Co-expression of guanine nucleotide-binding regulatory (G) protein-coupled receptors (GPCRs), such as the G\textsubscript{i/o}-coupled human 5-hydroxytryptamine receptor 1B (5-HT\textsubscript{1B}R), with the G\textsubscript{q/11}-coupled human histamine 1 receptor (H\textsubscript{1}R) results in an overall increase in agonist-independent signaling, which can be augmented by 5-HT\textsubscript{1B}R agonists and inhibited by a selective inverse 5-HT\textsubscript{1B}R agonist. Interestingly, inverse H\textsubscript{1}R agonists inhibit constitutively H\textsubscript{1}R-mediated as well as 5-HT\textsubscript{1B}R agonist-induced signaling in cells co-expressing both receptors. This phenomenon is not solely characteristic of 5-HT\textsubscript{1B}R; it is also evident with muscarinic M\textsubscript{2} and adenosine A\textsubscript{1} receptors and is mimicked by mastoparan-7, an activator of G\textsubscript{i/o} proteins, or by over-expression of G\textsubscript{q/11} subunits. Likewise, expression of the G\textsubscript{q/11}-coupled human cytomelanosirus (HCMV)-encoded chemokine receptor US28 unmasks a functional coupling of G\textsubscript{i/o}-coupled CCR1 receptors that is mediated via the constitutive activity of receptor US28. Consequently, constitutively active G\textsubscript{q/11}-coupled receptors, such as the H\textsubscript{1}R and HCMV-encoded chemokine receptor US28, constitute a regulatory switch for signal transduction by G\textsubscript{i/o}-coupled receptors, which may have profound implications in understanding the role of both constitutive GPCR activity and GPCR cross-talk in physiology as well as in the observed pathophysiology upon HCMV infection.

GPCRs,\textsuperscript{1} which can be activated by a diverse array of stimuli, represent the largest group of integral membrane proteins involved in signal transduction. As such, GPCRs are the primary therapeutic target for many of today’s drugs (1). The heterotrimeric G proteins mediate signaling from a large number of diverse GPCRs to a variety of intracellular effectors (see Refs. 2 and 3). A large body of work investigating the mechanisms underlying receptor-G protein interactions supports a network of interactions between signaling pathways that converge and diverge at multiple levels, enabling cells to coordinate responses to diverse environmental stimuli (4).

The hitherto existing knowledge of GPCR signal transduction pathways are founded largely upon experimental data obtained by the individual stimulation of the receptor of interest by specific ligands, either in heterologous expression systems or native tissues. Yet, under physiological conditions cells are permanently co-stimulated by various agonists. Investigations using receptor co-stimulation with agonists have recently shown cooperative effects of G\textsubscript{i/o} and G\textsubscript{s} (5), G\textsubscript{i/o} and G\textsubscript{q/11} (6), and G\textsubscript{s} and G\textsubscript{q/11} (7), the ability of G\textsubscript{i/o}-coupled receptors to activate G\textsubscript{s}-coupled receptors to transduce their signals by G\textsubscript{q/11} exchange (8), as well as G\textsubscript{q/11}-mediated glucocorticoid receptor transactivation (9). Such synergistic signaling mechanisms have been suggested to play an important role in signal adaptation (8) and may have a significant role in both physiological and pathophysiological processes (5).

Investigations of GPCRs expressed in surrogate cell systems have greatly modified our understanding of the pharmacological properties of GPCRs and consequently of the mechanistic drug-receptor models used to simulate drug action (10, 11). One of the prominent recent additions to our understanding of GPCR action is the occurrence of constitutive, agonist-independent GPCR activity (12–15). Currently, all models consider agonist-independent activity secondary to spontaneous isomerization of the receptors between the inactive and active receptor state(s), which couple(s) to the G protein; agonists are considered to preferentially bind to the active receptor state, whereas inverse agonists preferentially bind to the inactive receptor state (11). Despite initial concerns, the physiological relevance of constitutive GPCR activity is now accepted, and it appears not only to be a common property of GPCRs but, in various instances, also to be involved in the initiation or progression of disease (15). Various polymorphic GPCR variants have been shown to be highly constitutively active (15, 16), but also certain wild-type GPCRs, such as the histamine H\textsubscript{2} receptor, exhibit high constitutive activity in vivo (17). Moreover, viral infection of cells may also result in the expression of virally encoded GPCRs that exhibit high levels of constitutive activity (18, 19).

To date, the impact of constitutive GPCR activity on signaling properties on co-expressed GPCRs has not been studied in full detail, despite the potential (patho)physiological relevance. In the present study, we investigated the potential cross-talk between a variety of G\textsubscript{i/o} and constitutively active G\textsubscript{q/11}-coupled receptors using a heterologous expression system. Our data indicate that both activated histamine H\textsubscript{2}R and HCMV-encoded receptor US28 can result in the propagation of G\textsubscript{i/o}-coupled receptor dependent signaling. Therefore, active G\textsubscript{q/11}—
coupled receptors constitute a regulatory role in the regulation of Gα-coupled receptor signaling events. As a consequence, the synergistic activation of signaling cascades that is observed upon co-expression of constitutively active receptors may be modulated by ligands acting at either Gα/11 or Gα/1 coupled receptors.

