Molecular Pharmacology Fast Forward. Published on October 20, 2004 as doi:10.1124/mol.104.004549

Mol 4549

"RECIPROCAL REGULATION OF AGONIST AND INVERSE AGONIST SIGNALLING EFFICACY UPON SHORT-TERM TREATMENT OF THE HUMAN DELTA-OPIOID RECEPTOR WITH AN INVERSE AGONIST"

Graciela Piñeyro, Mounia Azzi, André deLéan, Peter W Schiller and Michel Bouvier.

Département de Biochimie (G.P., M.A., M.B.) and Département de Pharmacologie (A.D.), Faculté de Médecine, Université de Montréal, Montréal, Québec, Canada, H3C 3J7 and Institut de Recherches Cliniques de Montréal (P.W.S.), Université de Montréal, Montréal, Québec, Canada, H2W 1R7

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Reciprocal regulation of agonist and inverse agonist responses

Corresponding author: Graciela Piñeyro
7331 Rue Hochelaga
Centre de Recherche Fernand Séguin
Montréal, Québec, H1N 3V2
TEL: 514-251-4015 ext 2361
FAX: 514-251-2617
email: graciela.pineyro@crfs.umontreal.ca

Number of text pages: 28
Number of tables: 1
Number of figures: 9
Number of references: 40
Number of words in abstract: 250
Number of words in introduction: 604
Number of words in discussion: 1909

Abbreviations: B_2: bradykinin2 receptor; β_2AR; beta 2 adrenergic receptor; DCI: di-chloro-isoproterenol; δOR: Delta opioid receptor; DMEM: Dulbecco's modified Eagle medium; DMSO: di-methyl-sulfoxide; Emax: maximal effect; EC_{50}: effective concentration 50; GPCR: G protein-coupled receptors; IBMX: isobutyl-methyl-xanthine; n-DDM: n-Dodecyl maltoside; RGS: Regulators of G protein signaling

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Rapid regulation of receptor signaling by agonist ligands is widely accepted, whereas short-term adaptation to inverse agonists has been little documented. In the present study, [^{35}S]GTPγS binding and cAMP accumulation assays were used to assess the consequences of 30 min exposure to the inverse agonist ICI174864 (1μM), on delta opioid receptor (δOR) signaling efficacy. ICI174864 pretreatment increased maximal effect (Emax) for the partial agonist TIPP at the two levels of the signaling cascade while Emax for more efficacious agonists like SNC-80 and bremazocine, were increased in [^{35}S]GTPγS binding but not in cAMP accumulation assays. Preexposure to ICI174864 also induced a shift to the left in dose response curves for bremazocine and TIPP. Conversely, Emax for the inverse agonist TICPψ was reduced in both assays, but no changes in potency were observed. For the weaker inverse agonist naloxone, Emax in [^{35}S]GTPγS binding was drastically modified since the drug turned from inverse agonist to agonist following ICI174864 pretreatment. Similarly, ICI174864 turned from inverse agonist to agonist when tested in cAMP accumulation assays. In both cases, inversion of efficacy was concomitant with marked increase in potency for agonist effects. Together with functional changes, short-term treatment with ICI174864 reduced basal receptor phosphorylation and increased immunoreactivity for $G\alpha_{i3}$ in membrane preparations. Functional consequences of ICI174864 pretreatment were simulated in the cubic ternary complex model by increasing receptor-G protein coupling or G protein amount available for interaction with the receptor. Taken together, these data show that inverse agonists may induce rapid regulation in receptor signaling efficacy.

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

G protein-coupled receptors (GPCRs) are the single largest family of membrane proteins capable of transducing extracellular cues into cellular responses. Usually, this process consists of two identifiable phases: i) signal detection and transduction, and ii) adjusting of signaling efficacy. It is a well-established fact that agonist ligands induce receptor desensitization, a process that starts within minutes of agonist exposure. This regulatory response is triggered by phosphorylation of the receptor (Benovic et al., 1987; Lohse et al., 1990) leading to receptor uncoupling from the G protein, its targeting to clathrin-coated vesicles, its removal from the cell surface via dynamin-mediated endocytosis (von Zastrow, 2003) and finally, receptor degradation or recycling back to the cell surface (Tsao and Von Zastrow; 2000). If, instead of a few minutes, agonist exposure is prolonged for several hours, the total amount of receptor protein may be reduced not only by degradation (Tsao and Von Zastrow 2000, Valiquette et al., 1990) but also by decrease in transcription efficiency (Blaukat et al., 2003; Chau et al., 1994).

G proteins also undergo adaptive changes that contribute to desensitization of agonist responses, and despite the fact that these processes have been less well characterized than those of receptors, some of their regulatory mechanisms are starting to become widely accepted. For example, it is now known that, within minutes of receptor stimulation, regulators of G protein signaling (RGS) promote the inactivation of different $G\alpha$ subunits by stimulating GTP hydrolysis (De Vries et al., 2000). In the specific case of δOR and $G\alpha_{i3}$, before RGS interacts with the alpha subunit, the receptor and $G\alpha$ must translocate together from their initial location in non-clathrin domains of the plasma membrane into clathrin-coated pits (Elenko et al., 2003). Subsequently, the receptor is internalized while $G\alpha_{i3}$ stays at the membrane (Elenko et al., 2003). In other instances, translocation takes place between the plasma membrane and the cytosol as

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

has been described for $G\alpha_s$ following palmitoylation (Ransnas et al., 1989; Wedegaertner et al., 1996). On the other hand, if receptor stimulation takes place over prolonged periods of time, $G\alpha$ subunits will eventually be down-regulated by degradation (Shah et al., 1995).

For a long time, agonists were considered the only drugs capable of inducing pharmacological responses, but since the development of heterologous expression systems, the concept of efficacy has evolved to also include inverse agonism (Costa et al., 1992 Samama et al., 1993 Chidiac et al., 1994). While agonists are defined as drugs that enhance receptor activation, inverse agonists have been characterized as ligands that reduce constitutive receptor activity (see Kenakin 2004 for review). Like agonists, these new ligands also induce adaptive responses that modify signaling efficacy. Particularly, long-term exposure to an inverse agonist is known to produce receptor (Heinflink et al., 1995; MacEwan and Milligan, 1996; Lee et al., 1997) and G protein up-regulation (Berg et al., 1999), both of which result in enhanced responsiveness to agonist drugs. In contrast, little is known about more immediate, short-term adaptive responses elicited by inverse agonists.

Therefore, in the present study, we used the δOR to assess the capacity of short-term inverse agonist treatment (30 min) to modulate receptor signaling. It was found that pretreatment with the inverse agonist ICI174864 reduced receptor phosphorylation and increased $G\alpha_{i3}$ immunoreactivity associated with cell membranes. These changes were accompanied by functional sensitization of the system towards agonistic responses and reduction of inverse agonist effects, both of which could be simulated in the cubic ternary complex model by representing the effect produced by pretreatment with ICI174864 as an increase in receptor G protein coupling or an increase in the total amount of G protein available for interaction with the receptor.

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

EXPERIMENTAL PROCEDURES

Reagents. Buffer chemicals, protease inhibitors; naloxone; forskolin, isobutyl-methyl-xanthine (IBMX), anti-FLAG M2 affinity resin, and FLAG peptide were purchased from Sigma. [^{35}S]GTPγS; [^{3}H]adenosine and [^{32}P]ATP were from Perkin-Elmer. ICI174864 and SNC-80 were obtained from Tocris Cookson and bremazocine from Research Biochemical International. TIPP and TICPΨ were synthesized as previously described (Schiller et al., 1999). G418, Dulbecco's modified eagle medium (DMEM), fetal bovine serum, fungizone, glutamine, penicillin, streptomycin were purchased from Wisent.

DNA Constructs. The hδOR cDNA was subcloned into the pcDNA3 expression vector (Invitrogen) as previously described (Valiquette et al., 1996), and was tagged at the C-terminal end using the CLONTECH site-directed mutagenesis kit to remove the stop codon and introduce the sequence coding for the FLAG epitope (DYKDDDDK). The construction was verified and confirmed by restriction enzyme mapping and DNA sequencing. Pharmacological signaling properties of this construct have been repeatedly shown to be identical to those of the wild type δOR (Petaja-Repo et al., 2000; Pineyro et al., 2001).

Cell Culture and Transfection. HEK 293s cells were transfected using the calcium-phosphate precipitation method and clones stably expressing the hδOR-FLAG were selected using 400 µg/ml of G-418 (Wisent). Cells were grown and maintained in complete Dulbecco's modified Earl's medium (DMEM) containing 10% (v/v) fetal bovine serum, 1000 units/ml penicillin, 1 mg/ml streptomycin, and 1.5 µg/ml fungizone) in a humidified atmosphere of 5% CO_2 at 37 C.

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

Treatments. The day of the experiment cells were detached and incubated for 30 min in DMEM containing either vehicle (0.01% DMSO), 1 μM ICI174864 or 1 μM SNC-80. Reaction was stopped by dilution in cold PBS and cells washed three times for 5 min in 50 volumes PBS at RT. When experiments were conducted in the presence of an inhibitor of protein synthesis, cycloheximide was introduced 2 h before the experiment at a final concentration of 5μg/ml and ICI174864 (1μM final concentration) was added during the last 30 min of incubation.

Membrane preparation. Membranes were prepared as previously described (Piñeyro et al., 2001) following repeated washing to remove pretreatment drug. Briefly, cells were suspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine) and homogenized with a Polytron homogenizer (Ultra-Turrax T-25, Janke and Kunkel) using three bursts of 5 s at maximum setting. Homogenates were centrifuged at 700 × *g* for 5 min, and the supernatant was further centrifuged at 27,000 × *g* for 20 min. Pellets were washed twice in lysis buffer and were either immediately resuspended in [^{35}S]GTPγS assay buffer or in sample buffer for western blot analysis.

Displacement of[^{3}H]Bremazocine by naloxone. For competition binding assays, cell membranes (10 to 25 μg of protein) were incubated at 25°C for 1 h in Tris-HCl (50 mM, pH 7.4) with [^{3}H]-bremazocine (3 nM) and various concentrations of unlabelled naloxone in a final volume of 300 μl. Non-specific binding was defined with 10 μM naloxone. The reaction was terminated by rapid filtration using GF C filters and radioactivity determined by liquid scintillation counting. Affinity values (K_i) were calculated from a nonlinear least-squares analysis of displacement data using Prism (GraphPad Software, San Diego, CA).

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

[^{35}S]GTPγS binding assays. Crude membrane preparations were resuspended in assay buffer (50 mM Hepes; 200 mM NaCl; 1 mM EDTA; 5 mM $MgCl_2$; 1 mM DTT; 0.5% BSA and 3 μM GDP; pH 7.4) to yield 10 μg protein/tube. [^{35}S]GTPγS was used at a 30-50nM concentration and non-specific binding was determined in the presence of 100 μM GTP. Test compounds were introduced at a final concentration of 1 μM and incubation was allowed to proceed for one hour at RT. The reaction was terminated by rapid filtration onto Whatman GF/C glass filters pre-soaked in water. Filters were washed twice with ice-cold wash buffer (pH 7) containing 50 mM Tris, 5 mM $MgCl_2$ and 50 mM NaCl, and the radioactivity retained was determined by liquid scintillation.

cAMP accumulation assays. Cells were labeled overnight (16 hours) with complete DMEM and containing 1 μCi/ml of [^{3}H]adenine. The day of the experiment radioactive medium was replaced with fresh DMEM, cells mechanically detached, thoroughly washed (3 times) with PBS (4^{o}C) and viability assessed using trypan blue (mortality was never higher than 5%). 5 x 10^5 cells were then incubated for 20 min at 37°C in 300 μl assay mixture containing PBS, 25 μM forskolin, 2.5 μM IBMX, and different drugs at the indicated concentrations. At the end of the incubation period, the assay was terminated by adding 600 μl ice cold solution containing 5% trichloroacetic acid, 5mM ATP and 5mM cAMP. [^{3}H]ATP and [^{3}H]cAMP were separated by sequential chromatography on Dowex exchange resin and aluminum oxide columns. Results were expressed as the ratio of [^{3}H]cAMP/[^{3}H]ATP + [^{3}H]cAMP.

