

Tiotidine, a Histamine H₂ Receptor Inverse Agonist That Binds with High Affinity to an Inactive G-Protein–Coupled Form of the Receptor. Experimental Support for the Cubic Ternary Complex Model

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ABSTRACT

Knowing the importance for research and pharmacological uses of proper ligand classification into agonists, inverse agonists, and antagonists, the aim of this work was to study the behavior of tiotidine, a controversial histamine H₂ receptor ligand. We found that tiotidine, described previously as an H₂ antagonist, actually behaves as an inverse agonist in U-937 cells, diminishing basal cAMP levels. [³H]Tiotidine showed two binding sites, one with high affinity and low capacity and the other with low affinity and high capacity. The former site disappeared in the presence of guanosine 5'-O-(3-thio)triphosphate, indicating that it belongs to a subset of receptors coupled to G-protein, showing the classic binding profile for an agonist. Considering the occupancy models developed up to now, the only one that explains tiotidine dual behavior is the

cubic ternary complex (CTC) model. This model allows G-protein to interact with the receptor even in the inactive state. We showed by theoretical simulations based on the CTC model of dose-response and binding experiments that tiotidine biases the system to a G-protein–coupled form of the receptor that is unable to evoke a response. This theoretical approach was supported by experimental results in which an unrelated G-protein–coupled receptor that also signals through G α_s -protein (β_2 -adrenoreceptor) was impeded by tiotidine. This interference clearly implies that tiotidine biases the system to G α_s -coupled form of the H₂ receptor and turns G α_s -protein less available to interact with β_2 -adrenoreceptor. These findings not only show that tiotidine is an H₂ inverse agonist in U-937 cells but also provide experimental support for the CTC model.

Receptors coupled to G-proteins (GPCRs) play a major role in signal transduction and are the targets for a large number of therapeutic drugs. Although much is known about signal transduction pathways, the mechanism by which ligands bind to and activate GPCRs remains unclear. In an attempt to understand such mechanisms, several occupancy models have been developed. The first theoretical model of receptor function that included G-proteins was the ternary complex model of De Léan et al. (1980) in which the receptor possesses two binding sites, one for the ligand on the extracellular side and other for the G-protein in the intracellular side. This

model is a generalization of former models, in which only the free receptor and the receptor ligand complex existed, allowing receptors to interact with ligands as well as G-proteins. An important advancement in the understanding of GPCRs was the recognition that they could couple to and functionally activate G-proteins even in the absence of an agonist. This receptor feature was suggested by the identification of constitutively activated mutant forms of several receptors (CAM receptors), such as α_{1B} adrenoreceptor (Kjelsberg et al., 1992), thyrotropin receptor (Parma et al., 1993), and luteinizing hormone receptor (Shenker et al., 1993). These experimental results led to the extended ternary complex model (ETC) (Samama et al., 1993), 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

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ABBREVIATIONS: GPCR, G-protein coupled receptor; CAM, constitutively activated mutant; ETC, extended ternary complex model; CTC, cubic ternary complex model; H₂R, histamine H₂ receptor; GTP γ S, guanosine 5'-O-(3-thio)triphosphate; RPMI, Roswell Park Memorial Institute; IBMX, 3-isobutyl-1-methylxanthine; BU-E-75, (\pm)-N¹-[3-(3,4-difluorophenyl)-3-(pyrid-2-yl)propyl]-N²-[3-(1H-imidazol-4-yl)propyl]guanidine.

basal activity, and in CAM receptors, it is driven toward R*, increasing the response in the absence of drugs. A further modification of the ETC model is the cubic ternary complex model (CTC) (Weiss et al., 1996a,b,c), which completes the ETC model by allowing G-proteins to interact with receptors in both their active and inactive states (R*G, LR*G, and RG, LRG).

These two models account for the effects of different types of ligands on receptor signaling. Agonists increase receptor activity, neutral antagonists have no effect on it, and inverse agonists are able to reduce the activity of receptor systems that are active in the absence of agonists.

Although initially drugs were classified as agonists and neutral antagonist, now it is considered that ligands have an efficacy ranging from agonism through neutral antagonism to inverse agonism. In fact, many drugs with important therapeutic actions that were originally described as neutral antagonists are actually inverse agonists. Examples of this are cimetidine [selective histamine H2 receptor (H2r) ligand] (Smit et al., 1996), haloperidol (which acts on dopamine D₂ receptors) (Hall and Strange, 1997), prazosin (α_1 -adrenoreceptor ligand) (Rossier et al., 1999), timolol (which acts on β_2 -adrenoreceptor) (Chidiac et al., 1994), and clozapine (which acts on D₂ and 5-HT_{2C} receptors) (Westphal and Sanders-Bush, 1994; Hall and Strange, 1997).

H2r have the regulatory functions of histamine during cell proliferation, gastric acid secretion, airway and vascular smooth muscle relaxation and immune responses (Del Valle and Gantz, 1997). Furthermore, some histamine H2 antagonists are effectively used as therapeutical agents, such as ranitidine, which is effective in the modulation of gastric acid secretion. Taking into account that treatment with antagonists would not be effective for several pathologies associated with point mutations in GPCRs that lead to increased basal receptor activity and, on the other hand, that long-term administration of some inverse agonists leads to the development of tolerance and increased sensitivity after withdrawal, it is highly important to differentiate between neutral antagonists and inverse agonists.

The presence of H2r showing typical pharmacological profile and G-protein-mediated adenylyl cyclase stimulation has been described in U-937 promonocytic cell line, which make it an adequate model to study H2r signaling (Davio et al., 1995a). However, in these cells, tiotidine, previously reported as an H2 specific antagonist, showed inverse agonist characteristics (Monczor et al., 1998). The suitability of tiotidine has been a controversial issue (van der Goot and Timmerman, 2000) because of its complex binding since studies carried out in gastric mucosal cells (Batzri and Harmon, 1986) and kidney membranes (Rising and Norris, 1985) revealed the presence of several binding sites for [³H]tiotidine. On these bases, we aimed to study the atypical behavior of tiotidine in U-937 promonocytic cell line.

We found a two-site binding unusually sensitive to GTP γ S and an inverse agonist behavior that can be explained only in terms of the cubic ternary complex model. These observations suggest the existence of an inactive receptor species coupled to G-protein. These observations shed new light into the pharmacological classification of H2 antagonists and may offer a plausible explanation for the results previously reported by other authors and us.

