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Reciprocal Regulation of Agonist and Inverse Agonist Signaling Efficacy upon Short-Term Treatment of the Human δ-Opioid Receptor with an Inverse Agonist

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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, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding and cAMP accumulation assays were used to assess the consequences of 30-min exposure to the inverse agonist *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI174864) (1 μM) on δ-opioid receptor signaling efficacy. ICI174864 pretreatment increased maximal effect (E_{max}) for the partial agonist Tyr-1,2,3,4-tetrahydroisoquinoline-Phe-Phe-OH (TIPP) at the two levels of the signaling cascade, whereas E_{max} values for more efficacious agonists like (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC-80) and bremazocine were increased in [^{35}S]GTPγS binding but not in cAMP accumulation assays. Pre-exposure to ICI174864 also induced a shift to the left in dose-response curves for bremazocine and TIPP. On the other hand, E_{max} for the inverse agonist *H*-Tyr-Ticψ[CH_2NH]Cha-Phe-OH was reduced in both assays, but no changes in potency were observed. For the weaker inverse agonist naloxone, E_{max} in [^{35}S]GTPγS binding was drastically modified because the drug turned from inverse agonist to agonist after ICI174864 pretreatment. Likewise, 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.

G protein-coupled receptors are the single largest family of membrane proteins capable of transducing extracellular cues into cellular responses. This process usually consists of two identifiable phases: 1) signal detection and transduction, and 2) 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 (Valiquette et al., 1990; Tsao and von Zastrow, 2000), but also by a decrease in transcription efficiency (Chau et al., 1994; Blaukat et al., 2003).

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

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ABBREVIATIONS: δOR, δ opioid receptor; DMEM, Dulbecco's modified Eagle's medium; SNC-80, (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; ICI174864, *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)-triphosphate; TIPP, Tyr-1,2,3,4-tetrahydroisoquinoline-Phe-Phe-OH; TICPψ, *H*-Tyr-Ticψ[CH_2NH]Cha-Phe-OH; h, human; HEK, human embryonic kidney; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; CTL, control; ANOVA, analysis of variance.

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 promote the inactivation of different Gα subunits by stimulating GTP hydrolysis (De Vries et al., 2000). In the specific case of δOR and Gα_{i3}, before a regulator of G protein signaling interacts with the α subunit, the receptor and Gα must translocate together from their initial location in nonclathrin domains of the plasma membrane into clathrin-coated pits (Elenko et al., 2003). Thereafter, the receptor is internalized while Gα_{i3} stays at the membrane (Elenko et al., 2003). In other instances, translocation takes place between the plasma membrane, and the cytosol as has been described for Gα_s after palmitoylation (Ransnas et al., 1989; Wedegaertner et al., 1996). On the other hand, if receptor stimulation takes place over prolonged periods of time, Gα 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). Whereas agonists are defined as drugs that enhance receptor activation, inverse agonists have been characterized as ligands that reduce constitutive receptor activity (Kenakin, 2004). Like agonists, these new ligands also induce adaptive responses that modify signaling efficacy. In particular, 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 Gα_{i3} immunoreactivity associated with cell membranes. These changes were accompanied by functional sensitization of the system toward 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.

Materials and Methods

Reagents. Buffer chemicals, protease inhibitors, naloxone, forskolin, isobutyl-methyl-xanthine, anti-FLAG M2 affinity resin, and FLAG peptide were purchased from Sigma-Aldrich (St. Louis, MO). [^{35}S]GTPγS, [^{3}H]adenosine, and [^{32}P]ATP were from PerkinElmer Life and Analytical Sciences (Boston, MA). ICI174864 and SNC-80 were obtained from Tocris Cookson Inc. (Ellisville, MO), and bremazocine was from Sigma/RBI (Natick, MA). TIPP and TICPΨ were synthesized as described previously (Schiller et al., 1999). G418, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, fungizone, glutamine, penicillin, and streptomycin were purchased from WISENT Inc. (Saint-Jean-Baptiste de Rouville, Quebec, Canada).

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

Cell Culture and Transfection. HEK293s cells were transfected using the calcium-phosphate precipitation method, and clones stably expressing the hδOR-FLAG were selected using 400 μg/ml G418 (Wisent). Cells were grown and maintained in complete DMEM containing 10% (v/v) fetal bovine serum, 1000 U/ml penicillin, 1 mg/ml streptomycin, and 1.5 μg/ml fungizone in a humidified atmosphere of 5% CO_2 at 37°C.

Treatments. The day of the experiment, cells were detached and incubated for 30 min in DMEM containing either vehicle (0.01% dimethyl sulfoxide), 1 μM ICI174864, or 1 μM SNC-80. Reaction was stopped by dilution in cold PBS, and cells were washed three times for 5 min in 50 volumes of PBS at room temperature. 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 5 μg/ml, and ICI174864 (1 μM final concentration) was added during the last 30 min of incubation.

Membrane Preparation. Membranes were prepared as described previously (Pineyro et al., 2001) after repeated washing to remove pretreatment drug. In brief, 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 & Kunkel, Staufen, Germany) 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 unlabeled naloxone in a final volume of 300 μl. Nonspecific binding was defined with 10 μM naloxone. The reaction was terminated by rapid filtration using GF/C filters, and radioactivity was determined by liquid scintillation counting. Affinity values (K_i) were calculated from a nonlinear least-squares analysis of displacement data using Prism (GraphPad Software, Inc., San Diego, CA).

[^{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 dithiothreitol, 0.5% bovine serum albumin, and 3 μM GDP, pH 7.4) to yield 10 μg protein/tube. [^{35}S]GTPγS was used at a 30 to 50 nM concentration, and nonspecific 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 1 h at room temperature. The reaction was terminated by rapid filtration onto Whatman GF/C glass filters presoaked in water (Whatman, Clifton, NJ). 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 h) with complete DMEM containing 1 μCi/ml [^{3}H]adenine. The day of the experiment, radioactive medium was replaced with fresh DMEM, cells were mechanically detached and thoroughly washed (three times) with PBS (4°C), and viability was assessed using trypan blue (mortality was never higher than 5%). Cells (5×10^5) were then incubated for 20 min at 37°C in 300 μl of assay mixture

containing PBS, 25 μM forskolin, 2.5 μM isobutyl-methyl-xanthine, and different drugs at the indicated concentrations. At the end of the incubation period, the assay was terminated by adding 600 μl of ice-cold solution containing 5% trichloroacetic acid, 5 mM ATP, and 5 mM 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 2 h, after which [^{32}P]orthophosphoric acid was added at a final concentration of 1 mCi/ml, and incubation was allowed to proceed for an additional hour. At this time, SNC-80 (1 μM), ICI174864 (1 μM), or vehicle (0,01% dimethyl sulfoxide) was added to the incubation medium for a 30-min treatment. Cells were then recovered, membranes were prepared as indicated above, and they were finally suspended in solubilization buffer consisting of 0.5% *N*-dodecyl maltoside (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 phenylmethylsulfonyl fluoride, and 2 mM 1,10-phenanthrolene. After agitation at 4°C for 60 min, the solubilized fraction was centrifuged at 100,000*g* for 60 min, and the receptor was immunoprecipitated from the supernatant fraction using anti-FLAG M2 antibody resin. A 20-μl sample of antibody-coupled resin equilibrated in solubilization buffer and supplemented with 0.1% bovine serum albumin (w/v) was used to purify the receptor overnight at 4°C under gentle agitation. The next morning, the resin was pelleted and 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*-dodecyl maltoside (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/μl FLAG peptide. 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 Corporation, Billerica, MA). SDS sample buffer (25 μl) was then added, and samples were used for SDS-PAGE.

