Expression of a G Protein-coupled Receptor (GPCR) Leads to Attenuation of Signaling by Other GPCRs

EXPERIMENTAL EVIDENCE FOR A SPONTANEOUS GPCR CONSTITUTIVE INACTIVE FORM*

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The idea of G protein-coupled receptors (GPCRs) coupling to G protein solely in their active form was abolished when it was found that certain ligands induce a G protein–coupled but inactive receptor form. This receptor form interferes with signaling of other receptors by sequestering G protein. However, the spontaneous existence of this receptor species has never been established. The aim of the present work was to evaluate the existence of the spontaneous conformational change of the receptor inactive coupled to G protein able to interfere with the response of other GPCRs. According to the law of mass action, receptor overexpression should lead to increased amounts of all spontaneously occurring species. Based on this, we generated Chinese hamster ovary (CHO-K1)-derived cell lines expressing various amounts of the human histamine H2 receptor. In these systems, the signaling of other endogenously and transiently expressed GPCRs was attenuated proportionally to human H2 receptor expression levels. G protein transfection specifically reverted this attenuation, strongly suggesting hijacking of the G protein from a common pool. Similar attenuation effects were observed when the β2-adrenergic receptor was overexpressed, suggesting that this is a more general phenomenon. Moreover, in human mammary MDA-MB-231 cells, a consistent increase in the response of other GPCRs was observed when endogenous expression of β2-adrenergic receptor was knocked down using specific small interfering RNAs. Our findings show that GPCRs may interact with the signaling of other receptors by modulating the availability of the G protein and suggest the existence of GPCR spontaneous coupling to G proteins in an inactive form.

G protein-coupled receptors (GPCRs)2 form a large and functionally diverse superfamily of proteins that transduce signals across cell membranes. Although much is known about structural features of GPCRs involved in ligand recognition and G protein binding, the actual mechanism underlying GPCR signaling remains unclear.

Traditionally, agonist occupancy of GPCRs is believed to result in a conformational change in the receptor, leading to activation of G proteins (1). However, in genetically engineered systems where receptors can be expressed at high densities, Costa and Herz (2) noted that high levels of receptor expression uncovered the existence of a population of spontaneously (unliganded) active receptors, resulting in an elevated basal response in the system.

The histamine H2 receptor (H2R) is an extensively characterized member of the GPCR family, which in most systems couples to Gs proteins to activate adenylyl cyclase (3–6). Compared with other GPCRs, the H2R is unique in that the wild-type receptor possesses a remarkably high degree of constitutive activity. With a receptor density of 300 fmol/mg protein, constitutive H2 receptor activity could be detected in Chinese hamster ovary (CHO-K1) cells (7).

The notion that GPCRs also signal without an external chemical trigger, i.e. in a constitutive or spontaneous manner, resulted in a paradigm shift in the field of GPCR pharmacology. Before the discovery of constitutive GPCR activity, efficacy was considered only as a positive property (i.e. producing an increased receptor activity, and only ligand-induced activation of receptors was thought to induce G protein activity), but with the discovery of spontaneous activation of G proteins by unliganded receptors came the prospect of ligands that selectively inhibit this spontaneous activation, specifically denominated inverse agonists.

In an attempt to understand GPCR activation mechanisms, several receptor occupancy models have been developed (8). The first that explicitly considered constitutive activity was the extended ternary complex (ETC) model presented by Samama et al. (9), which includes two distinct conformational states of the receptor, an active (R*) and an inactive (R) state, that exist in equilibrium even in the absence of drugs. This spontaneous equilibrium determines the level of constitutive activity because in the ETC model, only R* is able to couple to the G protein and is considered the responsible of basal activity (R*G).
A further modification of the ETC model is the cubic ternary complex model (CTC) (10–12), that extends the ETC model by allowing G proteins to interact with receptors in both their active and inactive states (i.e. R*G, and RG). Although the development of the ETC model was made necessary by experimental observations, the CTC model was originally proposed in an attempt to explore theoretically the mathematical and pharmacological implications that can be derived from permitting G proteins to interact with receptors in their inactive and active forms. Thus, the CTC model was the culmination of a trend in increasing model complexity and statistical and thermodynamic completeness.