Materials and Methods

Materials

Cell culture medium, DEAE-dextran, 3-isobutyl-1-methylxanthine (IBMX), cAMP, isoproterenol, and forskolin were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum was obtained from Invitrogen (Carlsbad, CA). Tiotidine was obtained from Tocris Cookson Inc. (Ballwin, MO). [³H]cAMP and [³H]tiotidine were purchased from PerkinElmer Life Sciences (Boston, MA). BU-E-75 was generously provided by Dr. A. Buschauer (Regensburg Universität, Regensburg, Germany). All other chemicals were of analytical grade.

Plasmid

H2r was previously cloned into the eukaryotic expression vector pCEFL (Shayo et al., 2001). Plasmid purification was performed using reagents from QIAGEN (Valencia, CA) according to the manufacturer's instructions.

Cell Culture

U-937 and COS-7 cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ in RPMI 1640 medium and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% fetal calf serum and 50 μ g/ml gentamicin.

Transient Transfection of COS-7 Cells

Transient transfection was performed by the DEAE-dextran technique (Shayo et al., 2001). COS-7 cells plated in 35-mm dishes were transfected at 80% confluence using 1 μ g of plasmid. Assays were performed 48 h after transfection.

Stable Transfection of U-937 Cells

Cells were transfected as reported previously (Fernández et al., 2002). Briefly, cells were harvested by centrifugation from cultures in exponential growth phase, washed once in phosphate-buffered saline, and resuspended at 10⁶ cells/ml in fresh RPMI 1640 medium on ice. Linearized pCEFL-rH2 (10 μ g) with *SalI* was added to cell suspension (400 μ l) and kept on ice for 5 min. The cells and DNA were then subjected to a pulse of 150 V at a capacitance of 250 μ F using a Bio-Rad "Gene Pulser". Cells were returned to ice for 5 min and nonselective medium was added overnight. The next day, cells were plated on a 96-well culture tray, switching the medium to RPMI 1640 containing 0.8 mg/ml G-418. After 2 to 3 weeks, the surviving clones were amplified.

cAMP Assay

Suspension Cells. Cells in Hanks' solution were supplemented with 1 mM IBMX, at a density of 10⁶ cells/ml, preincubated for 3 min at 37°C, and exposed for 9 min to different ligand concentrations. The reaction was stopped by a 3-min centrifugation at 3,000g. For cAMP extraction, ethanol was added to the pellet and further centrifuged 5 min at 3000g.

COS-7 Cells. Cells were incubated 3 min in Dulbecco's modified Eagle's medium supplemented with 1 mM IBMX at 37°C, and exposed for 9 min to the ligand at the indicated concentrations. Cells were then washed with phosphate-buffered saline and subjected to ethanol extraction followed by a 5-min centrifugation at 3,000g.

The ethanol phase was dried and resuspended in 50 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin. cAMP content was determined by competition of [³H]cAMP for protein kinase A, as described previously (Davio et al., 1995b).

Radioligand Binding Assay

Suspension Cells. Triplicate assays were performed in polyethylene tubes in 50 mM Tris-HCl, pH 7.4. For saturation studies, increasing concentrations of [³H]tiotidine were incubated with 10⁶ cells/tube in the absence or presence of 1 mM histamine in a total volume of 200 μ l. After 40 min at 4°C, incubation was stopped by

dilution with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.4; rapid filtration onto Whatman GF/B glass-fibers filters was performed under reduced pressure, followed by three washes with 3 ml of ice-cold buffer.

COS-7 Cells. Triplicate assays were performed in COS-7 transfected cells in 24 multiwell plates. For saturation studies, increasing concentrations of [³H]tiotidine were incubated in the absence or presence of 1 μM tiotidine in a total volume of 200 μl of 50 mM Tris-HCl, pH 7.4. After 40 min at 4°C, incubation was stopped by dilution with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.4, followed by washes with ice-cold buffer.

Experiments on intact cells were carried out at 4°C to avoid internalization of the ligand. Kinetic studies showed that equilibrium was reached after 30 min and persisted for 4 h (data not shown).

Membrane-purified fraction was obtained by cell sonication in 50 mM Tris-HCl buffer, pH 7.4, and centrifuged for 15 min at 8,500g; the supernatant was further centrifuged for 15 min at 30,000g. The pellet obtained was resuspended in an adequate volume.

The membrane fraction was pretreated at 37°C with 10 μM GTPγS for 2 h in Hanks' medium.

Analytical Methods

Binding data were analyzed using a weighted, nonlinear, least-squares program that determines binding to multiple sites using the law of mass action (LIGAND program) as detailed previously (Munson and Rodbard, 1980). Sigmoidal dose-response fittings were done using Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).

Results

[³H]Tiotidine Binding Assay and H2r States Identification. As is well known, agonists and inverse agonists have differential affinities for the different receptor states. For that reason, radioligand binding allows the identification of those activation states.

Binding assays using [³H]tiotidine, a specific H2r ligand (described previously as antagonist), revealed the presence of two binding sites, one with higher affinity and lower capacity ($K_{d1} = 2.2 \pm 0.8$ nM; $Q_1 = 2,000 \pm 430$ sites/cell) and the other with lower affinity but higher capacity ($K_{d2} = 20 \pm 3$ nM; $Q_2 = 20,000 \pm 1,800$ sites/cell) (Fig. 1). Similar results were obtained when membrane fractions were used (Fig. 2A).

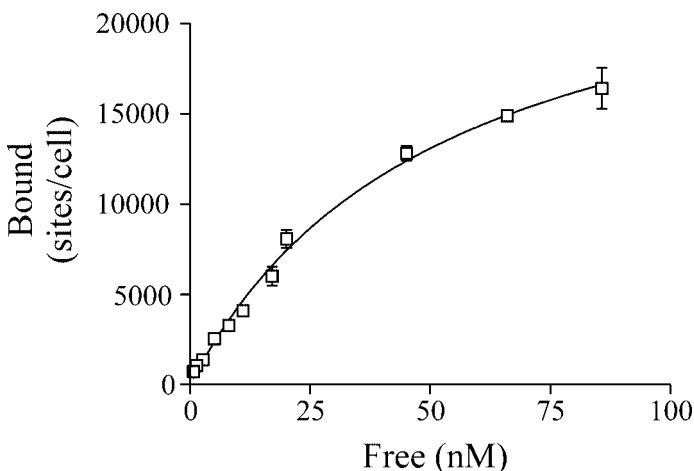


Fig. 1. [³H]Tiotidine binding assay in intact U-937 cells. Saturation assays for [³H]tiotidine. Data were calculated as the mean \pm S.D. of assay triplicates and analyzed using a weighted, nonlinear, least-squares program that determines binding to multiple sites using the law of mass action (LIGAND program), as described under *Materials and Methods*. Similar results were obtained in at least three independent experiments.

