# Signal transduction mechanism of biased ligands at histamine $H_2$ receptors.

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## ABSTRACT

**7TMR exists as conformational collections in which different conformations would lead to differential downstream behaviors such as receptor phosphorylation, G-protein activation and receptor internalization among others. In this context, a ligand may cause differential activation of some, but not all, of the signaling events, which are associated to a particular receptor, and it would lead to biased agonism. The aim of the present work is to study if histamine $H_2$ receptor ($H_2R$) ligands -described as inverse agonists because of their negative efficacy at modulating adenylyl cyclase- could display some positive efficacy concerning receptor desensitization, internalization or even signaling through an adenylyl cyclase independent pathway. Our present findings indicate that treatment with $H_2R$ inverse agonists leads to receptor internalization in HEK293T transfected cells, by a mechanism mediated by arrestin and dynamin but being independent of GRK2-mediated phosphorylation. On the other hand we prove that two of the $H_2R$ inverse agonists tested, ranitidine and tiotidine, also induce receptor desensitization. Finally, we show that these ligands are able to display positive efficacy towards ERK1/2 pathway by a mechanism that involves Gβγ and PI3K mediated signaling in both HEK293T transfected cells and human gastric adenocarcinoma cells. These results point to the aspect of pluridimensional efficacy of $H_2R$ as a phenomenon that could be extended to naïve cells, and challenge previous classification of pharmacologically relevant histaminergic ligands.**

**Summary Statement:** In the present work we showed that inverse agonists at histamine $H_2$ receptors display positive efficacy regarding receptor desensitization/internalization and ERK1/2 phosphorylation. These findings demonstrated that histamine receptor ligands show functional selectivity respect to distinct receptor behaviors.

**Short title:** Pluridimensional efficacy at $H_2R$

**Key words:** 7TMR, $H_2R$ ligands, biased agonism, pluridimensional efficacy, GPCR internalization, MAPK.



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**Abbreviations used**: 7TMR, seven transmembrane receptor; $H_2R$, histamine receptor 2; IBMX, isobutylmethyl xanthine; MAPK, mitogen-activated protein kinase; BSA, bovine serum albumin; HEK293T, human embryonic kidney; AGS, human gastric cancer; cAMP, cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; GRKs, G protein-coupled receptor kinase; PKA, protein kinase A; AC, adenylyl cyclase; EGFR, Epidermal growth factor receptor; Gαt, Gα transducin; PH, pleckstrin homology domain of GRK2; PTX, pertussis toxin.

## INTRODUCTION

Seven transmembrane receptors (7TMR) represent the largest family of cell surface receptors. They mediate signaling across the plasma membrane of a wide variety of stimuli. The canonical linear sequence of 7TMR signaling begins when an extracellular stimulus binds and switches the receptor from an inactive state to an active state conformation which in turn leads to activation of its coupled heterotrimeric G-protein that dissociates from the receptor and both Gα subunit and the Gβγ dimer, modulates the activity of an effector enzyme or ion channel. In the classical models, signaling by 7TMR is terminated by receptor phosphorylation, principally mediated by GRKs; arrestin binding to the phosphorylated receptor which leads to uncoupling from G protein and consequent receptor desensitization; and finally internalization by clathrin-coated vesicles [1].

Since 7TMR are key regulators of almost every known function of eukaryotic cells, they have emerged as the most commonly targeted receptors for human therapeutic. At present they represent the target of approximately 27% of all FDA-approved drugs [2, 3]. It is often assumed that treatment with agonists leads to tachyphylaxis but 7TMR desensitization is not considered when antagonists or inverse agonists are used. This fact mainly results from the traditional classification of ligands which considers that 7TMR represent switches that alternate between "off" and "on" states. Therefore, agonists and inverse agonists promote an active or inactive state of receptors whereas antagonists block the cellular signaling coupled to the receptor. However increasing evidence supports that 7TMR exist as conformational collections where each conformation promotes different downstream effects such as receptor phosphorylation, G-protein activation or receptor internalization among others. In this context, ligand binding stabilizes the different conformations through a process known as conformational selection [4]. A consequence is biased agonism, where a ligand is able to cause differential activation of some signaling events associated to a particular receptor, resulting in differential activation of specific signal transduction pathways [5]. This functional selectivity supports that it is the ligand-receptor complex which governs the ultimate downstream signaling event and not the receptor itself [6]. Therefore it is likely that an antagonist or an inverse agonist stabilizes a particular conformation that fails to stimulate a G-protein signaling but results in desensitization, internalization or even G-protein independent signaling. Since Galandrin and Bouvier proposed in 2006 this pluridimensional characteristic of efficacy, the term efficacy is understood in the context of the different behaviors modulated by a 7TMR [7, 8]. Biased agonism has been mainly studied regarding adrenergic receptors, but little is known about other ligands therapeutically used [9, 10]. Cimetidine and ranitidine are clinically employed to control gastric acid secretion and rank among the most widely prescribed and over the counter-sold drugs in the world [11, 12]. Therefore, in the present study we sought to establish whether $H_2$ receptor ligands widely used



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and classified as inverse agonists for their negative efficacy in modulating adenylyl cyclase display positive efficacy regarding receptor desensitization, internalization or adenylyl cyclase independent signaling.
We show that ranitidine and tiotidine but not cimetidice induced $H_2R$ desensitization in HEK293T transfected cells mediated by arrestin, clathrin and dynamin leading to receptor down-regulation. Furthermore, the three inverse agonists induced ERK 1/2 activation not only in HEK293T cells but also in human gastric adenocarcinoma cells as histamine and the specific $H_2R$ agonist, amthamine.
The present study shows that the $H_2R$ ligands classified as inverse agonists display positive efficacy regarding receptor desensitization and internalization as well as mitogen-activated protein kinases (MAPKs) activation. Our findings may have relevant clinical implications given that some of these $H_2R$ ligands are clinically used in long-term treatments so they may explain therapeutic differences and side effects of histaminergic ligands.

**Experimental Procedures**

*Materials*. Cell culture medium, antibiotics, isobutylmethyl xanthine (IBMX), cAMP, bovine serum albumin (BSA), cycloheximide, amthamine, cimetidine, ranitidine, Tyrphostin *AG1478* and pertussis toxin were obtained from Sigma Chemical Company (St. Louis, MO). Tiotidine and Ly294002 were from Tocris Cookson Inc. (Ballwin, MO). [$^3$H]cAMP, and [$^3$H]tiotidine were purchased from Perkin Elmer Life Sciences (Boston, MA). Fetal calf serum was from Natocor (Argentina). Other chemicals used were of analytical grade and obtained from standard sources.
*Plasmid Constructions.* pcDNA3-β1arrestin (arrestin 2), pcDNA3-β2arrestin (arrestin 3), pcDNA3-HA-dynaminK44A, pcDNA3- β1-arrestin(319–418) and pcDNA3-GRK2-K220R were generous gifts from Dr. J. Benovic (Thomas Jefferson University, Microbiology and Immunology Department, Kimmel Cancer Center, Philadelphia, PA). pEGFP-C2- Eps15 EH29 construct was a generous gift from Dr. Benmerah (Universite´ Paris 5, Institut Cochin, Departement de Maladies Infectieuses, Paris, France). GRK2, -3,-5, and -6 cDNAs were subcloned into the pCEFL vector (pCEFLGRK2,-3, -5, and -6) as previously described [13]. pCEFLHA-$H_2R$ was previously generated in our laboratory [13]. pCEFL-Gα transducin was kindly provided by Dr S. Gutkind (Oral and Pharyngeal Cancer Branch, National Institutes of Health, Bethesda). The plasmid containing the PH domain of GRK2 was constructed by PCR amplification of bovine GRK2. To obtain the PH construct, the sequence coding for residues 553–651 was amplified and inserted into EcoRI/Xba site of pCEFL-HA.
*Cell Culture.* HEK293T (Human embryonic kidney) and AGS (human gastric cancer) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and Kaighn's Modification of Ham's F-12 medium (F12K) respectively, supplemented with 10% fetal calf serum and 5 µg/ml gentamicin at 37°C in humidified atmosphere containing 5% $CO_2$.
*Transient Transfection.* For transient transfection of HEK293T, cells were grown to 80-90% confluency. cDNA constructs were transfected into cells using LipofectAMINE 2000. The transfection protocol was optimized as recommended by the supplier (Invitrogen). Assays were performed 48 h after transfection and the expression of the constructs was confirmed by immunobloting using specific antibodies.