However, there is a growing body of evidence suggesting that the CTC model is the only one capable of explaining some experimental observations concerning the mechanism of action of certain inverse agonists. Inverse agonists may act by binding to an inactive, G protein-coupled form of the receptor, decreasing basal activity of the specific GPCR of interest but also in some cases the activity of other GPCRs that signal through the same G protein, via a proposed “molecular kidnap- ping mechanism” (13–15).

According to the law of mass action, receptor overexpression leads to an increased amount of all spontaneously occurring species. Hence, receptor overexpression should uncover a receptor species spontaneously coupled to G protein but inactive, able to interfere with other GPCRs that signal through the same G protein pool.

In this study, aiming to characterize inactive spontaneously GPCR species experimentally, we generated five CHO-K1 cell clones stably transfected with the human histamine H2R. These clones express different and increasing amounts of the receptor and respond to ligand stimulation with an unaltered pharmacological profile.

Surprisingly, in these clones the signaling of other Gs-coupled receptors is attenuated proportionally to the H2R expression levels. Similar results were obtained when another Ga-coupled receptor, β2-adrenergic receptor (βAR), was overexpressed, indicating that this phenomenon is not restricted to histamine receptors. Moreover, G protein transfection specifically reverted this interference, strongly suggesting that the mechanism is related to G protein hijacking. Finally, we observed that knocking down the expression of endogenously expressed βAR leads to an increased ligand-induced response of other Ga-coupled receptors, indicating that this phenomenon is not restricted only to overexpression systems.

These results indicate that the CTC model prediction, stating that GPCRs spontaneously exist not only as a constitutive active form (R*G) but also as a constitutive inactive form (RG), was verified experimentally by its ability to sequester G protein and interfere with the signaling of other GPCRs. This phenomenon could have serious physiological implications because it was observed not only in genetically manipulated systems, but also with endogenously expressed receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—CHO-K1 dhfr− and MDA-MB-231 cells were obtained from the American Type Culture Collection. Cell culture medium, antibiotics, isobutylmethylxanthine (IBMX), cAMP, HT medium supplement, G418, and bovine serum albumin were obtained from Sigma. Amthamine, isoproterenol, prostaglandin E2 (PGE2), salmon calcitonin (sCT), and tiotidine were from Tocris Bioscience (Ellisville, MO). [3H]cAMP (31 Ci/mmol), [3H]tiotidine (75 Ci/mmol), and [3H]CGP12177 (30 Ci/mmol) were purchased from PerkinElmer Life Sciences. Three siRNAs for βAR were purchased from Invitrogen (ADR2 Stealth Select RNAi™, HSS100258, HSS100259, and HSS100260). Other chemicals used were of analytical grade.

pcDNA3-βAR was a generous gift from Dr. M. Levin (INGEBI, CONICET, Argentina). pcDNA3Gαs plasmids were generous gifts from Dr. O. Cosso (Facultad de Ciencias Exactas y Nucleares, Universidad de Buenos Aires, Argentina). Human H2R was previously cloned into the eukaryotic expression vector pCEFL (16).

**Cell Culture and Transfection**—All cells were grown at 37 °C in a humidified 5% CO2 incubator. CHO-H2R and CHO-mock cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM l-glutamine, 100 μM hypoxanthine, 16 μM thymidine, 50 g/ml Gentamicin, and 0.8 mg/ml G418. Parental CHO-K1 cells were cultured in the same medium without G418. MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium-F12 medium containing 10% fetal calf serum and 50 g/ml Gentamicin.

For transfection CHO-K1 cells were grown to 80–90% confluence. cDNA constructs were transfected into cells using Lipofectamine 2000. The transfection protocol was optimized as recommended by the supplier (Invitrogen). After transfection, five stable clones with different H2R levels were established by G418 selection. A separate single clone containing the empty vector was selected under the same conditions (CHO-mock).