EXPERIMENTAL PROCEDURES

Materials—pNP-KB-Luc was obtained from Stratagene (La Jolla, CA). ATP disodium salt, bovine serum albumin, CGS-12066A maleate, chloroquine diphosphate, chola toxin, DEAE-dextran (chloride form), histamine dihydrochloride, mastoparan-7, mepymazine (pyrimilamine maleate), myo-inositol, myo-inositol hexaphosphate (10 mM), myo-inositol pentaphosphate, myo-inositol tripentenaminomethane hydrochloride, 3,3',5,5'-tetramethylbenzidine liquid substrate system for ELISA, tripolidine hydrochloride, and Tween 20 were purchased from Sigma. The human chemokine RANTES (CCL5) was obtained from Peprotech (Rocky Hill, NJ), n-Luciferin was obtained from Dufcheca Biochemie BV (Haarlem, The Netherlands), glycerol from Riedel-de-Haen (Germany), Triton X-100 from Fluka (Switzerland), and goat anti-mouse horseradish peroxidase conjugate from Bio-Rad. Cell culture media, penicillin, and streptomycin were obtained from Invitrogen. Fetal calf serum was obtained from Integro B.V. (Dieren, The Netherlands) and dailed fetal calf serum from HyClone® Laboratories Inc. (H)Mepyramine (30 mM/ml), (H)5-HT (20 mM/ml), and myo-inositol hexaphosphate (10 mM/ml) was obtained from Amersham Biosciences.

The gifts of aacervinase (The Wellcome Foundation Ltd.), GR-127935 (Dr. P. R. Saxena), mianserin hydrochloride (Organon N.V., Oss, The Netherlands), (R)- and (S)-cetirizine hydrochloride (UCB Pharma), SA22429 (SmithKline Beecham), and pDEF (Dr. J. Langer) and of the cDNAs encoding bovine Glh5 (Dr. M. Lohse), Glh2 (Dr. I. Fyengar), Clos- tridium botulinum c exoenzyme (Dr. S. Narumiya), US28 (encoded by VHL/HCMV strain, GenBank™ accession number L29501, bases 219000–220263) in pDNA. 14C, the human CR1 receptor in pDN. (Dr. C. Tensen), the human serotonin 5-HT1R receptor in pKCREH (Dr. N. Stam), the human muscarinic M1 receptor in pcD (Dr. R. Maggio), the human adenosine A1 receptor in pcDNA (Dr. S. A. Rikvde), the porcine α2A-adrenergic receptor and the adrenergic toxin (PTX-insensitive mutant rat GαS-C351I (α2A-GαS-C351I) (20) (Dr. G. Milligan), and the human histamine H1 receptor (Dr. H. Fukui) are gratefully acknowledged.

DNA Constructs—3–22)–US28 and the HA-tagged versions of both wild-type and 3–22)–US28 were generated by PCR as described previously (18). The single amino acid mutation for US28R129A was introduced using the Altered Sites II in vitro mutagenesis system (Promega, Madison, WI) as described earlier (22). All constructs were verified by dyeo sequencing.

Cell Culture and Transfection—COS-7 African green monkey kidney cells were maintained at 37 °C in a humidified 5% CO2, 95% atmosphere of H1R-binding buffer. The COS-7 cell homogenates were incubated with ice-cold H1-binding buffer. The COS-7 cell homogenates were incubated for 30 min at 25 °C in 50 mM Na2/H-phosphate buffer (pH 7.4) in 400 μl with 1 μM mepymazine. The nonspecific binding was determined in the presence of 1 μM mianserin. The incubations were stopped by rapid dilution with 3 ml of ice-cold 50 mM Na2/H-phosphate buffer (pH 7.4). The bound radioactivity was separated by filtration through Whatman GF/C filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3 ml of buffer, and radioactivity retained on the filters was measured by liquid scintillation counting.

5-HTgR Binding Studies—COS-7 cells used for 5-HTgR binding studies were harvested 48 h after transfection and homogenized in ice-cold 5-HT-gelbinding buffer (50 mM Tris-HCl (pH 7.4), containing 4 mM CaCl2, 100 μM ascorbic acid, and 10 μM pargyline). 5-HTgR binding studies were performed using ~7 nM [125I]H-5-HT. The COS-7 cell home- genates that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3 ml of buffer, and radioactivity retained on the filters was measured by liquid scintillation counting. A Kd of 6.8 nm was subsequently used to calculate the expression levels of the 5-HT-gR (25).

US28 Receptor Binding Studies—The transfected COS-7 cells used for radioligand binding studies were seeded in 24-well plates; 48 h after transfection, binding was performed on whole cells for 3 h at 4 °C using [125I]CC15 (RANTES) in binding buffer (50 mM HEPES, pH 7.4, 1 mM CaCl2, 5 mM MgCl2, and 0.5% bovine serum albumin). After incubation, cells were washed four times at 4 °C with binding buffer supplemented with 0.5 mM NaCl. Nonspecific binding was determined in the presence of 0.1 μM unlabeled CCL5.

