme heterogeneity in responses, the all-or-nothing response pattern for overexpressed receptors, and the many factors that can contribute to the calcium signal (Berridge et al., 2000), we deemed these measurements not suitable for the generation of concentration-response curves. Similarly, we observed a transient response for the cAMP biosensor, hindering the determination of the potency of the H2R. In general, a requirement for obtaining concentration-response curves from single-cell data is that the response of the biosensor reaches a plateau on a timescale of seconds. This seems to be a general feature of the heterotrimeric G-protein biosensors, highlighting the need for this kind of FRET sensor for Goa and Go12/Go13.

In conclusion, we characterized the canonical G-protein signaling profiles of the four histamine receptor subtypes using FRET biosensor data. Moreover, we show that it is feasible to produce concentration-response curves from single-cell measurements, and that FRET biosensors can be used to screen for antagonist specificity. We expect that FRET-based biosensor measurements provide a valuable addition to the existing palette of quantitative cell-based methods for measuring GPCR activation.

Acknowledgments

The authors thank H. Vischer (VU University) for providing cDNA encoding H2R, and A. Pietraszewska (University of Amsterdam) for the cloning of N1-H2R-RmCherry and N1-H2R-p2a-mCherry. We are grateful to Y. Wu (UConn Health, Farmington, CT) for sharing the DORA RhoA sensor, and to Andrea Strasser and Sigurd Elz (University of Regensburg, Germany) for providing methylhistadipriden. The authors thank Carsten Hoffmann (University of Würzburg, Würzburg, Germany) for critically reading the manuscript.

Authorship Contributions

Participated in research design: van Unen, Postma, Gadella, Goedhart.
Conducted experiments: van Unen, Rashidfarrokh.
Performed data analysis: van Unen, Hoogendoorn, Postma, Goedhart.
Wrote or contributed to the writing of the manuscript: van Unen, Rashidfarrokh, Hoogendoorn, Gadella, Goedhart.

References

Signaling Downstream of Histamine Receptor Subtypes 175
Supplementary information

Quantitative single cell analysis of signaling pathways activated immediately downstream of histamine receptor subtypes

Jakobus van Unen, Ali Rashidfarrokhi, Eelco Hoogendoorn, Marten Postma, Theodorus W.J. Gadella Jr., Joachim Goedhart

*Molecular Pharmacology*
Supplemental Figure 1

Representative images of the mCherry fluorescence in HeLa cells transfected with either Histamine-1, Histamine-2 or Histamine-3 receptors fused to p2A-mCherry. Width of the individual images corresponds to 73µm.
HEK293 cells transfected with H₁R-p2A-RFP and the DORA RhoA biosensor show a fast and reversible change in YFP/CFP ratio upon stimulation with histamine and mepyramine (black). Control cells transfected with the DORA RhoA biosensor do not show any change in YFP/CFP ratio upon stimulation with histamine and mepyramine (grey). HEK293 cells were stimulated with histamine at $t = 32s$ and the response was antagonized by the addition of mepyramine at $t = 152s$. Time traces show the average ratio change of YFP/CFP fluorescence (± s.e.m).
HEK293 cells transfected with H$_2$R-p2A-RFP and the Ycam biosensor show a fast transient change in YFP/CFP ratio upon stimulation with histamine and a slightly sustained increased ratio. Subsequent stimulation with Carbachol shows another fast transient change in the YFP/CFP ratio, resulting in a sustained elevated ratio (*black*). Control cells transfected with the Ycam biosensor do not show any change in YFP/CFP ratio upon stimulation with histamine and show a fast change in YFP/CFP ratio upon stimulation with carbachol (*grey*). Cells treated for 1 hour with the G$q$ inhibitor FR900359, and transfected with H$_2$R-p2A-RFP and the Ycam biosensor do not show any change in YFP/CFP ratio upon stimulation with histamine or carbachol. HEK293 cells were stimulated with histamine at $t = 40s$ and stimulated with carbachol at $t = 160s$. Time traces show the average ratio change of YFP/CFP fluorescence (± s.e.m).
Supplemental Figure 4

