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Mepyramine, a Histamine H_1 Receptor Inverse Agonist, Binds Preferentially to a G Protein-coupled Form of the Receptor and Sequesters G Protein*

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Accurate characterization of the molecular mechanisms of the action of ligands is an extremely important issue for their appropriate research, pharmacological, and therapeutic uses. In view of this fact, the aim of the present work was to investigate the mechanisms involved in the actions of mepyramine at the guinea pig H_1 receptor stably expressed in Chinese hamster ovary cells. We found that mepyramine is able to decrease the basal constitutive activity of the guinea pig H_1 receptor, to bind with high affinity to a $G_{q/11}$ protein-coupled form of the receptor and to promote a G protein-coupled inactive state of the H_1 receptor that interferes with the $G_{q/11}$-mediated signaling of the endogenously expressed ATP receptor, as predicted by the Cubic Ternary Complex Model of receptor occupancy. The effect of mepyramine on ATP-induced signaling was specifically neutralized by $G\alpha_{11}$ overexpression, indicating that mepyramine is able to reduce G protein availability for other non-related receptors associated with the same signaling pathway. Finally, we found a loss of mepyramine efficacy in decreasing basal levels of intracellular calcium at high $G\alpha_{11}$ expression levels, which can be theoretically explained in terms of high H_1 receptor constitutive activity. The whole of the present work sheds new light on H_1 receptor pharmacology and the mechanisms H_1 receptor inverse agonists could use to exert their observed negative efficacy.

Traditionally, ligands acting at G protein-coupled receptors (GPCRs)[1] were classified as agonists, accounting for ligands with observable positive efficacy, or as antagonists, for ligands with no observable efficacy. However, the identification of constitutively active receptors led to the identification of ligands with intrinsic negative efficacy, characterized by their ability to decrease basal constitutive receptor activity. In view of this property, those ligands were defined as inverse agonists (1). Considering the importance accurate ligand classification has in terms of proper pharmacological use, the mechanism(s) by which these ligands exert their effects has to be extensively and carefully characterized (2).

In an attempt to understand such mechanisms, several receptor occupancy models have been developed (3–7). For all of the currently accepted models, basal activity is proportional to the population of receptors active and coupled to G protein. The simplest explanation for the negative efficacy observed for inverse agonists is, then, the selective affinity of the ligand for an inactive state of the receptor. Binding of the ligand would then deplete the levels of active receptors coupled to G protein, resulting in a decrease in receptor constitutive activity. Alternatively, ligands with observed negative efficacy could promote uncoupling from G proteins, leading to G protein-free receptor species, which are unable to evoke response. However, the Cubic Ternary Complex Model (CTC) predicts a third mechanism accounting for negative efficacy (5–7). According to the CTC, a ligand may bind with high affinity to a G protein-coupled but inactive form of the receptor, resulting in a decrease of spontaneous receptor activity. The distinctive feature of this proposed mechanism is that, by promoting receptor-G protein coupling, binding of the ligand would result in a reduction of the G protein availability to other receptors associated with the same pathway, thus interfering with the signaling of those GPCRs (8). Importantly, experimental evidence supporting all the three aforementioned mechanisms has been reported for different inverse agonists.

Mepyramine, traditionally classified as a histamine H_1 receptor antagonist, is the reference ligand for studying H_1 receptor pharmacology (9). Together with other formerly named H_1 antagonists, it has been recently reclassified as an inverse agonist, in view of its negative efficacy at the constitutively active human H_1 receptor observed in transiently transfected COS-7 cells (10). Taking into account the clinically widespread use of histamine H_1 receptor antihistamines in the treatment of human diseases, such as allergic rhinitis, conjunctivitis, and dermatitis, proper classification of these ligands and accurate characterization of their mechanism(s) of action is of great importance.

The aim of the present work was to identify the mechanisms

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[1] The abbreviations used are: GPCR, guanine nucleotide binding regulatory protein (G protein)-coupled receptor; CTC, Cubic Ternary Complex Model; gp-H_1r, guinea pig histamine H_1 receptor; GTPγS, guanosine 5′-O-(3-thio)triphosphate; mepyramine, 2-((2-(dimethylamino)ethyl)(*p*-methoxybenzyl)amino)-pyridine; triprolidine, (*E*)-2-[1-(4-methylphenyl)-3-(1-pyrrolidinyl)-1-propenyl]pyridine; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; PTX, pertussis toxin; InsP, inositol phosphate.

involved in mepyramine actions at the guinea pig H_1 receptor (gp-H_1r) stably expressed in Chinese hamster ovary (CHO-K1) cells. We found that mepyramine acts as an inverse agonist decreasing the basal spontaneous activity of the gp-H_1r. Moreover, mepyramine inhibited histamine-induced inositol phosphate (InsP) production and intracellular calcium mobilization with a marked decrease in the maximal response to histamine at high mepyramine concentrations. This effect was not observed with triprolidine, another H_1 receptor inverse agonist. A strong interference of mepyramine with the $G_{q/11}$-mediated signaling of the endogenously expressed ATP receptor was observed. This indicates that mepyramine may, at least in part, exert its action by promoting a G protein-coupled inactive state of the H_1 receptor as predicted by the CTC model. Accordingly, the effect of mepyramine was neutralized by overexpression of $G\alpha_{11}$ protein, indicating that mepyramine is able to reduce the G protein availability for other non-related receptors associated with the same signaling pathway. Finally, we found a decrease in the efficacy of mepyramine to diminish the basal levels of intracellular calcium at high $G\alpha_{11}$ expression levels. This G protein-dependent efficacy observed for mepyramine can be explained in terms of the high H_1 receptor constitutive activity. The whole of the present work sheds new light into the understanding of H_1 receptor pharmacology and the mechanisms inverse agonists could utilize to exert their observed negative efficacy.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Mepyramine and *myo*-[^{3}H]inositol were purchased from PerkinElmer Life Sciences. Histamine dihydrochloride, mepyramine hydrochloride, *myo*-inositol, pertussis toxin (PTX), cAMP, fura-2 AM (fura-2 pentakis(acetoxymethyl) ester), bovine serum albumin, bicinchoninic acid method reagents, and ethidium bromide were purchased from Sigma. Triprolidine was from Tocris Cookson Inc. (Ballwin, MO). Dowex AG-1X8 formate form and Chelex 100 resins were obtained from Bio-Rad. DMEM, fetal calf serum, agarose, and antibiotics were purchased from Invitrogen. *Taq* DNA polymerase and all the restriction endonucleases were from Promega Corp. (Madison, WI). pcDNA3Gα_q, pcDNA3Gα_{11}, pcDNA3Gα_{i1}, and pcDNA3Gα_S plasmids were generous gifts from Dr. O. Cosso (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina). All other chemicals were of analytical grade.

Cloning and Sequencing of the Guinea Pig Histamine H_1 Receptor—Guinea pig histamine H_1 receptor was cloned following recently described procedures (11) with some modifications. Briefly, two 25-bp oligonucleotides derived from the sequence of the intronless gene encoding the gp-H_1r (12) (GenBank™ accession number S68706) were used to amplify a 1501-bp cDNA fragment from guinea pig genomic DNA, obtained from guinea pig liver (5′-ct<u>aagctt</u>gtc**attatgg**ctttcct-3′ (sense primer), where the underlined sequence indicates a HindIII site, and the sequence in *bold* indicates a Kozac's sequence, and 5′–ctgcctc-c<u>tctaga</u>tgcaccctcaa-3′ (antisense primer), where the underlined sequence indicates a XbaI site containing an internal stop codon).