ELISA—48 h after transfection, receptor expression in COS-7 cells was measured using an ELISA as described previously (18). A mouse anti-HA monoclonal antibody was used as primary antibody and a goat anti-mouse-horseradish peroxidase conjugate as secondary antibody. The 3,3',5,5'-tetramethylbenzidine liquid substrate system for ELISA was used as substrate, and the optical density was measured in a Victor2 at 450 nm.

Analytical Methods—All data shown are expressed as means ± S.E. The data from radioligand-binding and functional assays data were evaluated by a nonlinear, least squares curve-fitting procedure using Graphpad Prism® (GraphPad Software, Inc., San Diego, CA).

RESULTS

Constitutive H1r Activity and Inverse Agonist Properties of Antihistamines—Transient expression of human H1R in COS-7 cells resulted in a high affinity binding site for the H1R radioligand [3H]mepyramine (Kd = 1.7 ± 0.2 nM, Bmax = 4.6 ± 0.1 pmol/mg protein, data not shown). H1R activates both PLC- and NF-κB-mediated enhancement of gene transcription as measured by the accumulation of [3H]inositol phosphates and NF-κB-driven reporter gene assays, respectively, in an agonist-dependent and -independent manner (Fig. 1). Moreover, the H1R-selective antagonist mepyramine acts as an inverse H1R agonist for the constitutive H1R-mediated activation of these signal transduction pathways. These data are in agreement with previous results indicating the constitutive H1R activity and inverse H1R agonist properties of various antihistamines (24, 26). The H1R-mediated effects on PLC and NF-κB activation are PTX-insensitive (see also Fig. 2A), indicating that the Gαi family of G proteins is not involved in these H1R-mediated responses (24, 26). Moreover, we have shown that constitutive H1R-mediated activation of these pathways can be enhanced selectively via co-expression of G proteins that belong to the Gαi family of G proteins (24), confirming that the H1R is coupled to Gαi proteins for modulation of these signaling events in these cells.

Constitutive Activation of NF-κB by the h5-HT3R—We evaluated the human 5-HT3R for constitutive activity upon transient expression of this receptor in COS-7 cells. Expression of the receptor was verified by [3H]5-HT radioligand binding studies, indicating 5-HT3R expression levels of ~ 310 fmol/mg protein (data not shown). We also assessed the capacity of
5-HT1B Rs to mediate activation of NF-κB. In 5-HT1B R-expressing cells the selective 5-HT1B agonist CGS-12066A (CGS) (27) and 5-HT stimulate NF-κB activation only ~0.6-fold (Fig. 1D). Despite the functional 5-HT1B R expression in these cells, we did not detect constitutive 5-HT1B R activation in our reporter gene assay, as the selective inverse 5-HT1B agonist mepyramine (10 μM) was without effect on basal NF-κB activation in 5-HT1B R-expressing cells (data not shown). Consistent with the notion of a Gxi/o-coupled 5-HT1B R receptor, 5-HT1B R-mediated NF-κB activation was PTX-sensitive (Fig. 1D), indicating the involvement of Gxi/o proteins in 5-HT1B R-mediated NF-κB activation.

5-HT1B R Signaling in Cells Co-expressing H1 R—In agreement with previous reports on agonist-mediated signaling (25, 29), stimulation of COS-7 cells co-expressing H1 R and 5-HT1B R with 10 μM CGS, a selective 5-HT1B R-agonist (27), results in a marked elevation of the inositol phosphate accumulation (Fig. 1C). Surprisingly, however, treatment of cells co-expressing both H1 R and 5-HT1B R with 10 μM CGS resulted in a robust PLC and NF-κB activation, yielding almost the same efficacy as histamine (His), which was completely sensitive to the inverse H1 R agonist mepyramine (10 μM, Fig. 1, C and D), indicating the importance of constitutive H1 R activity for the observed response to CGS. CGS is without effect on H1 R-mediated NF-κB activation in cells transfected with only H1 R-cDNA (Fig. 3A), whereas mepyramine (10 μM) does not influence CGS-induced 5-HT1B R-mediated NF-κB activation in cells transfected with only the 5-HT1B R-cDNA (data not shown), indicating that these ligands are selective for their respective receptors. Moreover, also in 3[H]5-HT and 3[H]mepyramine binding studies both ligands turned out to behave as selective ligands (data not shown). H1 R expression levels were unaffected by co-expression of 5-HT1B R as determined by 3[H]mepyramine radioligand binding studies (Bmax = 4.6 ± 1.3 pmol/mg protein, data not shown).

As seen in Figs. 2A and 3, A and C, co-expression of both GPCRs not only results in CGS-induced 5-HT1B R-mediated NF-κB activation but also in a 2–3-fold increase in basal NF-κB activation. The fold over basal activation of NF-κB by histamine is reduced from 4.8 ± 0.4-fold (n = 76), when H1 R is expressed alone, to 3.3 ± 0.5-fold (n = 9-fold), upon co-expression of the 5-HT1B R. These data do not reflect a suppression of the response to histamine when H1 R is co-expressed with 5-HT1B R; however, because of the increase in the level of basal signaling, it does result in a reduced fold of stimulation. The constitutive NF-κB activation observed upon co-expression of both GPCRs is partially PTX-sensitive but was completely inhibited by mepyramine (Fig. 2A). PTX treatment of the cells
had no effect on mepyramine-treated cells (Fig. 2A). H₁R is known to exhibit stereospecificity toward the enantiomers of the partial inverse H₁R agonist cetirizine (24, 26). Indeed, the inhibition of CGS-induced NF-κB activation by the enantiomers of cetirizine is found to be stereospecific in cells co-expressing H₁R and 5-HT₁B receptors (Fig. 3A).