Because tiotidine specifically binds H2rs, these results can be explained in terms of tiotidine binding to two distinct receptor states. It is worth noting that in competitive binding experiments performed with [³H]tiotidine and various concentrations of unlabeled tiotidine, famotidine, and BU-E-75, the maximal inhibition of [³H]tiotidine binding by each compound did not differ significantly from that obtained in the presence of 10 μM tiotidine (data not shown). These results verify that tiotidine-labeled sites are histamine H2 sites.

In assays performed on membrane fractions of cells previously incubated with 10 μM GTPγS, the Scatchard plot showed only one binding site corresponding to the low affinity site. As it can be observed, the number of sites with low affinity is equal to the total amount of sites without GTPγS showed in Fig. 1 ($K_d = 20 \pm 3$ nM, $Q = 22,000 \pm 1,900$ sites/cell $\cong Q_1 + Q_2$) (Fig. 2A).

These data suggest that the high-affinity site corresponds to a subset of receptors coupled to G-protein, because it disappeared in the presence of GTPγS. This is supported by

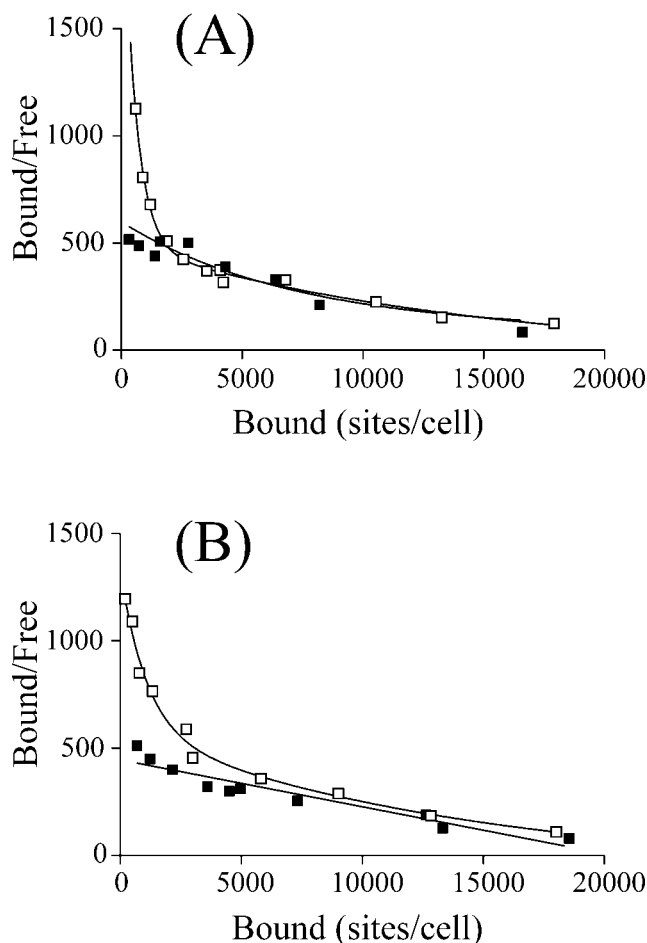


Fig. 2. [³H]Tiotidine binding assay in U-937 cells. A, Scatchard plot of saturation assays for [³H]tiotidine in U-937 membrane purified fractions treated (■) or not (□) with 10 μM GTPγS for 2 h. Data were calculated as the mean \pm S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. B, Scatchard plot of saturation assays for [³H]tiotidine in U-937 cells pretreated (■) or not (□) with 10 μM amthamine for 2 h. Data were calculated as the mean \pm S.D. of assay triplicates. Data were analyzed using a weighted, nonlinear, least-squares program that determines binding to multiple sites using the law of mass action (LIGAND program) as detailed under *Materials and Methods*. Similar results were obtained in at least three independent experiments.

the fact that binding assays in cells pretreated for 2 h with H2 agonist, which induces H2r desensitization or uncoupling from G-protein, only showed the site with low affinity (Fig. 2B). Based on classic ligand-receptor occupancy models (the ETC model of Samama et al., 1993) in which a ligand that has more affinity for the receptor coupled to G-protein (R^*G) instead of receptor alone (R or R^*) necessarily bias the system to a response-evoking receptor state (LR^*G), we can consider the tiotidine binding profile to be expected for an agonist. This conception is in opposition to the well established idea of tiotidine as the reference H2 antagonist. Therefore, we attempted to assess the ability of tiotidine to induce a second messenger signal and to block an H2 agonist-induced response.

Tiotidine Dose-Response Assays. In dose-response experiments carried out with BU-E-75, a specific H2 agonist, tiotidine not only produced a right shift on the EC_{50} (700 ± 120 nM versus $1,900 \pm 300$ nM) but also diminished the maximal response achieved with the agonist from 212.8 ± 7.7 pmol/ 10^6 cells to 112.8 ± 6.7 pmol/ 10^6 cells, suggesting that tiotidine behavior is more complex than expected for a neutral antagonist. We next evaluated the effect of tiotidine on cAMP accumulation through H2r by dose-response assays. Tiotidine is able to reduce cAMP basal levels in a dose-dependent manner, from 7.0 ± 0.4 pmol/ 10^6 cells to 1.3 ± 0.2 pmol/ 10^6 cells with an $EC_{50} = 11.5 \pm 4.5$ nM (Fig. 3, A and B).

To improve the sensitivity to this reduction in basal levels, different strategies that allow increasing spontaneous activity so that the negative effect of inverse agonists becomes clearly evident have been described. To achieve this, there are at least two experimental approaches; the first one involves the addition of a chemical agent that increases second messenger levels and the second one consists of receptor transfection in an overexpression system (Kenakin et al., 1995).

The first approach is based on evidence showing that inactivation of $G_{\alpha s}$ subunits decreases forskolin-induced cAMP levels. Leurs and coworkers (2002) proposed the modulation of the forskolin response by H2rs as a quite sensitive and

useful tool to study inverse agonism at H2rs. The last approach is based on evidence showing that when the receptor number is increased, there is a concomitant increase in the likelihood of spontaneous receptor coupling to G-protein, which in turns leads to an increase in basal levels (Milligan, 1996).

