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Short-Term Inverse-Agonist Treatment Induces Reciprocal Changes in δ-Opioid Agonist and Inverse-Agonist Binding Capacity

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ABSTRACT

This study assessed the effects of short-term treatment (30-min) with inverse agonists on receptor protein levels and on the ability of agonists, inverse agonists, and neutral antagonists to bind to the human δ-opioid receptor (hδOR). Incubation of human embryonic kidney 293s cells stably expressing hδOR with the inverse agonist ICI174864 (1 μM) induced reciprocal changes in agonist and inverse-agonist binding. The total number of binding sites recognized by the agonists [^{3}H]bremazocine and [^{3}H][D-Pen^2,D-Pen^5]-enkephalin was reduced by 33 and 57%, respectively, whereas binding capacity for the radiolabeled inverse-agonist [^{3}H]Tyr-TicY[CH$_2$NH]Cha-Phe-OH increased by 44%. In contrast, total receptor protein and sites labeled by neutral antagonists [^{3}H]naltrindole and [^{3}H]Tyr-D-Tic-Phe-Phe-OH remained unchanged. Pertussis toxin (PTX) and 5-guanylylimidodiphosphate (GppNHp) mimicked the outcome of ICI174864 pretreatment in promoting the loss of agonist binding sites. The lack of an additive effect on [^{3}H]bremazocine binding when these three agents were combined indicates that inverse agonists may, in part, share the mechanism by which GppNHp and PTX reduce agonist binding capacity. Spontaneous recovery of maximal agonist binding capacity after inverse-agonist treatment was slow, suggesting a decrease in the isomerization rate between agonist- and inverse agonist–preferring conformations. Overall, the data presented are consistent with the idea that hδORs exist in multiple states capable of discriminating among ligands of different levels of efficacy and show that, after short-term treatment with an inverse agonist, the receptor ability to adopt conformations preferentially induced by agonist ligands is reduced.

Numerous studies using heterologous expression systems have revealed that native G protein-coupled receptors (GPCRs) (Costa and Herz, 1989; Barker et al., 1994; Chidiac et al., 1994; Samama et al., 1994) as well as naturally occurring or laboratory-constructed mutants (Lefkowitz et al., 1993; Scheer and Cotecchia, 1997) may undergo agonist-independent activation. These observations prompted an updating in receptor theory, leading to the idea that receptors exist in different, interconverting conformations differentially stabilized by specific ligands and protein-protein interactions. For example, in the extended version of the ternary complex model, the receptor is believed to isomerize between active (R*) and inactive (R) states, and the G protein is considered to stabilize the active conformation (R*G; Samama et al., 1994). According to this model, receptor subpopulations corresponding to distinct activation states will coexist even under basal conditions and will be differentially recognized by ligands with distinct efficacies. Active conformations are preferentially bound and stabilized by agonists, whereas the inactive ones are best recognized by drugs currently known as inverse agonists (Milligan and Bond, 1997; de Ligt et al., 2000). Neutral antagonists for their part do not discriminate among subpopulations of receptor states.

It is well known that GPCRs adapt to agonist exposure by reducing cellular responses induced by receptor stimulation (Lefkowitz, 1998). Rapid phosphorylation of the receptor by G protein-coupled receptor kinases (Premont et al., 1995) and second-messenger–dependent kinases (Benovic et al., 1988; Hausdorff et al., 1989; Kobilka, 1992) is believed to be at the center of this response, characterized by receptor-G protein uncoupling (Lohse et al., 1990; Pals-Rylaarsdam et al., 1995;

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ABBREVIATIONS: GPCRs, G protein-coupled receptors; R, inactive receptor; R*, active receptor; G, G protein; hδOR, human δ-opioid receptor; IBMX, 3-isobutyl-1-methylxanthine; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; GppNHp, 5-guanylylimidodiphosphate; TIPP, Tyr-D-Tic-Phe-Phe-OH; TICPψ, Tyr-TicY [CH$_2$NH]Cha-Phe-OH; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; ANOVA, analysis of variance; PTX, pertussis toxin; AR, free receptor; ARG, coupled receptor; M, spontaneous interaction between R and G; K_{app}, apparent dissociation constant.