The PTX-sensitive increase in basal H₁R-mediated NF-κB activation is that observed upon 5-HT₁₃R co-expression, but not the constitutive H₁R activity itself, is potently inhibited by the inverse 5-HT₁₃R agonist SB-224289 (28) (Fig. 3, C and D). SB-224289 inhibited constitutive NF-κB activation in cells co-expressing H₁ and 5-HT₁B receptors to the level of basal NF-κB activation observed in H₁R-expressing cells not expressing the 5-HT₁₃R.
activity upon co-expression with H1R (Fig. 3C). The observed pIC50 value of 8.0 ± 0.2 is in good agreement with literature data for SB-224289 acting on 5-HT1R (28). SB-224289 is without effect on H1R-mediated NF-κB activation in cells only expressing the H1R, indicating that SB-224289 does not exert its effects of inhibition of constitutive NF-κB activation in cells co-expressing H1 and 5-HT1B receptors by acting at H1Rs. Effects of Co-expression of Other Gq11-coupled Receptors on Constitutive H1R Activity—To test whether these observations are exclusive for 5-HT1R, we co-expressed H1R with other Gq11-coupled receptors: the adenosine A1 and the muscarinic M2 receptors. Co-expression of H1R with the A1 or M2 receptor indeed resulted in a similar increase in basal NF-κB activation, which was largely PTX-sensitive (Fig. 2).

As observed after co-expression with the 5-HT1A R, PTX-insensitive NF-κB activation is fully inhibited by mepyramine, indicating that the increased NF-κB activation depends on constitutive H1R signaling. Stimulation of the M2 receptor with the muscarinic agonist carbachol results in a further increased mepyramine- and PTX-sensitive NF-κB activation (Fig. 2B). We also tested the ability of a Gq11-coupled α2A adrenergic receptor, in which the C terminus is fused to a Goα subunit that harbors a mutation that renders the Goα insensitive to PTX (α2A-GoαC351I) (20), to enhance NF-κB activation of co-expressed H3Rα. As expected, stimulation of the α2A receptor with the specific agonist clonidine resulted in a PTX-insensitive increase in NF-κB activation that could be fully inhibited by mepyramine, indicating that the PTX-insensitive GoαC351I subunit fused to the α2A receptor mediates NF-κB activation via H1R (data not shown).

Effects of Co-expression of Gq11 and Gq111-coupled Chemokine Receptors—To investigate whether the observed responses are H1R-specific or more generally relevant, we tested the effect of the co-expression of two chemokine receptors on their signaling properties. We co-expressed the Gq111-coupled virally encoded chemokine receptor US28, a chemokine receptor homolog encoded by HCMV, which binds with high affinity the CC-chemokine CCL5 (RANTES) (Fig. 4, together with the human Gq11-coupled chemokine receptor CCR1. We previously reported the high constitutive activity of US28 in activation of both PLC- and NF-κB-mediated enhancement of gene transcription as measured by the accumulation of [3H]inositol phosphates and NF-κB-driven reporter gene assays, respectively (23) (Fig. 4A, B, and E). CCL5 binds with high affinity to receptor US28 but does not increase the level of activation of receptor US28, and therefore CCL5 acts as a neutral antagonist for this receptor (Fig. 4A) (23). In contrast, CCL5 binds to CCR1 with high affinity and acts as a CCR1 agonist, inducing calcium mobilization in a PTX-sensitive manner (see Ref. 30). When tested for activation of NF-κB, CCR1 does not exhibit basal NF-κB activation, and stimulation of CCR1 with CCL5 results in a small, PTX-sensitive NF-κB activation. Upon co-expression of receptor US28 and CCR1, however, CCL5 elicits a robust agonist response that is completely PTX-sensitive, indicating the involvement of Gq proteins in CCL5-mediated NF-κB activation (Fig. 4A). The binding of CCL5 to receptor US28 is not involved in the observed phenomenon as shown by co-expression of CCR1 with an N-terminal truncation mutant of receptor US28 (∆(2–22)-US28), in which the first 22 residues of receptor US28 are deleted. The N-terminal truncation mutant ∆(2–22)-US28 receptor does not exhibit [125I]CCL5 binding as observed by [125I]CCL5 saturation binding analysis (Fig. 4D), in line with reports that chemokine binding to chemokine receptors is critically dependent on the N terminus of the receptor (31). We used an ELISA to confirm cell surface expression of ∆(2–22)-US28 (45% of wild-type receptor US28, Fig. 4C). ∆(2–22)-US28 still activates both NF-κB (Fig. 4B) and PLC constitutively (Fig. 4E), and co-expression of ∆(2–22)-US28 with CCR1 resulted in CCL5-induced CCR1-mediated activation of NF-κB (Fig. 4B). In contrast, a mutant US28 receptor (US28-R129A) (22), which is expressed at the cell surface (Fig. 4, C and D), and which binds CCL5 with unchanged affinity in comparison with wild-type receptor US28 (Fig. 4D) but is devoid of constitutive activity (Fig. 4, B and E), does not transduce CCR1-mediated NF-κB activation upon co-expression with CCR1 (Fig. 4B); this indicates the importance of the constitutive activity of receptor US28 for the observed response to CCL5.