*cAMP assays.* For concentration-response assays, cells were incubated 3 min in basal culture medium supplemented with 1 mM IBMX at 37°C, followed by 9 min exposure to different concentrations of ligands.



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For desensitization assays, cells were pretreated with 10 µM $H_2R$ ligands in the absence of IBMX for different periods of time as shown in the figures. Cells were then washed and resuspended in fresh medium containing 1 mM IBMX, incubated for 3 min, and exposed to 10 µM amthamine or 100 µM histamine for 9 min to determine whether the system was able to generate a cAMP response.

In all experiments, the reaction was stopped by ethanol addition followed by centrifugation at 2000 x g for 5 min. The ethanol phase was then dried and the residue resuspended in 50 mM Tris-HCl pH 7.4, 0.1% BSA. cAMP content was determined by competition of $[^3H]$cAMP for PKA, as previously described [14].

*Radioligand Binding Assay.* Saturation binding experiments were carried out by incubating the cells for 40 min with increasing concentrations of $[^3H]$tiotidine, ranging from 0.4 up to 240 nM in the absence or presence of 1 µM unlabeled tiotidine. The incubation was stopped by dilution with 3 ml of ice-cold 50 mM Tris-HCl pH 7.4 and the bound fraction was collected in 200 µl of ethanol. Experiments on intact cells were carried out at 4°C to avoid ligand internalization. The kinetic studies performed with 2 nM $[^3H]$tiotidine at 4°C showed that the equilibrium was reached at 30 min and persisted for 4 h (data not shown).

*Receptor internalization and recovery.* HEK293T cells were incubated at different times with 10 µM inverse agonists and the number of receptor sites was analyzed by radioligand binding assay. The recovery of binding sites was evaluated by saturation binding assays at 60 min after washing the cells previously exposed to 10 µM inverse agonists for 90 min. In assays performed with 50 µM cycloheximide, the inhibitor was added 30 min before ligand treatment.

*Western Blot Assays*. For Western blot assays, cells were lysed in 50 mM Tris–HCl pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue and sonicated to shear DNA. Total cell lysates were resolved by SDS-PAGE, blotted and incubated with the primary antibodies anti- dynamin, -GRK2, -GRK3, -GRK5, -GRK6, -ERK1/2, -pERK, -actin (Santa Cruz Biotechnology, CA), -arrestin (BD Biosciences PharMingen, San Diego, CA) or -GFP (Invitrogen), followed by horseradish peroxidase conjugated anti-rabbit or anti-mouse (Santa Cruz Biotechnology, CA) and developed by enhanced chemiluminesence (ECL) following the manufacturer's instructions (Amersham Life Science, England). Films were scanned and quantified using Scion Image® software from National Institutes of Health (NIH).

*Statistical Analysis.* Statistical analysis was performed from at least three independent experiments. Binding data, sigmoidal dose-response, desensitization fittings and comparison of best fit values according to extra-sum of squares F test were performed with GraphPad Prism 5.00 for Windows, GraphPad Software (San Diego, CA). One-way ANOVA followed by the Dunnett's post-test was performed using GraphPadInStat version 3.01, GraphPad Software (San Diego CA). Specific binding was calculated by subtraction of nonspecific binding from total binding. Statistical of densitometric western blot analysis were carried out by one-way ANOVA or T-test followed by the Dunnett's or Tukey's Multiple Comparison post-test performed with GraphPad Prism 5.00 for Windows, GraphPad Software.

## Results

***$H_2R$ inverse agonist induced $H_2R$ internalization***. The effect of cimetidine, ranitidine and tiotidine on cAMP accumulation was evaluated in concentration-response assays performed in transiently $H_2R$ transfected HEK293T cells. As previously reported [15, 16] all ligands inhibited



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amthanine-induced cAMP accumulation and reduced cAMP levels in a concentration-dependent fashion, an effect not blocked by pertussis toxin pretreatment (Fig. 1A, 1B and 1C). These results confirm the negative efficacy of the ligands regarding cAMP regulation.

We next evaluated $H_2R$ internalization in HEK293T transfected cells as cell surface receptors loss following cimetidine, ranitidine or tiotidine exposure. In saturation binding assays we found that despite the absence of positive signaling through Gs-AC-cAMP pathway in the presence of the ligands, treatment with ranitidine and tiotidine led to significant $H_2R$ internalization (Fig. 2A). The extent and rate of $H_2R$ internalization was similar to amthamine-promoted endocytosis (Fig. 2B). On the other hand, cimetidine-treated cells, displayed a considerable slower kinetic showing a modest reduction in $H_2R$ membrane sites which was significant following 2 h ligand exposure (Fig. 2A and B).

We have previously reported that amthamine treatment evokes $H_2R$ internalization into clathrin-coated vesicles by GRK2 kinase activation as well as arrestin-3, dynamin and Eps15 [17]. In order to study the machinery involved in inverse agonists-induced $H_2R$ internalization, cells were cotransfected with the $H_2R$ and dominant negative mutants for arrestin (DNArr), dynamin (DNDyn), Eps15 (EH29) or GRK2 kinase inactive (DNGRK2). Saturation binding assays were performed after 90 min exposure to ranitidine or tiotidine, given that at this time significant $H_2R$ internalization was achieved. The proper expression of these constructs in HEK293T cotransfected cells was confirmed by western blot (Fig. 3A).

DNDyn, DNArr or EH29 abolished $H_2R$ internalization suggesting that the process is dependent on dynamin, arrestin as well as the correct assembly of clathrin-coated vesicles through Eps15. Surprisingly, DNGRK2 expression failed to induce ranitidine or tiotidine-evoked $H_2R$ internalization revealing that GRK2-mediated $H_2R$ phosphorylation is not necessary for inverse agonist promoted $H_2R$ internalization (Fig. 3B).

Following endocytosis, 7TMR may be either recycled to the plasma membrane or sorted for lysosomal degradation. To determine the fate of $H_2R$ sites after internalization, we evaluated the recovery of membrane $H_2R$ sites in the presence or absence of the well-characterized protein synthesis inhibitor, cycloheximide. Cells were exposed to ranitidine and tiotidine for 90 min to assess $H_2R$ sites after cell washing and incubation for 60 min in fresh medium. The removal of the stimulus led to a rapid recovery of the number of $H_2R$ binding sites that was abolished by cycloheximide (Fig. 4). Conversely, cycloheximide treatment did not completely dampen the recovery of internalized $H_2R$ after amthamine treatment (Fig. 4 inset). These findings indicate that the presence of $H_2R$ sites in the plasma membrane following the removal of the inverse agonists represent *de novo* $H_2R$ protein synthesis and not $H_2R$ recycling [17], suggesting that ranitidine and tiotidine induce $H_2R$ down-regulation.

***$H_2R$ inverse agonists induce receptor desensitization***. In order to evaluate whether these ligands displayed $H_2R$ desensitization, $H_2R$ transfected HEK293T cells were exposed to 10 µM amthamine, cimetidine, ranitidine or tiotidine at different time periods. After carefully washing, cells were re-challenged with the agonist and cAMP response was evaluated. The cAMP response evoked by amthamine in cells previously exposed to the inverse agonists was 41±7% for ranitidine and 36±9% for tiotidine whereas cimetidine did not modify $H_2R$ response to the agonist. The extent of receptor desensitization appeared to be ligand dependent since amthamine induced a 90% $H_2R$ desensitization (Fig. 5A). It is worth noting that similar results were obtained in response to the endogenous ligand, histamine (Fig. 5B).