Receptor phosphorylation and immunoprecipitation of flag-tagged hδORs. The day of the experiment, cells were incubated for 2 h in phosphate-free DMEM for two h, after which [^{32}P]orthophosphoric acid was added at a final concentration of 1 mCi/ml and incubation allowed to proceed for an additional h. At this time, SNC-80 (1μM), ICI174864

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

(1μM) or vehicle (0,01% DMSO) were added to the incubation medium for a 30 min treatment. Cells were then recovered, membranes prepared as indicated above and finally suspended in solubilization buffer: 0.5% n-DDM (w/v), 25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 140 mM NaCl, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, 2.μg/ml aprotinin, 0.5 mM PMSF, and 2 mM 1,10?-phenantroline. Following agitation at 4°C for 60 min, the solubilized fraction was centrifuged at 100,000 × *g* for 60 min and the receptor immunoprecipitated from the supernatant fraction using anti-FLAG M2 antibody resin. 20 μl of antibody-coupled resin equilibrated in solubilization buffer and supplemented with 0.1% BSA (w/v) were used to purify the receptor overnight at 4°C under gentle agitation. The next morning the resin was pelleted, washed twice with 500 μl of solubilization buffer and four times with 500 μl of modified solubilization buffer (containing 0.1% instead of 0.5% n-DDM (w/v)). The receptor was then eluted by incubating the resin for 10 min at 4°C with 100 μl of modified solubilization buffer containing 175 μg of FLAG peptide/ml. This elution was repeated three times, and the eluates were combined and concentrated down to 25 μl by membrane filtration over Microcon-30 concentrators (Millipore). 25 μl of SDS sample buffer were then added and samples used for SDS-PAGE.

SDS-PAGE and Western Blotting. SDS-PAGE was performed as described by Laemmli using a 4% stacking gel and 10% separating gel. Proteins resolved in SDS-PAGE were then transferred (50 mA, 16 h, Bio-Rad Mini-Trans Blot apparatus) from the gels onto nitrocellulose (Amersham Pharmacia Biotech;). In the case of phosphorylation studies, membranes were first used for autoradiography (BIOMAX films; KODAK) and then probed with antisera directed against the FLAG M2 antibody (1:1000; Sigma-Aldrich). When assessing changes in G protein immunoreactivity, membranes were incubated with antibodies directed against highly divergent domains of either $G_{\alpha o}$; $G_{\alpha i1}$;

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

$G_{\alpha i2}$; or $G_{i\alpha 3}$ (1:1000; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech) and enhanced chemiluminescence detection reagents (NEN Life Science Products) were used to reveal the blotted proteins. The relative intensities of the labeled bands were analyzed by densitometric scanning.

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Effect of short-term inverse agonist pretreatment on pharmacological responses elicited by different δOR ligands. The effect of short-term inverse agonist pretreatment on the efficacy of receptor signaling was determined in HEK293s cells stably expressing hδORs. To do so, cells were exposed to the inverse agonist ICI174864 (1 μM) for 30 min, following which they were washed three times with PBS before different receptor ligands were tested. Pharmacological responses were obtained in [^{35}S]GTPγS binding or cAMP accumulation assays so as to evaluate the influence of inverse agonist pretreatment at two consecutive levels of the δOR signaling cascade. Figure 1A, shows that pre-exposure to ICI174864 modified both, basal and ligand-dependent [^{35}S]GTPγS binding. In the case of basal binding ICI174864 increased values by 25%, shifting the amount of [^{35}S]GTPγS bound from 113 ± 1.6 fmole/mg of protein in controls, to 141 ± 6 fmole/mg of protein following pretreatment with ICI174864 (n=4; $p<0.05$; *Inset*). To avoid the confounding effect of this increase in basal levels, subsequent results describing the effect of different δOR ligands were expressed as the percentage change in [^{35}S]GTPγS binding with respect with the corresponding basal values. Thus, pretreatment with ICI174864 enhanced maximal [^{35}S]GTPγS binding for the agonist SNC-80 from 105 ± 13 % to 150 ± 16 % ($p < 0.05$; n=8) and that of bremazocine from 35 ± 5 % to 66 ± 8 % ($p < 0.01$; n = 18; fig 1A). Interestingly, these increases were correlated to the efficacy of the compound tested since the ability of SNC-80 to promote guanine nucleotide binding was increased 1.4 fold while that of the weaker agonist bremazocine (Piñeyro et al., 2001) was enhanced 1.9 fold. In the case of TIPP, which induced no significant [^{35}S]GTPγS binding in the control situation, pretreatment with the inverse agonist turned its response into that of partial agonist (-5 ±

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

5 % vs. 30 ± 9%; p < 0.01; n=13; fig 1A). The enhancing effect of ICI174864 pretreatment on bremazocine signaling efficacy was also observed in cells previously exposed to the protein synthesis inhibitor cycloheximide (see Table 1), indicating that this effect was not due to an increase in the synthesis of receptor protein or other proteins of the signaling cascade. In order to assess whether ICI174864 pretreatment modified potency values, dose response curves were obtained for SNC-80 and bremazocine, the two tested compounds producing detectable [^{35}S]GTPγS binding in control conditions. As shown in figure 1B, EC_{50} for bremazocine was shifted to the left by a factor of 24 while that of SNC-80 remained unchanged.

Results from cAMP accumulation assays appear in figure 2 and show that pretreatment with ICI174864 did not modify the dose response curve for SNC-80 but increased bremazocine potency by 6.6 fold, an effect that was associated with an increase in the Hill coefficient (Hill slope values in controls: -0.4 ± 0.1; Hill slope values following ICI174864: -0.9 ± 0.1; p < 0.05; n=4;). Only in the case of the weakest agonist TIPP, exposure to ICI174864 enhanced both, maximal cAMP inhibition (CTL: 29± 2%; inhibition in cAMP accumulation; following pretreatment with ICI174864: 44 ± 3 inhibition in cAMP accumulation) and potency (EC_{50} in CTL: 4.6 ± 0.2 pM; EC_{50} following ICI174864: 0.7 ± 0.02 pM; p< 0.001; n=8).

Comparison of Emax values obtained in [^{35}S]GTPγS binding (figure 1A) and in cAMP accumulation assays (figure 2) in the non-pretreated condition indicates that progression along the signaling cascade was associated with amplification of the signals induced by the different agonists. For example, though the maximal response elicited by bremazocine in [^{35}S]GTPγS binding was significantly smaller that the one obtained with SNC-80 (SNC-80: 105 ± 13 %; Brem 35 ± 5 %, p < 0.001), this difference was no longer

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

evident in cAMP accumulation assays (SNC-80: 70 ± 8 % inhibition in cAMP accumulation; Brem: 75 ± 6 % inhibition in cAMP accumulation; compare figures. 1A; 2A and 2B). TIPP, which in control conditions did not induce measurable changes in [^{35}S]GTPγS binding (Emax: -5 ± 5 %; figure 1A), displayed clear agonist behavior when tested in cAMP accumulation assays (Emax: 29 ± 3% inhibition in cAMP accumulation; $p < 0.01$, n= 8; figure 2C). On the other hand, signal amplification reduced the impact of inverse agonist pretreatment on Emax, as exemplified by the fact that pre-exposure to ICI174864 increased Emax for SNC-80 and bremazocine in [^{35}S]GTPγS binding but not in cAMP accumulation assays.

The consequences of ICI174864 pretreatment on inverse agonist behavior was determined next, using TICPΨ, ICI174864 and naloxone as test compounds. Results show that the effect of pretreatment was specific for each of the inverse agonists used (figure 3A). TICPΨ, the drug that produced the greatest reduction in [^{35}S]GTPγS binding, produced a non-significant inhibition following ICI174864 pretreatment (CTL: 24 ± 5 % inhibition in [^{35}S]GTPγS binding; following ICI174864: 6 ± 6 inhibition in [^{35}S]GTPγS binding; $p < 0.05$; n=12; figure 3A). Similar reduction in TICPΨ response was observed if pretreatment with ICI174864 was done in the presence of cycloheximide. (see Table 1). Naloxone, which induced the weakest inverse response in control conditions (11 ± 5 % inhibition in [^{35}S]GTPγS binding), turned into a partial agonist following exposure to ICI174864 (23 ± 9 % increase in [^{35}S]GTPγS binding; figure 3A), an effect that was also observed in cycloheximide-treated cells (see Table 1). Finally, pretreatement with ICI174864 reduced its own inverse efficacy ($p < 0.05$; n=17, CTL: 16 ± 5 % inhibition in [^{35}S]GTPγS binding; following ICI174864: 5 ± 8% increase in [^{35}S]GTPγS binding, fig 3A). Figure 3B shows dose response curves for compounds where EC_{50} values could be

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

calculated in control and pretreated conditions. In the case of TICPΨ potency remained unchanged, but for naloxone the switch from inverse agonist to agonist behavior entailed a dramatic shift in EC_{50} values (EC_{50} CTL: 174 ± 5 nM; following ICI174864: 0.01 ± 0.001 pM). In addition, displacement experiemnts in which increasing concentrations of naloxone were used to displace [^{3}H]bremazocine indicated that changes in potency were paralleled by changes in binding parameters. Indeed, figure 3C shows that naloxone bound to the δOR at a high and a low affinity site, and that pretreatment with ICI174864 left the low affinity site unchanged but increased naloxone's affinity for the high affinity site by a factor of 1000.

When considering the effect of ICI174864 pretreatment on cAMP responses (figure 4), the maximal effect produced by TICPΨ was significantly reduced by pretreatment with ICI174864 (CTL: 55 ± 12% increase in accumulated cAMP; following ICI174864: 26 ± 5 increase in accumulated cAMP; $p < 0.05$; n=6). In contrast, the inverse response elicited by ICI174864 was turned into that of an agonist shifting from a 35 ± 7% increase in controls to a 22 ± 3% reduction in cells that had been previously exposed to ICI174864 itself ($p < 0.001$; n=3, fig 2B). It is also worth noting that similar to naloxone, inversion of efficacy for ICI174864 was accompanied by a marked change in potency values. EC_{50} values for ICI174864 dropped from 1.5 ± 0.5 nM in controls to 0.02 ± 0.01 pM ($p < 0.001$; n=3; figure 2B) in pretreated cells. Concerning naloxone's behavior in cAMP accumulation assays, the observed effects in non-treated cells was that of a partial agonist (18 ± 5% reduction in cAMP accumulation). Following pretreatment with ICI174864 both, Emax values (28 ± 5 reduction in cAMP accumulation; $p < 0.001$; n=4) and drug potency were increased (EC_{50} CTL: 0.3 ± 0.05 pM; following ICI174864: 0.05 ± 0.01; $p < 0.001$; n=4; figure 2C), as had been previously observed for TIPP.

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

Comparison of [^{35}S]GTPγS binding results with results from cAMP accumulation assays in the non pretreated condition showed that naloxone behaved either as an inverse agonist or a partial agonist, depending on the assay in which its effects were assessed. This dual behavior is in keeping with protean properties displayed by the drug following ICI17484 pretreatement. In contrast, and despite its protean behavior following short-term inverse agonist pretreatment, the effect of ICI174864 in the non pretreated condition was that of an inverse agonist along the two consecutive levels of the signaling cascade, as was also the case for TICPψ (figure 2). Comparison of control Emax values for ICI174864 and TICPψ in [^{35}S]GTPγS binding (figure 1A; 1B) and cAMP accumulation shows that at both levels ICI174864 produced an effect that was two thirds that of TICPψ, arguing against signal amplification for these drugs.

Receptor phosphorylation and changes in δOR responsiveness to different ligands. It is well established that phosphorylation plays a role in the dynamic regulation of receptor signaling by agonists (Benovic et al., 1987; Lohse et al., 1990). It was therefore deemed important to determine whether functional changes that follow short-term inverse agonist treatment correlated with modifications in receptor phosphorylation. For this purpose, cells were pre-labelled with ^{32}P-orthophosphoric acid and exposed or not to SNC-80 or ICI174864 (30 min, 1 μM). Receptors were then immunopurified and the purification product resolved on SDS-PAGE. Following transfer onto nitrocellulose, membranes were first exposed for autoradiography and then blotted with anti-FLAG M2 antibody (figure 5A). Immunoblots revealed two broad bands at ≅55 and ≅40 kDa, corresponding to the monomeric mature and immature forms of the receptor, respectively (Petaja-Repo et al., 2001. Note: the slight increase in immunoreactivity of the immature form of the receptor following pretreatment with ICI174864 was a non-

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

reproducible, one time observation.). Autoradiograms showed that exposure to the agonist SNC-80 induced a 4.4 ± 0.5 increase in ^{32}P incorporation by the ≅55 kDa species (n=3; $p < 0.001$) while similar pretreatment with ICI174864 produced an opposite effect, reducing basal phosphorylation of this band by 45 ± 3% (n=4; $p < 0.05$). In contrast, ^{32}P incorporation by the ≅40 kDa band was not modified by either treatment, consistent with the notion that this species corresponds to an immature form of the receptor (Petaja-Repo et al., 2001).