SDS-PAGE and Western Blotting. SDS-PAGE was performed with the Laemmli method 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; Bio-Rad, Hercules, CA) from the gels onto nitrocellulose (Amersham Biosciences Inc., Piscataway, NJ). In the case of phosphorylation studies, membranes were first used for autoradiography (BIOMAX films; Eastman Kodak, Rochester, NY) 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 $G_{\alpha o}$, $G_{\alpha i1}$, $G_{\alpha i2}$, or $G_{i\alpha 3}$ (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies (Amersham) and enhanced chemiluminescence detection reagents (PerkinElmer) were used to reveal the blotted proteins. The relative intensities of the labeled bands were analyzed by densitometric scanning.

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, after 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 fmol/mg protein in controls to 141 ± 6 fmol/mg protein after pretreatment with ICI174864 ($n = 4$, $p < 0.05$) (Fig. 1A, 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% ($n = 8$, $p < 0.05$) and that of bremazocine from 35 ± 5 to 66 ± 8% ($n = 18$, $p < 0.01$) (Fig. 1A). It is interesting that these increases were correlated to the efficacy of the compound tested because the ability of SNC-80 to promote guanine nucleotide binding was increased 1.4-fold, whereas that of the weaker agonist bremazocine (Pineyro 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 ± 5 versus 30 ± 9%; $n = 13$, $p < 0.01$) (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 (Table 1), indicating that this effect was not caused by an increase in the synthesis of receptor protein or other proteins of the signaling cascade. 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 Fig. 1B, the EC_{50} value for bremazocine was shifted to the left by a factor of 24, whereas that of SNC-80 remained unchanged.

Results from cAMP accumulation assays appear in Fig. 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 after ICI174864, −0.9 ± 0.1; $n = 4$; $p < 0.05$). Only in the case of the weakest agonist, TIPP, did exposure to ICI174864 enhance both maximal cAMP inhibition (CTL, 29 ± 2% inhibition in cAMP accumulation; after pretreatment with ICI174864, 44 ± 3 inhibition in cAMP accumulation) and potency (EC_{50} in CTL, 4.6 ± 0.2 pM; EC_{50} after ICI174864, 0.7 ± 0.02 pM; $n = 8$, $p < 0.001$).

Comparison of E_{max} values obtained in [^{35}S]GTPγS binding (Fig. 1A) and in cAMP accumulation assays (Fig. 2) in the nonpretreated condition indicates that progression along the signaling cascade was associated with amplification of the signals induced by the different agonists. For example, although 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%; bremazocine, 35 ± 5%; $p < 0.001$), this difference was no longer evident in cAMP accumulation assays (SNC-80, 70 ± 8% inhibition in cAMP accumulation; bremazocine, 75 ± 6% inhibition in cAMP accumulation) (compare Figs. 1A and 2, A and B). TIPP, which in control conditions did not induce measurable

changes in [^{35}S]GTPγS binding (E_{max}, −5 ± 5%) (Fig. 1A), displayed clear agonist behavior when tested in cAMP accumulation assays (E_{max}, 29 ± 3% inhibition in cAMP accumulation; $n = 8$, $p < 0.01$) (Fig. 2C). On the other hand, signal amplification reduced the impact of inverse agonist pretreatment on E_{max}, as exemplified by the fact that pre-exposure to ICI174864 increased E_{max} 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 (Fig. 3A). TICPΨ, the drug that produced the greatest reduction in [^{35}S]GTPγS binding, produced a nonsignificant inhibition after ICI174864 pretreatment (CTL, 24 ± 5% inhibition in [^{35}S]GTPγS binding; after ICI174864, 6 ± 6% inhibition in [^{35}S]GTPγS binding; $n = 12$, $p < 0.05$) (Fig. 3A). Similar reduction in TICPΨ response was observed if pretreatment with ICI174864 was performed in the presence of cycloheximide (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 after exposure to ICI174864 (23 ± 9% increase in [^{35}S]GTPγS binding) (Fig. 3A), an effect that was also observed in cycloheximide-treated cells (Table 1). Finally, pretreatement with ICI174864 reduced its own inverse efficacy (CTL, 16 ± 5% inhibition in [^{35}S]GTPγS binding; after ICI174864, 5 ± 8% increase in [^{35}S]GTPγS binding; $n = 17$, $p < 0.05$) (Fig. 3A). Figure 3B shows dose-response curves for compounds in which EC_{50} values could be 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 (CTL EC_{50}, 174 ± 5 nM; after ICI174864, 0.01 ± 0.001 pM). In addition, displacement experiments 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, Fig. 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 (Fig. 4), the maximal effect produced by TICPΨ was significantly reduced by pretreatment with ICI174864 (CTL, 55 ± 12% increase in accumulated cAMP; after ICI174864, 26 ± 5% increase in accumulated cAMP; $n = 6$, $p < 0.05$). In contrast, the inverse response elicited by