The effects of CCL5 that are observed upon co-expression of CCR1 and receptor US28 are similar to the effects observed for 5-HT1A R agonists in cells co-expressing H1R and 5-HT1B R. The co-expression of the Gq11-coupled receptor US28 with the Gq11-coupled CCR1 results in the alteration of CCR1 signaling capabilities. By co-expressing receptor US28 and CCR1, CCR1 acquires the capability to signal through NF-κB upon agonist (CCL5) stimulation. However, it does not appear to result in increased constitutive receptor activation, as we did not observe modulation of basal NF-κB activation in cells expressing receptor US28 upon co-expression of CCR1 (Fig. 4A).

Mechanism of Synergistic NF-κB Activation by Co-expressed Gq11 and Gq111-coupled Receptors—Various GPCRs may activate signal transduction pathways leading to NF-κB activation (see Fig. 7). Both the H1R and receptor US28 are reported to activate G proteins belonging to both the Gq11 and Gq111 families of G proteins (23, 24, 26, 32–35). Yet, Goα subunits are not implicated in the H1R or receptor US28-mediated activation of phospholipase C or NF-κB, as PTX treatment of the cells did not alter H1R or receptor US28-mediated [3H]inositol phosphate production or NF-κB activation (23, 24) (see also Figs. 2A and 4A).

Co-expression of H1R or receptor US28 with Goα or Go111 resulted in a Go expression level-dependent increase in constitutive H1R- or receptor US28-mediated NF-κB activation and inositol 1,4,5-trisphosphate production (23, 24) (Fig. 5A). Scavenging of Gβγ subunits by co-expression with either Goα (Fig. 5) or GRK2 (data not shown) effectively reduced constitutive H1R and receptor US28-mediated NF-κB activation, whereas co-expression with Gβγ subunits resulted in the elevation of the constitutive receptor activity of both H1R and receptor US28 (23, 24) (Fig. 5A). In line with published data on GPCR-mediated activation of NF-κB (see also Fig. 7) these data imply both the Goq111 and Gβγ subunits, which are released upon H1R or receptor US28-mediated activation of Gq111 proteins, in the activation of signal transduction pathways resulting in activation of NF-κB (23, 24).

Activation of H1R (36–38) and Goα signaling (39, 40), as well as NF-κB activation, have been linked to the activation of small G proteins and especially the Rho-like G proteins (41–45). We therefore investigated the role of Rho signaling in the H1 receptor-mediated activation NF-κB. C. botulinum cε exoenzyme, which prevents the coupling of RhoA, -B, and -C to its downstream effectors by irreversible ADP-ribosylation, has often been used to demonstrate the involvement of Rho in GPCR signaling, including NF-κB activation (42). Co-expression of the cε exoenzyme abrogates all, both constitutive as well as agonist-induced, human H1R-mediated NF-κB activation (Fig. 5B).

We have previously reported that constitutively activated (Q205L) Goq11 proteins do not enhance H1R-mediated NF-κB activation (24), which indicates that activation of Gq11-coupled...
Reciprocal Signaling by Co-expressed G\textsubscript{q11}/G\textsubscript{i0}-coupled GPCRs

Receptors most likely result in the release of G\beta\gamma subunits from activated G\textsubscript{i0} proteins, which then may serve to potentiate signaling mediated by G\textsubscript{q11} coupled receptors. To mimic activation of G\textsubscript{i0}-coupled receptors we used mastoparan-7 (M7), a relatively stable analogue of mastoparan, to directly activate G\textsubscript{i0} proteins and to stimulate both G\textsubscript{q11} and G\beta\gamma-mediated signal transduction pathways (46). M7 induced a PTX-sensitive NF-κB activation in cells expressing H\textsubscript{1}R to a similar extent as the 5-HT\textsubscript{1A}R agonist CGS (Fig. 6A). M7 also induced NF-κB activation in cells expressing H\textsubscript{1}R; this effect is effectively blocked by the inverse H\textsubscript{1}R agonist mepyramine (Fig. 6B), demonstrating that activated G\textsubscript{i0} proteins may enhance constitutive H\textsubscript{1}R-mediated NF-κB activation. Mepyramine did not affect M7-induced NF-κB activation in cells that do not express H\textsubscript{1}R (Fig. 6A).