PCR amplification was carried out using *Taq* DNA polymerase, and the resulting PCR products were gel-purified and cloned into the pCEFL vector. The 1501-bp insert was sequenced using specific primers for the vector, and the sequence was found to be 100% homologous to the reported gp-H_1r. For functional expression, the insert was excised by double digestion with HindIII-XbaI and cloned into the multiple cloning site of the mammalian expression vector pcDNA 3 (Invitrogen) at the HindIII-XbaI sites to give the correct orientation for transcription. The identity and orientation of the insert was verified by sequencing. The resulting plasmid was used to transfect CHO-K1, and a single stable clone with high histamine H_1 receptor expression (CHO-gpH_1) was established by neomycin selection. A separate single clone containing the empty vector was selected under the same conditions (CHO-mock). Transient and stable transfections were performed with LipofectAMINE (Invitrogen) following the manufacturer's instructions.

Cell Culture—All cells were grown at 37 °C in a humidified 5% CO_2 incubator. CHO-gpH_1 and CHO-mock cells were cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine, 50 μg/ml gentamicin, and 0.8 mg/ml G418. Parental CHO-K1 cells were cultured in the same medium without G418.

Membrane Preparation and H_1 Receptor Binding Assays—Membrane-purified fraction from CHO-gpH_1 and CHO-mock cells were prepared, and binding assays were performed as described (8). CHO-gpH_1 cells were cultured in 10-cm plastic plates for 48 h (70–80% confluence) and lifted from the plates using a plastic cell lifter (Costar®, Corning Inc., Corning, NY) in 5 volumes of ice-cold binding buffer (50 mM Tris-HCl, pH 7.4). The cells were then disrupted by sonication in binding buffer and centrifuged at 1000 × *g* for 10 min to remove nuclei and unbroken cells. The supernatant was centrifuged for 15 min at 30,000 × *g*, and the pellet was suspended in an adequate volume of binding buffer. Protein concentration was determined by the bicinchoninic acid method, and the pellet was resuspended in binding buffer at a final protein concentration of 1 mg/ml and stored at −70 °C.

For saturation binding experiments membrane fractions (10–20 μg of protein) were incubated in triplicate with different concentrations of [^{3}H]mepyramine ranging from 0.05 to 10 nM in a final volume of 200 μl for 45 min. For competition binding experiments, membranes were incubated with [^{3}H]mepyramine (2 nM) in assay triplicates in the presence of different concentrations of competitors in a final volume of 200 μl for 45 min. For dissociation kinetics of [^{3}H]mepyramine binding, assay preparations were done in a larger volume to allow extraction of several aliquots of 1 ml at adequate time points over a time interval of 90 min. Membranes were preincubated with [^{3}H]mepyramine for 45 min before measuring radioligand dissociation by the addition of 10 μM cold mepyramine (13). In all cases assay mixtures were incubated at 25 °C in polypropylene tubes. To study the effect of GTPγS on [^{3}H]mepyramine binding, membrane fractions were pretreated at 37 °C for 1 h in Hanks' balanced salt solution in the presence of increasing concentrations of GTPγS. Nonspecific binding was determined in the presence of 10^{-4} M triprolidine. Kinetic studies demonstrated that equilibrium was reached after 15 min of incubation and persisted for 4 h (data not shown). After the incubation samples were filtered through 0.3% polyethyleneimine-soaked GF/B filters and washed 3 times with ice-cold binding buffer. Filters were suspended in 5 ml of Hi-Safe scintillation mixture (PerkinElmer), and radioactivity was counted in a Wallac 1410 liquid scintillation counter.

[^{3}H]Inositol Phosphate Production—Total inositol phosphate production was measured as previously described (14). Briefly, cells were seeded in 24-well cluster dishes and cultured for 24 h (70–80% confluence) in DMEM. Cells were then washed, and the medium was replaced for DMEM without calf serum plus the addition of *myo*-[^{3}H]inositol (2 μCi/ml) and cultured for 24 h. Thereafter, the medium was aspirated and replaced with DMEM without calf serum containing 10 mM LiCl and incubated for 20 min. Cells were then stimulated for 20 min with histamine in concentrations ranging from 1 nM to 100 μM in a final volume of 300 μl in the presence or absence of specific H_1 antagonists in the concentrations indicated in each particular experiment. The incubation was stopped by the addition of 900 μl of cold chloroform, methanol, 0.12 M HCl (1:2:1 v/v, freshly prepared), and phases were split by the addition of 300 μl of water and 300 μl of chloroform. The mixture was then centrifuged at 1500 × *g* for 10 min, and the total water-soluble inositol phosphate fraction was purified by anion exchange chromatography. Radioactivity of the eluted fractions was measured using a Wallac 1410 liquid scintillation counter. Results were expressed as the ratio obtained when total [^{3}H]inositol phosphate radioactivity was normalized to total [^{3}H]inositol radioactivity recovered from the initial water wash of the columns.

Ca^{2+} Measurements—Changes in intracellular Ca^{2+} concentration were measured by the standard method described elsewhere (15). Briefly, cells were cultured in 10-cm-diameter plastic dishes for 24 h (70–80% confluence). Thereafter, cells were detached with trypsin, EDTA (0.5 g/liter of trypsin, 0.2 g/liter of EDTA, and 0.85 g/liter NaCl) and resuspended in loading buffer (2 mM $CaCl_2$, 145 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM $MgSO_4$, and 10 mM HEPES, pH 7.4). The cells were then loaded in the presence of 5 μM fluorescent Ca^{2+} indicator fura-2 acetoxymethyl ester (fura-2 AM) for 20 min at 37 °C. The cells were 5-fold-diluted and incubated for 10 min at 37 °C to facilitate the hydrolysis of the ester to the acid form. Excess dye was removed by two cycles of centrifugation/resuspension in 2 ml of loading buffer containing 0.1 mM EGTA. Each Ca^{2+} measurement was preceded by a rapid cycle of centrifugation/resuspension in 2 ml of loading buffer containing 0.1 mM EGTA. Fluorescence was measured in a Jasco FP750 spectrofluorometer (Jasco, Tokyo, Japan) equipped with the CA-261 accessory to measure Ca^{2+} with continuous stirring. The wavelength was set at 340/380 nm, and detection was at 500 nm. After 10 s of initial recording to determine basal levels, agonists were added in 20-μl final volumes, and the time course of intracellular Ca^{2+} mobilization was recorded for 70 s. At the end of the time course, $CaCl_2$ (20 mM) followed by Triton

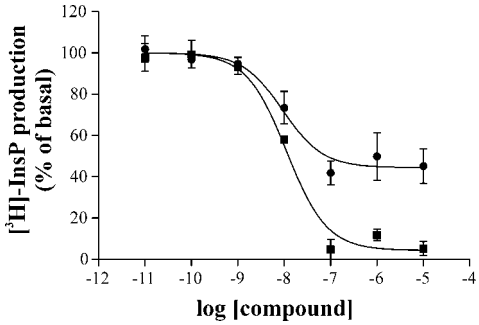

FIG. 1. **Effects of mepyramine and triprolidine on basal InsP production.** CHO-gpH_1 cells were treated for 20 min with increasing concentrations of mepyramine (■) or triprolidine (●) in presence of 10 mM LiCl. Data are expressed as the mean ± S.E. of assay triplicates. Similar results were obtained in at least three independent experiments.

X-100 (0.25% v/v) was added to determine F_{max} followed by EGTA (6.25 mM, pH 8.6) to determine F_{min}. Autofluorescence was quantified by measuring the fluorescence produced by an equivalent suspension of not-loaded cells. Using these values intracellular Ca^{2+} concentrations were calculated as described by Grynkiewicz *et al.* (16). Antagonists were preincubated for 10 min. Results were expressed as the percentage of the maximal response of the system, measured in the presence of 20 mM $CaCl_2$, 0.25% Triton X-100. Basal levels determined in the first 10 s were considered 0% in all of the cases.