Diviani et al., 1996), internalization, and eventually receptor-protein degradation (Sibley and Lefkowitz, 1985, 1987; Bouvier et al., 1988). In contrast, the adaptive response of GPCRs to treatment with inverse agonists has been less well characterized. No information on the effect of short-term treatment (minutes) is presently available and, for long-term treatments (hours to days), both up-regulation (Heinflink et al., 1995; MacEwan and Milligan 1996a,b; Smit et al., 1996; Lee et al., 1997; Samama et al., 1997) and reduction in receptor number (commonly referred to as atypical down-regulation; Barker et al., 1994; Labrecque et al., 1995) have been reported. These changes have been traditionally attributed to increase or decrease in receptor protein levels as a consequence of either increased stabilization or degradation, respectively. However, the notion that distinct receptor conformations may be differently recognized by agonists and inverse agonists may introduce an extra level of complexity in interpreting the capacity values in radioligand binding studies. For example, if the isomerization capacity of a given receptor is modified and interferes with the interconversion from a very low-affinity state to a high-affinity state for the labeling drug, the effect would appear as a decrease in B_{max} value and could be erroneously interpreted as a loss in the total amount of receptor protein.

In the present study, the effect of short-term inverse-agonist pretreatment on membrane receptors was assessed by monitoring levels of total receptor protein and the ability of the receptor to be recognized by ligands with different efficacies. Given its rich pharmacology and the availability of agonist, inverse agonist, and antagonist radioligands, the human δ-opioid receptor (hδOR) was chosen for this purpose. Results revealed that after a 30-min treatment with the inverse agonist ICI174864, the total amount of receptor protein and the number of receptors recognized by neutral antagonists remained unchanged, the number of binding sites labeled by an inverse agonist was significantly increased, and agonist binding was reduced. The process was shown to be reversible, although recovery of agonist binding capacity was slow. These observations were interpreted as an indication that exposure to an inverse agonist reduces the rate of receptor isomerization, resulting in its decreased ability to adopt conformations that may be readily recognized by agonist ligands. Changes in binding capacity could not be accounted for by simulating receptor-G protein uncoupling in the fully extended version of the ternary complex model (cubic), but they were consistent with changes in the relative frequency of non–readily interconverting sites of a multistate model.

Materials and Methods

Reagents. Buffer chemicals, protease inhibitors, forskolin, 3-isobutyl-1-methylxanthine (IBMX), GppNHp, pertussis toxin, DPDPE, naltrindole, and anti-FLAG M_2 antibody were purchased from Sigma (St. Louis, MO). [^{3}H]Adenosine, [^{3}H]naltrindole (26 Ci/mmol), [^{3}H]DPDPE (33 Ci/mmol), and [^{3}H]bremazocine (26.5 Ci/mmol) came from PerkinElmer Life Sciences (Boston, MA), and [^{3}H]TICP (111.3 Ci/mmol) and [^{3}H]TIPP (50 Ci/mmol) were purchased from the Institute of Isotopes (Budapest, Hungary). TIPP and TICP were synthesized as described previously (Schiller et al., 1999a). ICI174864 was obtained from Tocris Cookson (St. Louis, MO) and bremazocine from Sigma/RBI. G418, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, Fungizone, glutamine, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Nitrocellulose was obtained from Schleicher & Schuell (Keene, NH), and chemiluminescence was assessed using Renaissance Plus kit (PerkinElmer Life Sciences).

Receptor Expression and Cell Culture. Tagged with the FLAG epitope at the carboxyl terminus, hδORs were synthesized as described previously (Petäjä-Repo et al., 2000), were stably expressed in HEK293s cells using a calcium phosphate precipitation procedure, and were selected with G418 (500 μg/ml). Cells were grown as monolayers in 75-cm^2 Starstedt plastic flasks containing DMEM supplemented with 10% fetal bovine serum, 1 mM glutamine, 500 units/ml penicillin, and 500 units/ml streptomycin in an atmosphere of 95% air/5% CO_2 at 37°C. Before treatment, attached cells (80% confluence) were washed with warm DMEM. They were then incubated for 30 min in 10 ml of DMEM containing either vehicle (1 μl of 50% dimethyl sulfoxide and 50% H_2O) or the indicated doses of ICI174864 or TICP. They were then detached, diluted in cold PBS (4°C) to stop treatment, and washed three times in 50 volumes of PBS at room temperature. When pertussis toxin was used, cells were incubated for 16 h with 50 ng/ml, followed by a similar washing procedure. All binding assays were carried out on whole cells, except when the effect of GppNHp was assessed, in which case a membrane preparation was used.