Transfections with double stranded siRNA targeting βAR at 20 nM concentration were also performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The three different sequences provided were used separated or pooled. As control, nontargeting scrambled siRNA was used. Transiently cDNA or siRNA-transfected cells were assayed 48 h after transfection. Rpeptor expression was evaluated by specific radioligand binding assay as described below.

**cAMP Assays**—Concentration-response assays were performed by incubating the cells for 3 min in culture medium supplemented with 1 mM IBMX at 37 °C, followed by a 7-min exposure to different concentrations of ligands. The reaction was stopped by the addition of ethanol. The ethanol phase was then dried and the residue resuspended in 50 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin. cAMP content was determined by competition of [3H]cAMP for protein kinase A, as described previously (17).

**Radioligand Binding Assay**—Triplicate assays were performed in 50 mM Tris-HCl, pH 7.4. For saturation studies, 104 CHO-mock, CHO-H2R, or MDA-MB-231 cells/well of a 48-well cluster plate were incubated for 40 min at 4 °C with increasing concentrations of [3H]tiotidine, ranging from 0.4 to 240 nM in the absence or in the presence of 1 μM unlabeled tiotidine or for 4 h at 4 °C with increasing concentrations of [3H]CGP12177, ranging from 20 to 0.02 nM in the absence or in the presence of 100 nM isoproterenol. The incubation was
stopped by dilution with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.4. After three washes with 3 ml of ice-cold buffer, the bound fraction was collected in 200 μl of ethanol. Experiments with intact cells were performed at 4 °C to avoid ligand internalization. The kinetic studies performed with 2 nM [3H]tiotidine at 4 °C showed that the equilibrium was reached at 30 min and sustained for 4 h (data not shown).

**Statistical Analysis**—Binding data and sigmoidal dose-response fittings were performed with GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA). One-way analysis of variance followed by the Dunnett’s post test was performed using GraphPad InStat version 3.01. Specific binding was calculated by subtraction of nonspecific binding from total binding.

**RESULTS**

**H2R Overexpression Interferes with the Signaling of Other Endogenously Expressed GPCRs**—H2R constitutive activity (7) and the triggering of cellular mechanisms tending to compensate the activity of the signaling pathways when it is overexpressed (18) have been described. With the aim of characterizing the effect of H2R overexpression on signaling of other Gs-coupled receptors, we established CHO-K1 cells clones stably transfected with cDNA encoding H2R. Several clones were isolated, and five were selected based on their H2R amounts (C1, C2A, C2B, C3, and C4). [3H]Tiotidine binding assays performed on these clones yielded different Bmax values ranging from about 1.3 × 10⁵ to 2 × 10⁶ sites/cell (Table 1). We observed for C1, C2A, and C2B the two different binding sites previously described for H2 (14, 19, 20): the high affinity site corresponding to the G protein-coupled forms of the H2R (about 20% of total sites number) and the low affinity site corresponding to the G protein-uncoupled states. However, in C3 and C4 clones that expressed the highest amounts of receptors, we observed only the low affinity binding site (Table 1). The lack of the high affinity site in these clones can be interpreted assuming that G pro-

<table>
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<td>C1</td>
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<td>C2B</td>
<td>50.9 ± 5.5</td>
<td>1.74 ± 0.21</td>
<td>237.6 ± 19.0</td>
<td>21.64 ± 2.41</td>
<td>17.60</td>
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<td>C3</td>
<td>1254 ± 49</td>
<td>0.47</td>
<td>30.76 ± 2.98</td>
<td>23.54 ± 4.67</td>
<td>17.33</td>
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*Statistical Analysis*—Binding data and sigmoidal dose-response fittings were performed with GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA). One-way analysis of variance followed by the Dunnett’s post test was performed using GraphPad InStat version 3.01. Specific binding was calculated by subtraction of nonspecific binding from total binding.