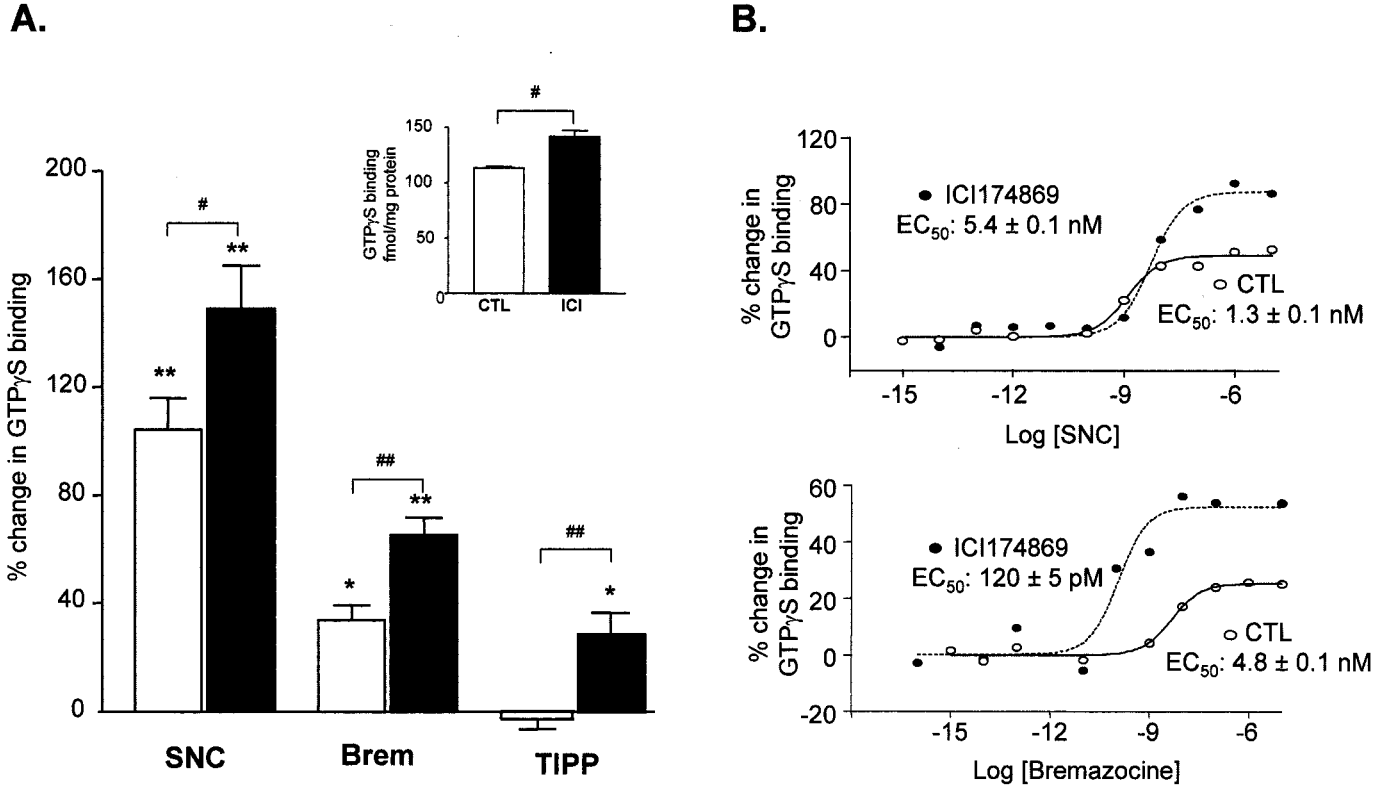

Fig. 1. Effect of pretreatment with ICI174864 on [^{35}S]GTPγS binding. HEK293s cells expressing hδOR-Flag were incubated (■) or not (□) with ICI174864 (1 μM for 30 min) and used to prepare membranes for [^{35}S]GTPγS binding. A, E_{max} values for different δOR ligands (1 μM). Values are expressed as percentage change in [^{35}S]GTPγS binding observed in the presence of each δOR ligand compared with the binding observed in its absence (percentage change in GTPγ[^{35}S] binding = {[GTPγ[^{35}S]$bound_{ligand}$ − GTPγ[^{35}S] $bound_{no\ ligand}$]/GTPγ[^{35}S]$bound_{no\ ligand}$}×100) and represent the mean ± S.E.M. of 8 to 18 independent experiments carried out in triplicate. 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 percentage drug effects in control and treated membranes (#, $p < 0.05$; ##, $p < 0.01$). Inset, absolute values for basal [^{35}S]GTPγS binding in controls (□) or after pretreatment with ICI174864 (■). Results are expressed as femtomoles per milligram of protein, and statistical significance of changes induced by ICI174864 pretreatment was established using unpaired t tests 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 (○) and after pretreatment with ICI174864 (●). SNC, SNC-80; Brem, bremazocine; ICI, ICI174869.

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 exposed previously to ICI174864 itself ($n = 3$, $p < 0.001$) (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 decreased from 1.5 ± 0.5 nM in controls to 0.02 ± 0.01 pM ($n = 3$; $p < 0.001$) (Fig. 2B) in pretreated cells. Concerning naloxone's behavior in cAMP accumulation assays, the observed effect in nontreated cells was that of a partial agonist (18 ± 5% reduction in cAMP accumulation). After pretreatment with ICI174864, both E_{max} values (28 ± 5% reduction in cAMP accumulation; $n = 4$, $p < 0.001$) and drug potency were increased (EC_{50} CTL, 0.3 ± 0.05 pM; after ICI174864, 0.05 ± 0.01 pM; $n = 4$, $p < 0.001$) (Fig. 2C), as had been observed previously for TIPP.

Comparison of [^{35}S]GTPγS binding results with results from cAMP accumulation assays in the nonpretreated 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 after ICI17484 pretreatement. In contrast and despite its protean behavior after short-term inverse agonist pretreatment, the effect of ICI174864 in the nonpretreated condition was that of an inverse agonist along the two consecutive levels of the signaling cascade, as was also the case for TICPψ (Fig. 2). Comparison of control E_{max} values for ICI174864 and TICPψ in [^{35}S]GTPγS binding (Fig. 1, A and B) 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 prelabeled with [^{32}P]orthophosphoric acid and exposed or not to SNC-80 or ICI174864 (1 μM for 30 min). Receptors were then immunopurified, and the purification product was resolved on SDS-PAGE. After transfer onto nitrocellulose, membranes were first exposed for autoradiography and then blotted with anti-FLAG M2 antibody (Fig. 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 that the slight increase in immunoreactivity of the immature form of the receptor after pretreatment with ICI174864 was a nonreproducible, 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 ($n = 3$, $p < 0.001$) whereas 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-

TABLE 1
Effect of pretreatment with ICI174864 (1 mM for 30 min) in the presence or absence of cycloheximide (5 mg/ml)
E_{max} values for the different ligands were obtained in [^{35}S]GTPγS binding assays. Values are expressed as the percentage of unstimulated GTPγS binding ± S.E.M. and correspond to a minimum of three experiments.

Ligands	No Pretreatment	ICI174864	p
Control			
Bremazocine	35 ± 5	66 ± 8	0.002
Naloxone	−11 ± 4	23 ± 9	0.002
TICPΨ	−24 ± 5	−5 ± 5	0.035
Cycloheximide			
Bremazocine	41 ± 6	71 ± 2	0.04
Naloxone	−22 ± 8	17 ± 15	0.006
TICPΨ	−36 ± 7	−4 ± 5	0.016