We carried out tiotidine dose-response experiments in forskolin pretreated U-937 cells and in the heterologous system of H2r-transfected COS-7 cells. In both assays, we observed an evident decrease in cAMP levels when cells were treated with increasing tiotidine concentrations (Fig. 4, A and B). Similar results were obtained with U-937 cells overexpressing H2rs, indicating that this effect was not dependent on an intrinsic property of the COS-7 heterologous system (Fig. 4C). It is worth noting that in both COS-7 and U-937 cells overexpressing H2r, [3H]tiotidine binding profile is the same that in U-937-naive cells (data not shown).

Up to this point, the results obtained in dose-response experiments provide enough evidence to consider tiotidine as an inverse agonist. However, we can associate its binding profile with the characteristic for agonists. As can be observed, the results from binding assays disagreed with what we expected based on the classic models for inverse agonists and challenged us to analyze them using another model capable of explaining them.

Cubic Ternary Complex Receptor-Occupancy Model: a Possible Explanation for Tiotidine Behavior. The only described model capable of predicting a higher affinity for the receptor G-protein complex even for an inverse agonist is the CTC model (Fig. 5). In this way, if we consider that the species able to generate physiological responses are R^*G and LR^*G , we can define a normalized factor called f^* as the addition of that species over the total receptor amount:

$$f^* = \frac{[R^*G] + [LR^*G]}{[R] + [R^*] + [RG] + [R^*G] + [LR] + [LR^*] + [LRG] + [LR^*G]} \quad (1)$$

Taking into account the model-assumed equilibrium, its related constants, and conservation equations for each compo-

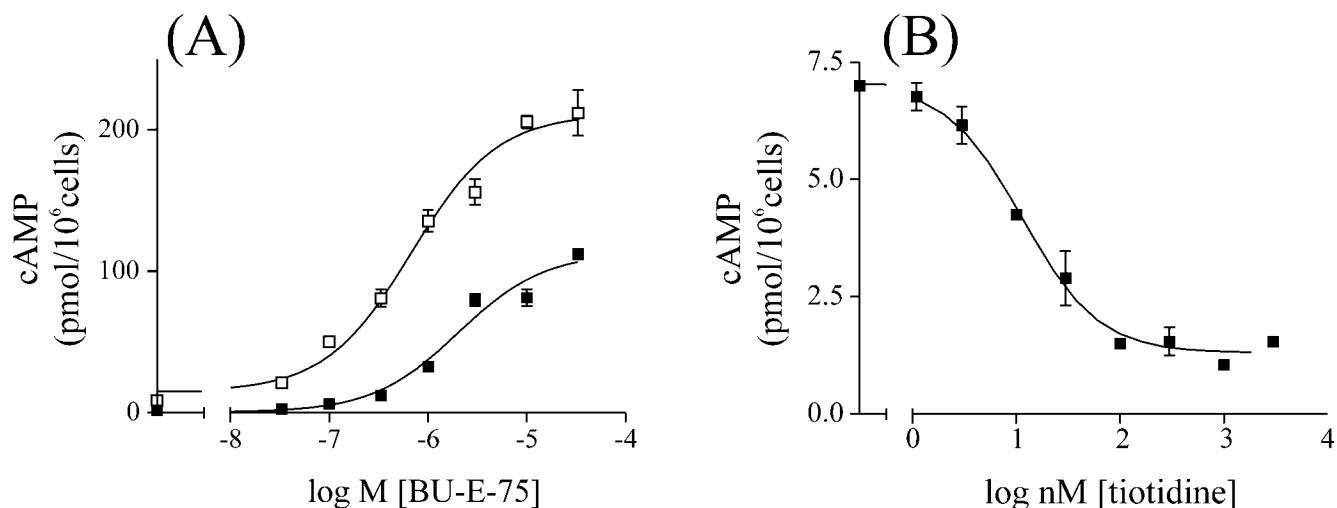


Fig. 3. Dose-dependent cAMP reduction by tiotidine treatment. A, U-937 cells were preincubated (■) or not (□) for 9 min with 33 nM tiotidine, incubated for 9 min with increasing concentrations of BU-E-75 at 37°C in the presence of 1 mM IBMX, and cAMP levels were determined. B, U-937 cells were incubated for 9 min with increasing concentrations of tiotidine at 37°C in the presence of 1 mM IBMX, and cAMP levels were determined. Data were calculated as the means \pm S.D. of assay triplicates. Similar results were obtained in at least three independent experiments.