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**TABLE 1**

Binding of [3H]tiotidine in CHO-K1-H2R clones

The Kd and Bmax values were calculated using the equation for one or two binding sites. The simpler model was chosen using the extra sum-of-squares F test unless p < 0.05. The table shows the mean ± S.E.; the number of determinations (n) is in parentheses.

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**FIGURE 1.** Dose-dependent cAMP production by amthamine treatment in H2-transfected CHO cells. A, control cells (mock transfected, ◦) and clones C1 (●), C2A (□), C2B (▲), C3 (■), and C4 (■) cells were incubated for 7 min with increasing concentrations of amthamine at 37 °C in the presence of 1 mM IBMX, and cAMP levels were determined. Data are the mean ± S.D. (error bars) of triplicate assays and representative of at least six independent experiments. B, variation of fitted parameters for cAMP dose-response curves (maximal responses (●), basal levels (▲), and pEC50) with H2R number is shown. Data are the mean ± S.E. of six independent experiments and are best fit by a hyperbola (maximal responses) or a straight line (basal levels and pEC50) with slope significantly different from zero (p < 0.01). Dotted lines represent the 95% confidence interval of the curve.
proteins are in limiting quantity with respect to the amounts of receptor overexpressed.

Concentration-response curves performed with the specific H2 agonist amthamine showed an increase in cAMP basal and stimulated levels and a decrease in pEC50 values according to the increment on receptor amount. This behavior agrees with predictions made, using simpler operational models (21) (Fig. 1).

As stated in the Introduction, if a receptor is overexpressed, all spontaneous species should be incremented as well, according to its probability of occurrence. Considering this, receptor overexpression may also lead to an increase in the hypothetical species corresponding to an unliganded inactive G protein-coupled form of the receptor.

According to the results obtained for [3H]tiotidine binding, G protein amounts are in a limiting number regarding H2 receptors. As a consequence, overexpression of a particular receptor may cause a G protein kidnapping and an interference in the response of other GPCRs that signal through the same subfamily of G proteins.

Hence, to test whether H2R expression affects the signaling of other GPCRs that transduce their signals through the same G protein, we evaluated the ability of signaling of CHO-K1 endogenously expressed Gaα,S-coupled receptors. To do this, we confirmed the presence and the functionality of CT and PGE2 receptors that were previously described on the CHO-K1 cell line (22, 23) (Fig. 2).

The only presence of the H2R is able to reduce, in a receptor number-dependent manner, the CTR and PGE2R signaling. This interference consists in a reduction of the ligand-induced maximal responses without significantly affecting the pEC50 (Fig. 2 insets). This can be predicted with any model of receptor occupancy considering a limiting and diminishing G protein amounts available for signaling. As shown in Fig. 2 insets, the decrease in maximal responses is best fit to an exponential decay equation, and the interference is more intense for the PGE2 system, indicating that the propensity to be interfered is different for each GPCR. It is worth noting that, in saturation binding assays, the number and the affinity constants of the aforementioned receptors remained unchanged (data not shown).

Overall, these results may be explained by the kidnapping of available G protein in an inactive form by overexpressed H2R. To confirm this hypothesis, we attempt to overexpress G protein to increase its availability.

Gaα Overexpression Reverts the H2R Interference on Calcitonin and PGE2 Signaling—The results described above may be explained by the kidnapping of available Ga protein in an inactive form by overexpressed H2R. If this hypothesis was right, an increase in the amounts of G proteins of this family would counteract the effect of H2R on the sCT and PGE2 response in CHO clones.