As GRK2 and GRK3 were previously described to mediate $H_2R$ homologous desensitization [13], we next evaluated whether GRKs were involved in inverse agonists-induced $H_2R$



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desensitization. For that purpose, HEK293T were co-transfected with the $H_2R$ and each of the most ubiquitous members of GRK family of proteins (GRK2, 3, 5 and 6). The overexpression of GRKs was evaluated by western blot (Fig. 6A) and had no significant effect on inverse agonist-mediated $H_2R$ desensitization (Fig. 6B). Our findings reveal that the GRK family is not-involved in the mechanism by which $H_2R$ lose its ability to respond to amthamine following inverse agonist exposure.

In an attempt to determine whether $H_2R$ internalization mediated inverse agonist-induced $H_2R$ desensitization, desensitization assays with HEK293T cells cotransfected with a dominant-negative mutants shown to block $H_2R$ endocytosis (Fig. 2B) as well as with wild type arrestin 2 (Arr2) or arrestin 3 (Arr3) were carried out. Neither dominant negative mutants nor arrestins overexpression had a drastic effect on $H_2R$ desensitization (Fig. 6C) suggesting that internalization is not responsible for inverse agonists-induced $H_2R$ desensitization, and ruling out uncoupling from G-protein by arrestin.

***Modulation of ERK1/2 by $H_2R$ inverse agonists.*** Diverse 7TMR activate MAPK pathways through G-protein dependent or independent mechanisms involving Gβγ activity, 7TMR internalization, arrestin and/or dynamin recruitment or even EGFR transactivation [18]. Therefore, we explored whether $H_2R$ ligands with negative efficacy at modulating the adenylyl cyclase pathway displayed positive efficacy concerning MAPK modulation. As shown in Figure 7A and B, p-ERK levels were increased by all ligands, being the maximum activation observed at 5 min following ligand treatment. Given that amthamine and histamine increase cAMP levels and cimetidine, ranitidine and tiotidine diminish them (Fig. 7C) these findings show that regardless the efficacy towards the adenylyl cyclase pathway, all ligands displayed positive efficacy respect to ERK1/2 modulation, thus behaving as agonists concerning this signaling pathway.

Arrestin and/or dynamin recruitment by diverse 7TMR leads to MAPK activation [19] so we evaluated their role on ERK1/2 modulation. HEK293T cells were co-transfected with $H_2R$ and DNDyn or DNArr and stimulated with $H_2R$ ligands. Results showed that arrestin did not mediate ERK1/2 modulation by any of the assayed ligands. On the other hand, only amthamine- induced ERK activation mediated by dynamin (Fig. 8A and B). These findings show that although the ligands induced ERK activation, the mechanism involved is different from that of amthamine. It is worth noting that AG1478, the pharmacological inhibitor of EGFR, had no effect on ERK1/2 activation by these ligands (data not shown) ruling out the possibility that EGFR transactivation might be responsible for ERK1/2 activation as previously described for other 7TMR [20, 21]. Gβγ signaling was previously found to stimulate the PI3K signaling pathway leading to downstream activation of ERK1/2 [22]. In order to evaluate the potential implication of Gβγ in MAPK activation by $H_2R$ ligands, ERK1/2 modulation was evaluated in HEK293T cells co-transfected with $H_2R$ and Gα transducin (Gαt), a widely used scavenger of Gβγ [23, 24]. In the presence of Gαt, cimetidine, ranitidine and tiotidine-induced ERK1/2 phosphorylation was significantly inhibited (Fig. 9A). These results were confirmed by the pleckstrin homology (PH) domain of GRK2, another well described scavenger of Gβγ [25, 26] (Fig. 9B). Moreover, Ly294002 inhibitor of PI3K, also blocked cimetidine, ranitidine and tiotidine induced ERK1/2 phosphorylation (Fig. 9C). In addition, the increase in p-ERK levels following amthamine treatment was not dampened by Gαt, confirming the central role of dynamin on amthamine induced ERK1/2 activation (Fig. 9D). Overall, these results show that ligands previously described as $H_2R$ inverse agonists regarding the Gs-AC-cAMP pathway display positive efficacy towards ERK1/2 through a pathway involving the Gβγ dimer and PI3K activation.



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***cAMP accumulation and MAPK activation by $H_2R$ ligands in human gastric adenocarcinoma AGS cells***. AGS cells not only endogenously express $H_2R$ but also represent a relevant model concerning $H_2R$ histaminergic ligands and their clinical use. Regarding cAMP accumulation histamine and amthamine displayed positive efficacy while cimetidine, ranitidine and tiotidine did not significantly reduced cAMP basal levels (Fig. 10A). Although constitutive activity of H2R was consistently reported, its modulation is difficult to achieve on naïve systems [15]. Interestingly, positive efficacy of cimetidine, ranitidine and tiotidine, towards ERK1/2 modulation was also found in AGS cells where the highest response was observed at 10 min. (Fig. 10). Moreover, cimetidine-induced phosphorylation of ERK1/2 was similar to that stimulated by amthamine (Fig. 10B and C). These findings clearly show that histamine $H_2R$ ligands classically classified as antagonist or inverse agonists based on cAMP modulation may induce ERK1/2 phosphorylation through an adenylyl cyclase independent pathway not only in overexpression models but also in human gastric adenocarcinoma cells that endogenously express $H_2R$.

**Discussion**

In the context of pluridimensional efficacy where receptors exhibit diverse behaviors and ligands display different efficacies depending on the readout chosen as receptor response, the major finding of our work was the identification of different efficacies displayed by $H_2R$ ligands towards receptor desensitization/internalization, Gαs-AC, and Gβγ-ERK1/2 pathways.

Histamine $H_2$ receptor ligands were traditionally classified according to their ability to modulate cAMP levels. Thus, cimetidine, ranitidine and tiotidine were originally considered neutral antagonists, but when receptor constitutive activity became evident they were reclassified as inverse agonists, with negative efficacy [15, 16]. However, the diversity of effectors considered in this work, challenged this simple scheme, making it difficult to categorize these ligands according to unique efficacy terms.

In the present work we show that not only amthamine, but also ranitidine and tiotidine induced significant receptor desensitization and internalization (Fig. 2 and 3). It is worth noting that attenuation mechanisms of 7TMR signaling have been originally described as adaptive processes evoked by agonists to prevent receptor overstimulation, but despite the lack of positive cAMP response evoked by these ligands, they induced substantial internalization and desensitization of $H_2R$.

Receptor desensitization triggered by ranitidine and tiotidine was independent of arrestin-2, -3 and the GRKs, whereas receptor internalization was mediated by arrestin, dynamin, and clathrin, but not by GRK2 phosphorylation. Moreover, inverse agonist-induced $H_2R$ internalization appeared to mediate receptor down-regulation rather than recycling, as previously observed for the agonist amthamine [17]. These findings support that inverse agonist induced receptor desensitization/internalization protein-partners profile and cellular fate once the receptor is internalized, is strikingly different from that observed when the processes are triggered by agonists. Although ligand efficacies are shared, the mechanisms involved differ, supporting that the receptor partners engaged in a certain pathway are strongly dependent on the ligand.