Western Blots—Standard Western blotting was performed as previously described (17). Briefly, cells were lysed in sample buffer (50 mM Tris-HCl, 2% SDS, 100 mM 2-mercaptoethanol, 10% glycerol, and 0.05% bromphenol blue, pH 6.8) and sonicated to shear DNA. Samples were boiled for 5 min, and aliquots were electrophoresed in 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The residual binding sites were blocked with 5% nonfat powdered milk in PBST (phosphate-buffered saline containing 0.05% Tween 20), and membranes were incubated with 1 μg/ml anti-Gα_{11} rabbit antibody or anti-actin goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBST. All subsequent washes were performed with the same buffer. Reactivity was developed using an anti-rabbit or anti-goat polyclonal antibody linked to horseradish peroxidase and enhanced chemiluminescence reagents, according to the manufacturer's instructions (Amersham Biosciences).

Analytical Methods—Binding data and dose-response curve fitting were done using Prism 3.00 for Windows (GraphPad Software, San Diego, CA). Specific binding was calculated by subtraction of nonspecific binding from total binding. One-way analysis of variance with Dunnett's post test was performed using InStat version 3.01 (GraphPad Software, San Diego, CA).

RESULTS

Mepyramine Binding and Inverse Agonism at the Stably Expressed Guinea Pig Histamine H_1 Receptor—We first aimed to study the binding and signaling profile of mepyramine at the gp-H_1r heterologously expressed in CHO-K1 cells. To examine this, we generated a stable cell line expressing gp-H_1r named CHO-gpH_1. In these cells, mepyramine displayed specific, reversible, and saturable binding. Interestingly, saturation binding experiments disclosed two binding sites for mepyramine ($p < 0.05$, F test), one with higher affinity (0.16 ± 0.02 nM) and lower capacity (32.8 ± 2.8 fm/mg of protein) and a second one with lower affinity (2.4 ± 0.4 nM) and higher capacity (445.8 ± 19.9 fm/mg of protein). This is consistent with previous observations from several other groups (18–20).

Most of the ligands previously classified as H_1 antagonist were recently reclassified as inverse agonists in view of the reported constitutive activity of the human histamine H_1 receptor (10). To corroborate this observation made in COS-7 cells, we tested the effects of mepyramine on InsP levels in CHO-gpH_1 cells. As anticipated, mepyramine was able to dose-dependently decrease basal InsP levels (log EC_{50} = −7.94 ± 0.11), (Fig. 1). To confirm this observation we tested the effect of triprolidine, another formerly designated H_1 antagonist, on basal InsP levels in CHO-gpH_1 cells. As observed with mepyramine, triprolidine was able to dose-dependently decrease the basal InsP production in CHO-gpH_1 cells (log EC_{50} = −8.03 ± 0.23) (Fig. 1). Importantly, although the potency of both inverse agonists was similar, triprolidine could only inhibit 60% of the basal levels of InsP, indicating that triprolidine is a less efficacious ligand than mepyramine and, thus, might be classified as a partial inverse agonist, whereas mepyramine may be classified as full inverse agonist at the gp-H_1r expressed in CHO cells. Both tested compounds inhibited the constitutive activity of the gp-H_1r, displaying the negative efficacy expected for inverse agonists, in accordance with previous observations made with the human H_1 receptor (21).

Effect of GTPγS on Mepyramine Binding at the gp-H_1r—In the theoretical representation of ligand-receptor interaction denoted by the extended ternary complex model (4), the negative efficacy observed for inverse agonists is explained by a selective affinity of the ligand for a non response-evoking state of the receptor (2). Under this assumption, the affinity of an inverse agonist should be increased by uncoupling the receptor from the G protein (4).

To evaluate the effect of receptor uncoupling from G protein, membranes from CHO-gpH_1 cells were incubated with increasing amounts of GTPγS. Under these conditions, GTPγS dose-dependently inhibited mepyramine binding with a pEC_{50} value of 4.72 ± 0.18 and a maximal effect of 64.9 ± 4.6% inhibition at 1×10^{-3} M GTPγS (Fig. 2*A*). We then proceeded to further study the effect of G protein uncoupling on mepyramine binding using a concentration of 3×10^{-4} M GTPγS. Preincubation of CHO-gpH_1 cell membranes with this GTPγS concentration induced a significant decrease in the affinity of mepyramine for the gp-H_1r, shifting the homologous displacement curves of mepyramine from a pIC_{50} value of 8.77 ± 0.08 in the absence of GTPγS to a pIC_{50} value of 7.44 ± 0.07 in the presence of GTPγS ($p < 0.01$, $n = 5$). These results are in agreement with previous observations made with the gp-H_1r expressed in atrial membranes (22). Overexpression of Gα_{11} proteins had the opposite effect, inducing a leftward shift in the homologous displacement curves to a pIC_{50} value of 9.68 ± 0.08 ($p < 0.01$ *versus* non transfected cells, $n = 8$) (Fig. 2*B*). Conversely, under the same experimental conditions the affinity of triprolidine for the gp-H_1r was only marginally affected, with observed pIC_{50} values of 8.83 ± 0.05 in the absence of GTPγS *versus* 8.51 ± 0.07 in the presence of GTPγS and 8.99 ± 0.10 in the Gα_{11} overexpression system ($p > 0.05$ *versus* non-treated non-transfected cells, $n = 8$) (Fig. 2*C*).

These results indicated that even though mepyramine and triprolidine displayed intrinsic negative efficacy at the gp-H_1r, they may recognize functionally different receptor subpopulations. Triprolidine binding was insensitive to uncoupling of the receptor from G protein. In contrast, mepyramine affinity for the gp-H_1r was significantly decreased by pretreatment with GTPγS and increased when Gα_{11} protein was overexpressed, indicating a higher affinity for receptor species coupled to G protein. We have recently reported similar observations with the histamine H_2 receptor inverse agonist tiotidine (8). Pretreatment of membranes from U-937 cells with GTPγS drastically decreased the affinity of tiotidine for the human H_2 receptor, indicating that binding of the inverse agonist could bias the system toward a G protein-coupled but inactive receptor state, predicted only by the CTC model of receptor occupancy (7). To test if binding of mepyramine could induce a similar receptor state of the H_1 receptor, we decided to measure the effects of mepyramine on histamine intrinsic efficacy at the H_1 receptor expressed in CHO-gpH_1 cells.