Given that pre-exposure to the agonist SNC-80 and the inverse agonist ICI174864 had opposite effects on ^{32}P incorporation, the next series of experiments was carried out to determine whether these two treatments also had opposing actions at the functional level. To do so, cells were treated in parallel either with SNC-80 (1 μM; 30 min) or ICI174864 (1 μM; 30 min) following which [^{35}S]GTPγS binding assays were run. Figure 5B shows that even though ICI174864 and SNC-80 modified Emax for bremazocine in opposite directions, the same does not apply for TICPΨ, where both treatments produced a similar reduction in the inverse agonist response.

Effect of inverse agonist pretreatment on G protein immunoreactivity from crude membrane preparations. The total amount of G protein available for interaction with the receptor (Kenakin; 1997a) and the proportion of each specific Gα subtype (Yang and Lanier, 1999; Moon et al., 2001) are known to determine the effects produced by a drug. Thus, experiments were designed to assess whether pretreatment with an inverse agonist could modify the type or the amount of G protein available for interaction with the receptor at the plasma membrane. For this purpose membranes were prepared from cells exposed or not to ICI174864 (1 μM; 30 min) and then resolved on SDS-PAGE. Western blot analyses were carried out using antibodies capable of

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

discriminating between the different subtypes of Gi/o proteins that may potentially couple to δOR (α_o and $\alpha_{i1-3;}$ figure 4). In all cases, immunoblots revealed monomeric bands located in the vicinity of 40 kDa. Bands recognized by antibodies specific for α_o or with preferential affinity for $G\alpha_{i1}$ or $G\alpha_{i2}$ subunits revealed no change following exposure to ICI174864. However, the antibody that preferentially recognized $G\alpha_{i3}$ showed that pretreatment pretreatment with the inverse agonist produced an increase in immunoreactivity. Although this antibody may also react to a lesser degree with the other $G\alpha_i$ subunits, the fact that inverse agonist pretreatment did not change bands labeled by antibodies with preferential affinity for $G\alpha_{i1}$ and $G\alpha_{i2}$ confirms that pre-exposure to ICI174864 selectively increased $G\alpha_{i3}$-immunoreactivity in membrane preparations.

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

The present study assessed the ability of ICI174864 to regulate the efficacy of δOR signaling within a short treatment period (30 min) and found that pre-exposure to ICI174864 produced an overall sensitization to agonist responses (figures 1 and 2) and reduced or abolished inverse agonist effects (figure 3 and 4). Functional changes were accompanied by a reduction in basal receptor phosphorylation (figure 5A) and an increase in $G\alpha_{i3}$ immunoreactivity from membrane preparations (figure 6).

The impact of ICI174864 pretreatment on the effects of different ligands was dependent on the efficacy of the compound tested. For example, potency and maximal response for SNC-80 were modified to a much lesser extent than those of bremazocine and TIPP (figure 1). In this sense, SNC-80 could be classified as an “efficacy-driven” agonist, characterized by its ability to produce a marked effect at low occupancy, and by its low sensitivity to changes occurring at receptor and G protein levels (Kenakin; 1997b). This efficacy profile could also account for the lack of effect of inverse agonist pretreatment on cAMP regulation by SNC-80 (figure 2A). Most probably, because SNC-80 induced maximal inhibition in the control situation, there was no room left for sensitization following ICI174864 pretreatment. On the other hand, bremazocine and TIPP had an efficacy profile corresponding to “affinity-driven” drugs. This profile is characterized by considerable ligand affinity for the receptor but modest effects, thus requiring high occupancy to produce maximal response (Kenakin; 1997). Unlike “efficacy-driven drugs”, the effects of “affinity-driven” ligands are expected to be much more sensitive to signal amplification and changes affecting receptor-G protein stoicheometry. This would explain why TIPP and bremazocine were much more sensitive than SNC-80 to ICI174864 pretreatment. Moreover, such high sensitivity to signal amplification also explains why TIPP could have induced an agonistic effect in

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

cAMP accumulation assays despite the fact that no response was detected at the less amplified level of Gprotein signalling (figures 1 and 2). SNC-80 and bremazocine are two non-peptidic ligands for δORs, and as suc share common interaction sites within the receptor's binding pocket. This makes it unlikely that the distinct type of behavior that was observed for the two drugs could be explained on a structural basis. Moreover, responses for bremazocine and the peptidic ligand TIPP were both highly sensitive to ICI174864, further supporting the idea that the distinct impact of inverse agonist pretreatment is not likely a direct consequence of differences in chemical structure.

Concerning drugs that produced inverse agonist responses, TICPΨ was the only one to maintain its inverse agonist profile following pre-exposure to ICI174864. Yet, its Emax was considerably reduced (figures 3 and 4), as would be expected for an "affinity-driven" ligand. In the case of naloxone and ICI174864 the effects of inverse agonist pretreatment were much more complex, including not only a change in magnitude but also a change in the direction of efficacy. For naloxone a shift from inverse agonist to agonist behavior was evident at the G protein level (figure 3) but for ICI174864, the agonistic properties of the ligand only became apparent when the effects of inverse agonist pretreatment were assessed in cAMP accumulation assays (figure 4B). In addition to the effect of pretreatment with ICI174864, the protean nature of naloxone was also revealed in the control situation, since the drug behaved as a weak inverse agonist in [^{35}S]GTPγS binding assays but turned into a partial agonist when tested for cAMP accumulation. Independent on whether "protean" or "dual" behavior was observed because of different assay conditions or due to pretreatment with ICI174864, reversal of drug efficacy may be explained by changes in the spontaneous activity of the system producing the response (see below).

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

From a phenomenological point of view inverse agonists have been recognized as drugs that produce effects opposite to those of agonists, leading to the idea that one type of drug was the reciprocal or mirror image of the other. In fact, inverse agonist behavior was first explained by a two-state model (Costa et al., 1992; Leff, 1995) that proposed the existence of two opposite receptor states: active and inactive. While inverse agonists would recognize and stabilize the inactive state, agonists would prefer the complementary, active conformation of the receptor (Costa et al., 1992; Leff, 1995). However, since their initial description, evidence has accumulated showing that some inverse agonists are complex drugs capable of activating receptor signalling in specific conditions (Azzi et al., 2003; Chidiac et al., 1996; Gbahou et al., 2003; this study). For this type of drug, the idea that inverse efficacy is the opposite of agonist efficacy can no longer be held. Moreover, results presented herein show that ICI174864 and the agonist SNC-80 do not induce reciprocal adaptive responses since short term treatment with either of them reduced inverse response for TICP. These new set of observations call for an alternative model that would take into account the “dual” or “protean” (Kenakin, 2004) nature of certain drugs. According to this alternative concept, receptors would exist not just as dichotomous conformations with opposing signaling properties but as a population of states capable of inducing responses that span the complete spectrum of efficacy (Kenakin, 2004; Ghanouni et al., 2001). Within this heterogeneous population the possibility that the specific state stabilized by a protean may produce an agonist or inverse agonist response is given in part by the spontaneous activity of the system (Kenakin, 2004).

Spontaneous activity of δORs, as that of any other GPCR, is determined by the total amount of receptors, their ability to become spontaneously active, the total amount of G protein available for interaction with the receptor and the effectiveness of receptor-

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

G protein coupling. Receptor-G protein coupling is a highly regulated process greatly dependent on receptor phosphorylation (Benovic et al., 1987; Lohse et al., 1990). For δORs, it has been well documented that phosphorylation interferes with $G\alpha_{i/o}$-dependent signalling (Kouhen et al., 2000; Law et al., 2000), due in part to uncoupling of the receptor from the G protein. Thus, the observation that short-term exposure to ICI174864 reduced basal hδOR phosphorylation (figure 5A) is consistent with the possibility that ICI174864 pretreatment may have enhanced receptor G protein coupling, and increased the spontaneous activity of the system. This idea is further supported by the fact that pre-exposure to ICI174864 increased ligand-independent [^{35}S]GTPγS binding by 25% ± 0.8%, an observation that is also consistent with the observed increase in $G\alpha_{i3}$ immunoreactivity in membrane preparations following pretreatment with the inverse agonist. (figure 6). Moreover, the effects of ICI174864 pretreatment on Emax of all ligands tested may be simulated by assuming an increase in spontaneous activity secondary either to enhanced hδOR-G protein coupling or to increased Gprotein availability for interaction with the receptor. (See annex for predictions from the cubic ternary complex model concerning possible mechanisms underlying the functional effects of ICI174864 pretreatment). On the other hand, there are a number of observations that allow to exclude the possibility that the observed functional effects could have been related to incomplete wash out of pretreatment drug. First, pretreatment with ICI174864 induced an increase in basal [^{35}S]GTPγS binding, an effect opposite to what would be expected if removal of the pretreatment drug had been incomplete. Second, in all cases where EC_{50} values were modified, potency was increased, an observation that is also opposite to what would be expected if the pretreatment drug had been incompletely removed. Third, exposure to ICI174864

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

induced an increase in naloxone binding affinity and not a decrease, as would be expected if the pretreatment drug had not been properly washed away. Finally, it has been previously shown that similar exposure to ICI174864 (1 mM; 30 min) and wash out procedure produced no significant change in affinity values for [^{3}H]naltrindole (Pineeyro et al., 2001).

Pretreatment with an inverse agonist has been previously shown to enhance receptor responsiveness to agonist drug actions. However, treatment duration in the preceding reports was much longer than the one used in the present study (12-48 hours), and the observed effects were linked to an increase in the expression levels either of the receptor (Heinflink et al., 1995; McEwan and Milligan, 1996; Samama et al., 1997) or the G protein (Berg et al., 1999). In contrast, functional effects associated with 30 min exposure to ICI174864 were not dependent on *de novo* protein synthesis, since they could also be observed in the presence of protein synthesis inhibitor cycloheximide (Table 1). Nonetheless, an increase in $G\alpha_{i3}$ immunoreactivity was observed in membranes prepared from cells that had been exposed to ICI174864 (figure 6), suggesting that pretreatment with the inverse agonist may have caused a redistribution of $G\alpha_{i3}$ proteins, causing them to accumulate at the membrane level. A possible explanation for this accumulation could be that since ICI174864 pre-treatment reduces receptor phosphorylation, δORs may have a tighter precoupling with its interacting Gproteins. Another possibility could be that ICI174864 stabilizes the receptor in conformation that that keeps the $G\alpha_{i3}$ subunit in interaction with the receptor, an idea that has also been suggested for cannabinoid$_2$ or histamine$_1$ receptors and their respective inverse agonists (Bouaboula et al., 1999; Fitzsimons et al., 2004). In either case, enhanced interaction between δOR and $G\alpha_{i3}$ could then prevent translocation of

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

this specific Gα subtype into a different compartment, causing it to accumulate at the membrane, and priming the system for subsequent activation by other ligands. In addition, a tighter interaction between δOR and the $G\alpha_{i3}$ subtype may have reduced the loss of this specific subtype during membrane preparation, contributing to its increased detection in membrane preparations.

Together with modifications in Emax, short-term exposure to ICI174864 produced changes in potency (figures 1B; 1C; 2B and 2C). For bremazocine or TIPP, these changes were in the range of one order of magnitude, a shift that is predicted by the ternary complex model following modifications in receptor/G protein coupling or G protein contents (Lee et al., 1986; Costa et al., 1992). On the other hand, potency changes associated with naloxone and ICI174864 were of several orders of magnitude, and cannot be explained by changes in $G\alpha_{i3}$ immunoreactivity (Figure 6) or δOR phosphorylation (figure 5A). However, and consistent with its increase in potency, naloxone also displayed a 1000 fold increase in affinity for its high affinity binding site (figure 3C). The magnitude of this increase *per se* does neither explain the large shift in EC_{50} values, but the fact that naloxone recognizes at least two different binding sites may provide a clue to this behavior. Of these two sites, only the high affinity one was modified by inverse agonist pretreatment, indicating that the two sites recognized by the drug are functionally distinct. Since different conformations need not share the same signalling properties, it is possible that one of the receptor states may couple to a given Gα subtype to produce agonist responses while the other couples to a different Gα subtype to produce inverse agonist effects. In such case, naloxone could have induced its inverse agonist effect prior to ICI174864 primarily via the low affinity site, and its agonistic effects via the high affinity site following treatment. If pharmacological actions of naloxone were more powerful at the high affinity than the low affinity site, such

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

difference together with the marked increase in affinity and modifications in receptor Gprotein coupling or Gprotein content specifically favoring signalling via the high affinity site, may have shifted the weight of naloxone's actions away from the low affinity site and account for the observed reversal of efficacy and the increase in $EC_{50.}$ This possibility is analyzed in the appendix.