**DISCUSSION**

We co-transfected COS-7 cells with cDNAs encoding various GPCRs and examined agonist-induced as well as constitutive GPCR-mediated stimulation of PLC- and NF-κB-regulated gene expression. Expression of either the human histamine H\textsubscript{1}R or the HCMV-encoded receptor US28 in COS-7 cells results in the activation of both PLC- and NF-κB-regulated gene expression in an agonist-dependent (H\textsubscript{1}R and receptor US28) (23, 24, 26). H\textsubscript{1}R- or receptor US28-mediated activation of PLC and NF-κB is PTX-insensitive (23, 24) (Figs. 1, 2, and 4), indicating that G\textsubscript{i0} proteins are not involved in these H\textsubscript{1}R- and receptor US28-mediated responses in COS-7 cells. By co-expression of a variety of G protein subunits, we have shown earlier that G\textsubscript{q11} as well as G\textsubscript{q11}/G\textsubscript{i0} subunits are involved in NF-κB activation via H\textsubscript{1}R (23, 24) (Fig. 7), whereas for both receptors, the activation of PLC is mediated via the classical G\textsubscript{q11} pathway (23, 24). In the present study we have investigated the effects of co-expression of various G\textsubscript{i0} coupled receptors on agonist-induced and constitutive activity of the G\textsubscript{q11}/G\textsubscript{i0} H\textsubscript{1}R and receptor US28 using a heterologous expression system.

In agreement with reports on synergistic agonist-induced PLC activation in cells co-expressing H\textsubscript{1}R and G\textsubscript{i0}-coupled 5-HT\textsubscript{1A}R (25, 29), stimulation of cells co-expressing both receptors with the selective 5-HT\textsubscript{1A}R agonist CGS-12066A (27) results in a marked synergistic elevation of inositol phosphate accumulation (Fig. 1). Moreover, upon co-expression of 5-HT\textsubscript{1A}R with H\textsubscript{1}R, CGS elicited a robust PTX-sensitive activation of NF-κB, whereas CGS had no such effects on NF-κB activation in cells lacking 5-HT\textsubscript{1A}Rs (Fig. 1).

Unexpectedly, we found that H\textsubscript{1}R-mediated effects of H\textsubscript{1}R-selective inverse H\textsubscript{1}R agonists such as mepyramine and cetirizine include the inhibition of CGS-induced 5-HT\textsubscript{1A}R-mediated activation of both PLC and NF-κB in cells co-expressing both 5-HT\textsubscript{1A}R and H\textsubscript{1}R (Figs. 1, 2, and 4). In contrast, inverse H\textsubscript{1}R agonists had no such effects on 5-HT\textsubscript{1A}R-mediated signaling in cells lacking H\textsubscript{1}Rs. Radioligand binding studies confirmed the H\textsubscript{1}R selectivity of the inverse H\textsubscript{1}R agonist, indicating that inverse H\textsubscript{1}R agonists do not modulate 5-HT\textsubscript{1A}R signaling via a direct interaction with either G\textsubscript{i0} proteins. We concluded from

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**FIG. 4.** Effects of CCL5 on NF-κB activation in cells expressing either CCR1 or US28 or co-expressing both CCR1 and US28 receptors compared with control cells (Mock). A, effects of co-expression of wild-type CCR1 and US28 receptors and PTX treatment

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(100 ng/ml) on CCL5-induced (100 nm) NF-κB activation. RLU, relative light unit. B, effects of co-expression of CCR1 and wild-type US28, Δ(2–22)-US28, or US28-R129A on CCL5-induced (100 nm) NF-κB activation. Similar results have been obtained using N-terminal HA-tagged US28 receptors. C, cell surface expression of N-terminal HA-tagged US28 receptors as monitored by ELISA using HA-specific antibodies. WT, wild type. D, cell surface expression of US28, US28-R129A, and Δ(2–22)-US28 receptors was monitored by whole cell [\textsuperscript{125}I]CCL5 binding. E, effects of wild-type US28, Δ(2–22)-US28, and US28-R129A on constitutive inositol phosphate accumulation.
these results that the interaction of inverse \( H_1 \)R agonists with \( H_1 \)Rs induces biological responses that negatively interfere with 5-HT1B-R signaling. Because these experiments were performed in triplicate. Dashed lines indicate the basal levels of NF-\( \kappa \)B activation observed in control cells (Mock) and in cells transiently expressing human \( H_1 \)R (\( H_1 \)) or the virally encoded chemokine receptor US28. Also shown are the effects of the \( H_2 \) agonist histamine (10 \( \mu M \)) and inverse \( H_1 \)R agonist mepyramine (10 \( \mu M \), Mep) on \( H_1 \)-mediated NF-\( \kappa \)B activation upon co-expression of \( \alpha_1 \), and \( \alpha_2 \), proteins and \( \alpha_3 \) subunits. B, effects of co-expression of the Rho inhibitor C. botulinum c\(_e\) exoenzyme (C3) on constitutive and agonist-induced (10 \( \mu M \) His) \( H_1 \)-mediated NF-\( \kappa \)B activation in COS-7 cells. Co-expressions were performed by co-transfection of equal amounts of the cDNA encoding \( H_1 \)R and the respective cDNAs coding for \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \), and the \( c_3 \) exoenzyme, respectively, whereas the total amount of transfected cDNA was maintained constant using pcDEFc.