Effects of Mepyramine on Histamine Intrinsic Efficacy at the gp-H_1r—To evaluate the effect of mepyramine on histamine

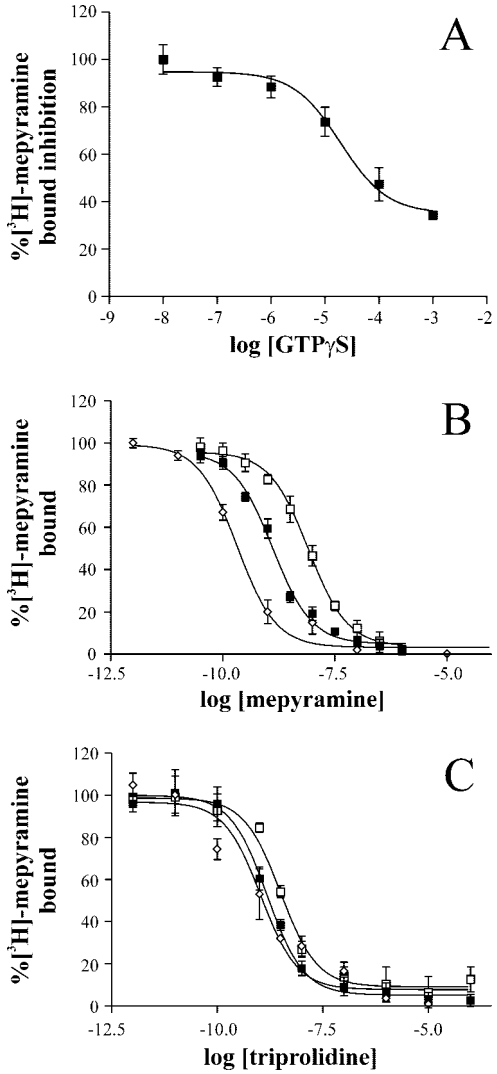

FIG. 2. **Effects of GTPγS pretreatment and $G\alpha_{11}$ overexpression on mepyramine- or triprolidine-induced displacement of [^{3}H]mepyramine binding.** *A*, membrane fractions obtained from CHO-gpH_1 cells were exposed to increasing concentrations of GTPγS before performing homologous competition binding experiments as described under "Experimental Procedures." *B*, membranes from CHO-gpH_1 cells (■), CHO-gpH_1 cells transiently overexpressing $G\alpha_{11}$ (◇), and CHO-gpH_1 cells preincubated with 3×10^{-4} M GTPγS (□) were exposed to 2 nM [^{3}H]mepyramine and increasing concentrations of unlabeled mepyramine as described under "Experimental Procedures." Results are expressed as the mean ± S.E. of assay triplicates. Similar results were obtained in five independent experiments. *C*, membranes from CHO-gpH_1 cells (■), CHO-gpH_1 cells transiently overexpressing $G\alpha_{11}$ (◇), and CHO-gpH_1 cell membranes preincubated with 3×10^{-4} M GTPγS (□) were exposed to 2 nM [^{3}H]mepyramine and increasing concentrations of unlabeled triprolidine as described under "Experimental Procedures." Results are expressed as the mean ± S.E. of assay triplicates. Similar results were obtained in eight independent experiments.

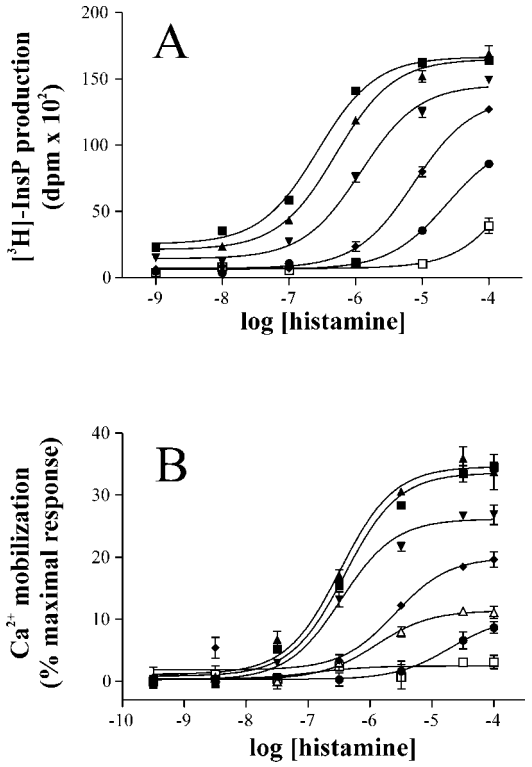

FIG. 3. **Effect of mepyramine on histamine-induced InsP production and intracellular Ca^{2+} mobilization.** *A*, CHO-gpH_1 cells were treated with increasing concentrations of histamine in the absence (■) or in the presence of 10^{-9} M (▲), 10^{-8} M (▼), 10^{-7} M (♦), 10^{-6} M (●), or 10^{-5} M (□) mepyramine, and InsP production was measured as described under "Experimental Procedures." Data were calculated as the mean ± S.E. of assay triplicates. Similar results were obtained in at least four independent experiments. *B*, CHO-gpH_1 cells were treated with increasing concentrations of histamine in the absence (■) or in the presence of 10^{-9} M (▲), 10^{-8} M (▼), 10^{-7} M (♦), $10^{-6.5}$ M (△), 10^{-6} M (●), or 10^{-5} M (□) mepyramine, and intracellular Ca^{2+} levels were measured as described under "Experimental Procedures." Data were calculated as the mean ± S.E. of assay triplicates. Similar results were obtained in at least four independent experiments.

intrinsic efficacy at gp-H_1r, different assays were used to rule out bias of the experimental readout on the observations. Histamine intrinsic efficacy was drastically decreased by incubation with increasing concentrations of mepyramine (Fig. 3, *A* and *B*). Mepyramine induced marked depressions of the maximal histamine response along with rightward shifts of histamine dose-response curves in both assays used to evaluate its effects on histamine response. Interestingly, similar effects of mepyramine on histamine maximal response have been recently described using a c-*fos*-luciferase reporter-gene assay (15). Although the magnitude of the reduction in the maximal response to histamine observed with that method was smaller than the reduction we report herein, that is to be expected from a measure done on a downstream effector (23).

In view of the differences observed in binding profiles, we decided to test the effects of triprolidine on histamine intrinsic efficacy at the gp-H_1r. As depicted in Fig. 4, *A* and *B*, triprolidine induced a rightward shift of histamine dose-response curves without affecting maximal response. This indicated that although both ligands displayed intrinsic negative efficacy at the gp-H_1r, the mechanisms that mepyramine and triprolidine use to induce this effect are different.

Interestingly, similar effects on histamine-induced maximal response have been recently reported for other formerly designated H_1 antagonists (24). These observations were explained in part by a lack of equilibrium of histamine and its antagonists at the H_1 receptor within the short time intervals used for intracellular calcium measurements, resulting in an apparent non-competitive antagonism. To address this point, we decided to study the kinetics of mepyramine dissociation from gp-H_1r. In our hands, [^{3}H]mepyramine could rapidly dissociate from the gp-H_1r upon the addition of 10 μM mepyramine with a half-life of 3.85 ± 0.24 min. This is in accordance with the common notion that mepyramine is a competitive ligand at the histamine H_1 receptor, previously demonstrated by several other groups (22).

Although the half-life value determined for mepyramine binding at the gp-H_1r could suggest that in the short times of calcium experiments histamine may not completely displace

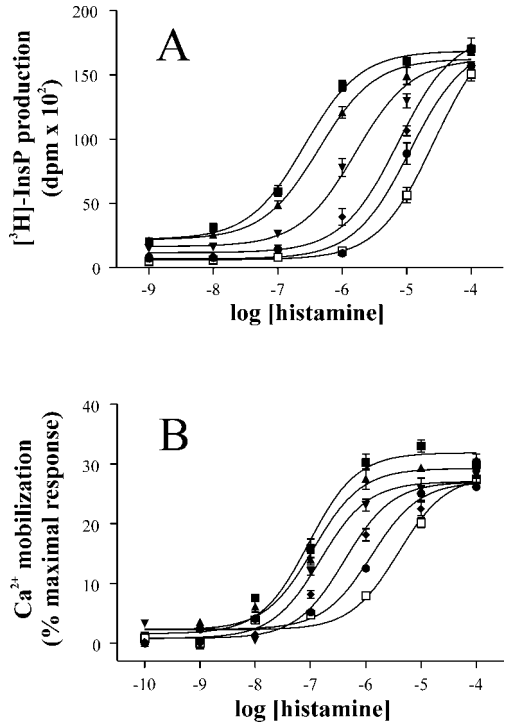

FIG. 4. **Effect of triprolidine on histamine-induced InsP production and intracellular Ca^{2+} mobilization.** *A*, CHO-gpH_1 cells were treated with increasing concentrations of histamine in the absence (■) or in the presence of 10^{-9} M (▲), 10^{-8} M (▼), 10^{-7} M (♦), 10^{-6} M (●), or 10^{-5} M (□) triprolidine, and InsP production was measured as described under "Experimental Procedures." Data were calculated as the mean ± S.E. of assay triplicates. Similar results were obtained in at least four independent experiments. *B*, CHO-gpH_1 cells were treated with increasing concentrations of histamine in the absence (■) or in the presence of 10^{-9} M (▲), 10^{-8} M (▼), 10^{-7} M (♦), 10^{-6} M (●), or 10^{-5} M (□) triprolidine. Intracellular Ca^{2+} levels were measured as described under "Experimental Procedures." Data were calculated as the mean ± S.E. of assay triplicates. Similar results were obtained in at least four independent experiments.