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

APPENDIX

This section will use the fully extended (cubic) version of the ternary complex model in order: i) to better understand how different determinants of constitutive activity may modify pharmacological behavior of a protean ligand, and ii) to assess how an increase in the total amount of G protein or an increase in receptor/G protein coupling may contribute to the changes in drug responsiveness associated with ICI174864 pretreatment.

The model. The fully extended ternary complex model is presented here as a modification of its initial simple form (De Léan et al., 1980). In its early conception the ternary complex model was described in terms of: i) ligand binding to the free receptor [AR] (K or K_{min} = [AR]/[A].[R]), ii) ligand binding to the coupled receptor [ARG] (K_{max} = [ARG]/[A].[RG] = αK_{min}), iii) interaction between the receptor R and a membrane regulatory component G (M = [RG]/[R].[G]), and iv) the factor α, characterizing the extent to which agonist binding to the receptor promotes receptor/G protein coupling (αM = [ARG]/ [AR].[G]). In this early version, the only active receptor species, [ARG], was promoted by agonist binding.

Later, in its extended version, the model incorporated the ability of the receptor to spontaneously isomerize (J) to an active state (R*; Samama et al., 1994). The fully extended form of the model (cubic model shown in Figure 1 of the annex) then allowed for the interaction of the G-protein not only with this active receptor form (R*G), but also with its inactive conformation (RG; Weiss et al., 1996). In this complete version preferential binding of the agonist to R* over R is given by the factor β; and higher tendency of the active (R*) over the inactive (R) receptor to form the R*G complex is given by γ. An additional constant δ, represents the synergistic effect of agonist binding

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

and G protein coupling on receptor activation, or that of agonist binding to the active conformation on the receptor's ability to couple to the G-protein (see appendix figure 1). The coupling constant M is corrected to M' = M(1+γJ/1+J) which accounts for the higher tendency of the activated receptor R* (over the inactive R conformation) to interact with the Gprotein. The coupling efficiency factor α is also modified, to incorporate the enhanced ability of an agonist to promote coupling between the activated state of the receptor and the G protein (α' = α(1+J/1+βJ)(1+δBγJ/1+γJ)). Also, new affinity constants K'_{min} and K'_{max} are introduced to account for increased agonist binding ability of the uncoupled active state over the inactive conformation (K'_{min}=K(1+βJ/1+J)), and for enhanced agonist affinity that is conferred by the coupling between the active receptor and the G protein (K'_{max}=αK(1+$\beta\delta\gamma$J/1+γJ) = α'K'_{min}).

Influence of different determinants of constitutive activity on the pharmacological behavior of a protean ligand. As stated in the discussion section determinants of spontaneous activity include the total amount of receptors (R_{tot}) and G proteins (G) present in the system, the ability of the receptor to spontaneously isomerize into an active conformation (J) and its tendency to couple to the G protein (M). Hence, to determine how activating or deactivating changes may modify the behavior of protean ligands, values given to each of these parameters were systematically changed. The pharmacological actions of a protean ligand defined by $\alpha = 2.2$; $\beta = 10$; and $\delta = 0.175$ were then determined using the following equation:

$$RG^*+ARG^* = (\gamma J/1+\gamma J)\ (M'G/1+M'G)\ (1+\alpha\beta\delta K[A]/1+K'_{app}\ [A])\ R_{tot}$$

$$(K'_{app} = K'_{min}+K'_{max}M'G/1+M'G)$$

to calculate the amount of active coupled receptors (RG* + ARG*) present under the different spontaneous activity conditions. Figure 2 shows the expected behaviour that

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

this protean would display following changes in the different determinants of spontaneous activity. As can be seen, diverse types of activating changes resulting from an increase in R_{tot} (total amount of receptors), G (total amount of G proteins) or M (tendency to couple to G protein), may all turn this protean from inverse agonist to agonist. Interestingly, a reduction in spontaneous activity associated with a decrease in J (isomerization constant), also predicts a reversal from inverse agonist to agonist effects. The fact that the inverse agonism turned into agonist behavior following either an increase or decrease in spontaneous activity points to the intricate relationship between spontaneous activity and perceived pharmacological actions. Indeed, as suggested by figure 2, the perceived pharmacological actions of a protean drug would depend not only on an increase or decrease in spontaneous activity, but also in the specific parameters driving these changes. This complexity is not only theoretical and is exemplified by different reports in the literature. For example, the ligand di-chloro-isoproterenol (DCI) for the beta2 adrenergic (β_2AR) receptor was shown to turn from partial agonist to inverse agonist in two opposing conditions (Chidiac et al., 1994; 1996). In one instance, the reversal of efficacy was associated with an increase in spontaneous activity secondary to changes in assay conditions (Chidiac et al., 1994) and in the other, the same reversal occurred following deactivation due to receptor desensitization (Chidiac et al., 1996). Another example corresponds to the bradykinin$_2$ (B_2) receptors for which two different activating mutations induced either agonist or inverse agonist behavior for the ligand HOE140 (Fathy et al., 1999; Marie et al., 1999). These apparently contradictory observations may be explained if one takes into account that the pharmacological response displayed by protean ligands depends on the level of spontaneous activity of the system in which the drug is studied, as well as on the specific parameters that contribute to determine the system's activity. Thus, a protean

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

drug may display opposite behaviour after different mutations that increase intrinsic activity if one of the mutations enhances receptor-Gprotein and the other increases isomerization.

Simulation of functional changes induced by ICI174864 pretreatment. As discussed previously, pretreatment with ICI174864 induced a decrease in δOR phosphorylation compatible with an increase in spontaneous receptor/G protein coupling, and an increase in $G\alpha_{i3}$ immunoreactivity that was interpreted as an increase in the total amount of G protein available for receptor interaction. Hence changes in receptor phosphorylation were simulated by increasing the coupling constant M (before pretreatment: $3x10^{-2}$; following ICI174864: $7\ x10^{-1}$) and changes in $G\alpha_{i3}$ immunoreactivity by increasing the total amount of G protein available for interaction with the receptor (before pretreatment: 180; following ICI174864: 2500). Simulation of dose response curves for three different types of ligands representing bremazocine, naloxone and TICPΨ in pre and post treatment conditions were obtained as described above. Bremazocine was defined by $\alpha = 2$; $\beta = 6$; and $\delta = 0.4$ (figure 3A and 3D of the appendix); parameters for naloxone were $\alpha = 2.8$; $\beta = 8$; and $\delta = 0.16$; Figure 3B and 3E) and for TICPΨ $\alpha = 40$; $\beta = 112$; and $\delta = 0.009$ (figure 3C and 3F of the appendix). As can be seen from the curves generated, any of the two mechanisms could theoretically account for the observed increase in Emax for the agonist, Emax reduction for the inverse agonist and a reversal of efficacy for the protean. Changes in potency for "bremazocine" (figure 3A and 3D of the annex) could also be simulated by an increase in G or M. However, this was not the case for "naloxone" (figure 3B and 3E of the annex), since a simple increase in M or G could not simulate the change in EC_{50} that accompanied reversal in efficacy. To recreate this observation it was necessary to

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

assume that the drug produced its effects via the low affinity site before pretreatment but that following exposure to ICI174864 naloxone's effects were generated via the high affinity site. This was represented by increasing naloxone's affinity for the receptor by 5.7×10^4 fold (which is the difference between the low and high affinity values observed before and after pretreatment, respectively). In addition, in order to fully account for the increase in potency the high affinity site was represented as a state on which the pharmacological effect of naloxone (α=2300) was much more powerful than the effect displayed at the low affinity site (α=2.8). This simulation reproduces the complete experimental observations and appears in the inset of figures 3B and 3E of the annex.

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements:

The authors would like to thank Monique Lagacé for careful reading and edition of this manuscript.

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M, Pineyro G. Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. (2003) *Proc Natl Acad Sci U S A*. 100:11406-11.

Benovic JL, Regan JW, Matsui H, Mayor F Jr, Cotecchia S, Leeb-Lundberg LM, Caron MG, Lefkowitz RJ. (1987). Agonist-dependent phosphorylation of the alpha 2-adrenergic receptor by the beta-adrenergic receptor kinase. *J Biol Chem*. 262:17251-3.

Berg KA, Stout BD, Cropper JD, Maayani S, Clarke WP. (1999) Novel actions of inverse agonists on 5-HT2C receptor systems. *Mol Pharmacol*. 55:863-72.

Blaukat A, Micke P, Kalatskaya I, Faussner A, Muller-Esterl W. (2003) Downregulation of bradykinin B2 receptor in human fibroblasts during prolonged agonist exposure. *Am J Physiol Heart Circ Physiol*. 284:H1909-16.

Bouaboula M, Dussossoy D, Casellas P. (1999) Regulation of peripheral cannabinoid receptor CB2 phosphorylation by the inverse agonist SR 144528. Implications for receptor biological responses. *J Biol Chem*. 274:20397-405.

Chau LY, Peck K, Yen HH, Wang JY. (1994) Agonist-induced down-regulation of platelet-activating factor receptor gene expression in U937 cells. *Biochem J*. 301: 911-6.

Chidiac P, Hebert TE, Valiquette M, Dennis M, Bouvier M. (1994). Inverse agonist activity of beta-adrenergic antagonists. *Mol Pharmacol*. 45:490-9.

Chidiac P, Nouet S, Bouvier M. (1996) Agonist-induced modulation of inverse agonist efficacy at the beta 2-adrenergic receptor. *Mol Pharmacol*. 50:662-9.

Costa T, Ogino Y, Munson PJ, Onaran HO, Rodbard D. (1992) Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. *Mol Pharmacol*. 41:549-60

De Lean A, Stadel JM, Lefkowitz RJ. (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. *J Biol Chem*. 255:7108-17.

De Vries L, Zheng B, Fischer T, Elenko E, Farquhar MG. (2000) The regulator of G protein signaling family. *Annu Rev Pharmacol Toxicol*. 40:235-71.

Elenko E, Fischer T, Niesman I, Harding T, McQuistan T, Von Zastrow M, Farquhar MG. (2003) Spatial regulation of Galphai protein signaling in clathrin-coated membrane microdomains containing GAIP. *Mol Pharmacol*. 64:11-20.

Fathy DB, Leeb T, Mathis SA, Leeb-Lundberg LM. (1999) Spontaneous human B2 bradykinin receptor activity determines the action of partial agonists as agonists or inverse agonists. Effect of basal desensitization. *J Biol Chem*. 274:29603-6.

Fitzsimons CP, Monczor F, Fernandez N, Shayo C, Davio CA. (2004) Mepyramine, a histamine H1 receptor inverse agonist, binds preferentially to a G protein coupled form of the receptor and sequesters G protein. *J Biol Chem*. Epub ahead of print.

Gbahou F, Rouleau A, Morisset S, Parmentier R, Crochet S, Lin JS, Ligneau X, Tardivel-Lacombe J, Stark H, Schunack W, Ganellin CR, Schwartz JC, Arrang JM. (2003) Protean agonism at histamine H3 receptors in vitro and in vivo. *Proc Natl Acad Sci U S A*. 100:11086-91.

Ghanouni P, Gryczynski Z, Steenhuis JJ, Lee TW, Farrens DL, Lakowicz JR, Kobilka BK. (2001) Functionally different agonists induce distinct conformations in the G protein coupling domain of the beta 2 adrenergic receptor. *J Biol Chem.* 276:24433-6.

Heinflink M, Nussenzveig DR, Grimberg H, Lupu-Meiri M, Oron Y, Gershengorn MC. (1995) A constitutively active mutant thyrotropin-releasing hormone receptor is chronically down-regulated in pituitary cells: evidence using chlordiazepoxide as a negative antagonist. *Mol Endocrinol*. 9:1455-60.

Kenakin T. (1997a) Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol Sci*. 18:456-64.

Kenakin T (1997b) Efficacy in “Pharmacologic analysis of drug receptor interactions” pp 289-330. Lippincott, Williams & Wilkins.

Kenakin T. (2004) Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Mol Pharmacol*. 65:2-11.

Kouhen OM, Wang G, Solberg J, Erickson LJ, Law PY, Loh HH. (2000) Hierarchical phosphorylation of delta-opioid receptor regulates agonist-induced receptor desensitization and internalization. *J Biol Chem*. 275:36659-64.