(A) HEK293 cells transfected with H$_2$R-p2A-RFP and the $^{\text{TEPAC}}$V biosensor show a fast change in YFP/CFP ratio upon stimulation with histamine and a minimal recovery after addition of ranitidine \textit{(black)}. Control cells transfected with the $^{\text{TEPAC}}$V biosensor show a fast change in YFP/CFP ratio upon stimulation with histamine and partial recovery after addition of ranitidine \textit{(grey)}. (B) Hela cells transfected with H$_1$R-p2A-RFP and the $^{\text{TEPAC}}$V biosensor show a small fast and reversible change in YFP/CFP ratio upon stimulation with histamine and mepyramine \textit{(black)}. Hela cells transfected with
H₁R-p2A-RFP and the TEMAC biosensor, and treated overnight with 100ng/ml Pertussis Toxin (PTX) or treated for 2 hours with 2µM of a Gαq inhibitor FR900359, also show a small fast and reversible change in YFP/CFP ratio upon stimulation with histamine and mepyramine (red and blue, respectively). HEK293 (A)/HeLa (B) cells were stimulated with histamine at t = 32s and the response was antagonized by the addition of ranitidine (A) at t = 172s or mepyramine (B) at t = 152s. Time traces show the average ratio change of YFP/CFP fluorescence (± s.e.m).

Supplemental Figure 5

[Graph showing YFP/CFP ratio changes for H₁R-p2A-RFP and Gαi biosensor with and without Pertussis Toxin (PTX).]

Hela cells transfected with H₁R-p2A-RFP and the Gαi biosensor show a fast and reversible change in YFP/CFP ratio upon stimulation with histamine and mepyramine (black). Cells treated overnight with 100ng/ml Pertussis Toxin (PTX), and transfected with H₁R-p2A-RFP and the Gαi biosensor do not show any change in YFP/CFP ratio upon stimulation with histamine and mepyramine (red). HeLa cells were stimulated with histamine at t = 32s and stimulated with mepyramine at t = 152s. Time traces show the average ratio change of YFP/CFP fluorescence (± s.e.m).
Supplemental Figure 6

A  H1R: Gq activation

B  H1R: Gq activation

C  H1R: Gq activation

D  H1R: Gq activation

E  H1R: RhoA activation

F  H2R: cAMP production

G  H3R: Gqi activation

H  H4R: Gqi activation
Single cell pharmacology measurements of the four histamine receptor isoforms. (A) Hela cells transfected with H1R-p2A-RFP and the Gaq biosensor were stimulated with the indicated cumulative concentrations of histamine at $t = 32s$, $t = 92$, $t = 152$, $t = 212$, and subsequently antagonized with mepyramine at $t = 292$ ($n = 26$). (B) Hela cells transfected with H1R-p2A-RFP and the Gaq biosensor were stimulated with the indicated cumulative concentrations of methylhistaprodifen at $t = 32s$, $t = 92$, $t = 152$, $t = 212$, $t = 272$, $t = 332$ and subsequently antagonized with mepyramine at $t = 412$ ($n = 17$). (C) Hela cells transfected with H1R-p2A-RFP and the Gai biosensor were stimulated with the indicated cumulative concentrations of histamine at $t = 32s$, $t = 92$, $t = 152$, $t = 212$, and subsequently antagonized with mepyramine at $t = 292$ ($n = 17$). (D) Hela cells transfected with H1R-p2A-RFP and the Gai biosensor were stimulated with the indicated cumulative concentrations of methylhistaprodifen at $t = 32s$, $t = 92$, $t = 152$, $t = 212$, $t = 272$, $t = 332$ and subsequently antagonized with mepyramine at $t = 412$ ($n = 10$). (E) Hela cells transfected with H1R-p2A-RFP and the DORA RhoA biosensor were stimulated with the indicated cumulative concentrations of histamine at $t = 32s$, $t = 92$, $t = 152$, $t = 212$, and subsequently antagonized with mepyramine at $t = 292$ ($n = 44$). (F) Hela cells transfected with H2R-p2A-RFP and the $^\text{TEPACVV}$ biosensor were stimulated with the indicated cumulative concentrations of histamine at $t = 32s$, $t = 92$, $t = 152$, $t = 212$, and subsequently antagonized with ranitidine at $t = 292$ ($n = 32$). (G) Hela cells transfected with H3R-p2A-RFP and the Gaii biosensor were stimulated with the indicated cumulative concentrations of histamine at $t = 32s$, $t = 92$, $t = 152$, $t = 212$, $t = 272$ and subsequently antagonized with thioperamide at $t = 292$ ($n = 29$). (H) Hela cells transfected with H4R-p2A-RFP and the Gaii biosensor were stimulated with the indicated cumulative concentrations of histamine at $t = 32s$, $t = 92$, $t = 152$, $t = 212$, $t = 272$ and subsequently antagonized with thioperamide at $t = 292$ ($n = 16$). Time traces show the average ratio change of YFP/CFP fluorescence ($\pm$ s.e.m).