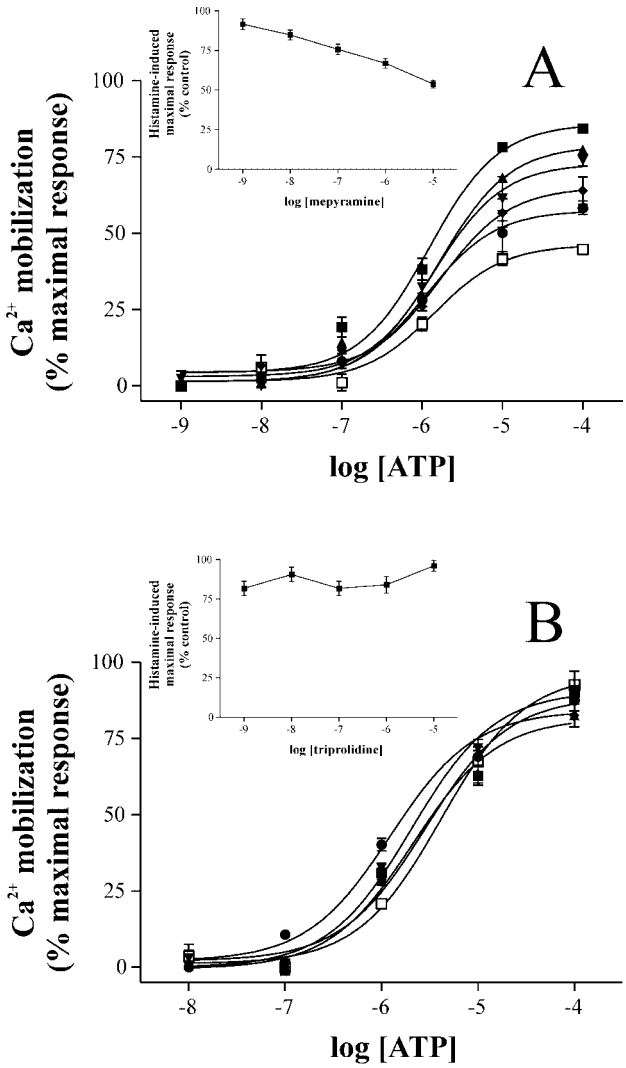

FIG. 5. **Effect of mepyramine and triprolidine on ATP-induced intracellular Ca^{2+} mobilization.** *A*, CHO-gpH_1 cells were treated with increasing concentrations of ATP in the absence (■) or in the presence of 10^{-9} M (▲), 10^{-8} M (▼), 10^{-7} M (♦), 10^{-6} M (●), or 10^{-5} M (□) mepyramine. Data were calculated as the mean ± S.E. of assay triplicates. Similar results were obtained in at least four independent experiments. *Inset*, variation of the ATP maximal responses with increasing concentrations of mepyramine. *B*, CHO-gpH_1 cells were treated with increasing concentrations of ATP in the absence (■) or in the presence of 10^{-9} M (▲), 10^{-8} M (▼), 10^{-7} M (♦), 10^{-6} M (●), or 10^{-5} M (□) triprolidine. *Inset*, variation of the ATP maximal responses with increasing concentrations of triprolidine. Intracellular Ca^{2+} levels were measured as described under "Experimental Procedures." Data were calculated as the means ± S.E. of assay triplicates. Similar results were obtained in at least four independent experiments.

mepyramine from a common binding pocket, the observations done with the [^{3}H]inositol phosphate assay, where ligands were co-incubated for 20 min, ruled out that possibility. Results indicate that our observations cannot be directly explained in terms of a supposed uncompetitive behavior of mepyramine at the gp-H_1r, suggesting that other previously designated H_1 antagonists may also induce a previously unidentified G protein-coupled inactive state of the H_1 receptor. To test the anticipated interference of such a receptor state with the signaling of other non-related GPCRs (8), we decided to measure the effects of mepyramine on the intrinsic efficacy of ATP at the purinergic receptor endogenously expressed in CHO cells (25).

Effects of Mepyramine on ATP Intrinsic Efficacy at the Purinergic Receptor Endogenously Expressed in CHO-gpH_1 Cells—ATP acts as an agonist at the purinergic receptor endogenously expressed in CHO cells, inducing activation of the receptor and the downstream signaling cascade. This results in G_q activation, phospholipase C activation, and intracellular calcium mobilization (25). Accordingly, the H_1 receptor has been classically associated with the same pathway (26). Considering these facts, we decided to study the effects of increasing concentrations of mepyramine on the concentration response curve induced by ATP. As depicted in Fig. 5A, increasing concentrations of mepyramine significantly decreased the maximal response to ATP in CHO-gpH_1 cells. This observation strengthened the conclusion that mepyramine is capable of stabilizing a G protein-coupled receptor state unable to evoke signal. Conversely, increasing concentrations of triprolidine had no significant effect on ATP maximal response (Fig. 5*B*), indicating again that both compounds may use different mechanism to inhibit the spontaneous activity of the gp-H_1r. Control experiments showed that neither histamine nor mepyramine alone or in combination with histamine had any detectable effect on Ca^{2+} basal levels in parental CHO-K1 (Fig. 6). Remarkably, mepyramine did not interfere with the ATP-mediated Ca^{2+} response observed in parental CHO-K1 cells (Fig. 6), indicating that the effects of mepyramine are specifically mediated by the exogenously expressed gp-H_1r and ruling out the possibility that mepyramine may have nonspecific effects on the ATP receptor or on G proteins, endogenously expressed in CHO-K1 cells. Moreover, the effects of ATP in CHO-K1 cells

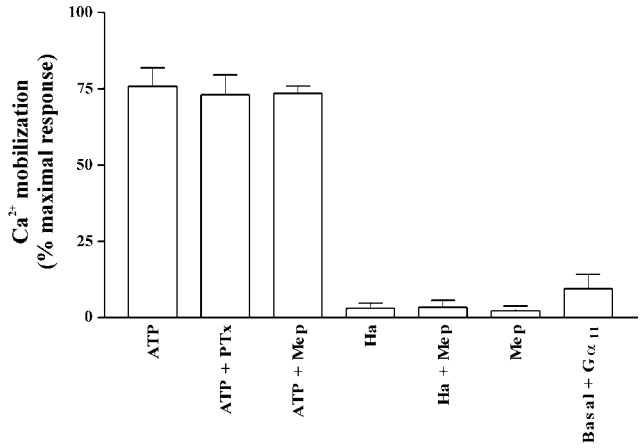

FIG. 6. **Effect of ATP, histamine, and mepyramine on intracellular [Ca^{2+}] mobilization in CHO-K1 cells.** CHO-K1 cells were treated with ATP (10^{-4} M), ATP + PTX (80 ng/ml, 24 h), ATP + mepyramine 10^{-5} M (*Mep*), histamine (10^{-4} M) (*Ha*), histamine + mepyramine 10^{-5} M, and mepyramine 10^{-5} M or were transiently transfected with 0.5 μg of $G\alpha_{11}$ cDNA (*Basal* + $G\alpha_{11}$). Intracellular Ca^{2+} levels were measured as described under "Experimental Procedures." Data were calculated as the mean ± S.E. of four independent experiments.

were completely insensitive to PTX (Fig. 6), suggesting that the signaling of the endogenous ATP receptor is most likely mediated by $G_{q/11}$ proteins as previously reported (27). Consistently, ATP- and histamine-induced calcium mobilization were completely PTX-insensitive in CHO-gpH_1 cells (data not shown).