Law PY, Kouhen OM, Solberg J, Wang W, Erickson LJ, Loh HH. (2000) Deltorphin II-induced rapid desensitization of delta-opioid receptor requires both phosphorylation and internalization of the receptor. *J Biol Chem*. 275:32057-65.

Lee TW, Sole MJ, Wells JW. (1986) Assessment of a ternary model for the binding of agonists to neurohumoral receptors. *Biochemistry*. 25:7009-20.

Leff P. (1995) The two-state model of receptor activation. *Trends Pharmacol Sci*. Mar;16(3):89-97.

Lohse MJ, Benovic JL, Caron MG, Lefkowitz RJ. (1990) Multiple pathways of rapid beta 2-adrenergic receptor desensitization. Delineation with specific inhibitors. *J Biol Chem*. 265:3202-11.

MacEwan DJ, Milligan G. (1996) Inverse agonist-induced up-regulation of the human beta2-adrenoceptor in transfected neuroblastoma X glioma hybrid cells. *Mol Pharmacol*. 50:1479-86.

Moon HE, Cavalli A, Bahia DS, Hoffmann M, Massotte D, Milligan G. (2001) The human delta opioid receptor activates G(i1)alpha more efficiently than G(o)alpha. *J Neurochem*. 76:1805-13.

Petaja-Repo UE, Hogue M, Laperriere A, Walker P, Bouvier M. (2000) Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. *J Biol Chem*. 275:13727-36.

Pineyro G, Azzi M, De Lean A, Schiller P, Bouvier M. (2001) Short-term inverse-agonist treatment induces reciprocal changes in delta-opioid agonist and inverse-agonist binding capacity. *Mol Pharmacol*. 60:816-27.

Ransnas LA, Svoboda P, Jasper JR, Insel PA. (1989) Stimulation of beta-adrenergic receptors of S49 lymphoma cells redistributes the alpha subunit of the stimulatory G protein between cytosol and membranes. *Proc Natl Acad Sci* 86:7900-3.

Samama P, Cotecchia S, Costa T, Lefkowitz RJ. (1993) A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem*. 268:4625-36.

Schiller P, Weltrowska G, Berezowska I, Nguyen T, Wilkes B, Lemieux C and Chung N (1999) The TIPP opioid peptide family: development of delta antagonists, delta agonists, and mixed mu agonist/delta antagonists. *Biopolymers*. 51: 411-425.

Shah BH, MacEwan DJ, Milligan G. (1995) Gonadotrophin-releasing hormone receptor agonist-mediated down-regulation of Gq alpha/G11 alpha (pertussis toxin-insensitive) G proteins in alpha T3-1 gonadotroph cells reflects increased G protein turnover but not alterations in mRNA levels. *Proc Natl Acad Sci*. 92:1886-90.

Tsao PI, von Zastrow M. (2000) Type-specific sorting of G protein-coupled receptors after endocytosis. *J Biol Chem*. 275:11130-40.

Valiquette M, Bonin H, Hnatowich M, Caron MG, Lefkowitz RJ, Bouvier M. (1990) Involvement of tyrosine residues located in the carboxyl tail of the human beta 2-adrenergic receptor in agonist-induced down-regulation of the receptor. *Proc Natl Acad Sci* 87:5089-93.

Valiquette M, Vu HK, Yue SY, Wahlestedt C, Walker P. (1996) Involvement of Trp-284, Val-296, and Val-297 of the human delta-opioid receptor in binding of delta-selective ligands. *J Biol Chem.* 271:18789-96.

von Zastrow M. (2003) Mechanisms regulating membrane trafficking of G protein-coupled receptors in the endocytic pathway. *Life Sci.* 74:217-24.

Wedegaertner PB, Bourne HR, von Zastrow M. (1996) Activation-induced subcellular redistribution of Gs alpha. *Mol Biol Cell.* ;7:1225-33.

Weiss JM, Morgan PH, Lutz MW, Kenakin TP. (1996) The cubic ternary complex receptor-occupancy model. III. Resurrecting efficacy. *J Theor Biol*. 181:381-97.

Yang Q, Lanier SM. (1999) Influence of G protein type on agonist efficacy. *Mol Pharmacol*. 56:651-6.

Footnotes:

This work was supported by grants from the Canadian Institute for Health Research (CIHR) and the Heart and Stroke Foundation of Canada (HSFC). G.P. was supported by a Postdoctoral Fellowship of the HSFC and M.A. by a Postdoctoral Fellowship of the Canadian Hypertension Society. M.B. holds the Hans Selye Chair in Molecular and Cell Biology and a Canada Research Chair in Signal Transduction and Molecular Pharmacology.

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

LEGEND TO THE FIGURES

Figure 1. Effect of pretreatment with ICI174864 on [^{35}S]GTPγS binding. HEK293s cells expressing hδOR-Flag were incubated (black) or not (white) with ICI174864 (30 min; 1 μM) and used to prepare membranes for [^{35}S]GTPγS binding. **(A)** Emax values for different δOR ligands (1 μM). Values are expressed as % change in [^{35}S]GTPγS binding observed in the presence of each δOR ligands as compared to the binding observed in its absence (% change in GTPγ[^{35}S] binding = {[GTPγ[^{35}S] bound$_{ligand}$ - GTPγ[^{35}S] bound$_{no\ ligand}$]/GTPγ[^{35}S] bound$_{no\ ligand}$}x100), and represent the mean ± SEM of 8-18 independent experiments carried out in triplicates. Statistical significance of drug effects at their maximal effective concentration was established using Student's t-test to compare absolute radioactivity levels in the presence and absence of the different ligands (* $p < 0.05$; ** $p < 0.01$). The statistical significance of changes induced by ICI174864 pretreatment was established using *t* test to compare percentual drug effects in control and treated membranes ($^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$). *Inset:* Absolute values for basal [^{35}S]GTPγS binding in controls (white) or following pretreatment with ICI174864 (black). Results are expressed as fmole/mg of protein and statistical significance of changes induced by ICI174864 pretreatment was established using unpaired *t* test to compare absolute values from control and ICI174864-treated conditions ($p < 0.05$; n=4) **(B):** Representative dose response curves for SNC-80 and bremazocine carried out in controls (o) and following pretreatment with ICI174864 (●).

Figure 2. Effect of pretreatment with ICI174864 on the regulation of cAMP accumulation by SNC-80 (SNC), bremazocine (Brem) and TIPP. Dose response curves for the indicated ligands were obtained in control cells and cells that had been previously exposed to ICI174864 (1 μM; for 30 min). Results are expressed as % change in cAMP

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

accumulation in the presence of the indicated ligand as compared to cAMP accumulation observed in its absence (% change in cAMP accumulation = {[$cAMP_{ligand}$ - $cAMP_{no\ ligand}$]/ $cAMP_{no\ ligand}$}x100). Each pair of control and treated curves were compared using two-way ANOVA to determine the significance of the effect of ICI174864 on the different ligands: SNC-80 non significant; bremazocine $p < 0.05$; TIPP $p < 0.01$. (o) Controls; (●) following pretreatment with ICI174864.

Figure 3. Effect of pretreatment with ICI174864 on the regulation of [^{35}S]GTPγS binding by naloxone (Nalox), ICI174864 and TICPΨ. HEK293s cells expressing hδOR-Flag were incubated (black) or not (white) with ICI174864 (30 min; 1 μM) and used to prepare membranes for [^{35}S]GTPγS binding. **(A)** Emax values for different δOR ligands (1 μM). Values are expressed as % change in [^{35}S]GTPγS binding observed in the presence of each δOR ligands as compared to the binding observed in its absence (% change in GTPγ[^{35}S] binding = {[GTPγ[^{35}S] $bound_{ligand}$ - GTPγ[^{35}S] $bound_{no\ ligand}$]/GTPγ[^{35}S] $bound_{no\ ligand}$}x100), and represent the mean ± SEM of 10-18 independent experiments carried out in triplicates. Statistical significance of drug effects at their maximal effective concentration was established using Student's t-test to compare absolute radioactivity levels in the presence and absence of the different ligands (* $p < 0.05$; ** $p < 0.01$). The statistical significance of changes induced by ICI174864 pretreatment was established using *t* test to compare percentual drug effects in control and treated membranes ($^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$). **(B):** Representative dose response curves for naloxone and TICPΨ carried out in controls (o) and following pretreatment with ICI174864 (●). **(C)** Displacement of [^{3}H]bremazocine (5 nM) by increasing concentrations of naloxone in control membranes (o) and in membranes obtained from cells that were pretreated with ICI174864 (1 μM; 30 min; ●). In both cases results were best fit by a two site model,

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

and calculated Ki values for high and low affinity sites appear in the figure. Statistical significance of the difference between the two curves was established using two-way ANOVA ($p < 0.01$; n=3).

Figure 4. Effect of pretreatment with ICI174864 on the regulation of cAMP accumulation by TICPΨ **(A)**, ICI174864 **(B)** and naloxone **(C)**. Dose response curves for the indicated ligands were obtained in control cells and cells that had been previously exposed to ICI174864 (1 μM; for 30 min). Results are expressed as % change in cAMP accumulation in the presence of the indicated ligand as compared to cAMP accumulation observed in its absence (% change in cAMP accumulation = $\{[cAMP_{ligand} - cAMP_{no\ ligand}]/ cAMP_{no\ ligand}\}x100$). Each pair of control and treated curves were compared using two-way ANOVA to determine the significance of the effect of ICI174864 pretreatment on the different ligands: TICPΨ $p < 0.05$; ICI174864 $p < 0.01$ and Nalox: $p < 0.01$; (ο) Controls; (●) following pretreatment with ICI174864.

Figure 5. (A): Effect of pretreatment with SNC-80 or ICI174864 (30 min; 1 μM) on the phosphorylation of hδORs. HEK293s cells stably expressing flag-tagged hδORs were metabolically labeled with ^{32}P and exposed for 30 min to the indicated drugs (1 μM). Solubilized hδORs were then purified by immunoprecipitation using anti-FLAG M2 antibody resin and subjected to electrophoresis on 10% SDS-PAGE (see *Experimental Procedures for details).* Representative autoradiographs and corresponding western blots for the different treatment conditions are shown. Bar graphs represent phosphorylation level of the receptors expressed as the mean ± SEM of the phospho/protein ratio calculated from densitometric analysis of each autoradiogram and its corresponding western blot (n=4-5). Statistical significance of drug effects was established by comparison with control values using Student's *t* test. **(B):** Comparison of

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

the effects of pretreatment with SNC-80 or ICI174864 on the regulation of [^{35}S]GTPγS binding by δ-OR ligands of different efficacies. HEK293s cells expressing hδORs were incubated or not with SNC-80 or ICI174864 (30 min; 1 μM), and used to prepare membranes for [^{35}S]GTPγS binding assays. Results are expressed as detailed before, and represent the mean ± SEM of 3-4 independent experiments carried out in triplicates. The statistical significance of changes induced by either SNC-80 or ICI174864 pretreatment appear in the figure and was established by comparing percentual effects of drugs in controls and the corresponding pretreatment group using Student's t test.

Figure 6. Effect of ICI174864 pretreatment (30 min; 1 μM) on the immunoreactivity of different $G\alpha_{i/o}$ subunits. HEK293s cells expressing hδORs were incubated (black) or not (white) with ICI174864, membranes prepared and resolved on SDS-PAGE (40 μg/well). Representative examples of immunoblots obtained for the different $G\alpha_{i/o}$ subunits are shown. Bar graph represents the mean ± SEM of optic density values obtained for each $G\alpha_{i/o}$ in four independent experiments. Statistical significance of changes induced by pretreatment with ICI174864 was established by using Student's *t* test to compare optic density units form control and treated blots.

Appendix, figure1. Fully extended ternary complex model (cubic model). See text for details.

Appendix, figure 2. Influence of different determinants of constitutive activity on the pharmacological behavior of a protean ligand defined by α=2.2; β=10; δ=0.175. Figure shows simulated dose response curves for parameter changes that results in inversion of efficacy. **(A)** Initial condition: R_{tot}=200; G=180; M=0.03; J=5; γ = 0.06. **(B)** Increase in receptor number: R_{tot}=2000; **(C)** Increase in G protein contents: G=2500; **(D)** Increase in receptor G protein coupling: M=0.7 **(E)** Decrease in receptor activation:

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

J=0.5. Drug effects are expressed as change in total amount of active coupled receptor species (RG* + ARG*).