Futhermore an additional level of selectivity was revealed when the signaling cascades leading to ERK1/2 activation were examined. Again, although all ligands evaluated displayed positive efficacy concerning ERK1/2 phosphorylation, the underlying mechanisms differed. Amthamine-stimulated ERK1/2 phosphorylation was mediated by dynamin (Fig. 8), as previously described for dimaprit, another $H_2R$ agonist [27], while cimetidine, ranitidine and tiotidine led to an



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increase in pERK levels by a mechanism independent of dynamin, arrestin, $H_2R$ internalization or even EGFR transactivation, but mediated by Gβγ.
Intriguingly, tiotidine, ranitidine, and cimetidine seemed to inactivate Gαs, diminishing cAMP levels, but stimulating Gβγ dependent pERK pathway. Considering the paradigm of heterotrimeric G protein activation, an apparent discrepancy in the activation of the Gβγ dimer seems to exist while the Gαs subunit remains inactive. However, the way in which G proteins are activated and propagate the signal has been challenged. Originally, Gα was thought to be the only G protein subunit able to govern the direct interaction with effector molecules but Gβγ has signal transduction properties of its own [28]. Therefore, subunit dissociation produces two signal transduction molecules when heterotrimeric G proteins are activated. However, the mechanism by which divergent signaling pathways within the cell are controlled by an unique receptor remains controversial. Thus, subunit dissociation is a critical event of the signal transduction mechanisms and it is essential that the subunit dissociation hypothesis be unequivocally established. Although this hypothesis is generally accepted [29, 30], there is evidence supporting that G protein activation occurs without dissociation and that subunit dissociation occurs without activation [31]. Our findings support that G protein heterotrimer do not need to be fully activated and that Gβγ pathway activation do not necessarily implies Gαs activity.
Overall, these results indicate that different ligands can lead to recruitment of distinct subsets of signaling effectors to activate a single pathway. This is reminiscent of other cases where distinct effectors were selectively engaged by different ligand/receptor pairs to stimulate a common downstream signaling integrator [32].
Nowadays 7TMRs are thought as allosteric machines where a punctual modification in the free energy of the receptor is transmitted to the rest of the protein affecting the receptor response to the cytosolic signaling proteins. Molecular dynamic modeling of proteins predicts that numerous protein conformations can exist and that receptors exist in “ensembles” of multiple conformations [33, 34]. Under these circumstances, ligand binding alters the receptor ensemble formation, causing the stabilization of different receptor conformations with different properties and behaviors. The machinery involved in inverse agonist stimulated-internalization was independent of GRK2-induced phosphorylation.Therefore ranitidine and tiotidine may induce or stabilize a conformation that exposes a receptor domain which recruits arrestin and the machinery involved in $H_2R$ internalization. In consequence, GRK2 phosphorylation may not be necessary to induce inverse agonist-mediated $H_2R$ internalization. This idea suggests that the conformation induced by the $H_2R$ inverse agonist is similar to that induced by GRK2 mediated-phosphorylation. Similar results concerning phophorylation independent antagonist-stimulated endocytosis of GPCRs were previously reported for cholecystokinin receptor [35]. Moreover, Haribabu and coworkers [36] reported that a carboxyl-terminal truncation mutant of human chemokine receptors CXCR4 could also internalize upon agonist challenge, although this mutant receptor showed no phosphorylation. In addition, Zhang X and coworkers [37] demonstrated that agonist-stimulated δ opioid receptor (DOR) internalization includes both receptor phosphorylation-dependent and phosphorylation-independent mechanisms and both were mediated by clathrin and β-arrestins.
The $H_2R$ ligands tested in the present study displayed distinct efficacy profiles towards adenylyl cyclase and MAPKs, demonstrating the existence of functional selectivity of $H_2R$ ligands. Whereas amthamine behaves as full agonist concerning both adenylyl cyclase and MAPK signaling, cimetidine, ranitidine and tiotidine displayed negative and positive efficacy for these pathways. Our results show that while amthamine-induced receptor conformation triggers the classical pathways involving Gαs activation and GRK2-βarrestin-dynamin-clathrin recruitment



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for receptor desensitization/internalization leading to ERK1/2 activation and receptor recycling to cell membrane, ranitidine and tiotidine may stabilize a conformation that exposes a receptor domain which activates Gβγ-ERK1/2 pathway, recruits arrestin and the the machinery involved in $H_2R$ desensitization and internalization without GRK2 phosphorylation and changing the receptor fate from recycling to degradation. Conversely, cimetidine induced a conformation engaged in Gβγ-ERK1/2 activation but not in receptor desensitization/ internalization. Overall, our results show that efficacy is not linear (i.e., ligands do not facilitate all behaviors of receptors) but rather is collateral, whereby only a subset of receptor behaviors can be activated or even inactivated. There are many possible receptor-based efficacies, and ligands may have varying subsets of efficacies. Similarly, it has been previously shown that β2-adrenergic, V2-vasopressin, serotonin 5-HT2C, and δ-opioid receptor ligands can act as inverse agonists on the adenylyl cyclase pathway but as agonists for MAPK signaling [38-41].

The observations made in AGS cells, that endogenously express $H_2R$, suggest that the pluridimensionality of signaling efficacies may be extended to naïve cells making our findings pharmacologically relevant. Recent data provide evidence of the existence of ligand-specific $H_2R$ conformations that explain the differences among these ligands' affinities, potencies and efficacies observed in neutrophils and eosinophils [42]. Nevertheless, additional studies are needed to assess the extent to which the effector-dependent signaling efficacies are detected in normal and pathophysiological conditions.

Since cimetidine and ranitidine profiles as biased ligands were described in *in-vitro* studies; the therapeutic impact of this phenomenon *in-vivo* cannot be inferred. These ligands are currently marketed and used to treat duodenal ulcers and prevent their recurrence. They are also used to treat gastric ulcers and Zollinger-Ellison disease [43]. However, these ligands are used because they inhibit $H_2R$ coupled cAMP response, but while both induce ERK activation like histamine, only ranitidine promotes the depletion of receptors from the membrane surface. Therefore the outcome of a long term treatment with these ligands would not be alike.

Although the signaling bias shown in this work runs counter to classic concepts of ligand efficacy, those aspects of drug receptor mechanisms has become firmly established and has been demonstrated for many receptors [44]. In the same way, present findings stress the relevance of studying the different efficacies of a ligand that appears to induce an inactive state of the receptor, further if accurate evaluation and understanding of its pharmacological behavior is intended.

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**Author Contribution**

Natalia Alonso carried out the experiments, analyzed data and assisted the writing of the paper; Federico Monczor and Emiliana Echeverría contributed to perform experiments and analyzed data; Carlos Davio and Carina Shayo contributed to analyze data and to design the experiments and Natalia Fernandez planed the research, conducted some experiments and wrote the paper. All authors participated in editing the paper in its final form.

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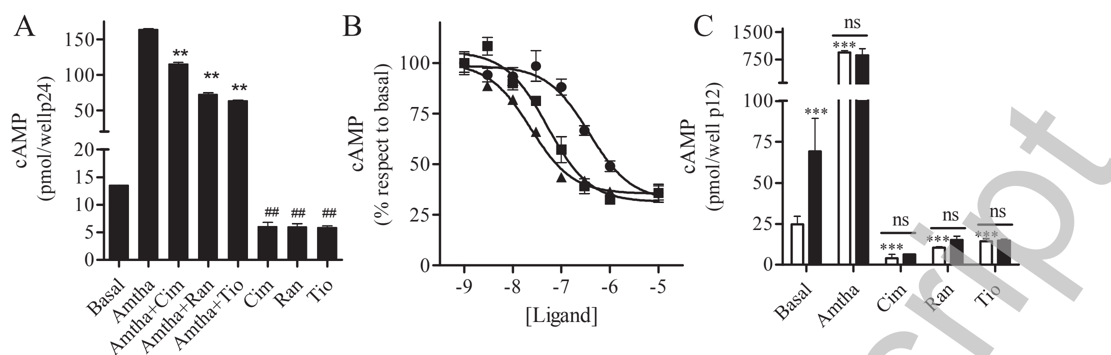


***Figure 1. Negative efficacy of cimetidine, ranitidine and tiotidine. (A)*** $H_2R$ transfected HEK293T cells were exposed to 10 µM amthamine alone or in combination with inverse agonists for 9 min, in the presence of IBMX. ** p<0.01 with respect to amthamine; ## p <0.01 with respect to basal. ***(B)*** Cells were exposed for 9 min to increasing concentrations of cimetidine (●), ranitidine (■) or tiotidine (▲) at 37°C in the presence of 1 mM IBMX. ***(C)*** Cells were pretreated for 6 h with (***black bars***) or without (***white open bars***) 100 ng/ml pertussis toxin and exposed to 10 µM specific ligand for 9 min, in the presence of IBMX. *** p<0.001 with respect to basal; *ns,* with respect to the same treatment without pertussis toxin. ***(A-C)*** Cyclic AMP levels were determined as detailed under Experimental Procedures. Data were calculated as the means ± SD of assay duplicates. Similar results were obtained in at least three independent experiments.