Effects of Heterologous G Proteins Overexpression on the Actions of Mepyramine—Because the results described above are not easily explained by simple receptor occupancy models, we decided to further investigate the possible association of mepyramine with a receptor state bound to G proteins of the $G_{q/11}$ family, resulting in G protein sequestration. If that is true, increasing the amounts of G proteins of this family would counteract the effect of mepyramine on ATP response in CHO-gpH_1 cells. Importantly, the histamine H_1 receptor has been shown to couple efficiently to $G\alpha_{11}$ subunits (21, 28).

As shown in Fig. 7*A*, the effects exerted by mepyramine on ATP maximal response (Fig. 5) were reversed by introduction of 0.5 μg of $G\alpha_{11}$ cDNA/10^6 cells (100.3 ± 3.0 and 95.8 ± 2.4% in the absence and in the presence of 10^{-5} M mepyramine). Interestingly, with 1 μg of $G\alpha_{11}$ cDNA transfected/10^6 cells, the basal levels were increased to an extent that ATP was unable to induce response over those basal values (Fig. 7*B*). This is consistent with increased constitutive activity as shown for other constitutively active receptors (29). Under these experimental conditions, the effect of mepyramine was to significantly decrease basal levels of intracellular calcium, allowing the system to respond again to increasing concentrations of ATP, with a pEC_{50} value similar to the one obtained in the parental cell line (5.53 ± 0.11 *versus* 5.55 ± 0.23, respectively) (Fig. 7*B*). Similar results were obtained using $G\alpha_q$ cDNA, although higher cDNA concentrations were necessary to reach the same effects (data not shown). Similarly, when equivalent amounts of either $G\alpha_s$ cDNA or $G\alpha_{i1}$ cDNA were used, no significant recovery of the maximal response to ATP was achieved (Fig. 7*C*).

These results clearly indicate that overexpression of $G_{q/11}$ proteins could reverse the negative effects of mepyramine on ATP intrinsic efficacy. Even more, the high basal levels of intracellular calcium induced by the overexpression of $G\alpha_{11}$ were markedly decreased by mepyramine, indicating that they were most likely induced by the constitutive activity of the

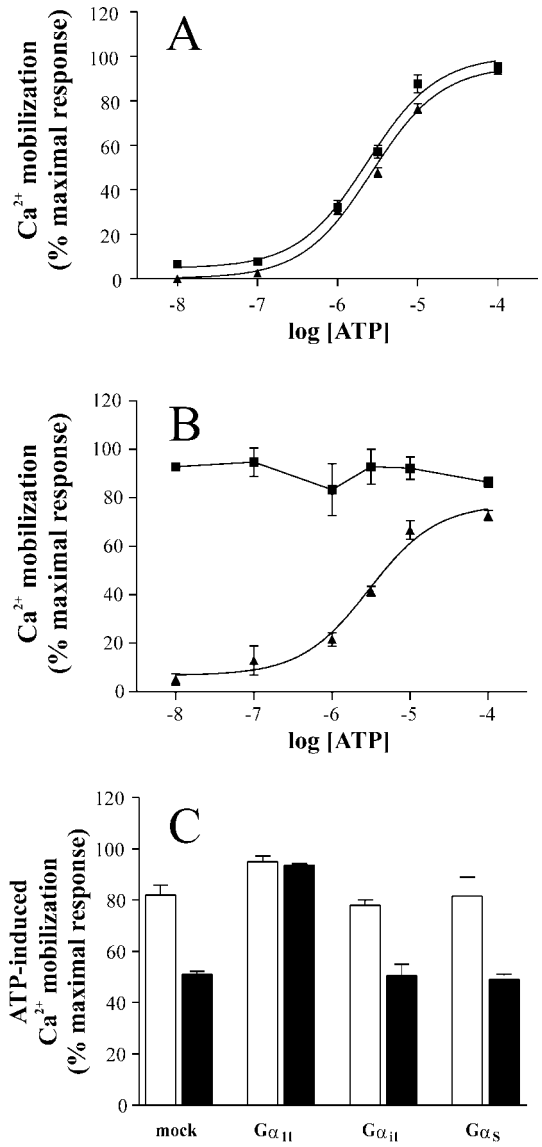

FIG. 7. **Effect of various Gα subunits on ATP-induced intracellular Ca^{2+} mobilization in CHO-gpH_1 pretreated with mepyramine.** *A*, CHO-gpH_1 cells transiently transfected with 0.5 μg of $G\alpha_{11}$ cDNA/10^6 cells were treated with increasing concentrations of ATP in the absence (■) or in the presence of 10^{-6} M (▲) mepyramine, and intracellular Ca^{2+} levels were measured as described under "Experimental Procedures." Data were calculated as the mean ± S.E. of assay triplicates. Similar results were obtained in at least four independent experiments. *B*, CHO-gpH_1 cells transiently transfected with 1 μg of $G\alpha_{11}$ cDNA/10^6 cells were treated with increasing concentrations of ATP in the absence (■) or in the presence of 10^{-6} M (▲) mepyramine, and intracellular Ca^{2+} levels were measured as described under "Experimental Procedures." Data were calculated as the mean ± S.E. of assay triplicates. Similar results were obtained in at least four independent experiments. *C*, CHO-gpH_1 cells transiently transfected with 0.5 μg of empty vector (*mock*), $G\alpha_{11}$, $G\alpha_{i1}$, or $G\alpha_S$ cDNA/10^6 cells were treated with ATP (10^{-6} M) in the absence (*open bars*) or in the presence (*filled bars*) of mepyramine (10^{-6} M), and intracellular Ca^{2+} levels were measured as described under "Experimental Procedures." Data were calculated as the mean ± S.E. of four independent experiments.

gp-H_1r, since the expression of $G\alpha_{11}$ in the parental CHO-K1 cells had no significant effect on basal levels (Fig. 6). These observations indicate that mepyramine stabilizes a G protein-coupled receptor state that is unable to evoke a response. Because this conformation is only predicted by the CTC, at least under certain conditions this model could more accurately predict the behavior of constitutively active GPCRs.