Fig. 3 shows that the interference was abolished on C1, C2A, and C2B clones, both for CTR and PGE2R ligand-induced signaling but that this recovery effect lost efficacy on the clones where H2R number is higher (C3 and C4 clones). These results
G Protein Sequestering by GPCRs

**FIGURE 3. Effect of Gs subunit transfection on sCT- and PGE2-induced cAMP response in CHO-H2 clones.** Control cells (black bar), CHO-H2 clones transfected with empty vector (empty bars), and CHO-H2 clones transfected with a vector encoding for the Gs protein subunit (gray bars) were incubated for 7 min with increasing concentrations of sCT (A) and PGE2 (B) at 37 °C in the presence of 1 mM IBMX, and cAMP levels were determined. Data are expressed as the span of the sigmoidal dose-response fitted curves. Data were calculated as the mean ± S.E. (error bars) of four independent experiments. **p < 0.01.

confirm that the interference observed could be due to the kid-
napping of the G protein by H2R and strengthen the concept that the stoichiometry of the different signaling partners is cru-
ial to determine the signaling ability of a system.

**H2R Expression Also Attenuates the Signaling of Other Exog-
enuously Expressed GPCRs**—To evaluate further the attenuating
effect of H2R expression, considering a role of an endogenous
regulation as partially responsible for the observed interfer-
ence, we also studied the signaling of a heterologously
expressed GPCR, the βAR. When βAR was transiently
transfected into the different CHO-H2R clones we observed no dif-
ferences on receptor expression by [3H]CGP12177 saturation
binding experiments (data not shown). However, we could
observe that its signaling is also attenuated in an H2R number-
dependent manner. When plotted as isoproterenol maximal
response versus H2R number, the data also best fit a one-phase
exponential decay, but the curve was shifted to the right, indi-
cating that H2R is less efficacious in interfering with the βAR
signaling (Fig. 4). When βAR is expressed on CHO cells, its
presence is also able to attenuate sCT and PGE2 signaling (Fig.
5, A and B), and when expressed on CHO-H2R cells it is also
able to interfere with H2R-mediated response (Fig. 5C), indic-
ating that this interference phenomenon is not restricted to
any chosen receptor pair.

As shown before, H2R overexpression led to a concomitant
increase on second messenger basal levels (Fig. 1B). However,
unexpectedly, the same effect was not observed when βAR is
overexpressed. In the latter case, the basal levels of all clones
were unchanged or diminished (Fig. 4A). To evaluate this strik-
ing effect better, we overexpressed Gα protein. Under these
experimental circumstances, we were able to observe the
expected increase on cAMP levels, but βAR co-expression was
capable of diminishing this magnified basal response (Fig. 6).
This was tested using three different Gα and βAR plasmid
concentrations, and the results were reproducible for every
condition (data not shown). These results may be indicative of
the natural tendency of a GPCR to adopt distinct spontaneous
conformations, showing that βAR has more tendency than H2R
to adopt a spontaneous conformation able to bind G proteins in
an inactive state.

**Knockdown of Endogenous βAR Augments the Response of
Other GPCRs**—Previously, it has been reported that although
heterologously transfected GPCRs share a common G protein
pool, endogenously expressed receptors by naïve cells activate
different pools of G protein (24). Therefore, to evaluate whether
this interference phenomenon is restricted to exogenously
expressed receptors, we utilized a cell line that endogenously
expresses the set of Gα-coupled GPCRs examined in this work.
We chose MDA-MB-231 cells, a human mammary carcinoma
cell line that endogenously expresses βAR and H2R (25, 26). In
this cell line, transfection with siRNA targeted against βAR
diminished membrane receptor number approximately 80%
when measured by saturation binding experiments and
decreased isoproterenol-induced cAMP levels 60%. However,
although siRNA transfection did not change the H2R number,
the H2R response was significantly increased (22.01 ± 2.73 ver-
sus 51.82 ± 3.47 pmol/well), consistent with our hypothesis
(Fig. 7). Furthermore, the potentiating effect of the βAR-spe-
cific siRNA was observed as well for other endogenously
expressed receptors such as CTR and PGE2R (27, 28) (Fig. 7).
These results support the fact that the only presence of a GPCR
can affect the response of another receptor not only in geneti-
cally manipulated cells, but also in endogenous expression
systems.