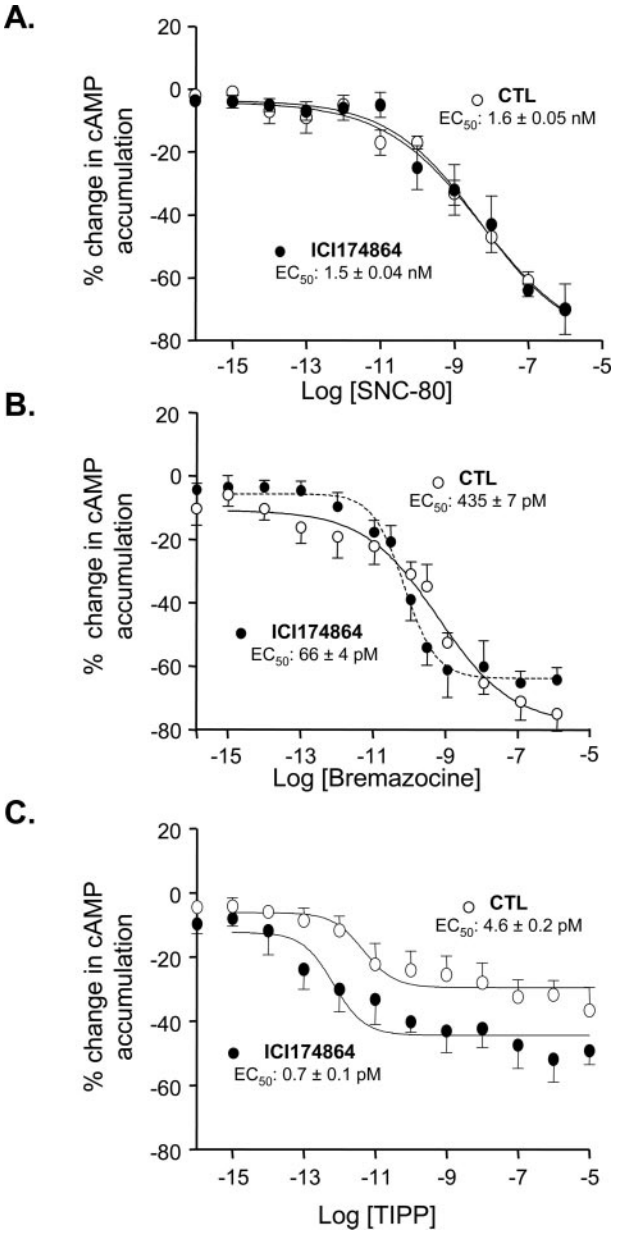

Fig. 2. Effect of pretreatment with ICI174864 on the regulation of cAMP accumulation by SNC-80, bremazocine, and TIPP. Dose-response curves for the indicated ligands were obtained in control cells and cells that had been previously exposed to ICI174864 (1 μM for 30 min). Results are expressed as the percentage change in cAMP accumulation in the presence of the indicated ligand compared with cAMP accumulation observed in its absence (percentage change in cAMP accumulation = {[$cAMP_{ligand}$ − $cAMP_{no\ ligand}$]/$cAMP_{no\ ligand}$} × 100). 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, nonsignificant; bremazocine, $p < 0.05$; TIPP, $p < 0.01$. ○, controls; ●, after pretreatment with ICI174864.

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 for 30 min) or ICI174864 (1 μM for 30 min), after which [^{35}S]GTPγS binding assays were run. Figure 5B shows that even though ICI174864 and SNC-80 modified E_{max} for bremazocine in opposite directions, the same does not apply for TICPΨ, in which 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 for 30 min) and then resolved on SDS-PAGE. Western blot analyses were carried out using antibodies capable of discriminating between the different subtypes of $G_{i/o}$ proteins that may potentially couple to δOR (α_o and α_{i1-3}) (Fig. 4). In all cases, immunoblots revealed monomeric bands located in