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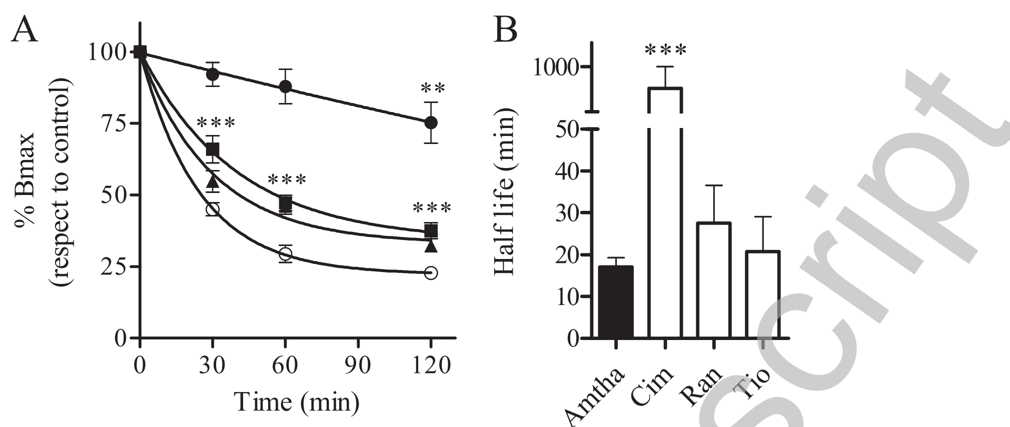


***Figure 2. Inverse agonists induced $H_2R$ internalization. (A)*** $H_2R$-transfected HEK293T cells were exposed to 10 μM cimetidine (●), ranitidine (■), tiotidine (▲) or amthamine (○) for different time periods and $H_2R$ binding sites were determined by saturation assays as described under Experimental Procedures. Data were calculated as the means ± SD of assay duplicates. Similar results were obtained in at least three independent experiments. ** $p<0.01$; *** $p<0.001$ with respect to 100%. ***(B)*** Half-life constants of the internalization kinetics were derived by curve fitting of experiments in ***A***, the data represent the means ± SE (n=3).



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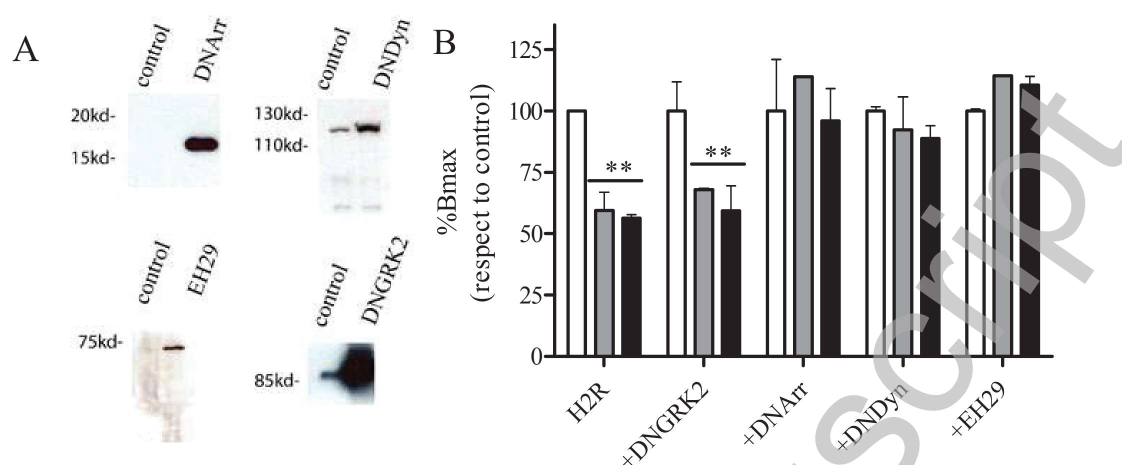


***Figure 3. Mechanisms involved in $H_2R$ inverse agonists- induced internalization.*** HEK293T cells were co-transfected with $H_2R$ and Arrestin 319-418 (DNArr), Dynamin K44A (DNDyn), EH29 or GRK2K220R (DNGRK2) dominant negative constructs. ***(A)***, analysis of constructs expression in HEK293T-transfected cells. Cells were lysed, and equal amounts of proteins were subjected to SDS-PAGE and analyzed by Western Blot using: ***upper panel left***, anti-arrestin 2/3 antibody; ***upper panel right***, anti-dynamin antibody; ***down panel left***, anti-GFP antibody; ***down panel right***, anti-GRK2 antibody. Data are representative of at least three independent experiments. ***(B)***, Transfected cells were treated for 90 min with 10 µM ranitidine (***grey bars***) or 10 µM tiotidine (***black bars***) and $H_2R$ membrane sites are shown. Data represent % Bmax fitted by nonlinear regression of $[^3H]$Tiotidine saturation assay, expressed as the means ± SE (n=3). 100% corresponds to transfected cells without treatment (***white bars***). ** $p<0.01$ with respect to untreated cells.



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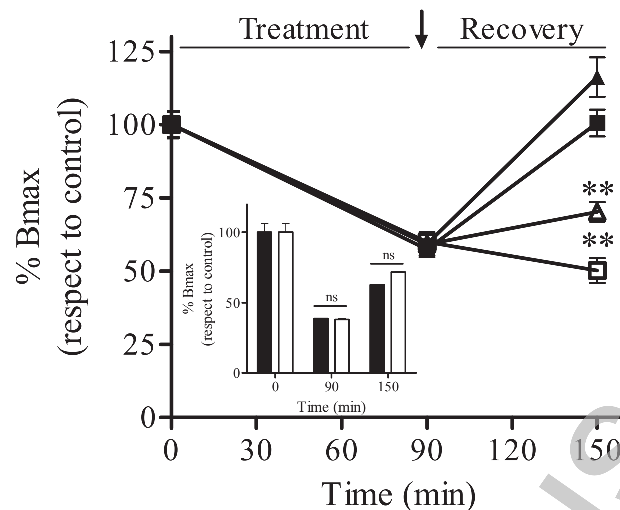


***Figure 4. $H_2R$ internalization and recovery in the presence of cycloheximide.*** [$^3$H]Tiotidine saturation assays were performed in $H_2R$-transfected HEK293T cells treated for 90 min with 10 µM ranitidine (■) or 10 µM tiotidine (▲), washed (↓), and further incubated for 60 min in fresh medium. Open symbols represent the same treatment in the presence of 50 µM cycloheximide. Data represent the percentage Bmax value fitted by nonlinear regression of [$^3$H]Tiotidine saturation assay, calculated as the means ± SE (n = 3). ** $p<0.01$ vs. similar assay in the absence of cycloheximide; 100% correspond to untreated cells. ***Inset.*** $H_2R$-transfected HEK293T cells were treated for 90 min with 10 µM amthamine (90), washed and further incubated for 60 min in fresh medium (150). Assays were carried out in the absence (***black bars***) or presence of cycloheximide (***white bars***) and results were expressed as mentioned above, no significant differences are observed between cycloheximide treated or untreated cells (*ns*).