**DISCUSSION**

Three main conclusions can be drawn from our studies. First,
human H2R overexpression in CHO-K1 cells shows that H2R is
able to interfere with CTR and PGE2R signaling. Second, tran-
sient expression of βAR shows that this is not exclusive of H2R.
Third, experiments performed knocking down the endogenous
expression of βAR in MDA-MB-231 cells show that this phe-
nomenon is not restricted to overexpression systems and that it
can be evidenced in physiological conditions.

We have previously described that certain inverse agonists
acting on histaminergic receptors interfere with the signaling of
other receptors that share common Gα subunits (14, 15). This
interference is thought to be caused by the ligand-induced sta-
bilization of a G protein-coupled form of the receptor, which is
unable to evoke a response. Such receptor conformation could be responsible for a G protein kidnapping that may invoke the aforementioned interference.

It could be tempting to explain the interference on other GPCR signaling observed in H2R overexpression systems in terms of the induction of adaptive mechanisms such as phosphodiesterase activity augmentation or an increased general receptor internalization. However, the first possibility was ruled out because in all cases cAMP levels were evaluated in presence of the phosphodiesterase inhibitor IBMX. Likewise, the assessed GPCRs that showed a decreased response showed no modification in protein levels and $K_d$ values (data not shown), and similarly, when the βAR was knocked down, the H2R response was increased without variation on receptor number as well. Overall, our results are better interpreted considering the spontaneous existence of a receptor conformation coupled to G protein but inactive, which was confirmed by the reversion of this effect when the specific Gαi4 overexpression, strengthening our proposal of the G protein-coupled but inactive receptor form.

The competition of two or more receptors for the same pool of G proteins could be assumed to be the cause of certain previously observed effects, such as several types of synergism and cross-signaling. For example, there have been documented synergistic interactions between D1 and D2 dopamine receptors (29), δ and κ opioid receptors (30), and muscarinic and α2-adrenergic receptors (31). Although receptor dimerization, oligomerization, and co-localization have all been proposed as mechanisms for these cross-signaling processes, it appears that other mechanisms can also lead to GPCR signaling modulation.

Assuming the collision-coupling model of membrane receptor signaling (32, 33), which allows for receptors and G proteins to diffuse freely in the membrane, if the latter are in a limiting number, it is possible to anticipate an interference of a GPCR with the signaling of other receptors based on the redistribution of the subabundant G proteins. That may be the case for the reported ligand competitive behavior, in which the stimulation achieved by the addition of two agonists acting on different GPCRs is less than the sum of the activation caused by the individual receptors alone (e.g. CB1 and μ opioid receptors) (24).

Remarkably, our experiments show that solely the expression of a GPCR dampens the agonist-induced signaling of endogenously or heterologously expressed receptors. Moreover, overexpression of the βAR is able to diminish elevated cAMP levels resulting from Gαi4 overexpression, strengthening our proposal of the G protein-coupled but inactive receptor form.

In line with our results, it has been described that 5HT7 serotonin receptor attenuates adenylyl cyclase activation by βAR and prostanoid EP receptor. However, in that case, neither Gαi nor adenylyl cyclase overexpression is able to reverse the interference effect, indicating that the mechanism underlying their observations is different (36). Furthermore, Stephan and coworkers have shown that the constitutive abnormal signaling of mutated yeast pheromone receptors Ste2p and Ste3p is suppressed upon co-expression with wild-type but not G protein coupling-defective receptors, suggesting that wild-type receptors may sequester a limiting pool of G proteins (37). Considering the results obtained on the carcinoma cell line, the phenomenon herewith described could have serious implications regarding the effects of an unbalance of protein expression on receptor signaling.
Hasseldine and co-workers (38) have described the signaling of the \( \beta_2 \)-adrenergic system in TG4 mice, a strain that specifically overexpresses \( \beta_2 \)AR in cardiac tissue. In this system, as a result of its overexpression, \( \beta_2 \)AR couples simultaneously to \( G_s \) and \( G_i \) pathways, but for yet unknown reasons cardiac signaling is dampened in the transgenic mice. This surprising result could be interpreted in terms of the G protein-hijacking mechanism proposed, bringing out the possible physiological relevance.