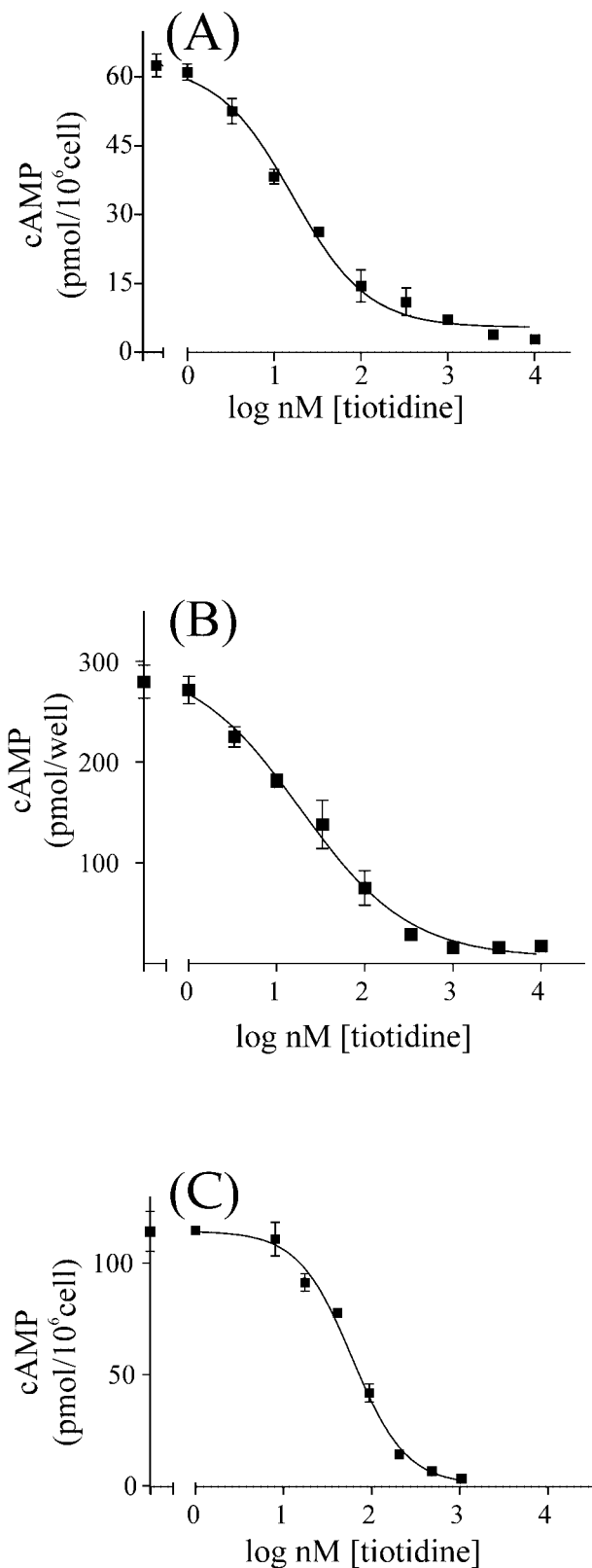


Fig. 4. Dose-dependent cAMP reduction by tiotidine treatment in systems with increased basal cAMP levels. U-937 cells pretreated with 33 μ M forskolin for 3 min (A), COS-7 cells transfected with pCEFL-H2r (B), and U-937 cells transfected with pCEFL-H2r (C) were incubated for 9 min with increasing concentrations of tiotidine at 37°C in the presence of 1 mM IBMX, and cAMP levels were determined. Data were calculated as the means \pm S.D. of assay triplicates. Similar results were obtained in at least three independent experiments.

ment (R, G, L), we can obtain the equation that describes f^* variation as a function of ligand concentration (see Appendix I).

$$f^* = \frac{\beta \cdot K_g \cdot K_{act}[G] + \alpha\beta\gamma\delta \cdot K_a \cdot K_{act} \cdot K_g[L]}{1 + K_{act} + K_g[G] + \beta \cdot K_g \cdot K_{act}[G] + (K_a + \alpha \cdot K_a \cdot K_{act} + \gamma \cdot K_a \cdot K_g[G] + \alpha\beta\gamma\delta \cdot K_a \cdot K_{act} \cdot K_g[L]) \cdot [L]} \quad (2)$$

It is worth noting that the concentration of the free G-protein [G] is a function of the ligand present in the system, and its value must be solved for each L concentration (see Appendix 2 for further details).

If we represent f^* as a function of $\log [L]$, we can simulate

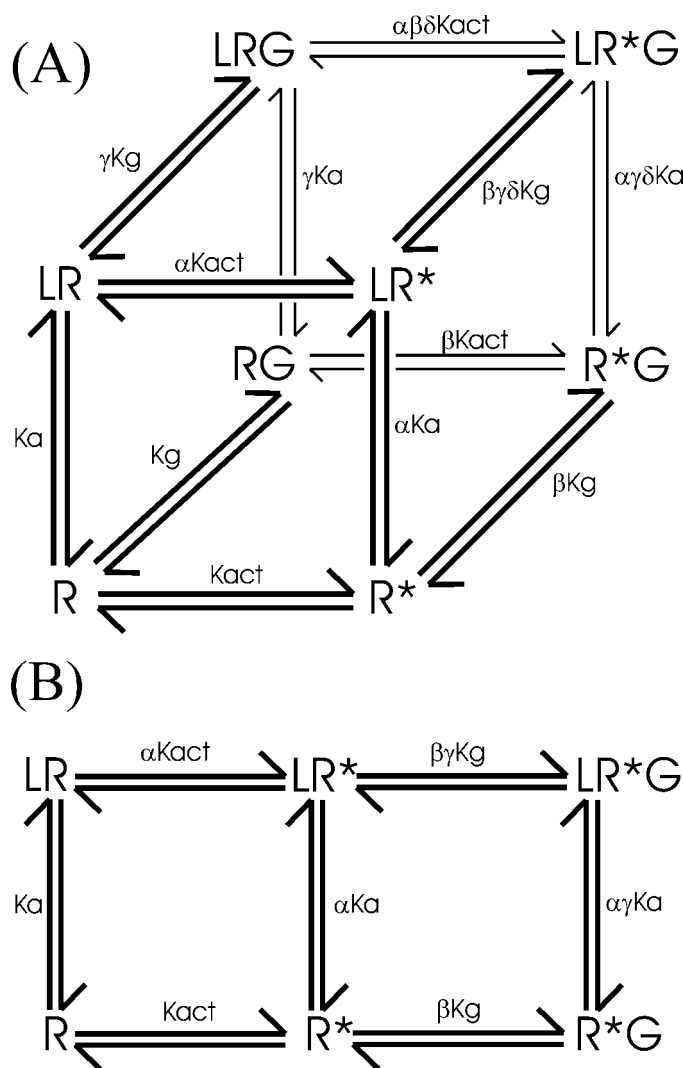


Fig. 5. A, cubic ternary complex model. CTC model whereby both inactive and active states of the receptor are allowed to interact with the G-protein, but only LR*G mediates response (adapted from Weiss et al., 1996a,b,c). B, extended ternary complex model. ETC model assumes that only the active state of the receptor (R*) can interact with the G-protein either spontaneously (to form R*G) or through ligand binding (to form LR*G) (adapted from Samama et al., 1993). L represents the ligand; K_a and K_g represent the association constants to ligand and G-protein, respectively; K_{act} is the allosteric constant; and α , β , and γ are the modifiers of affinity once the receptor is active, G-protein-bound, or ligand-bound, respectively. δ represents the joint effect of any two parameters (receptor activation, G-protein coupling, or the binding of ligand) on the third.

a theoretical dose-response assay for a given set of parameters. Among all parameters, δ describes the extent to which the joint effect of any two receptor modifications (i.e., receptor activation, G-protein coupling, and ligand binding) varies in relation to the third. In other words, it gives an idea of the interaction or synergism between two modifications in the receptor state over the third one. Considering that the ligand facilitates receptor activation ($\alpha > 1$), that the active receptor has more affinity for G protein than the inactive form ($\beta > 1$), and that the ligand improves G protein coupling ($\gamma > 1$), we are in the presence of an agonist for the ETC model.