Appendix, figure 3. Simulation of the effect of ICI174864 pretreatment on the pharmacological behavior of ligands representing "bremazocine", "naloxone" and "TICPΨ". Parameters for each ligand appear on the figure. Panels A, B, C show the functional consequences if pretreatment is simulated by an increase in total G protein contents: before treatment G=180 (full line) and following ICI174864 G=2500 (dashed line). Panels D, E, F show the effect of an increase in M, which represents enhanced receptor/G protein coupling: before treatment M=0.03 (full line) and following ICI174864 M=0.7 (dashed line). The rest of the system parameters were kept constant: R_{tot}= 80; J=5; γ=0.06. *Insets 3B and 3E*: show the simulations for naloxone following adjustments to account for increase in potency. See text for details. Drug effects are expressed as the results obtained in the previous section, by calculating the % change in the amount of signaling receptor complexes in the presence of drug $(RG^* + ARG^*)_{drug}$ as compared to the amount of complex present in its absence $(R^*G)_{basal}$.

% change in total amount of signaling complex $=\{[(RG^* + ARG^*)_{drug} - (R^*G)_{basal}]/(R^*G)_{basal}\}x100$.

Molecular Pharmacology Fast Forward. Published on October 20, 2004 as DOI: 10.1124/mol.104.004549
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Effect of pretreatment with ICI174864 (1 mM; 30 min) in the presence (CHX) or absence (CTL) of cycloheximide (5 mg/ml). Emax values for the different ligands were obtained in [35S]GTPγS binding assays.

[1] values expressed as % of unstimulated GTPγS binding and correspond to a minimum of at least 3 experiments

		Emax ± SEM[1]		
	Ligands	**No pretreatment**	**ICI174864**	
CTL	Bremazocine	35 ± 5	66 ± 8	p = 0.002
	Naloxone	-11 ± 4	23 ± 9	p = 0.002
	TICPΨ	-24 ± 5	-5 ± 5	p = 0.035
CHX	Bremazocine	41 ± 6	71 ± 2	p = 0.04
	Naloxone	-22 ± 8	17 ± 15	p = 0.006
	TICPΨ	-36 ± 7	-4 ± 5	p = 0.016

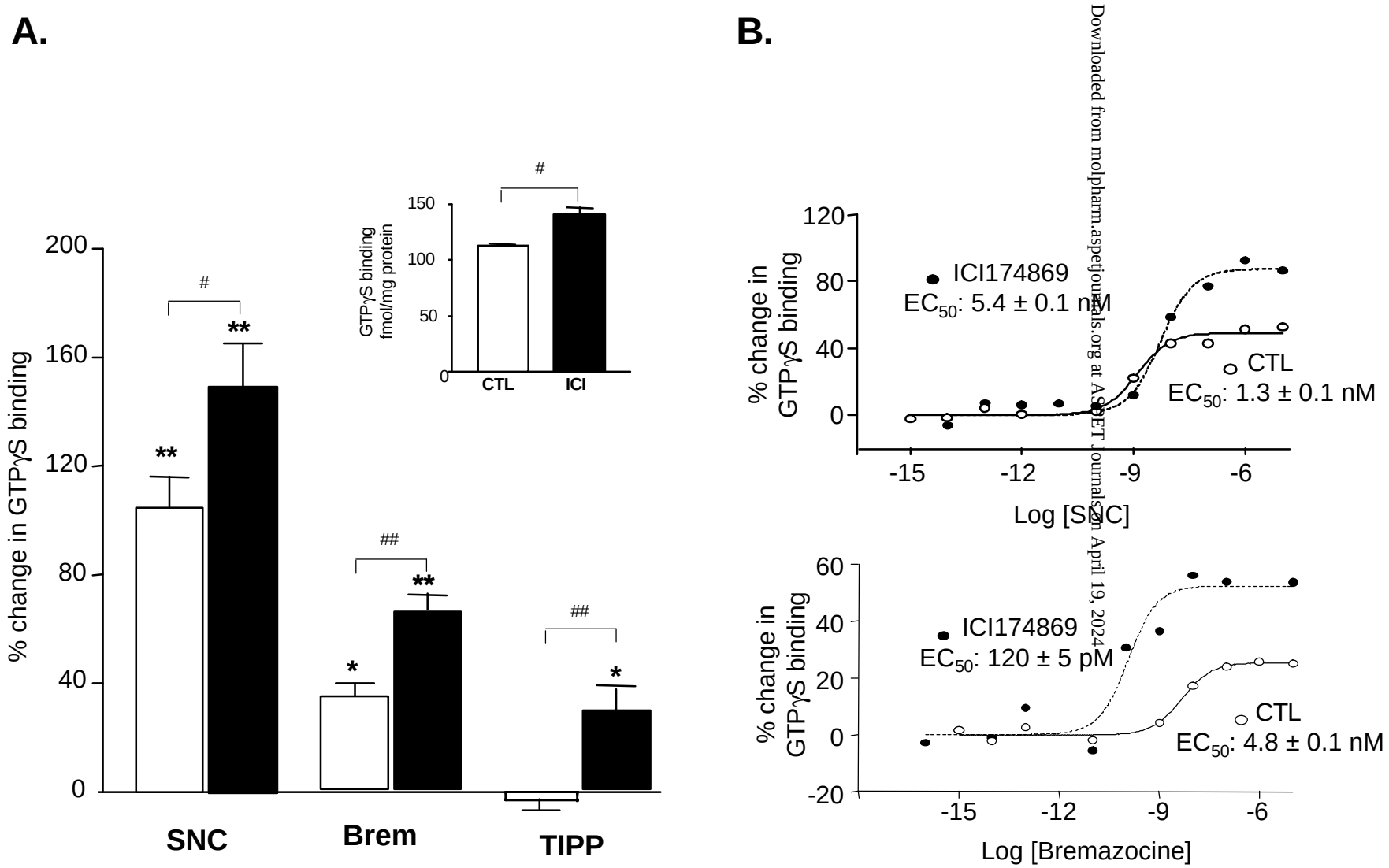


Figure 1

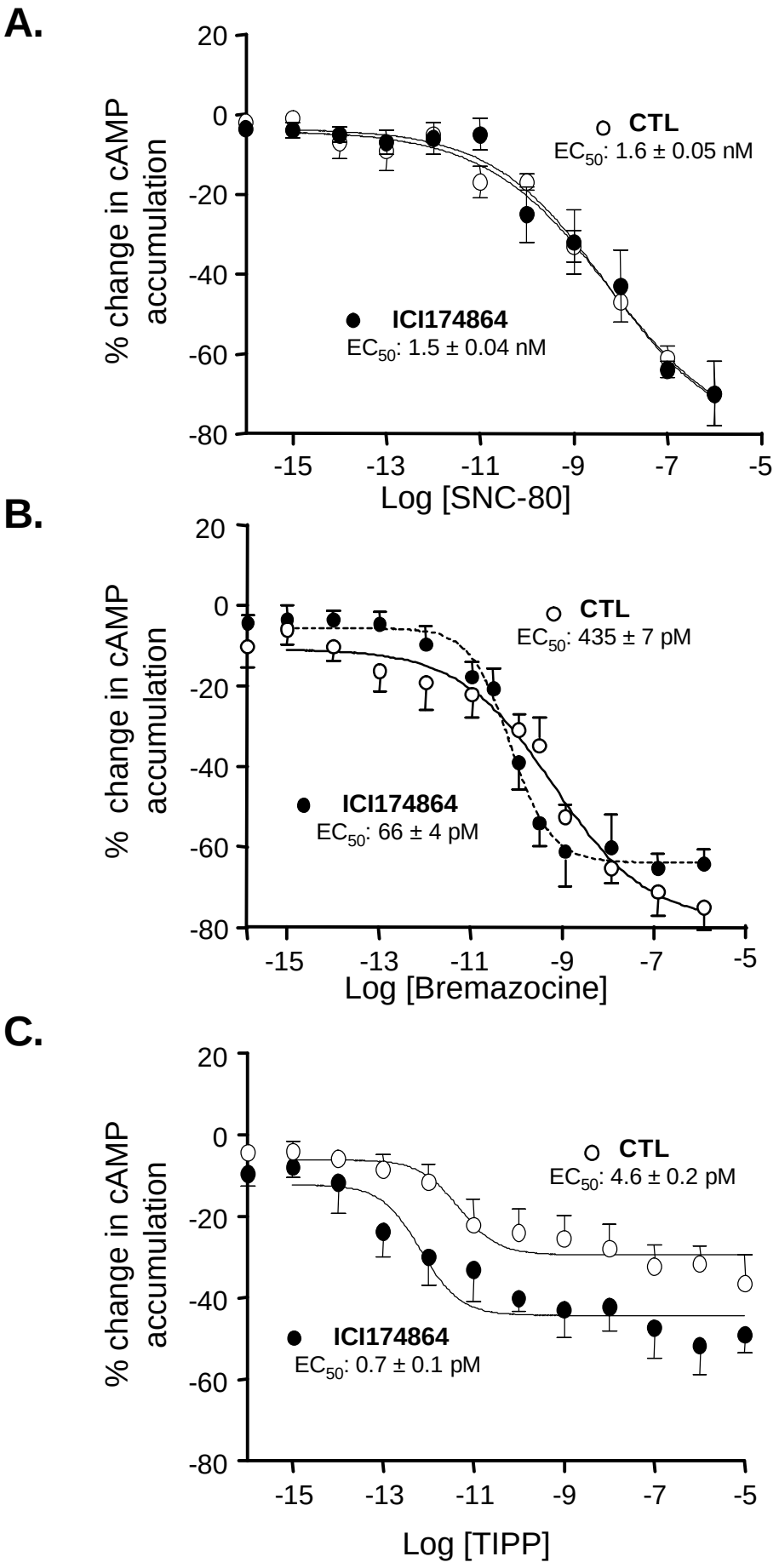

A.
20
0
-20
-40
-60
-80
% change in cAMP accumulation
CTL
EC50: 1.6 ± 0.05 nM
ICI174864
EC50: 1.5 ± 0.04 nM
-15
-13
-11
-9
-7
-5
Log [SNC-80]
B.
20
0
-20
-40
-60
-80
% change in cAMP accumulation
CTL
EC50: 435 ± 7 pM
ICI174864
EC50: 66 ± 4 pM
-15
-13
-11
-9
-7
-5
Log [Bremazocine]
C.
20
0
-20
-40
-60
-80
% change in cAMP accumulation
CTL
EC50: 4.6 ± 0.2 pM
ICI174864
EC50: 0.7 ± 0.1 pM
-15
-13
-11
-9
-7
-5
Log [TIPP]