Membrane Preparation and SDS-Polyacrylamide Gel Electrophoresis. Membranes were prepared as described previously (Rousseau et al., 1996). Briefly, washed cells were resuspended in buffer containing 5 mM Tris-HCl, pH 7.4, 2 mM EDTA, and protease inhibitors (5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine) and then lysed on ice with a Polytron homogenizer ultraturax (three 3-s bursts at maximal speed; IKA, Wilmington, NC). Lysates were centrifuged at 500*g* for 5 min at 4°C, and the resulting supernatant was centrifuged at 45,000*g* for 20 min at 4°C. The resulting pellet was resuspended in binding buffer (75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, 2 mM EDTA, and protease inhibitors as indicated above) and immediately used in binding assays or for gel electrophoresis. Protein content was determined using the Bio-Rad DC Protein Assay Kit (Richmond, CA). For SDS-polyacrylamide gel electrophoresis, membranes were denatured in sample buffer (62.5 mM Tris-HCl, 5% SDS, 50 mM dithiothreitol, 10% glycerol, 0.05% bromphenol blue) and separated on a 9% SDS-polyacrylamide gel, according to the method used by Laemmli (1970). Proteins were transferred onto nitrocellulose, and Western blot analysis was carried out using M_2 antiflag monoclonal antibodies, horseradish peroxidase-coupled anti-mouse antibody, and chemiluminescence. The amount of receptor in each lane was estimated by densitometric analysis of the autoradiogram using National Institutes of Health image software (http://rsb.info.nih.gov/nih-image/).

Radioligand Binding Assays. For saturation experiments, 10 to 25 μg of protein was diluted in PBS (whole-cell binding) or binding buffer (membranes) to a final volume of 300 μl and incubated with variable concentrations of [^{3}H]bremazocine (0.05–14 nM), [^{3}H]DPDPE (0.01–8 nM), [^{3}H]naltrindole (0.05–5 nM), [^{3}H]TIPP (0.05–5 nM), and [^{3}H]TICP (5–500 pM). Incubation was allowed to proceed at room temperature until equilibrium was reached (1 h, except in the case of [^{3}H]DPDPE, for which incubation time was 2 h). Nonspecific binding was determined in the presence of 10 μM naloxone; when indicated, GppNHp was used at a concentration of 500 μM. Incubation was terminated by rapid filtration in a cell harvester (Brandel Inc., Gaithersburg, MD) through GF/C filters (Whatman, Clifton, NJ) (presoaked in 0.1% polyethylenimine) using ice-cold PBS (for cells) or Tris-HCl (50 mM, pH 7.4, for membranes). Bound radioactivity was determined by scintillation counting. Unless otherwise stated, apparent K_d and B_{max} values were determined from a nonlinear least-squares analysis of saturation data using Prism (GraphPad Software, San Diego, CA). Statistical comparison of curves was performed with two-way ANOVA using the same program. When specified, hδOR maximal density was estimated from a single point, using near-saturation concentration, and statistical differences were

determined using either one-way ANOVA followed by Student-Newman-Keuls post hoc test or Student's t test, for multiple or simple comparisons, respectively.

cAMP Accumulation Assays. Cells were grown in 75-cm^2 flasks and labeled overnight (16 h) with DMEM supplemented with 10% fetal bovine serum and contained 1 μCi/ml of [^{3}H]adenine. Radioactive medium was then replaced with fresh DMEM, and the cells were mechanically detached and thoroughly washed (three times) with PBS (4°C). Viability was assessed using trypan blue (mortality was never higher than 5%). Then, 5×10^5 cells were resuspended in 300 μl of assay mixture containing PBS, 25 μM forskolin, 2.5 μM IBMX, and different drugs at the indicated concentrations and incubated for 20 min at 37°C. The assay was terminated by the addition of 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. Results were expressed as the ratio of [^{3}H]cAMP/[^{3}H]ATP + [^{3}H]cAMP. Statistical significance of drug effects was determined using one-way ANOVA followed by Student-Newman-Keuls post hoc test.