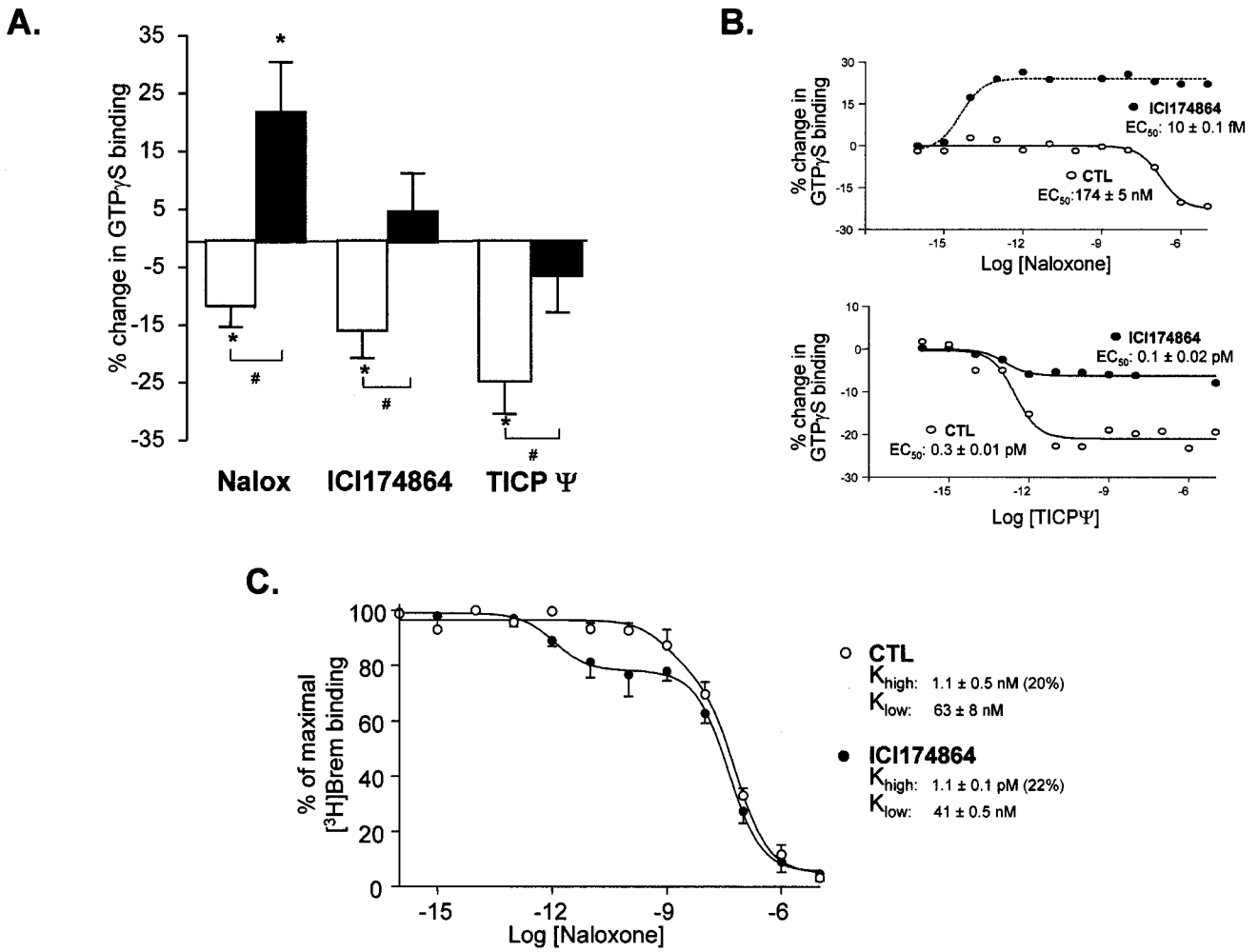

Fig. 3. Effect of pretreatment with ICI174864 on the regulation of [^{35}S]GTPγS binding by naloxone (Nalox), ICI174864, and TICPΨ. HEK293s cells expressing hδOR-FLAG were incubated (■) or not (□) with ICI174864 (1 μM for 30 min) and used to prepare membranes for [^{35}S]GTPγS binding. A, E_{max} values for different δOR ligands (1 μM). Values are expressed as the percentage change in [^{35}S]GTPγS binding observed in the presence of each δOR ligands compared with the binding observed in its absence (percentage change in GTPγ[^{35}S] binding = {[GTPγ[^{35}S] $\text{bound}_{\text{ligand}}$ − GTPγ[^{35}S]$\text{bound}_{\text{no ligand}}$]/GTP$\gamma$[^{35}S] $\text{bound}_{\text{no ligand}}$} × 100) and represent the mean ± S.E.M. of 10 to 18 independent experiments carried out in triplicate. 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 (⋆, $p < 0.05$). The statistical significance of changes induced by ICI174864 pretreatment was established using t tests to compare percentage drug effects in control and treated membranes (#, $p < 0.05$). B, representative dose-response curves for naloxone and TICPΨ carried out in controls (○) and after pretreatment with ICI174864 (●). C, displacement of [^{3}H]bremazocine (5 nM) by increasing concentrations of naloxone in control membranes (○) and in membranes obtained from cells that were pretreated with ICI174864 (1 μM for 30 min; ●). In both cases, results were best fit by a two-site model, and calculated K_i values for high- and low-affinity sites are shown. Statistical significance of the difference between the two curves was established using two-way ANOVA ($n = 3$, $p < 0.01$).

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 after exposure to ICI174864. However, the antibody that preferentially recognized $G\alpha_{i3}$ showed that 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, inverse agonist pretreatment did not change bands labeled by antibodies with preferential affinity for $G\alpha_{i1}$ and $G\alpha_{i2}$, which confirms that pre-exposure to ICI174864 selectively increased $G\alpha_{i3}$ immunoreactivity in membrane preparations.

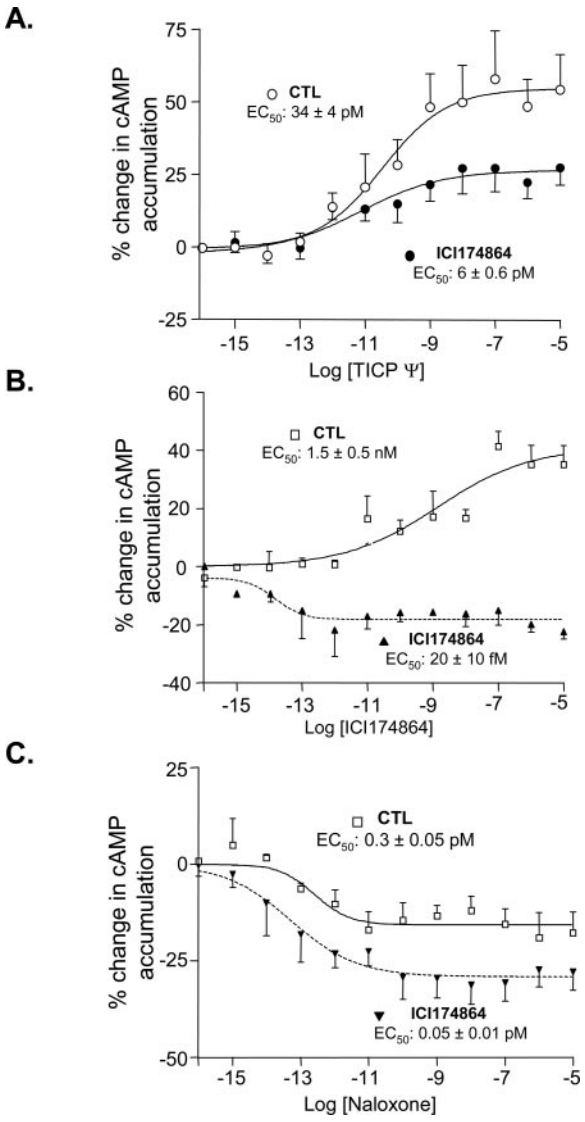

Fig. 4. Effect of pretreatment with ICI174864 on the regulation of cAMP accumulation by TICPΨ (A), ICI174864 (B), and naloxone (C). Dose-response curves for the indicated ligands were obtained in control cells and cells that had been exposed previously to ICI174864 (1 μM for 30 min). Results are expressed as the percentage change in cAMP accumulation in the presence of the indicated ligand compared with cAMP accumulation observed in its absence (percentage change in cAMP accumulation = $\{[cAMP_{ligand} - cAMP_{no\ ligand}]/cAMP_{no\ ligand}\} \times 100$). 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, $p < 0.01$; Naloxone, $p < 0.01$; ○, controls; ●, after pretreatment with ICI174864.

Discussion

This 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 (Figs. 1 and 2) and reduced or abolished inverse agonist effects (Figs. 3 and 4). Functional changes were accompanied by a reduction in basal receptor phosphorylation (Fig. 5A) and an increase in $G\alpha_{i3}$ immunoreactivity from membrane preparations (Fig. 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 (Fig. 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 (Fig. 2A). Most probably because SNC-80 induced maximal inhibition in the control situation, there was no room left for sensitization after 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; 1997b). 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 stoichiometry. 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 cAMP accumulation assays despite the fact that no response was detected at the less amplified level of G protein signaling (Figs. 1 and 2). SNC-80 and bremazocine are two nonpeptidic ligands for δORs, and as such 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 to be 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 after pre-exposure to ICI174864. Yet its E_{max} was considerably reduced (Figs. 3 and 4), as would be expected for an affinity-driven ligand. In the cases 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 (Fig. 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 (Fig. 4B). In addition to the effect of pretreatment with ICI174864, the protean nature of nal-

oxone was also revealed in the control situation, because 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 of whether "protean" or "dual" behavior was observed because of different assay conditions or because of pretreatment with ICI174864, reversal of drug efficacy may be explained by changes in the spontaneous activity of the system producing the response (see below).