Figure 2

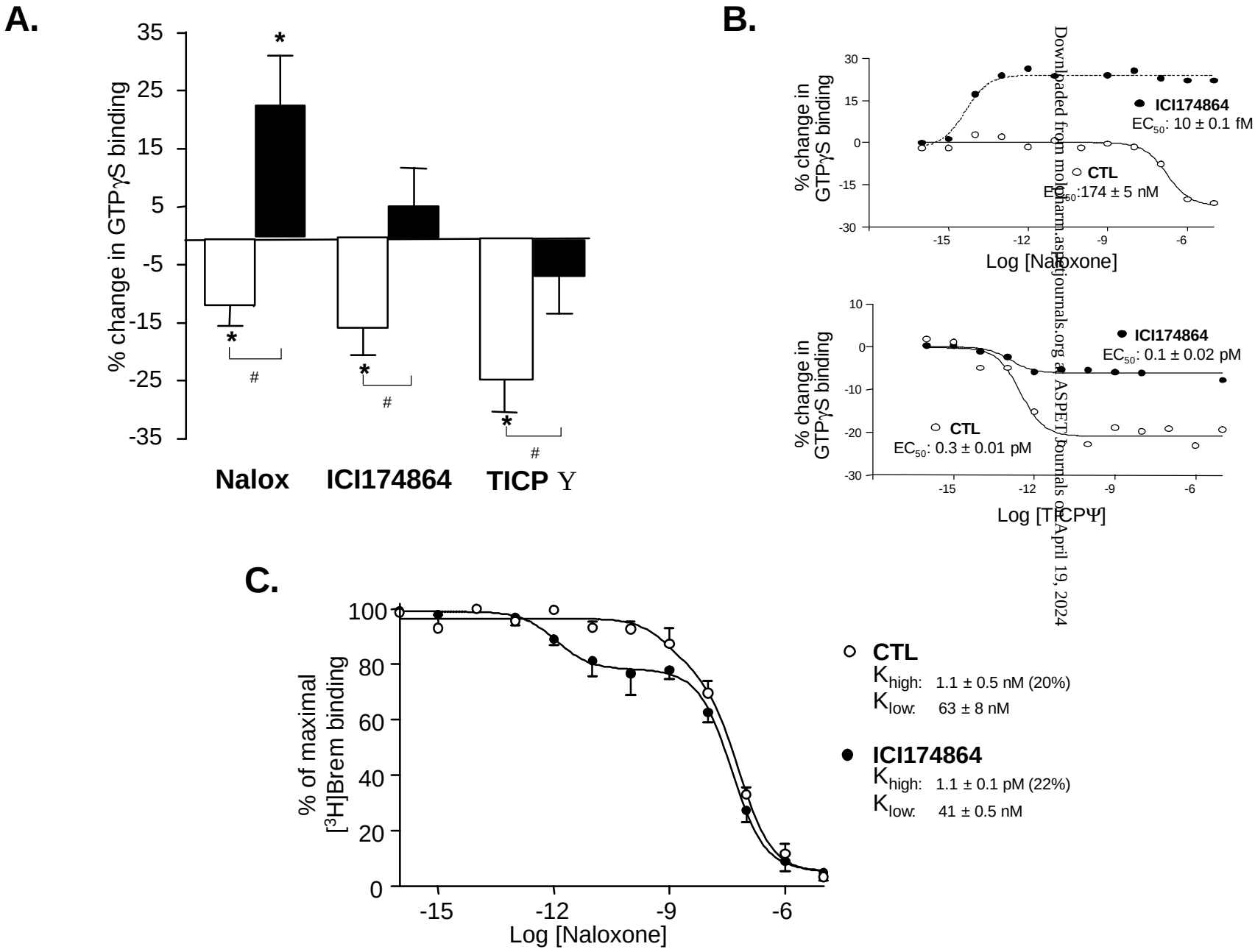


Figure 3

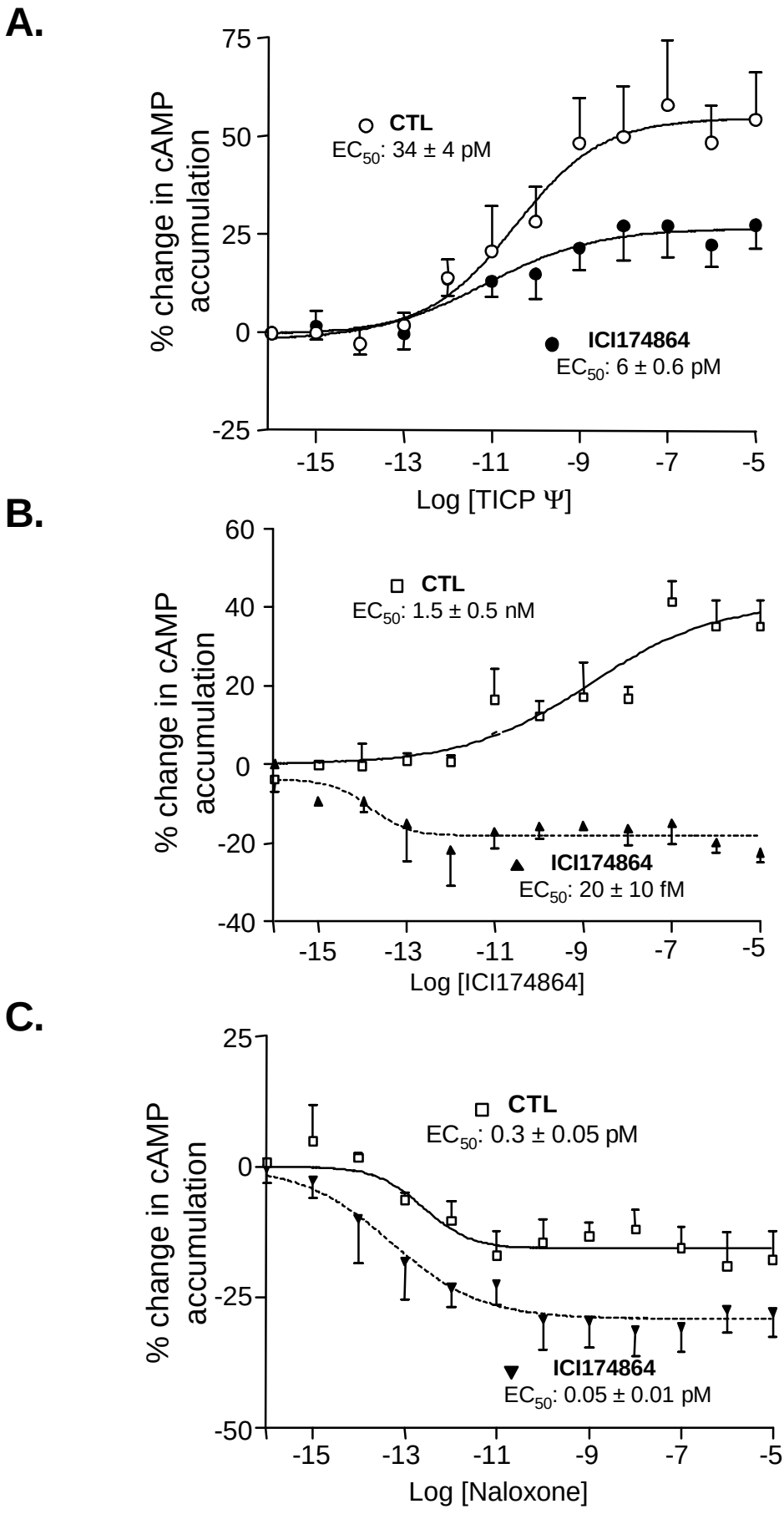

A.
% change in cAMP accumulation
75
50
25
0
-25
CTL
EC50: 34 ± 4 pM
ICI174864
EC50: 6 ± 0.6 pM
-15
-13
-11
-9
-7
-5
Log [TICP Ψ]
B.
% change in cAMP accumulation
60
40
20
0
-20
-40
CTL
EC50: 1.5 ± 0.5 nM
ICI174864
EC50: 20 ± 10 fM
-15
-13
-11
-9
-7
-5
Log [ICI174864]
C.
% change in cAMP accumulation
25
0
-25
-50
CTL
EC50: 0.3 ± 0.05 pM
ICI174864
EC50: 0.05 ± 0.01 pM
-15
-13
-11
-9
-7
-5
Log [Naloxone]