Results

Characterization of δOR Ligands. Drug efficacy was characterized for the expression system used in this study. cAMP Accumulation on HEK293s cells stably transfected with hδORs indicated that the cyclic enkephalin analog DPDPE (Mosberg et al., 1983) behaved as an agonist, inducing 55 ± 4% reduction in forskolin-stimulated cAMP (Fig. 1). The nonselective benzomorphan bremazocine (Romer et al., 1980) also behaved as an efficacious δ-agonist producing 58 ± 3% decrease in forskolin-stimulated cAMP accumulation. Consistent with signal amplification, EC_{50} values observed (Table 1) were 2 to 3 orders of magnitude lower than those previously reported in GTPase assays (Costa and Herz; 1989; Mullaney et al., 1996; Befort et al., 1999). The naltrexone derivative naltrindole and the deltorphin-related peptide TIPP, previously described as potent and selective δ-antagonists (Portoghese et al., 1988; Schiller et al., 1992), had no significant effect on cAMP production and thus were classified as neutral antagonists. Finally, ICI174864, an enkephalin analog (Cotton et al., 1984), and the pseudopeptide TICPΨ (Schiller et al., 1999a,b) behaved as inverse agonists, inducing respective increases of 46 ± 6% and 47 ± 9% over forskolin-stimulated cAMP accumulation (Fig. 1). TIPP (1 μM) blocked the effect of ICI174864 (1 μM), indicating that the effect of the inverse agonist was not caused by traces of endogenous opiates present in the incubation medium. The 28 ± 3% increase in cAMP accumulation induced by ICI174864 (1 μM) was reduced to 1 ± 6% when TIPP was also present ($p < 0.05$; $n = 4$). Basal values for cAMP accumulation in the presence and absence of TIPP were similar (48 ± 17 and 51 ± 17 arbitrary units, respectively).

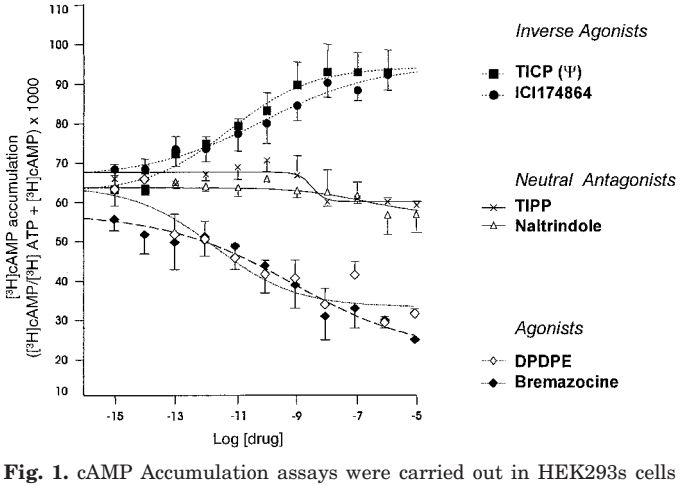

Fig. 1. cAMP Accumulation assays were carried out in HEK293s cells stably expressing hδORs (3–6 pmol/mg protein). Concentration response curves for TICP(Ψ), ICI174864, TIPP, naltrindole, bremazocine, and DPDPE were carried out in the presence of 25 μM forskolin and 2.5 μM IBMX. Data represent the mean ± S.E.M. of four to six experiments. One-way ANOVA followed by Student-Newman-Keuls post hoc tests were used to determine statistically significant differences from control for all treatments (naltrindole and TIPP were nonsignificant; remaining drugs, $p < 0.001$).