The co-expression of \( \alpha_{11} \) and \( \alpha_{10} \)-coupled receptors may not only give rise to agonist induced signaling of \( \alpha_{10} \)-coupled receptors but may also affect constitutive signaling. In fact, co-expression of \( H_1 \)R and 5-HT1B-R allows the detection of otherwise undetectable constitutive 5-HT1B-R activity. Co-expression of 5-HT1B-R together with \( H_1 \)R resulted in a significantly increased constitutive activation of NF-\( \kappa \)B (Figs. 2 and 3), which was completely inhibited by the inverse \( H_1 \)R agonist mepyramine but also, partially, by the inverse 5-HT1BR agonist SB-224289 (28). This inverse 5-HT1B-R agonist inhibited only the increased constitutive NF-\( \kappa \)B activation that is observed upon co-expression of the 5-HT1B-R but not the constitutive \( H_1 \)-R-mediated NF-\( \kappa \)B activation (Fig. 3). Based on these findings we conclude that the otherwise minimal 5-HT1B agonist-induced or undetectable constitutive 5-HT1B-R responses are enhanced through constitutive \( H_1 \)Rs.

We subsequently investigated whether the observed phenomena are receptor-specific by testing the effects of co-expression of other \( \alpha_{11} \) and \( \alpha_{10} \)-coupled receptors on NF-\( \kappa \)B activation. To test whether these phenomena were specific for 5-HT1B-R, we co-expressed several other \( \alpha_{11} \)-coupled receptors with \( H_1 \)R. Stimulation of cells co-expressing the muscarinic M2 receptor (M2R) with \( H_1 \)R with the M2R agonist carbachol resulted in a PTX-sensitive activation of NF-\( \kappa \)B. Similar to our findings upon \( H_1 \)-R/5-HT1B-R co-expression, the inverse \( H_1 \)R agonist mepyramine potently inhibited carbachol-induced M2R signaling in cells co-expressing \( H_1 \)-Rs (Fig. 2). As observed for co-expression of 5-HT1B-R, we saw an increased constitutive activation of NF-\( \kappa \)B upon co-expression of M2R with \( H_1 \)R. Because similar observations were made with the \( \alpha_{10} \)-coupled adenosine A1 (Fig. 2) and the adrenergic \( \alpha_2 \) receptors, constitutively active \( \alpha_{11} \)-coupled \( H_1 \)R seems to act as a regulatory switch for important cellular functions of \( \alpha_{10} \)-coupled GPCRs, including the activation of PLC- and NF-\( \kappa \)B-mediated gene transcription.

To assess whether the observed phenomenon is limited to \( H_1 \)R, we tested an unrelated GPCR, the virally encoded chemokine receptor US28, which is highly constitutively active (23), for its capacity to transduce signaling events mediated through activation of \( \alpha_{10} \)-coupled receptors. As observed in the experiments with cells co-expressing the 5-HT1B-M2, or \( \alpha_2 \) receptors together with \( H_1 \)R, activation of the human \( \alpha_{10} \)-coupled chemokine receptor CCR1 with the CCR1 agonist CCL5 induces a robust PTX-sensitive NF-\( \kappa \)B activation in cells co-expressing the constitutively active \( \alpha_{11} \)-coupled receptor US28 (Fig. 4). In contrast, CCL5 does not affect NF-\( \kappa \)B-medi-
ated gene transcription in cells that express CCR1 but not receptor US28. Moreover, CCL5 has no effect on cells that express receptor US28, for which CCL5 is a neutral antagonist (3). However, the binding of CCL5 to US28 is not involved in the observed phenomenon, as CCL5 also induces a robust PTX-sensitive NF-κB activation in cells co-expressing CCR1 together with the constitutively active H1R, c-Src, and β-arrestin 1-dependent pathway, or a PLC- and protein kinase C-independent (21) pathway. DAG, diacylglycerol; InsP3, inositol 1,4,5-trisphosphate.

Gq/11-coupled GPCRs may activate NF-κB via both Gq and Gβγ-activated pathways (23, 24, 59, 60), which may involve phosphatidylinositol 3-kinase (PI3K) (59), RhoA (44, 45, 61–64), Akt (60), and protein kinase C (PKC)-dependent pathways (65, 66). Gαi/o-coupled GPCRs may activate NF-κB via an Akt-dependent pathway (67), as well as Gβγ- and c-Src- and β-arrestin 1-dependent pathways, or a PLC- and protein kinase C-independent (21) pathway. DAG, diacylglycerol; InsP3, inositol 1,4,5-trisphosphate.

As shown by co-expression or scavenging of Gβγ subunits, NF-κB activation is dependent on the expression of constitutively active Gαq/11 receptors in COS-7 cells (23, 24). Gαq/11 proteins activate RhoA (40), which can lead to activation of NF-κB via PLC (47, 48). Previous studies have also reported RhoA as a signaling partner for H1R (49, 50). Rho-mediated signaling has been implicated in, for example, H1R-mediated airway hyper-responsiveness (38) and activation of PLC (43). Co-expression of C. botulinum C3 exoenzyme, which is known to inactivate the small G protein Rho (42), completely abolishes H1R-mediated NF-κB activation (Fig. 5), implying that Rho is required in the H1R-mediated activation of NF-κB.

Although the activation of Gαi/o-coupled receptors may result in a limited degree of NF-κB activation, the expression of activated Gαi proteins in COS-7 cells does not result in NF-κB activation (23, 24) or Rho activation (51). It has been suggested that Gαi proteins are not sufficient or necessary for GPCR-mediated signal transduction cascades.