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. Whereas 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 signaling in specific conditions (Chidiac et al., 1996; Azzi et al., 2003; 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 because short-term treatment with either of them reduced inverse response for TICP. These new sets 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 (Ghanouni et al., 2001; Kenakin, 2004). 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 G protein-coupled receptor, 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/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 signaling (Kouhen et al., 2000; Law et al., 2000), partly caused by uncoupling of the receptor from the G protein. Thus, the observation that short-term exposure to

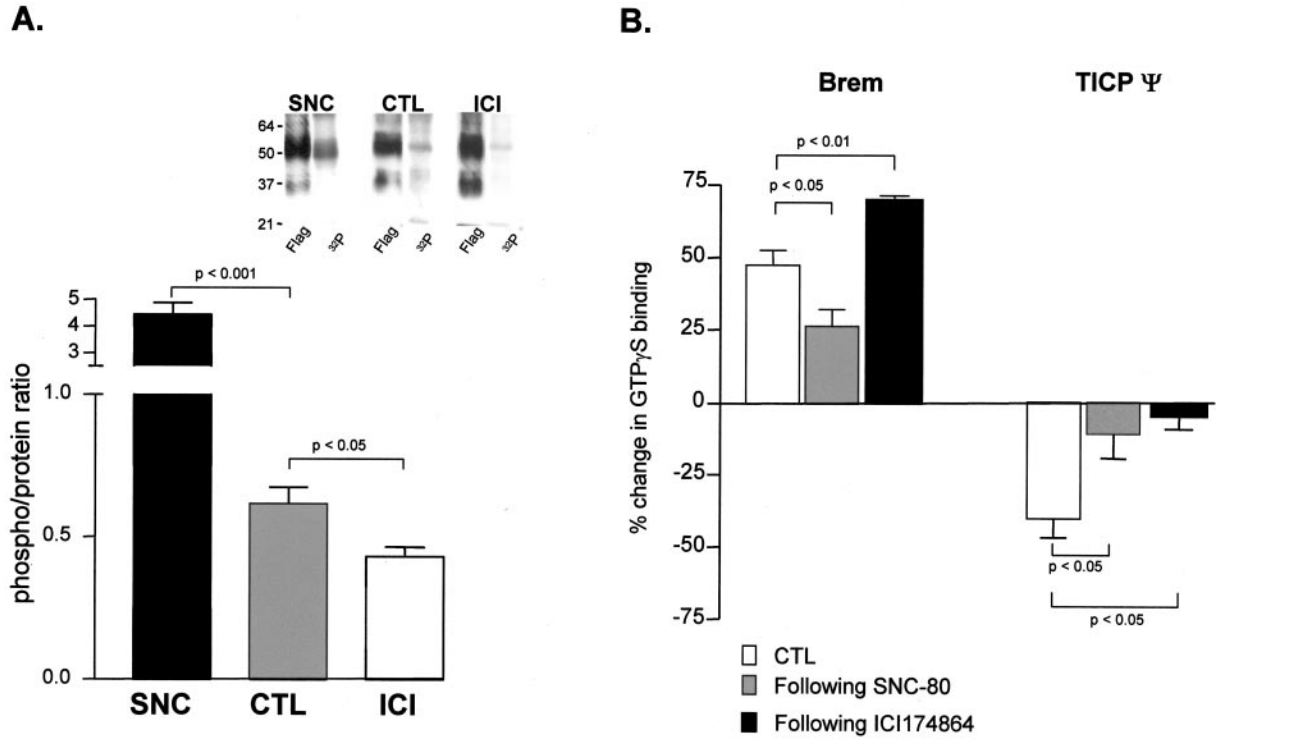

Fig. 5. A, effect of pretreatment with SNC-80 or ICI174864 (1 μM for 30 min) on the phosphorylation of hδORs. HEK293s cells stably expressing FLAG-tagged hδORs were metabolically labeled with ^{32}P and exposed for 30 min to the indicated drugs (1 μM). Solubilized hδORs were then purified by immunoprecipitation using anti-FLAG M2 antibody resin and subjected to electrophoresis on 10% SDS-PAGE (see *Materials and Methods*). Representative autoradiographs and corresponding Western blots for the different treatment conditions are shown. Bar graphs represent the phosphorylation level of the receptors expressed as the mean ± S.E.M. of the phosphorylation/protein ratio calculated from densitometric analysis of each autoradiogram (^{32}P) and its corresponding Western blot (Flag) (n = 4–5). Statistical significance of drug effects was established by comparison with control values using Student's t test. B, comparison of the effects of pretreatment with SNC-80 or ICI174864 on the regulation of [^{35}S]GTPγS binding by δOR ligands of different efficacies. HEK293s cells expressing hδORs were incubated or not with SNC-80 or ICI174864 (1 μM for 30 min) and used to prepare membranes for [^{35}S]GTPγS binding assays. Results are expressed as detailed before and represent the mean ± S.E.M. of three to four independent experiments carried out in triplicate. The statistical significance of changes induced by either SNC-80 or ICI174864 pretreatment is shown and was established by comparing percentage effects of drugs in controls and the corresponding pretreatment group using Student's t test. Brem, bremazocine.

ICI174864 reduced basal hδOR phosphorylation (Fig. 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 after pretreatment with the inverse agonist. (Fig. 6). Moreover, the effects of ICI174864 pretreatment on E_{max} 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 G protein availability for interaction with the receptor. (See *Appendix* 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 exclusion of the possibility that the observed functional effects could have been related to incomplete washout 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 in which 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 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 shown previously that similar exposure to ICI174864 (1 mM for 30 min) and washout procedure produced no significant change in affinity values for [^{3}H]naltrindole (Pineyro et al., 2001).

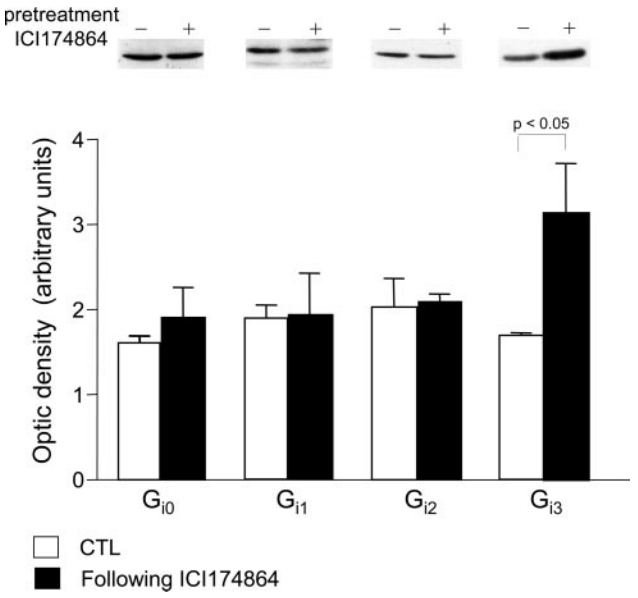

Fig. 6. Effect of ICI174864 pretreatment (1 μM for 30 min) on the immunoreactivity of different $G\alpha_{i/o}$ subunits. HEK293s cells expressing hδORs were incubated (■) or not (□) with ICI174864, and membranes were prepared and resolved on SDS-PAGE (40 μg/well). Representative examples of immunoblots obtained for the different $G\alpha_{i/o}$ subunits are shown. Bar graph represents the mean ± S.E.M. 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.