As mentioned above, GPCRs are overexpressed in various malignancies. For instance, there has been conducted an \textit{in silico} approach demonstrating overexpression of several GPCRs in primary tumor cells, including chemokine receptors and protease-activated receptors, neuropeptide receptors, adenosine A2B receptor, P2Y purinoceptor, calcium-sensing receptor, and metabotropic glutamate receptors. Analysis of cancer samples in different disease stages also suggests that some GPCRs, such as endothelin receptor A, may be involved in early tumor progression, and others, such as CXCR4, may play a critical role in tumor invasion and metastasis (39).

Besides cancer, there have been described other conditions in which receptors are overexpressed (e.g. schizophrenia and dopamine D4 receptors) (40) and presumably H2R and certain heart disease states (41)). In those cases, as well as some easily anticipated consequences (i.e. an elevation on second messen-
levels or promiscuous effects on G protein coupling) this plausible negative interference on other receptor signaling should be taken into account.

In summary, we have shown that the human H2R and βAR have the ability to block the signaling by other endogenous or exogenously expressed Gs-coupled receptors. Sequestration of G proteins by these receptors is well accommodated by CTC model, and our study suggests that GPCRs may act as proteins controlling the signaling of other receptors sharing a common and limiting G protein pool.

REFERENCES

FIGURE 7. Effect of endogenous βAR knockdown on H2R, CTR, and PGE2 cAMP response in MDA-MB-231 cells. A, maximal binding capacity of [3H]CGP12177 and [3H]tiotidine. Control cells (scramble siRNA transfected, open bars) or cells transfected with βAR-targeted siRNA (black bars) were exposed to increasing concentrations of [3H]CGP12177 or [3H]tiotidine as described under “Experimental Procedures,” and maximal binding parameters of one-site hyperbola fitting are represented. Results are expressed as percentage of control cells, and data are the mean ± S.E. (n = 3). ***, p < 0.01 with respect to control. Right, representative saturation binding experiment of control cells (■) and siRNA-transfected cells (▲). Control cells: [3H]CGP12177 Bmax 105,139 ± 6,370 sites/cell, Kd 0.31 ± 0.18 nM, [3H]tiotidine Bmax 40,262 ± 2,933 sites/cell, Kd 22.7 ± 3.4 nM. βAR-siRNA transfected cells: [3H]CGP12177 Bmax 40,398 ± 3,591 sites/cell, Kd 0.28 ± 0.13 nM, [3H]tiotidine Bmax 37,691 ± 2,531 sites/cell, Kd 21.22 ± 5.01 nM. B, cAMP maximal response to isoproterenol (ISO), amthamine (H2), sCT, and PGE2. Control cells (scramble siRNA-transfected, empty bars) or cells transfected with βAR-targeted pooled siRNA (black bars) were exposed to increasing concentrations of the different ligands, and cAMP levels were measured as described under “Experimental Procedures.” Maximal responses fitted from sigmoidal concentration-response equation are represented. Results are expressed as percentage of control cells, and data are the mean ± S.E. (error bars) of four independent experiments. **, p < 0.05; ***, p < 0.01 with respect to control. Right, representative concentration-response experiments: ISO (squares); H2 (circles); sCT (triangles), and PGE2 (diamonds) of control cells (open symbols) and siRNA-transfected cells (filled symbols). Control cells: maximum response ISO, 137.3 ± 15.3 pmol/well, H2, 22.1 ± 5.3 pmol/well, sCT, 39.3 ± 6.7 pmol/well, PGE2, 36.9 ± 4.1 pmol/cell. βAR siRNA-transfected cells: maximum response ISO, 51.3 ± 3.11 pmol/well, H2, 51.87 ± 4.3 pmol/well, sCT, 83.4 ± 2.9 pmol/well, PGE2, 78.5 ± 3.7 pmol/cell.

G Protein Sequestering by GPCRs

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