Figure 4

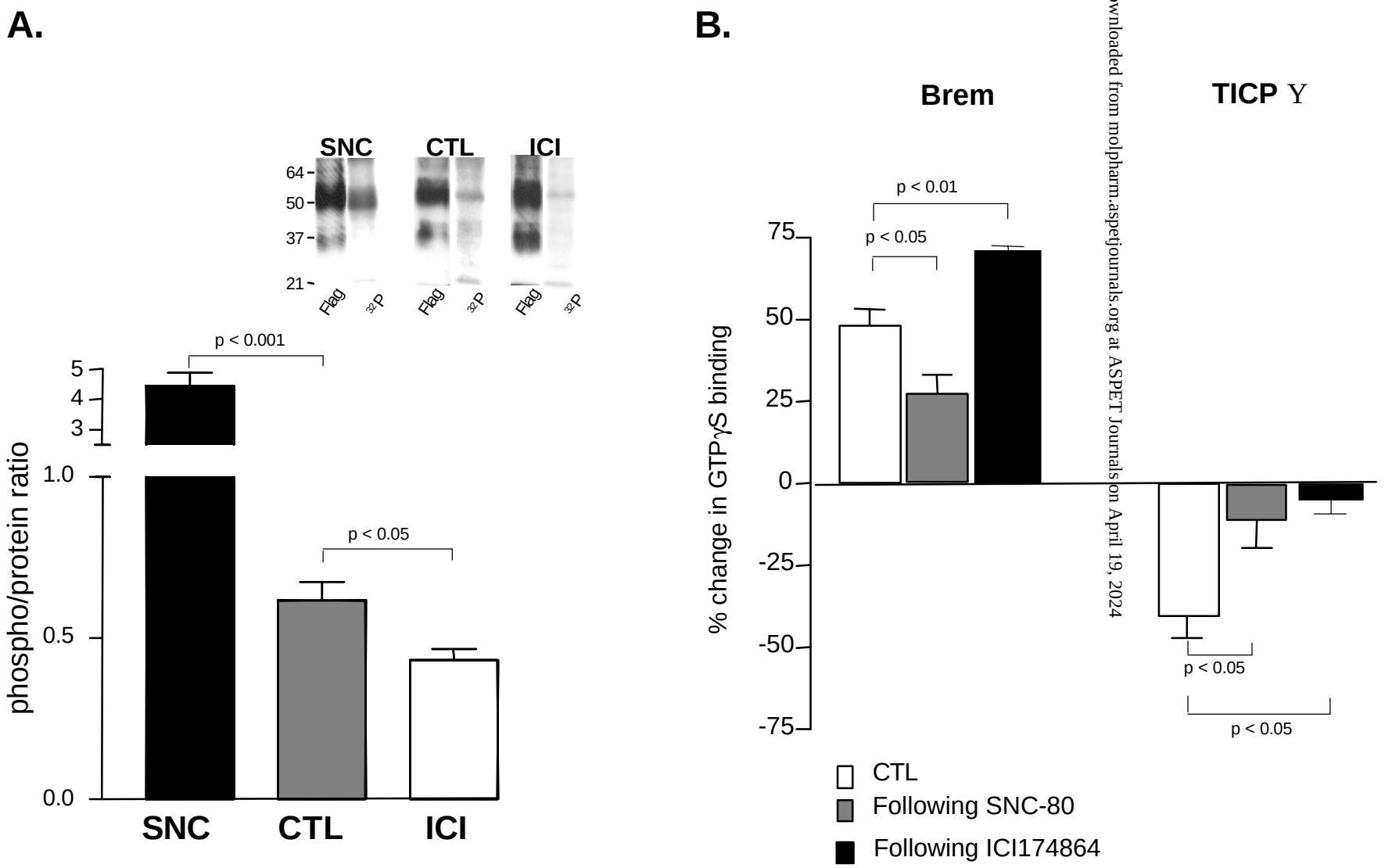


Figure 5

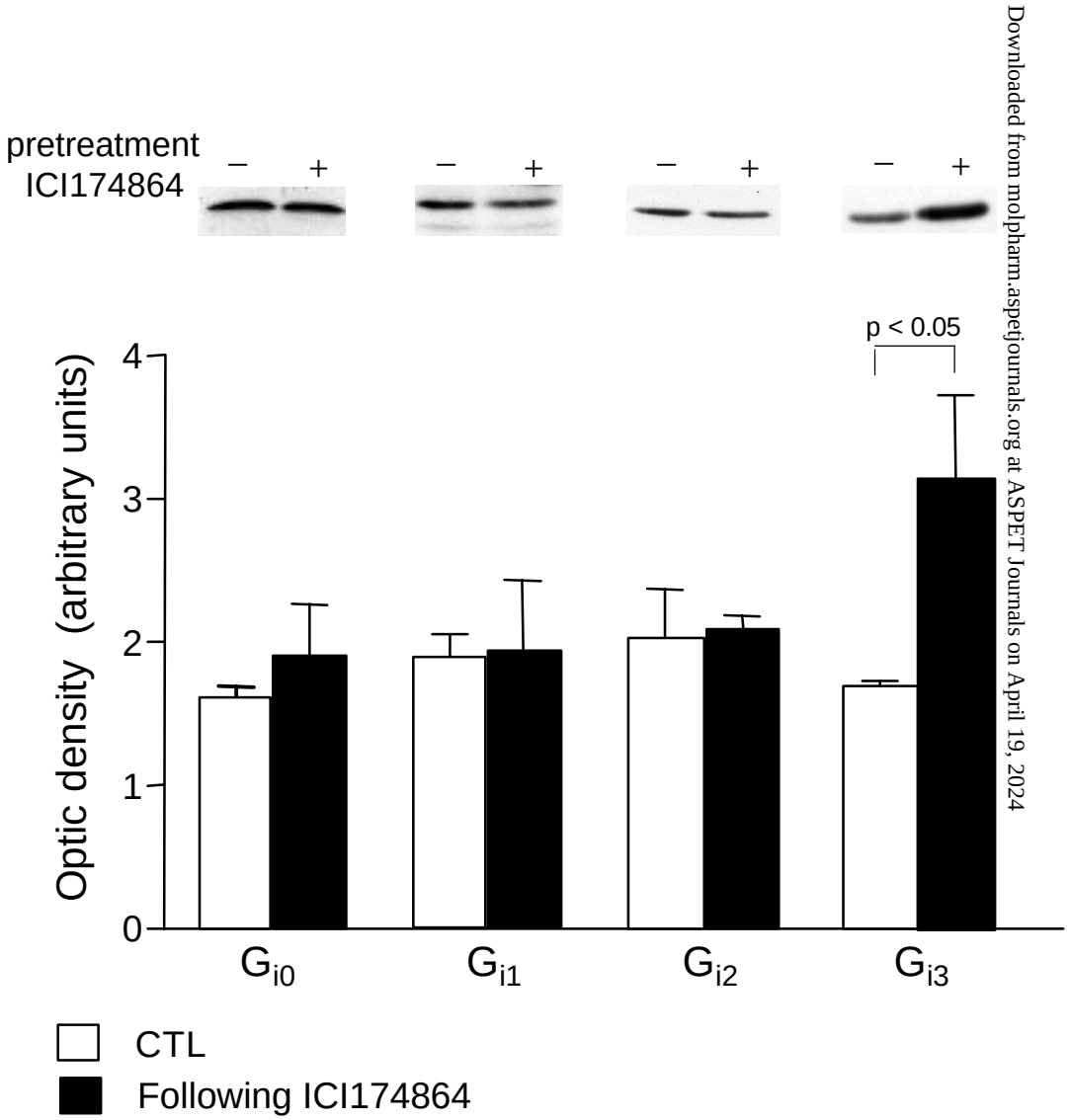
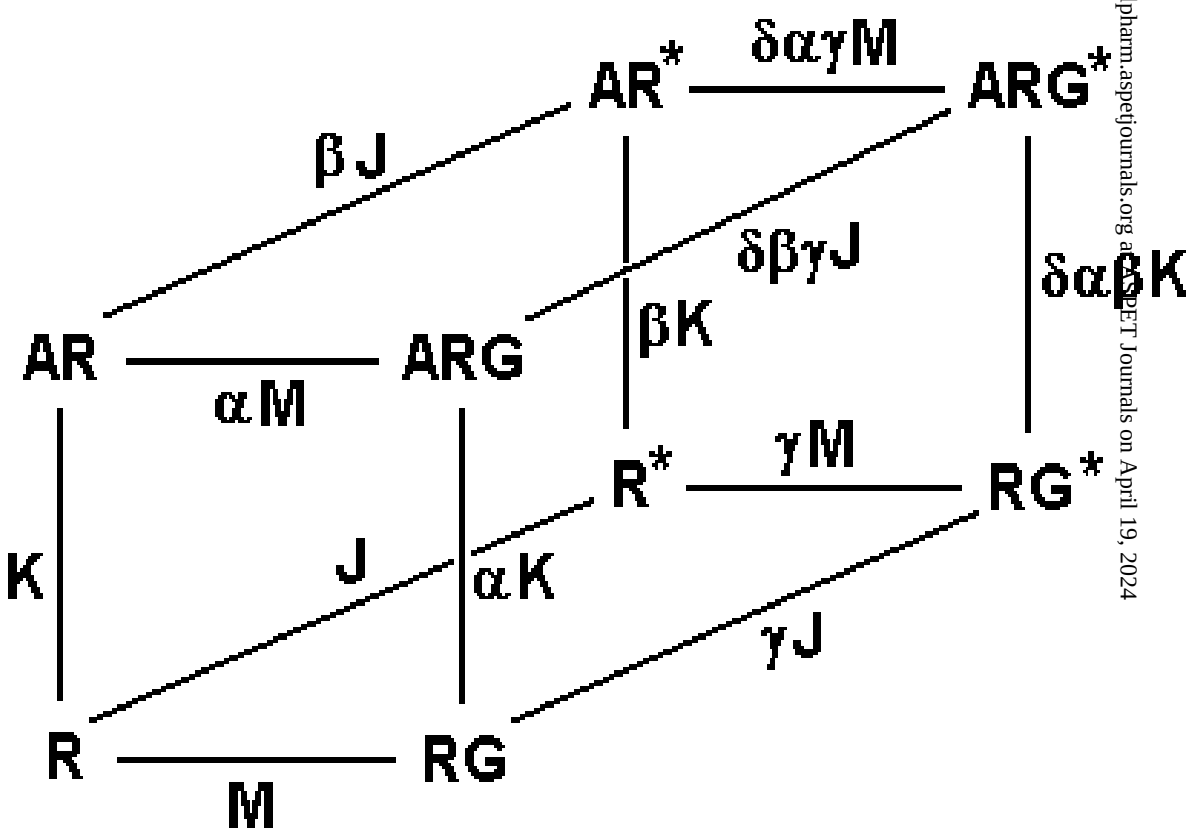
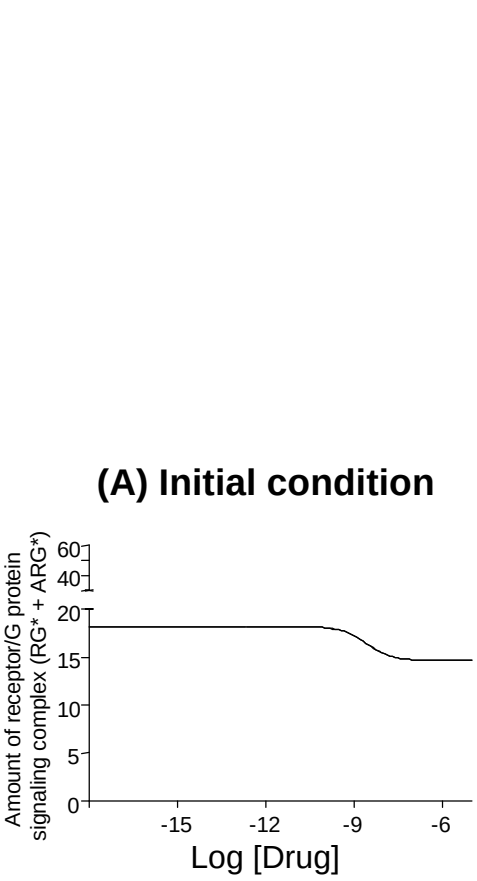
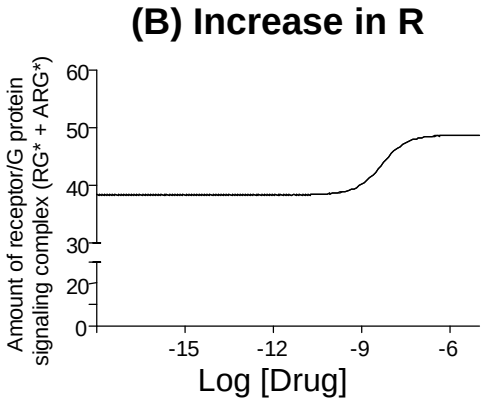


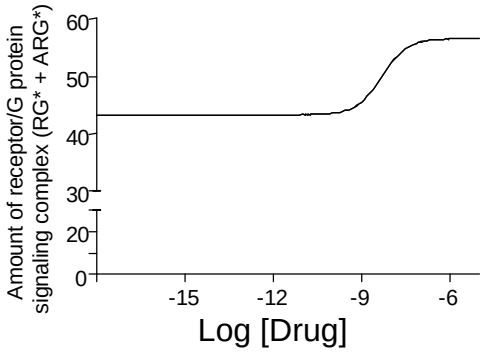
Figure 6

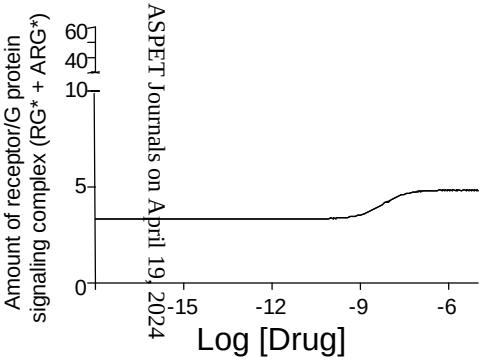


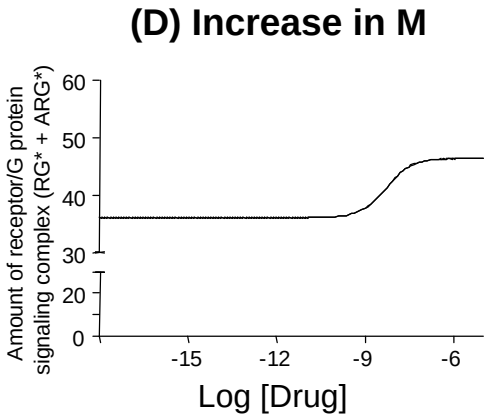
Appendix figure 1



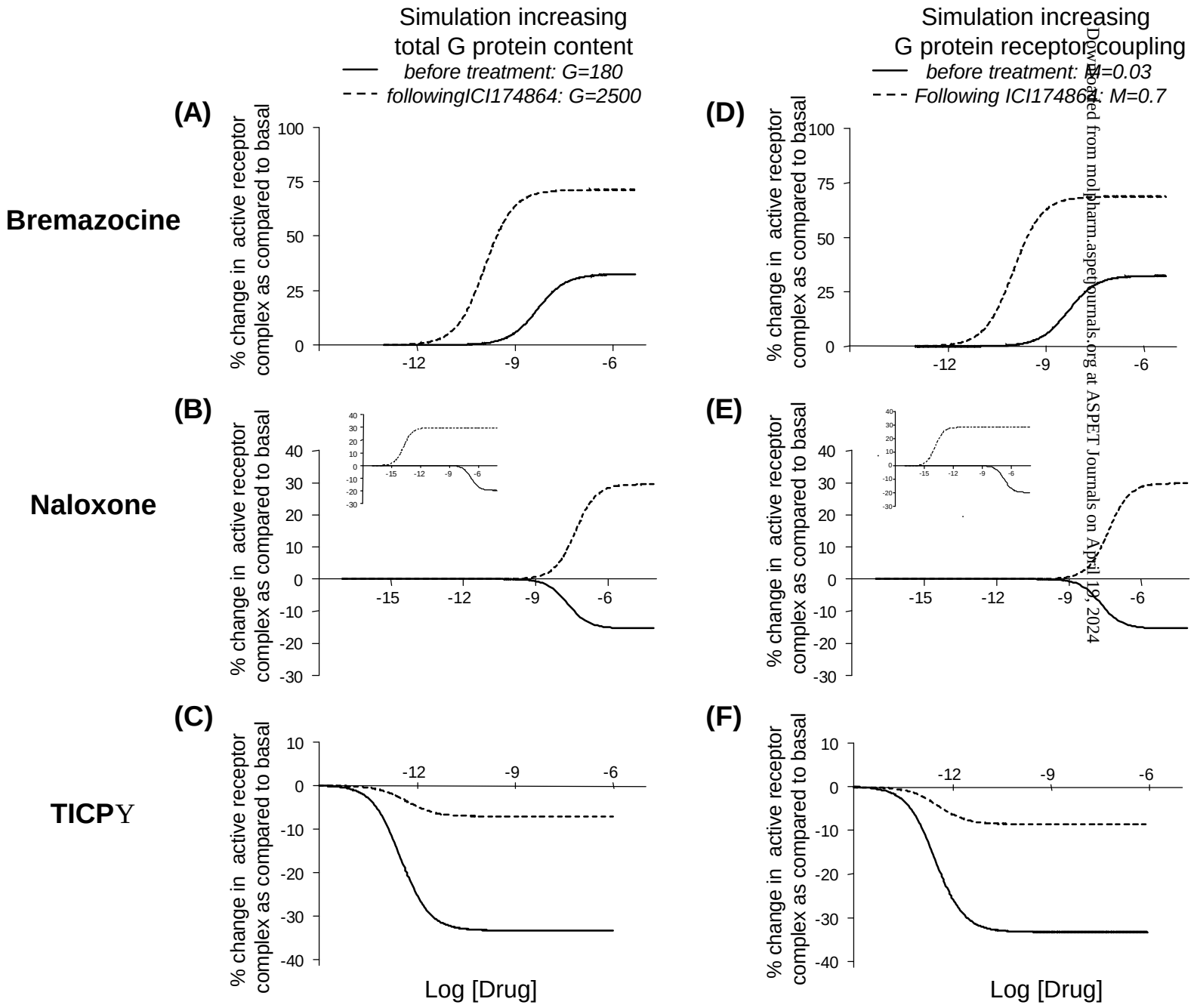








Appendix figure 2



Appendix figure 3