Saturation assays for different ligands that were conducted in parallel on the same population of cells are shown in Table 2. B_{max} values obtained under these controlled conditions indicate that the total number of receptors recognized by different ligands was different. A higher number of receptors ($p < 0.05$) was observed with lipophilic benzomorphan drugs (bremazocine and naltrindole) than with the peptidic ligands (DPDPE, TIPP, and TICPΨ), most likely because of the labeling of both cell surface and intracellular receptor sites by the former. Within the group of peptide derivatives, which, unlike bremazocine and naltrindole, preferentially bind to surface receptors (Childers et al., 1979; Arden et al., 1995; Schiller et al., 1999a,b), the number of labeled sites differed, suggesting the possibility of each ligand selectively recognizing different conformations. The neutral antagonist [^{3}H]TIPP labeled twice the number the sites recognized by the inverse agonist [^{3}H]TICP ($p < 0.05$), which in turn recognized twice the amount of receptors identified by the agonist [^{3}H]DPDPE ($p < 0.05$; Table 2). On the other hand, the difference observed between the number of sites labeled by the agonist bremazocine and the antagonist naltrindole did not attain significance.

Effect of Short-Term Inverse-Agonist Treatment on Binding Sites Recognized by Agonist Radioligands. Once the inverse efficacy of ICI174864 was established, it was used as a prototype to assess the effect of short-term inverse-agonist treatment on binding properties of hδORs, as detected by the agonists [^{3}H]bremazocine and [^{3}H]DPDPE. For this purpose, HEK293s cells expressing hδORs were incubated with ICI174864 (1 μM) for 30 min. Treatment was terminated by dilution in cold PBS (4°C), and cells were then washed three times (5 min per wash) to ensure complete elimination of the treatment drug. After washes, agonist binding was assessed. ICI174864 pretreatment induced 33% ($p < 0.05$) and 57% ($p < 0.001$) reduction in the total number of binding sites detected in saturation assays with [^{3}H]bremazocine and [^{3}H]DPDPE, respectively (Fig. 2).

To show that the observed loss in agonist binding sites was not unique to ICI174864 but rather was a property of the family of inverse-agonist ligands, the effect of TICPΨ on [^{3}H]bremazocine binding was also tested. Figure 3, A and B, respectively, shows that both ICI174864 and TICPΨ pretreatment induced a concentration-dependent decrease in [^{3}H]bremazocine B_{max} value. As in cAMP accumulation assays, TICPψ was more potent than ICI174864 in promoting the loss of agonist binding. Also in keeping with observations from cAMP accumulation, the effect of short-term ICI174864 treatment on [^{3}H]bremazocine binding sites could be blocked by TIPP (Fig. 3C).

The loss of agonist binding sites after pretreatment with an inverse agonist is not caused by the presence of residual drug. Indeed, for lingering ICI174864 to produce a 33% or 57% reduction in [^{3}H]bremazocine and [^{3}H]DPDPE B_{max} value, a 2- or 7-fold increase in their respective apparent K_d values would have been observed. The affinity of each of the compounds in control sites and after treatment was measured in saturation assays, and the results are shown in Table 1. The observed K_d value for [^{3}H]DPDPE was slightly reduced, whereas the affinity for neither [^{3}H]bremazocine nor any of the other radioligands tested was significantly modified by exposure to the inverse agonist (Table 1). Furthermore, the only tritiated inverse agonist available ([^{3}H]TICP) was used to directly assess the amount of remaining drug. It was observed that despite a 40% reduction in [^{3}H]bremazocine binding (from 2.5 to 1.4 pmol/mg), only 1% (28 fmol/mg) of the initial amount of [^{3}H]TICP-labeled receptors was still occupied by the radioligand.

Mechanism Involved in the Loss of [^{3}H]Bremazocine Binding Sites after Short-Term Treatment with Inverse Agonists. In the next series of experiments, the role of receptor protein down-regulation as a potential mechanism underlying the loss of agonist binding sites was investigated. Immunoblot analysis using FLAG-tagged hδORs revealed specific immunoreactive bands corresponding to the monomeric form of the mature receptor (55,000), immature receptor (39,000), and degradation products (27,000), as well as dimers either of the mature receptor (110,000) or of mature and immature species (94,000; Fig. 4). As shown in Fig. 4, A through C, for equal amounts of membrane protein obtained from control and ICI174864-treated cells, immunoreactivity for each of the receptor species was the same. Reciprocally, when loading an identical number of receptors, as measured by [^{3}H]bremazocine binding, immunoreactivity detected in membranes obtained from cells pretreated with ICI174864 was higher (Fig. 4D). This indicates that the reduction in agonist binding capacity was not accompanied by a decrease in the total amount of receptor protein; rather, receptors adopted a conformation not recognized by the agonist.