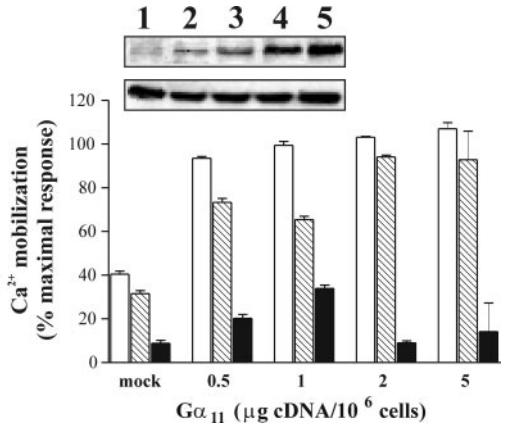

FIG. 8. **Effect of $G\alpha_{11}$ subunit overexpression on mepyramine efficacy.** *A*, CHO-gpH_1 cells transiently transfected with 5 μg of empty vector (*mock*) or 0.5, 1, 2, or 5 μg of $G\alpha_{11}$ cDNA/10^6 cells were treated with 10^{-6} M mepyramine (*hatched bars*) or not (*open bars*), and intracellular [Ca^{2+}] mobilization was measured as described under "Experimental Procedures." *Filled bars* represent the subtraction of basal levels and stimulated levels. Data were calculated as the means ± S.E. of four independent experiments. *Inset*, CHO-gpH_1 cells were transiently transfected with empty vector (5 μg) or 0.5, 1, 2, or 5 μg of $G\alpha_{11}$ cDNA/10^6 cells (*lanes 1–5*) and lysed as described under "Experimental Procedures." Samples were electrophoresed on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with polyclonal purified rabbit serum against $G\alpha_{11}$ (*top*) or with polyclonal purified goat anti-actin antibody (*bottom*).

Effects of Heterologous G Protein Overexpression and Mepyramine on Basal Ca^{2+} Levels—Transient transfection of CHO-gpH_1 cells with increasing amounts of $G\alpha_{11}$ cDNA resulted in increasing amounts of $G\alpha_{11}$ protein expression and in concomitantly increased basal levels of intracellular Ca^{2+} (Fig. 8), presumably by increasing the number of receptors coupled to $G\alpha_{11}$, consistent with the observed constitutive activity of the receptor. In all of the tested experimental conditions, mepyramine significantly decreased intracellular Ca^{2+} levels induced by the spontaneous activity of the gp-H_1r (Fig. 8). Because the Ca^{2+} basal levels are increased by the constitutive activity of the gp-H_1r in this surrogate system, a concomitant increase in the negative efficacy of mepyramine could be expected. However, mepyramine showed a bell-shaped efficacy curve (Fig. 8).

Such complex behavior could be interpreted with the aid of the CTC model (Fig. 9*A*). Considering R*G and LR*G as the species able to generate physiological responses and taking into account the equilibrium assumed by the model, its related constants, and conservation equations for each component (R, G, L), equations that describe the variation of basal levels (Equation 1) and maximal response elicited by the ligand (Equation 2) can be obtained as a function of G protein concentration,

$$f^*_{basal} = \frac{\beta K_{act} K_g [G]_0}{1 + K_{act} + K_g [G]_0 + \beta K_{act} K_g [G]_0} \quad \text{(Eq. 1)}$$

$$f^*_{L\to\infty} = \frac{\alpha\beta\gamma\delta K_{act} K_g [G]_\infty}{1 + \alpha K_{act} + \gamma K_g [G]_\infty + \alpha\beta\gamma\delta K_{act} K_g [G]_\infty} \quad \text{(Eq. 2)}$$

where $[G]_0$ and $[G]_\infty$ are the concentrations of free G protein in the absence or in the presence of saturating amounts of ligand, respectively. The concentration of the free G protein is a function of the ligand present in the system and must, therefore, be solved for each ligand concentration (see Ref. 8).

The subtraction of the former equations renders the ability of a ligand to modulate basal receptor activity, reflecting its efficacy, as described by Equation 3.

$$\text{Efficacy} = \frac{\beta K_{act} K_g [G]_0}{1 + K_{act} + K_g [G]_0 + \beta K_{act} K_g [G]_0} - \frac{\alpha\beta\gamma\delta K_{act} K_g [G]_\infty}{1 + \alpha K_{act} + \gamma K_g [G]_\infty + \alpha\beta\gamma\delta K_{act} K_g [G]_\infty} \quad \text{(Eq. 3)}$$

As shown in Fig. 9*B*, the efficacy of an inverse agonist with high affinity for inactive G protein-coupled states of the receptor, as mepyramine presumably is, varies according to the receptor activation constant (K_{act}). Interestingly, a bell-shaped efficacy curve could only be predicted for high K_{act} values (Fig. 9). Because K_{act} is the equilibrium constant for receptor transition from R to R*, bell-shaped efficacy could be expected for receptors with high constitutive activity. These predictions of the CTC give theoretical grounds to the observations reported herein. Thus, in this theoretical framework the conclusions that the gp-H_1r is constitutively active when expressed in CHO cells and that mepyramine acting as an inverse agonist at the gp-H_1r is able to stabilize a G protein-coupled receptor state that is unable to evoke response, are supported. Indeed, the CTC proved to be capable of explaining not only the ability of mepyramine to interfere with the ATP receptor signaling but also to predict the experimentally observed constitutive activity of the gp-H_1r.

DISCUSSION

In the present work we stably expressed the gp-H_1r in CHO-K1 cells to analyze the pharmacological behavior of mepyramine and its underlying mechanism of action as an inverse agonist. Three main conclusions could be drawn from these studies. First, the intrinsic negative efficacy of mepyramine is based on the stabilization of a G protein-coupled form of the H_1 receptor, which is unable to evoke a response. Second, as a consequence of such a mechanism mepyramine interferes with the signaling of at least another unrelated GPCR, the ATP receptor, by reducing the availability of $G_{q/11}$ proteins. As expected, this interference was abolished by overexpression of $G\alpha_{11}$ protein. This issue has particular relevance considering the potential therapeutic uses of inverse agonists. Finally, by analyzing the effects of mepyramine at high G protein concentrations, a high degree of constitutive activity could be predicted for the gp-H_1r with the aid of the CTC model. Similarly, a high degree of constitutive activity has been reported for the human H_1 receptor (10), indicating that constitutive activity could be a more general quality of the H_1 receptors. Based on these observations, we not only emphasize the importance of proper classification of ligands of constitutively active receptors but also the importance of elucidating the molecular mechanisms by which these ligands exert their actions in terms of their clinical applications and pharmacological effects.

In the last years many ligands formerly designated as H_1 antagonists have been reclassified as H_1 inverse agonists in view of the observed constitutive activity of the human H_1 receptor (10). The H_1 antihistamines are among the most widely prescribed and over the counter-sold drugs in the world. Because of their widespread use to treat non-life-threatening conditions, there is a trend to use them as long term therapy rather than restricting them to the treatment of short term manifestations of atopic diseases, allergic asthma, and allergic rhinitis. This makes the assessment of potential adverse or undesired effects of vital importance (30). Therefore, progress in the understanding of their mechanism of action is of crucial importance to improve their safety and specificity. Although several pharmacological differences between the human histamine H_1 receptor and the guinea pig histamine receptor have been recently reported (31), the guinea pig model has been classically used to test and design most of the currently used H_1