**Fig. 7.** Schematic representation of NF-κB activation by Gαq11- and Gαi/o-coupled GPCRs. Gαq11-coupled GPCRs may activate NF-κB via both Gq and Gβγ-activated pathways (23, 24, 59, 60), which may involve phosphatidylinositol 3-kinase (PI3K) (59), RhoA (44, 45, 61–64), Akt (60), and protein kinase C (PKC)-dependent pathways (65, 66). Gαi/o-coupled GPCRs may activate NF-κB via an Akt-dependent pathway (67), as well as Gβγ- and c-Src- and β-arrestin 1-dependent pathways, or a PLC- and protein kinase C-independent (21) pathway. DAG, diacylglycerol; InsP3, inositol 1,4,5-trisphosphate.
activation of Rho (52). To obtain evidence that the Gβγ subunits of Gq/11 proteins have a role in Gq/11-coupled receptor-mediated NF-κB activation, we stimulated COS-7 cells with mastoparan-7. Activation of heterotrimeric Gq/11 proteins using M7 results in the release of both the activated Goq/11 and Gβγ subunits (46). M7 induced PTX-sensitive NF-κB activation in COS-7 cells expressing 5-HT1B receptors to a similar extent as the 5-HT1B agonist CGS (Fig. 6). Moreover, M7 stimulated NF-κB activation in cells expressing the H1R to a similar extent as achieved by co-expression of Gβγ subunits (Figs. 5 and 6); this response was completely sensitive to the inverse H1R agonist mepyramine. Activation of Gi/o-coupled receptors may therefore provide additional free Gβγ subunits that synergize with constitutive H1R-mediated NF-κB activation.

While this manuscript was in preparation, constitutively active Goq/11-coupled cannabinoid CB1 receptors (CB1R) were shown to sensitize MAPK activation by the Goq/11-coupled orexin 1 receptor (OX1R), upon co-expression in Chinese hamster ovary cells (53). In this study orexin-mediated MAPK activation is shown to be sensitive to an inverse CB1R agonist (53). The authors explain the observed synergism by heterodimerization of the two GPCRs (53). In our view of the findings with M7 and the apparent lack of specificity for GPCR combinations, we do not consider heterodimerization a major determinant for our observations. However, our study does underscore the importance of constitutive GPCR activity in the modulation of cell signaling.

Our findings are consistent with the reported necessity for preactivation of various Goq/11-coupled receptors for Gi/o-coupled receptor-mediated signaling both in vitro and ex vivo (54). Pretreatment of blood vessels with histamine, for example, is known to yield an enhanced contractile response to 5-HT, which is mediated by previously “silent” 5-HT receptors (55). The conditional synergy might have important (patho)physiological significance, because constitutive activity of Goq/11-coupled receptors might be prominent in (patho)physiology (33).

Multiple mechanisms have previously been implicated in the NF-κB activation that is observed upon cytomegalovirus infection (56). HCMV infection of smooth muscle cells may result not only in the expression of constitutively active US28 receptors (18) but also in Goq/11-mediated (31) as well as PTK-sensitive NF-κB activation (57). Our present data suggest that expression of receptor US28 upon cytomegalovirus infection may be implicated in both Goq/11-mediated and PTK-sensitive NF-κB activation through the unmasking of signaling responses of Gi/o-coupled receptors. Therefore, these results provide a new mechanism by which the expression of constitutively active GPCRs, which may be constitutively active under physiological conditions (17) because of receptor mutation (15, 16) or viral infection (18, 19), may regulate signaling events through sensitization of cellular communication that may ultimately result in the initiation and/or progression of disease. These findings may also attribute potentially new roles to naturally occurring inverse agonists (see Ref. 58). Future studies will be required to address these issues in more detail.

In conclusion, our findings reveal a heretofore unrecognized role for constitutively active Goq/11-coupled receptors in the signaling events initiated by Gi/o-coupled receptors. Constitutively active Goq/11-coupled receptors constitute a regulatory switch for signal transduction by Gi/o-coupled receptors to unmask signaling events. The observed conditional synergy may be a key to the identification of the constitutive activity of native Goq/11-coupled GPCRs in vivo and could be useful as a sensitive screening strategy in drug discovery (e.g. for “de-orphanizing” receptors). Our findings suggest that endogenous levels of constitutive GPCR tone can be considerably higher than anticipated on cellular receptor number and expression levels of G proteins or effector molecules. Also, our data indicate that selective inverse agonists may affect signaling events that are induced upon activation of unrelated GPCRs. Although the implications of the present observations remain to be fully ascertained, these data clearly demonstrate the importance of cellular environment for GPCR function.

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27.在全球范围内，Goq/11-coupled GPCRs may play a role in the regulation of various cellular processes. The activation of these receptors has been linked to a variety of cellular responses, including cell proliferation, apoptosis, and inflammatory signaling pathways. The constitutive activity of Goq/11-coupled receptors has been shown to contribute to the development and progression of various diseases, such as cancer, inflammation, and neurodegenerative disorders. It has been suggested that the constitutive activity of Goq/11-coupled GPCRs may be a key to understanding these processes and developing effective therapeutic strategies. Further research is needed to elucidate the molecular mechanisms underlying the constitutive activity of Goq/11-coupled GPCRs and to identify potential pharmacological targets for the development of novel therapeutic agents.
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