Pretreatment with an inverse agonist has been shown previously 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 h), and the observed effects were linked to an increase in the expression levels either of the receptor (Heinflink et al., 1995; MacEwan 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, because 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 (Fig. 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 because ICI174864 pretreatment reduces receptor phosphorylation, δORs may have a tighter precoupling with its interacting G proteins. Another possibility could be that ICI174864 stabilizes the receptor in conformation 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 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 E_{max}, short-term exposure to ICI174864 produced changes in potency (Figs. 1, B and C, and 2, B and C). For bremazocine or TIPP, these changes were in the range of 1 order of magnitude, a shift that is predicted by the ternary complex model after 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 (Fig. 6) or δOR phosphorylation (Fig. 5A). However, and consistent with its increase in potency, naloxone also displayed a 1000-fold increase in affinity for its high-affinity binding site (Fig. 3C). The magnitude of this increase per se does not 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. Because different conformations need not share the same signaling properties, it is possible that one of the receptor states may couple to a given Gα subtype to produce agonist responses, whereas the other couples to a different Gα subtype to produce inverse agonist effects. In such a case, naloxone could have induced its inverse agonist effect before ICI174864 treatment primarily via the low-affinity site and its agonistic effects via the high-affinity site after treatment.

If pharmacological actions of naloxone were more powerful at the high-affinity than the low-affinity site, such difference together with the marked increase in affinity and modifications in receptor/G protein coupling or G protein content specifically favoring signaling 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} values. This possibility is analyzed in the *Appendix*.

Appendix

This section uses the fully extended (cubic) version of the ternary complex model to better understand how different determinants of constitutive activity may modify pharmacological behavior of a protean ligand and 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 Lean et al., 1980). In its early conception, the ternary complex model was described in terms of the following: 1) ligand binding to the free receptor [AR] (K or K_{min} = [AR]/[A] · [R]); 2) ligand binding to the coupled receptor [ARG] (K_{max} = [ARG]/[A] · [RG] = αK_{min}); 3) interaction between the receptor (R) and a membrane regulatory component (G) (M = [RG]/[R] · [G]); and 4) 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., 1993). The fully extended form of the model (cubic model shown in Fig. 7) 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 represented by the factor β, and higher tendency of the active (R*) over the inactive (R) receptor to form the R*G complex is represented by γ. An additional constant, δ, represents the synergistic effect of agonist binding 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 (Fig. 7). 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 G protein. 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 + $\delta\beta\gamma$J)/(1 + γJ)]]. In addition, 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)] = $\alpha' K'_{min}$].

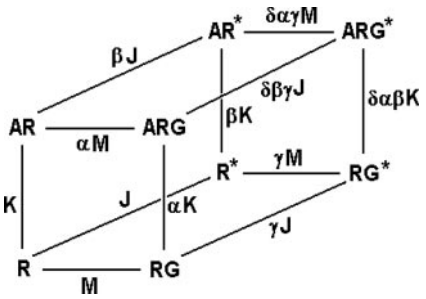

Fig. 7. Fully extended ternary complex model (cubic model). See *Appendix* for details.

Influence of Different Determinants of Constitutive Activity on the Pharmacological Behavior of a Protean Ligand. As stated under *Discussion,* 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 α = 2.2, β = 10, and δ = 0.175 were then determined using the equation RG* + ARG* = [γJ/(1 + γJ)][(M′G/(1 + M′G)][(1+ $\alpha\beta\delta K$ [A])/(1 + K'app[A])] R_{tot}, where 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 8 shows the expected behavior that this protean would display after 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. It is interesting that 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 after either an increase or decrease in spontaneous activity points to the intricate relationship between spontaneous activity and perceived pharmacological actions. Indeed, as suggested by Fig. 8, 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 for the β2 adrenergic 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 after deactivation because of receptor desensitization (Chidiac et al., 1996). Another example corresponds to the bradykinin 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 drug may display opposite behavior after different mutations that increase intrinsic activity if one of the mutations enhances receptor/G protein 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, 3×10^{-2}; after ICI174864, 7×10^{-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; after 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 $\delta = 0.4$ (Fig. 9, A and D); parameters for naloxone were $\alpha = 2.8$, $\beta = 8$, and $\delta = 0.16$ (Fig. 9, B and E) and for TICPΨ, $\alpha = 40$, $\beta = 112$, and $\delta = 0.009$ (Fig. 9, C and F). As can be seen from the curves generated, any of the two mechanisms could theoretically account for the observed increase in E_{max} for the agonist, E_{max} reduction for the inverse agonist, and a reversal of efficacy for the protean. Changes in potency for bremazocine (Fig. 9, A and D) could also be simulated by an increase in G or M. However, this was not the case for naloxone (Fig. 9, B and E), because 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 assume that the drug produced its effects via the low-affinity site before pretreatment but that after 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, to fully account for