However, by varying δ value in the CTC model, the ligand can behave as an agonist, an antagonist, or even as an inverse agonist (Fig. 6). In the same way, we can deduce another set of equations that describe the variation of each possible receptor state concentration (R, R*, RG, R*G, LR, LR*, LRG, LR*G) as a function of ligand dose. Keeping constant the parameters mentioned above and giving δ a value that makes the ligand work as an inverse agonist, we can observe in Fig. 7, A and B, that there is a significant increase in receptor species bound to ligand but unable to evoke a biological response ([LRG] and [LR*]).

If tiotidine biases the system to a G-protein-coupled but inactive H2r state, it can be inferred that it may interfere with the signal transduction pathway of an unrelated GPCR that also signals through a $G\alpha_s$ -protein. We tested this hypothesis by stimulating U-937 cells with a β_2 -adrenoreceptor agonist (isoproterenol) and variable doses of tiotidine. At isoproterenol EC_{50} (50 nM), tiotidine significantly diminishes isoproterenol-induced cAMP accumulation in a dose-dependent manner in more than a 60% (Fig. 8). These results agree with our prediction that tiotidine should interfere with the transduction cascade of an unrelated GPCR signaling through a $G\alpha_s$ -protein, such as the β_2 -adrenoreceptor.

We next evaluated the binding profile for a ligand with the parameters established above. To do that, we had to consider first that the affinity constant detected in a binding assay is an apparent constant, because we can measure only the fraction of receptors bound to ligands that also involves species uncoupled to G-protein ([LR], [LR*]) and coupled to G-protein ([LRG], [LR*G]). Therefore, even in this case, in which we can differentiate between receptor states coupled

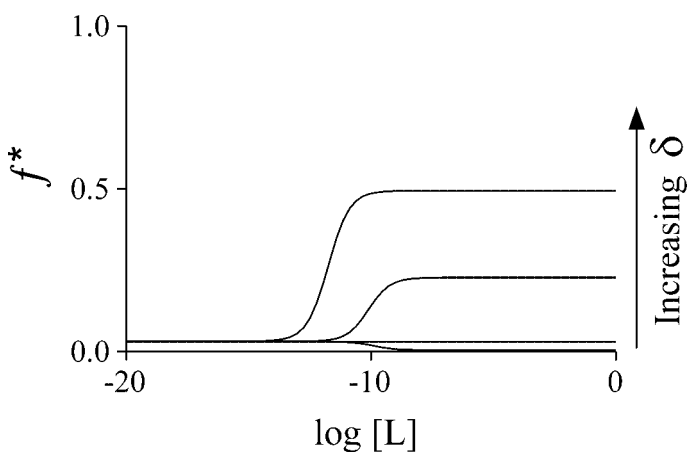


Fig. 6. Simulation of a dose-response experiment according to the CTC model. Simulation was based on Eq. 2 (see *Results*), using values of: $\alpha = \beta = \gamma = 10$; $K_a = K_g = 10^9$; $K_{act} = 10^{-2}$; $[G]_{total} = 0.5$; $[R]_{total} = 1$; $\delta = 0.01$; 0.078; 1; 100.

and uncoupled to G-protein, we have to measure the ligand affinity for more than one receptor species.

If $[R]_B$ is the receptor bound to ligand and $[R]_U$ the free receptor concentrations, we can define an affinity constant:

$$K_a = \frac{[R]_B}{[A][R]_U}$$

If we have more than one bound and free receptor species, the affinity constant corresponds to the previously mentioned apparent affinity constant, which can be expressed as:

$$K_{app} = \frac{\sum K_i \times [R_i]}{\sum [R_i]}$$

where K_i is the ligand affinity for the i^{th} unbound receptor state R_i . This K_{app} is a weighted average of the equilibrium association constants of the ligand for the unbound receptor species.

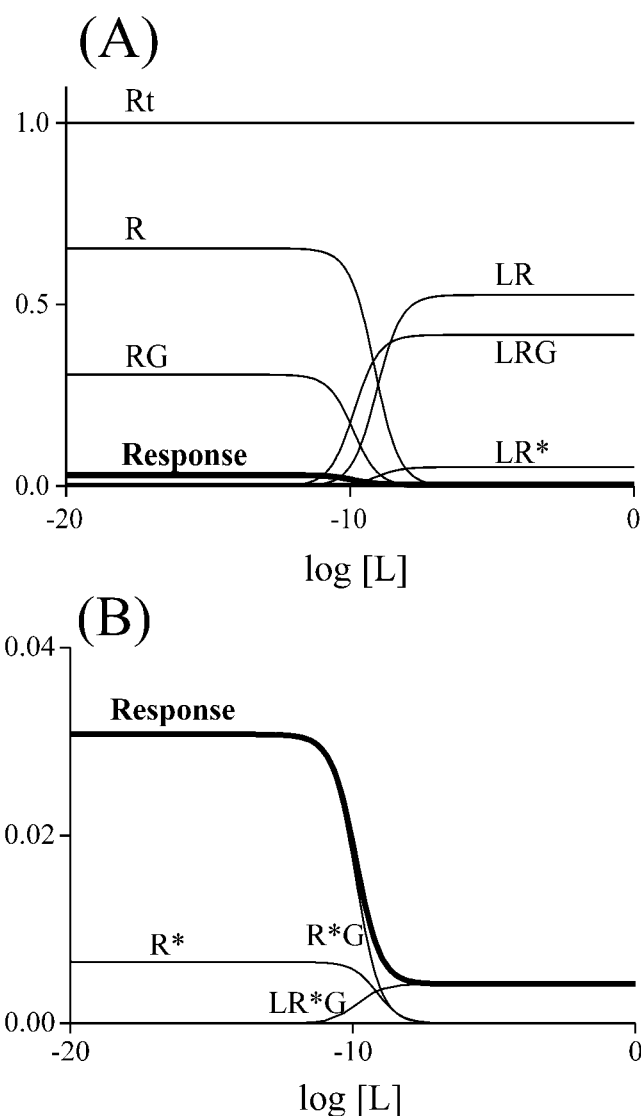


Fig. 7. A, simulation of receptor species variation depending on ligand concentration. f^* (assumed proportional to response) is represented in bold. Simulation was based on Eqs. 3 to 10 (see *Appendix 1*). $\alpha = \beta = \gamma = 10$; $K_a = K_g = 10^9$; $K_{act} = 10^{-2}$; $[G]_{total} = 0.5$; $[R]_{total} = 1$; $\delta = 0.01$. $[R]_{total}$ was calculated as the sum of every species and the plot was added as a control of model consistency. B, axis enlargement ranging from 0 to 0.04 was added for a better observation.