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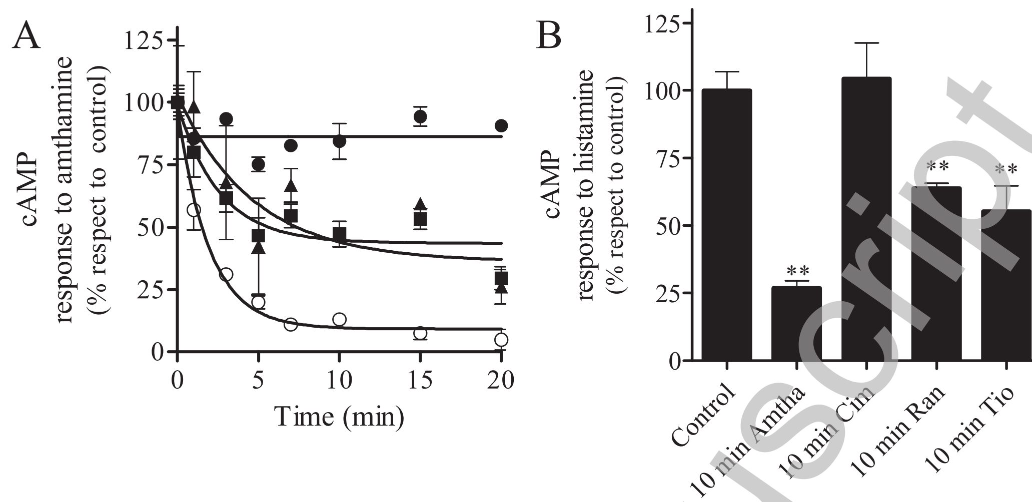


***Figure 5. Inverse agonists promoted desensitization.*** ***(A)***, $H_2R$-transfected HEK293T cells were exposed to 10 µM cimetidine (●), ranitidine (■), tiotidine (▲) or amthamine (○) for different time periods and cAMP response to amthamine was determined. ***(B)***, cells pre-treated for 10 min with 10 µM amthamine (Amtha), cimetidine (Cim), ranitidine (Ran) or tiotidine (Tio) were washed and exposed for 9 min to 100 µM histamine in the presence of IBMX. Cyclic AMP levels were determined as detailed under Experimental Procedures and expressed as stimulus to the agonist minus basal cAMP levels respect to the response of control cells without treatment. Data were calculated as the means ± SD of assay triplicates. Similar results were obtained in at least four independent experiments. ** $p<0.01$ with respect to untreated cells.



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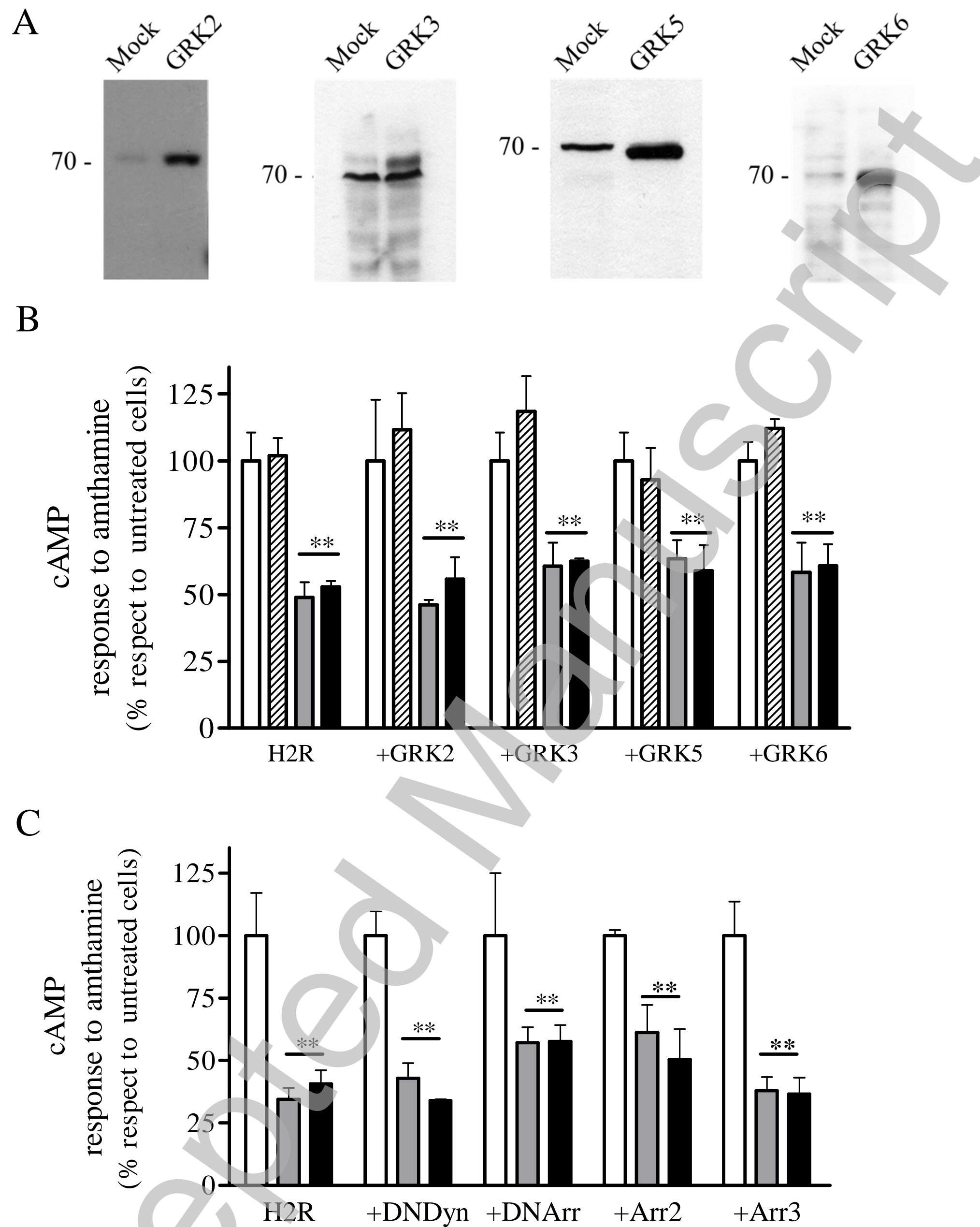


***Figure 6. Characterization of $H_2R$ desensitization promoted by inverse agonists. (A),*** analysis of GRKs expression in HEK293T-transfected cells. Cells were lysed, and equal amounts of proteins were subjected to SDS-PAGE and analyzed by Western Blot using specific antibodies against GRK2, 3, 5 or 6 (from left to right). ***(B)*** HEK293T cells transfected with $H_2R$ or co-transfected with different GRKs, were pre-treated for 10 min with 10 µM cimetidine (***slashed bars***), ranitidine (***grey bars***) or tiotidine (***black bars***)***, (C)*** cells transfected with $H_2R$ or co-transfected with dominant negative dynamine (DNDyn) and arrestin (DNArr) mutants or wild type arrestins were pre-treated for 10 min with 10 µM ranitidine (***grey bars***) or tiotidine (***black***



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***bars***). Cells were washed and exposed for 9 min to 10 µM amthamine in the presence of IBMX. Cyclic AMP levels were determined as detailed under Experimental Procedures and expressed as stimulus to the agonist minus basal cAMP levels respect to the response to amthamine of cells without treatment (***white bars***). Data were calculated as the means ± SD of assay triplicates. Similar results were obtained in three independent experiments. ** $p<0.01$ with respect to untreated cells.