To determine whether the reduction in agonist binding was paralleled by similar changes in inverse agonist and antagonist binding, the effect of ICI174864 pretreatment on the number of sites recognized by [^{3}H]TICP, [^{3}H]TIPP, and [^{3}H]naltridole was assessed. For the inverse agonist [^{3}H]TICP, a 44% increase in B_{max} values (control: 356 ± 4 fmol/mg versus ICI-treated: 511 ± 37 fmol/mg; $p < 0.01$; $n = 10$) was observed with ICI174864 treatment (Fig. 5). This is in contrast with the significant decrease in binding sites detected by [^{3}H]DPDPE (42 ± 11%; $p < 0.01$; $n = 4$) and [^{3}H]bremazocine (30 ± 5%; $p < 0.001$; $n = 16$) in the same cells. On the other hand, when the neutral antagonists [^{3}H]naltrindole or [^{3}H]TIPP were used as radioligands, B_{max} values remained unchanged by the treatment (Fig. 5A).

The effect induced by short-term inverse-agonist treatment was then compared with that induced by pretreatment with the agonist SNC-80 (30 min; 1 μM). As shown in Fig. 5B, the effect induced by SNC-80 was not reciprocal to that induced by ICI174864. When the membrane-impermeable peptide ligands were considered, SNC-80 produced a significant reduction of surface labeling by the agonist [^{3}H]DPDPE, the inverse agonist [^{3}H]TICPΨ, and the neutral antagonist [^{3}H]TIPP, most probably as a result of internalization of the receptor. For the nonpeptide ligands that can detect both cell-surface and intracellular sites, no change in the apparent B_{max} value was observed with the antagonist [^{3}H]naltrindole, but a marked reduction in binding was found for the agonist [^{3}H]bremazocine.

According to currently accepted isomerization models (Samama et al., 1994; Weiss et al., 1996; Leff et al., 1997), agonists preferentially recognize and stabilize the active, G protein-coupled form of the receptor (R*G), whereas inverse agonists favor the inactive, uncoupled conformation (R). Thus, it is possible that the reciprocal changes in agonist and inverse-agonist binding promoted by ICI174864 could have resulted from a relative increase in the uncoupled receptor conformation. To explore this possibility, [^{3}H]bremazocine binding was determined after uncoupling of hδOR from its cognate G protein by either treating cells with PTX or by adding GppNHp to membrane-binding assays. Figure 6A shows that a submaximal concentration of PTX by itself led to a significant loss in the number of sites recognized by [^{3}H]bremazocine, indicating that the receptor conformation induced by uncoupling of the receptor from the G protein does not bind the agonist with measurable affinity. Association of this submaximal concentration of the toxin to inverse-agonist pretreatment led to a more pronounced loss of binding sites ($p < 0.05$, $n = 6$). A similar cumulative tendency was observed when treatment with the inverse agonist was combined with GppNHp (500 μM). On the other hand, the effect of combining ICI174864 with either of the uncoupling agents was not larger than that produced by associating toxin treatment with guanine nucleotide, by combining the three treatment modalities (PTX–GppNHp–ICI174864), or by associating ICI174864 with a supramaximal dose of GppNHp (1 mM; Fig. 6A). The observed saturation in the responses elicited was interpreted as an indication that the three agents (PTX, GppNHp, and ICI1774864) share a common pathway in promoting the loss of agonist binding sites.

TABLE 1

Potency (EC_{50}) and affinity (K_d) of δ-opioid ligands assessed in cAMP accumulation and radioligand binding assays

Values were obtained by nonlinear regression analysis as described under *Experimental Procedures* and are expressed as mean ± S.E.M. of four to six independent experiments.

	Potency (EC_{50})	Affinity (K_d)	Efficiency Ratio (K_d/EC_{50})	Affinity (K_d) after ICI174869
TICPΨ	6 ± 0.3 pM	53 ± 11 pM	9	91 ± 18 pM
ICI174864	72 ± 6 pM	72 ± 1 nM	1000	85 ± 2 nM
Naltrindole	N.A.	152 ± 57 pM	N.A.	136 ± 56 pM
TIPP	N.A.	145 ± 24 pM	N.A.	133 ± 22 pM
Bremazocine	727 ± 65 pM	2.7 ± 0.3 nM	87	2.1 ± 0.3 nM
DPDPE	21 ± 1 pM	1.2 ± 0.5 nM	57	120 ± 42 pM*

N.A., not assessed; parameter estimation not possible within acceptable reliability ($\alpha = 0.05$).
* $p < 0.05$.