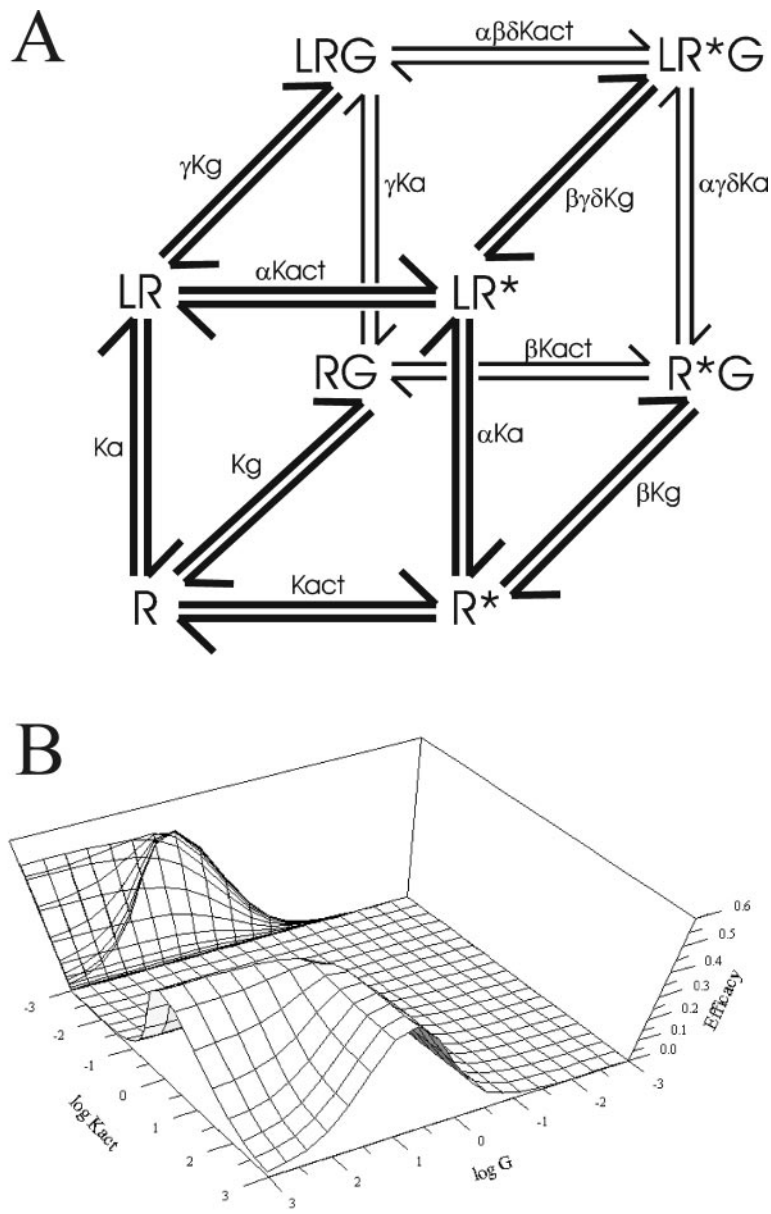

FIG. 9. **Effect of variation of G protein concentration and K_{act} on ligand efficacy.** *A*, Cubic Ternary Complex Model adapted from Weiss *et al.* (5). *R* and *R** represent the inactive and the active forms of the receptor, respectively; *L* represents the ligand; K_a and K_g represent the association constants to ligand and G protein, respectively; K_{act} is the allosteric constant; α, β, γ are the modifiers of affinity once the receptor is active or G protein or ligand-bound, respectively. δ represents the joint effect of any two of receptor activation, G protein-coupling, or the binding of ligand varies conditional on the level of the third. *B*, simulation of ligand efficacy (basal-stimulus) variation depending on G protein concentration and K_{act} value. On the log G-efficacy plane there is a projection of the curves for different values of K_{act}. Simulation was based on Equation 3 (see "Results") using values of $\alpha = \beta = \gamma = 10$, $\delta = 0.01$, and $K_a = K_g = 10^9$.

antihistamines (26) and still remains as one of the reference models to test H_1 antihistamines *in vivo* (32) and *ex vivo* (33). The study of the molecular mechanisms utilized by H_1 inverse agonists may help to predict and/or avoid some otherwise unexpected effects of antihistamines.

According to the currently accepted models, an inverse agonist may inhibit the constitutive activity of a GPCR by at least three different ways of action. First, it may act by binding preferentially to the inactive state of the receptor (3). Second, it could preferably bind to a G protein-uncoupled state of the receptor (4). A third possibility is that the observed intrinsic negative efficacy is achieved by biasing the receptor to an inactive, G protein-coupled conformation (2). Although the last mechanism is solely predicted by the CTC model because it allows the existence of an inactive form of the receptor coupled to G protein (7), it is worth noting that experimental evidence exists to support all of the three mechanisms mentioned above (4, 34–40).

According to the third mechanism, a ligand exerting its action by promoting a G protein-coupled inactive conformation of the receptor may not only diminish the activity of the specific receptor but may also interfere with the signaling of other GPCRs by sequestering G protein and, thus, reducing the availability of the G protein to those unrelated GPCRs (8). This may result in some unexpected effects, not observed with other inverse agonists that do not promote the same G protein-coupled state.

In this sense, although both triprolidine and mepyramine behaved as inverse agonists at the gp-H_1r, the differential binding sensitivity to GTPγS and Gα_{11} protein overexpression clearly indicates a difference in the mechanism used to exert their intrinsic negative efficacy. Particularly, mepyramine but not triprolidine induced a marked decrease on histamine-induced maximal response. Although the classic explanation to this kind of observations may suggest the existence of noncompetitive antagonism, several other models have been developed to explain similar ligand behaviors (41–44). In the context of the CTC model (Fig. 9*A*), RG could be considered an inactive form of the receptor that binds antagonists with high affinity, resulting in reduced signal transduction ability. Thus, the dissimilar effects of mepyramine and triprolidine on histamine-induced maximal response could be explained by differences in the mechanisms of action of both ligands at the gp-H_1r.

Both ligands displayed different effects on the ATP-induced

signaling at the endogenously expressed purinergic receptor as well. Mepyramine strongly interfered with the PTX-insensitive signaling pathway utilized by the ATP receptor to mobilize calcium, presumably by reducing the availability of $G_{q/11}$ proteins to the receptor. Conversely, triprolidine had no significant effect on ATP-induced signaling, again indicating a different mechanism of action. In accordance with our observations, mutational analysis of the gp-H_1r has shown that K200A mutation results in decreased potency of histamine and decreased affinity for mepyramine, whereas binding of triprolidine is unaffected (45), suggesting that mepyramine and triprolidine may not exactly recognize the same receptor state.

Similarly to what was described here for mepyramine at the gp-H_1r, observations made with antagonist of the μ-opioid receptor and with antagonists of the CB1 (and/or -2) cannabinoid receptor indicated that those ligands may promote G protein-coupled inactive states of the receptors as well. However, in the present work we theoretically inferred and experimentally demonstrated that the interference induced by mepyramine on the signaling of the ATP receptor expressed in CHO cells depends on the stoichiometric relationship between the components of the signaling pathway, since this effect was abolished by overexpression of $G\alpha_{11}$ protein. Therefore, variations in the signaling components stoichiometry, distinctive of each experimental system, might result in differential modulation of the interference phenomenon. Importantly, the stoichiometry of the different components of the activated signaling pathway is known to be relevant for the constitutive activity of GPCRs (23).

Our simulations done with the CTC model show that the abundance of the G protein influences the efficacy of a ligand such as mepyramine. For low K_{act} values, efficacy presents a monotone increasing curve, whereas for high K_{act} values efficacy presents a bell-shaped relation to G protein levels. The introduction of increasing amounts of $G\alpha_{11}$ allowed us to experimentally demonstrate that the efficacy of mepyramine as an inverse agonist of the gp-H_1r follows a bell-shaped curve, as predicted for a receptor with significant constitutive activity.

In conclusion, based on our observations we propose that the study of the efficacy of a ligand that promotes a G protein-coupled inactive receptor state, such as mepyramine, in systems with varying amounts of G protein is a suitable method to infer constitutive activity of the receptor. This indicates that the molecular composition of the system where mechanisms of action of a ligand are studied is an important factor to be taken into account if accurate evaluation and understanding of the pharmacological behavior is intended.

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