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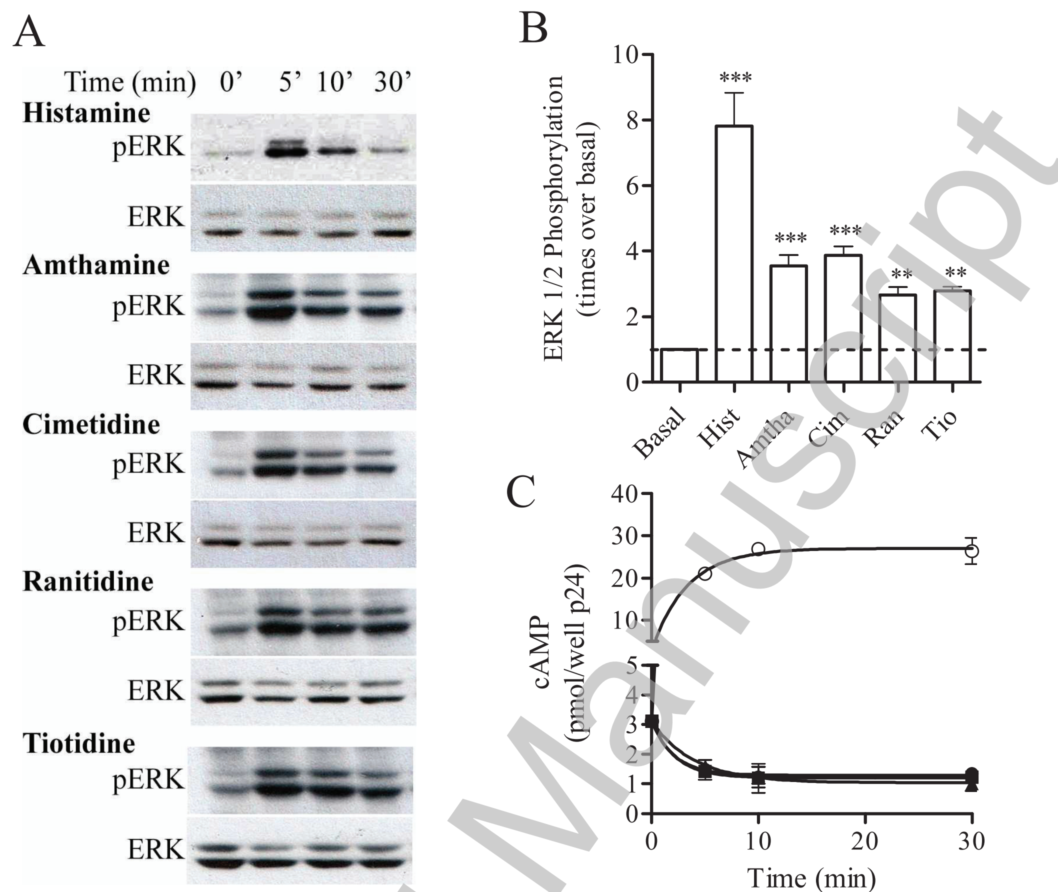


***Figure 7. ERK phosphorylation in response to $H_2R$ ligands. (A),*** $H_2R$ transfected HEK293T cells were treated during different time periods, lysed, and equal amounts of proteins were subjected to SDS-PAGE and analyzed by Western Blot. ***(B)***, Densitometric analysis of ERK phosphorylation at 5 min of treatment, normalized to the corresponding ERK total levels, obtained with the Scion Image Program. Data are expressed as times over basal p-ERK levels. Data are expressed as means ± SE (n=3). ** p<0.01; *** p<0.001 respect to basal levels. ***(C)***, cAMP kinetics following exposure to10 μM cimetidine (Cim) (●), ranitidine (Ran) (■), tiotidine (Tio) (▲), or amthamine (Amtha) (○).



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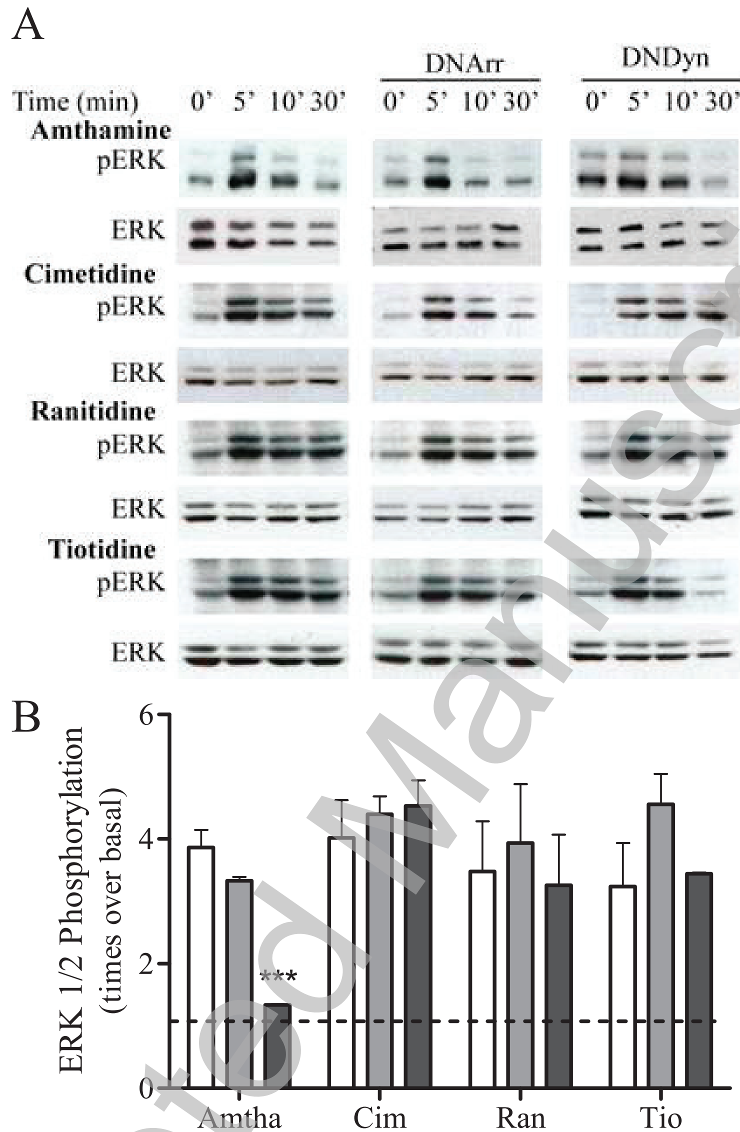


***Figure 8. Characterization of inverse agonists mediated ERK phosphorylation. (A),*** HEK293T cells transfected with $H_2R$ or co-transfected with Arrestin 319-418 (DNArr) or Dynamin K44A (DNDyn) dominant negative constructs were treated during different time periods with 10 µM cimetidine (Cim), ranitidine (Ran) or tiotidine (Tio), lysed, and equal amounts of proteins were subjected to SDS-PAGE and analyzed by Western Blot. ***(B),*** Densitometric analysis of ERK phosphorylation at 5 min of treatment in control (***white bars***) co-transfected with Arrestin 319-418 (***grey bars***) or Dynamin K44A (***black bars***), normalized to the corresponding ERK total levels, obtained with the Scion Image Program. Data are expressed as times over basal p-ERK levels. Data are expressed as means ± SE (n=3); *** $p<0.001$ respect to basal levels.