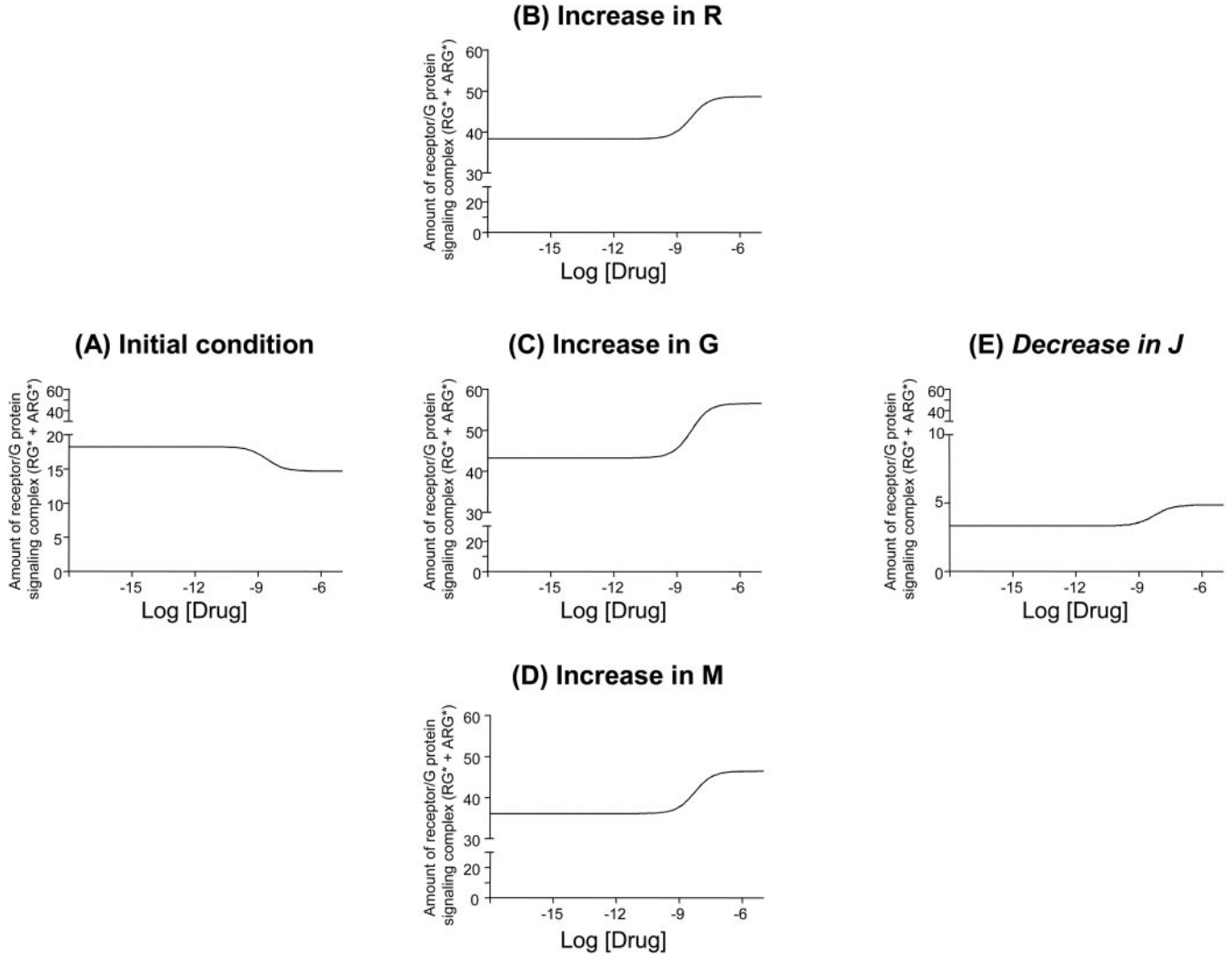

Fig. 8. Influence of different determinants of constitutive activity on the pharmacological behavior of a protean ligand defined by $\alpha = 2.2$, $\beta = 10$, and $\delta = 0.175$. Simulated dose-response curves for parameter changes that results in inversion of efficacy are shown. A, initial condition: $R_{tot} = 200$, G = 180, M = 0.03, J = 5, and $\gamma = 0.06$. 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: J = 0.5. Drug effects are expressed as change in total amount of active coupled receptor species (RG* + ARG*).

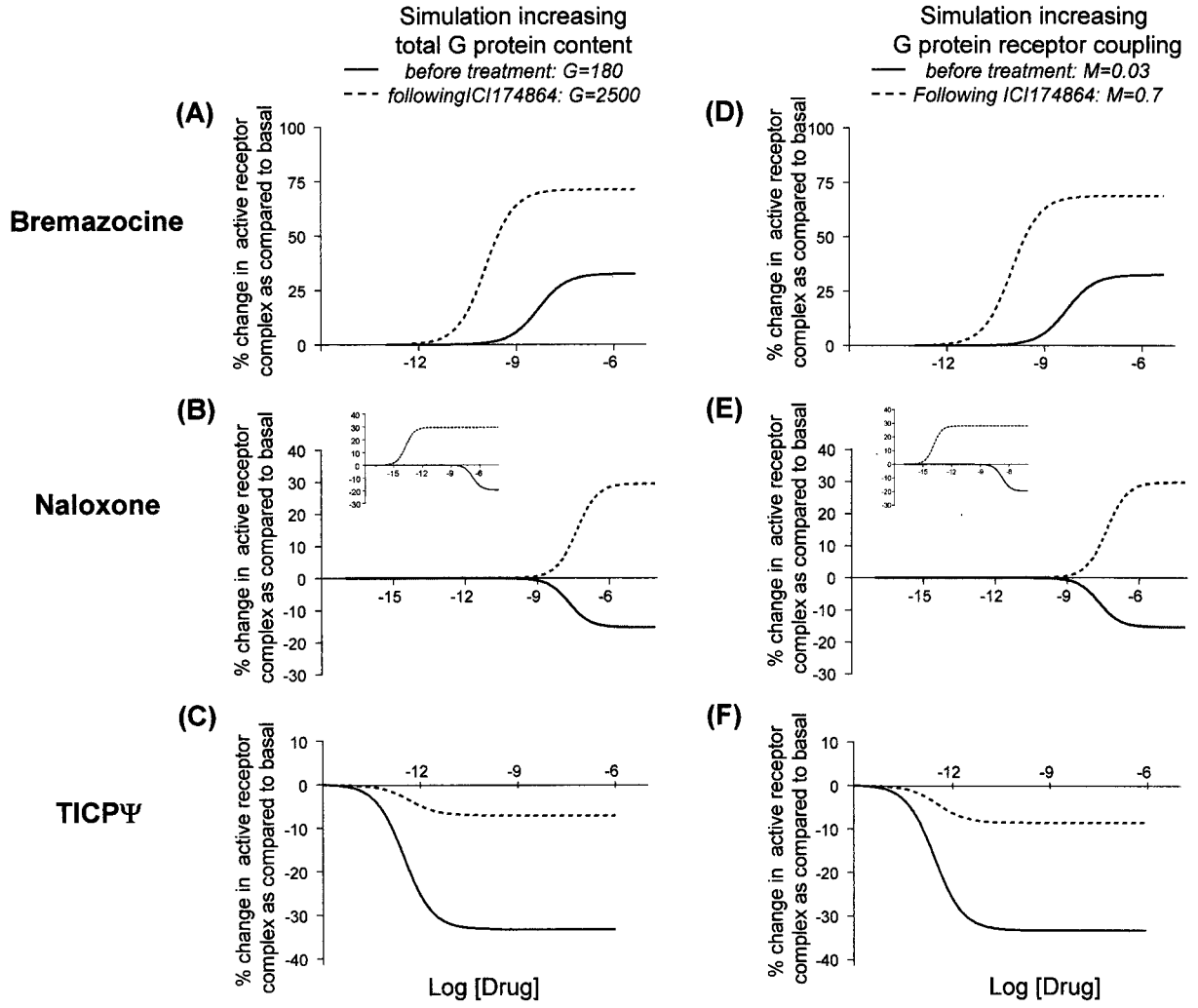

Fig. 9. Simulation of the effect of ICI174864 pretreatment on the pharmacological behavior of ligands representing bremazocine, naloxone, and TICPΨ. A, B, and C show the functional consequences if pretreatment is simulated by an increase in total G protein contents: before treatment, G = 180 (solid line) and after ICI174864, G = 2500 (broken line). D, E, and F show the effect of an increase in M, which represents enhanced receptor/G protein coupling: before treatment, M = 0.03 (solid line) and after ICI174864, M = 0.7 (broken line). The rest of the system parameters were kept constant: R_{tot} = 80, J = 5, and γ = 0.06. Insets to B and E show the simulations for naloxone after adjustments to account for increase in potency. See *Appendix* for details. Drug effects are expressed as the results obtained in the previous section, by calculating the percentage change in the amount of signaling receptor complexes in the presence of drug $(RG^* + ARG^*)_{drug}$ compared with the amount of complex present in its absence $(R^*G)_{basal}$. Percentage change in total amount of signaling complex = $\{[(RG^* + ARG^*)_{drug} - (R^*G)_{basal}]/(R^*G)_{basal}\} \times 100$.

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 Fig. 9, B and E.

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