Using this equation, the apparent affinity of the ligand for the receptor species coupled (K_{appG}) and uncoupled (K_{appN}) to G-protein can be calculated as follows:

$$K_{appG} = \frac{\gamma \times K_a(1 + \alpha \times \beta \times \delta \times K_{act})}{1 + \beta \times K_{act}}$$

$$K_{appN} = \frac{K_a(1 + \alpha \times K_{act})}{1 + K_{act}}$$

Considering this equation, and using the same values for the parameters as in Fig. 7, we can demonstrate the existence of at least one δ value for which the theoretical relation K_{appG}/K_{appN} (8.4) is quite similar to that experimentally obtained for [³H]tiotidine binding (9.1). This implies that a radiolabeled inverse agonist can label the uncoupled states with lower affinity and the coupled states with higher affinity, although it fails to signal.

Discussion

The major finding of this study is that tiotidine is an inverse agonist that binds with high affinity to a form of the receptor coupled to G-protein but inactive. Two lines of evidence strongly support our conclusion: 1) tiotidine binds with high affinity to a site that no longer exists in the presence of GTP γ S or in desensitized cells and 2) tiotidine leads to a dose-dependent decrease in cAMP basal levels in U-937 cells, an effect that is clearly evident in both homologous and heterologous overexpression systems, as well as in forskolin pretreated cells. Each of these points is discussed, and our results are then interpreted with the aid of the CTC model.

First of all, tiotidine was initially described as a potent H2 antagonist with reported pA2 values of 7.3 to 7.8 (guinea pig right atrium) (van der Goot et al., 1991). It was considered a selective tool to establish interactions with the H2r because of its low affinity for both the H1 and the H3 receptors (van der Goot and Timmerman, 2000). On the other hand, binding of [³H]tiotidine to gastric mucosal cells revealed the presence

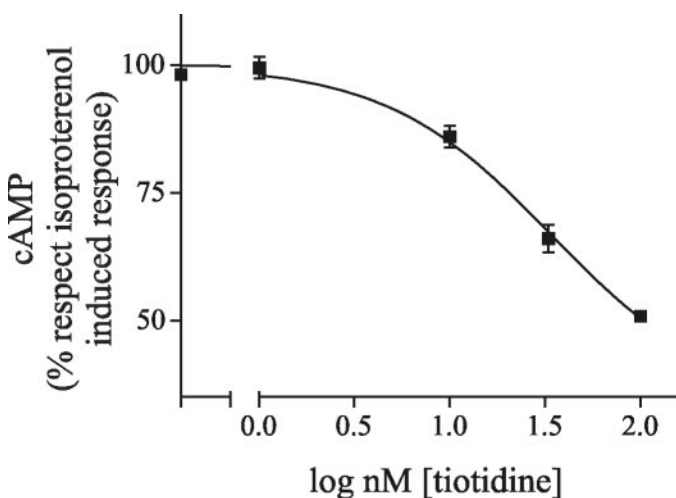


Fig. 8. Tiotidine dose-dependent reduction in isoproterenol-induced cAMP levels. U-937 cells were incubated for 3 min with increasing concentrations of tiotidine and 9 min with 50 nM isoproterenol at 37°C in the presence of 1 mM IBMX. Data are represented as the percentage of isoproterenol-induced cAMP levels and were calculated as the means \pm S.D. of assay triplicates. Similar results were obtained in three independent experiments.

of several binding sites (Batzri and Harmon, 1986). Therefore, the use of tiotidine for selectively labeling peripheral H2rs was considered rather limited. However, these findings can be explained with our first observation that tiotidine recognizes with different affinities the G-protein-coupled and -uncoupled forms of the H2r, as can be inferred from the vanishing of the high-affinity binding site in the presence of GTP γ S. This result was confirmed by studies in desensitized cells in which pretreatment with an H2 agonist that promotes a rapid receptor desensitization or uncoupling from heterotrimeric G-protein (Lemos et al., 2000) also leads to a single binding site for [³H]tiotidine.

GPCRs are affected by various receptor-specific ligands that modulate the GPCR activity in distinct ways. Originally, antagonists were thought to compete with the agonist for the same binding site but not to affect the GPCR activity directly. With the identification of CAM receptors, it has become clear that antagonists should be reclassified as neutral antagonists and inverse agonists.

Considering as an inverse agonist every ligand that is able to reduce the activity of receptor systems that are active in the absence of agonist, and taking into account our second observation, that tiotidine reduces cAMP basal levels in naive and surrogate systems, we believe that this ligand has to be reclassified as an H2 inverse agonist. Knowing that tiotidine may be regarded as an analog of cimetidine, a previously described H2 antagonist that was reclassified as an H2 inverse agonist (Smit et al., 1996), it is not surprising that this compound, which shares most of its structure with cimetidine, has a similar behavior.

It is widely assumed that inverse agonists stabilize certain states of the receptor with different functional activities. Based on the ETC model, they may act by binding to the R state of the receptor in preference to the R* state. Alternatively, they could bind to uncoupled states of the receptor (R and R*) in preference to the coupled state (R*G). A third possibility is that inverse agonists bias the receptor to an inactive conformation that can exist in G-protein coupled and uncoupled forms. The CTC model contains an inactive receptor conformation that can nevertheless couple to G-protein and makes it a suitable model to explain our findings about both tiotidine binding and its negative efficacy. To better explain what we discussed above, it is worth considering that for the ETC model, a ligand with high affinity for receptor species coupled to G-protein necessarily elicits a response. In contrast, the CTC model implies the existence of a receptor state coupled to G-protein, which is unable to evoke a response (RG), allowing a ligand with high affinity for the receptor form coupled to G-protein to behave as an antagonist or even as an inverse agonist (Fig. 6). This point is a distinctive feature of the CTC model.

Although the development of the ETC model was made necessary by experimental observation, the CTC model was originally proposed in an attempt to explore 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, our experimental findings can be explained solely in terms of the CTC model, justifying the use of this heuristic model despite its complexity. The main implication of the present study is

that it provides experimental support for the CTC model, showing that it has a practical use in addition to its theoretical use.

It is worth considering that another group obtained similar results for the CB1 cannabinoid receptor, which, after stimulation with a specific ligand, can couple to $G_{i/o}$ -protein without evoking a response (Bouaboula et al., 1997). Moreover, Brown and Pasternak (1998) showed that the binding of a specific μ -opioid receptor ligand, functionally classified as an antagonist, labels a G-protein-coupled state of the receptor with high affinity. These observations could also be explained using the CTC model.