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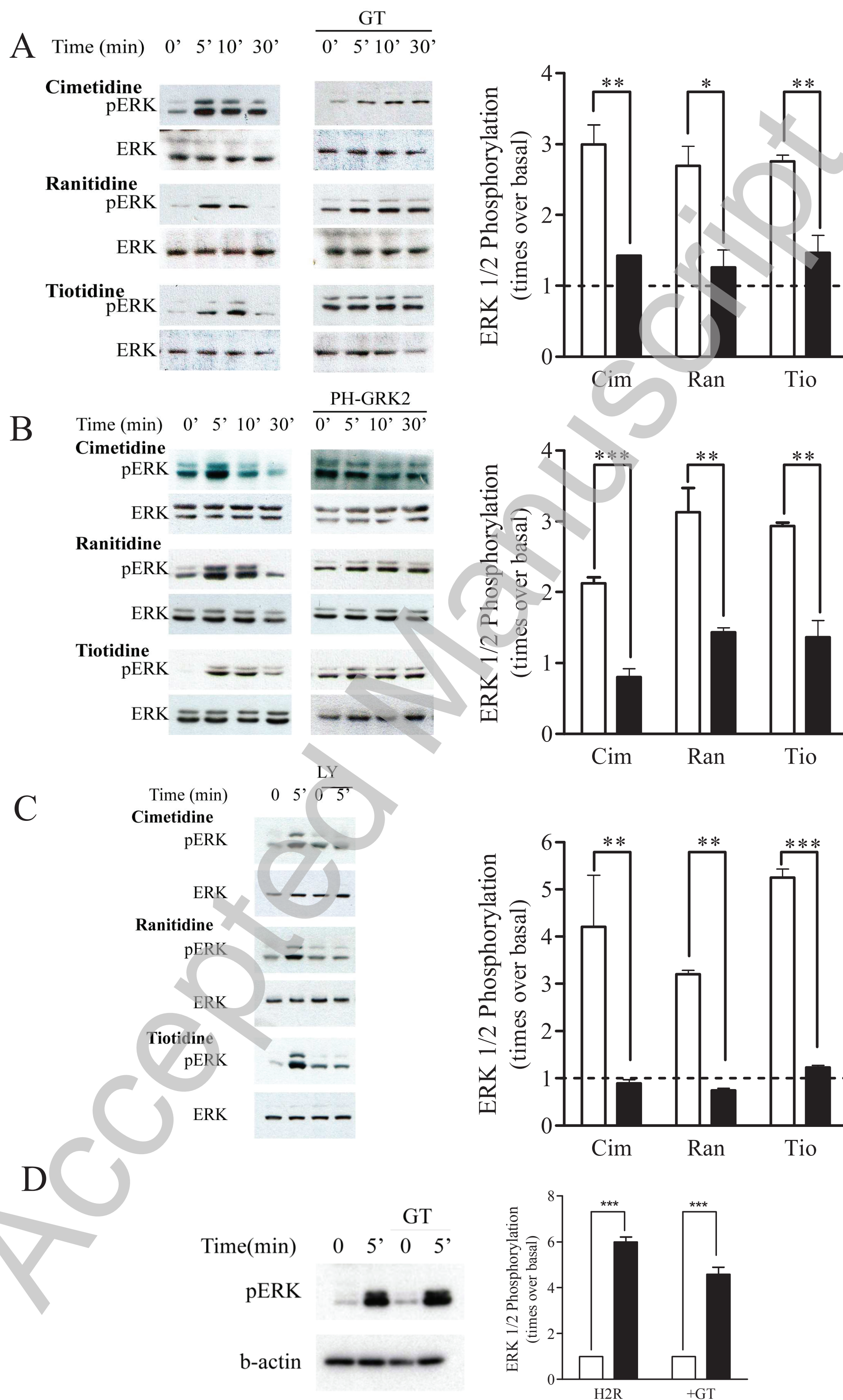


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***Figure 9. Gβγ involvement in inverse agonists mediated ERK phosphorylation. (A),*** HEK293T cells transfected with $H_2R$ or cotransfected with $G_\alpha$ transducin (GT) ***(B),*** or the PH domain (pleckstrin homology domain of GRK2) constructs ***(C)*** or pretreated for 30 min with 10 µM Ly294002 were treated during different time periods with 10 µM cimetidine (Cim), ranitidine (Ran) or tiotidine (Tio), lysed, and equal amounts of proteins were subjected to SDS-PAGE and analyzed by Western Blot. ***(D)*** HEK293T cells transfected with $H_2R$ or cotransfected with $G_\alpha$transducin (GT), were treated for 5 min with 10 µM amthamine, lysed, and equal amounts of proteins were subjected to SDS-PAGE and analyzed by Western Blot. ***Right panels***, Densitometric analysis of ERK phosphorylation at 5 min of treatment in control (***white bars***) co-transfected with PH, $G_{t\alpha}$ or pretreated with Ly294002 (***black bars***), normalized to the corresponding ERK total levels, obtained with the Scion Image Program. Data are expressed as times over basal p-ERK levels. Data are expressed as means ± SE (n=3).* $p<0.05$; ** $p<0.01$; *** $p<0.001$.



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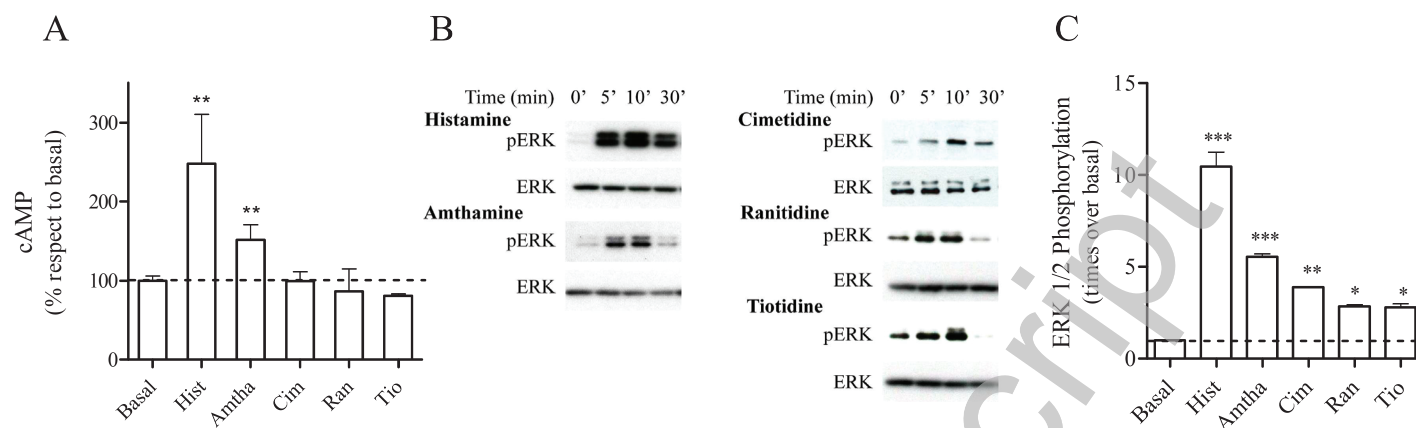


***Figure 10. Biased inverse agonism in human gastric AGS cells. (A)*** AGS cells were treated during 9 min with 100 µM histamine (Hist) or 10 µM amthamine (Amtha), cimetidine (Cim), ranitidine (Ran) or tiotidine (Tio) in presence of IBMX. Cyclic AMP levels were determined as detailed under Experimental Procedures. Data were calculated as the means ± SD of assay duplicates. ***(B)***, AGS cells were treated during different time periods with 100 µM histamine (Hist) or 10 µM amthamine (Amtha), cimetidine (Cim), ranitidine (Ran) or tiotidine (Tio), lysed, and equal amounts of proteins were subjected to SDS-PAGE and analyzed by Western Blot. ***(C),*** Densitometric analysis of ERK phosphorylation at 5 min of treatment, normalized to the corresponding ERK total levels, obtained with the Scion Image Program. Data are expressed as times over basal p-ERK levels. Data are expressed as means ± SE (n=3). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ respect to basal levels.



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