In terms of the CTC model, the fact that tiotidine interferes with the signal transduction pathway of the β 2-adrenoreceptor, which also involves $G\alpha$ s-protein, implicates that tiotidine is biasing the system to a G-protein-coupled form of the H2r and making the G-protein less available to other GPCRs. This result supports the speculations based on our theoretical simulations considering a limiting G-protein concentration.

Alewijnse et al. (2000) showed that, as seen for CAM receptors, the affinity of histamine for both R116A and R116N arginine H2r mutants was significantly increased over that of the wild-type receptor. However, this increase in agonist affinity was not accompanied by an increase in constitutive receptor activity. In contrast, basal cAMP levels of both arginine mutant H2rs were significantly decreased compared with the wild-type receptors. These mutant receptors seemed to be in an active conformation, but the ability to couple to or activate G-protein was decreased. We speculate that the form of the receptor obtained in the presence of tiotidine resembles the conformation of these mutated H2rs, which has a high affinity for G-protein but fails to evoke a response.

Taking into account that the ligand-receptor interaction is very complex, a further consideration is to analyze our results in terms of the proposed existence of receptor protein as collections of microconformational states that might be pharmacologically relevant (ensemble theory; Hilser et al., 1998). In this context, it is tempting to speculate that tiotidine behaves as a ligand that preferentially binds to a microconformation coupled to G-protein but inactive, biasing the system to a non-response-evoking state. This possibility could be partly a restatement of the CTC model, because it might provide a conformational explanation for the receptor species present in it.

These results, which enlarge the knowledge about inverse agonists, not only lead to the reclassification of tiotidine as an H2 inverse agonist with an unusual behavior but also provide evidence to consider the CTC an experimentally supported model.

Appendix 1

An equation that describes the variation of f^* as a function of ligand concentration can be obtained, and if we represent f^* as a function of $\log[L]$, we can simulate a theoretical dose-response assay for a given set of parameters (Fig. 6).

Taking into account Eq. 1 (see *Results*) and considering the model-assumed equilibrium, its related constants,

$$K_a = \frac{[LR]}{[L][R]} \quad K_{act} = \frac{[R^*]}{[R]} \quad K_g = \frac{[RG]}{[R][G]}$$

and the conservation equations for each component (R, G, L),

$$[R]_{total} = [R] + [R^*] + [LR] + [LR^*] + [RG] + [R^*G] + [LRG] + [LR^*G]$$

$$[G]_{total} = [G] + [RG] + [R^*G] + [LRG] + [LR^*G]$$

$$[L]_{total} = [L] + [LR] + [LR^*] + [LRG] + [LR^*G]$$

we can deduce Eq. 2 (see *Results*), which represents the variation in the concentration of the species capable of generating a physiological response as a function of ligand concentration $[L]$. In the same way, we can obtain the equations that describe the behavior of every species as a function of $[L]$. Let

$$Z = 1 + K_{act} + K_g[G] + \beta \times K_g \times K_{act}[G] + (K_a + \alpha \times K_a$$

$$\times K_{act} + \gamma \times K_a \times K_g[G] + \alpha\beta\gamma\delta \times K_a \times K_{act} \times K_g[G]) \times [L]$$

then

$$\frac{[R]}{[R]_{total}} = \frac{1}{Z} \quad (3)$$

$$\frac{[RG]}{[R]_{total}} = \frac{K_g[G]}{Z} \quad (4)$$

$$\frac{[LR]}{[R]_{total}} = \frac{K_a[L]}{Z} \quad (5)$$

$$\frac{[LRG]}{[R]_{total}} = \frac{\gamma \times K_a \times K_g[G][L]}{Z} \quad (6)$$

$$\frac{[R^*]}{[R]_{total}} = \frac{K_{act}}{Z} \quad (7)$$

$$\frac{[R^*G]}{[R]_{total}} = \frac{\beta \times K_{act} \times K_g[G]}{Z} \quad (8)$$

$$\frac{[LR^*]}{[R]_{total}} = \frac{\alpha \times K_a \times K_{act}[L]}{Z} \quad (9)$$

$$\frac{[LR^*G]}{[R]_{total}} = \frac{\alpha\beta\gamma\delta \times K_a \times K_{act} \times K_g[G][L]}{Z} \quad (10)$$

The model internal consistency was tested, verifying that the sum of the former quotients results in 1 for every L concentration (Fig. 7).

Appendix 2

As indicated in the text, $[G]$ represents the concentration of the free G-protein and, because it is a function of the concentration of the ligand present in the system, $[G]$ must be found for each L concentration. Because $[G]$ has a quadratic dependence on $[L]$, $[G]$ must be achieved by solving

$$\frac{-b \pm \sqrt{b^2 - 4 \times a \times c}}{2 \times a}$$

where

$$a = K_g + \beta K_g K_{act} + \gamma K_g K_a [L] + \alpha\beta\gamma\delta K_g K_{act} K_a [L] + K_g^2 + \beta K_g^2 K_{act} + \gamma K_g^2 K_a [L] + \alpha\beta\gamma\delta K_g^2 K_a K_{act} [L]$$

$$\begin{aligned}
b &= 1 + K_{\text{act}} + K_{\text{a}}[L] + \alpha K_{\text{a}} K_{\text{act}}[L] + K_{\text{g}} + K_{\text{g}} K_{\text{act}} + K_{\text{g}} K_{\text{a}}[L] \\
&\quad + \alpha K_{\text{g}} K_{\text{act}} K_{\text{a}}[L] + Rt(\beta K_{\text{g}} K_{\text{act}} + \gamma K_{\text{g}} K_{\text{a}}[L]) \\
&\quad + \alpha \beta \gamma \delta K_{\text{g}} K_{\text{act}} K_{\text{a}}[L] - G_{\text{total}}(K_{\text{g}} + \beta K_{\text{g}} K_{\text{act}} + \gamma K_{\text{g}} K_{\text{a}}[L]) \\
&\quad\quad\quad + \alpha \beta \gamma \delta K_{\text{g}} K_{\text{act}} K_{\text{a}}[L]) \\
c &= -G_{\text{total}}(1 + K_{\text{act}} + K_{\text{a}}[L] + \alpha K_{\text{act}} K_{\text{a}}[L])
\end{aligned}$$

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