TABLE 2

B_{max} values determined in radioligand binding saturation isotherm for δ-opioid drugs with different efficacies

Data are expressed as mean ± S.E.M. of four to six independent experiments.

Drug	B_{max}
	pmol/mg of protein
[^{3}H]Bremazocine	3.4 ± 0.8
[^{3}H]Naltrindole	4.1 ± 0.5
[^{3}H]DPDPE	0.3 ± 0.1
[^{3}H]TICPΨ	0.6 ± 0.1
[^{3}H]TIPP	2.3 ± 0.5

The next series of experiments was carried out to determine whether the effect of inverse-agonist treatment requires intact cells or whether it can be elicited on membranes. For this purpose, cells and membranes expressing hδORs were treated with ICI174864 (1 μM; 30 min), and [^{3}H]bremazocine binding was assessed. Agonist B_{max} value was decreased in membranes prepared from ICI174864-treated cells, but not in membranes that had been directly incubated with the inverse agonist (Fig. 6B), implying that cell integrity is a prerequisite for inverse-agonist pretreatment to effectively reduce agonist binding. Moreover, this

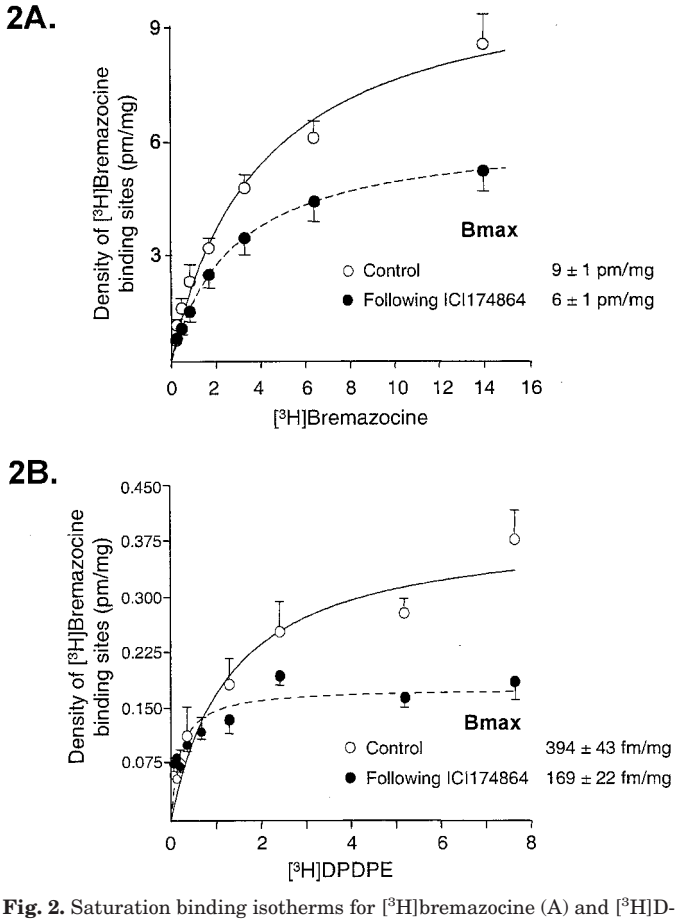

Fig. 2. Saturation binding isotherms for [^{3}H]bremazocine (A) and [^{3}H]D-PDPE (B) in control and ICI174864-treated cells. HEK293s cells stably expressing hδORs were incubated with ICI174864 for 30 min at 37°C and washed three times followed by [^{3}H]bremazocine or [^{3}H]DPDPE binding assays as described under *Experimental Procedures*. Values represent mean ± S.E.M. obtained in four experiments performed in triplicate and expressed in pmol/mg protein. Each pair of control/treatment curves were compared using two-way ANOVA to determine statistical significance of the effect of ICI174864 pretreatment on [^{3}H]bremazocine ($p < 0.05$) or [^{3}H]DPDPE ($p < 0.001$) binding.

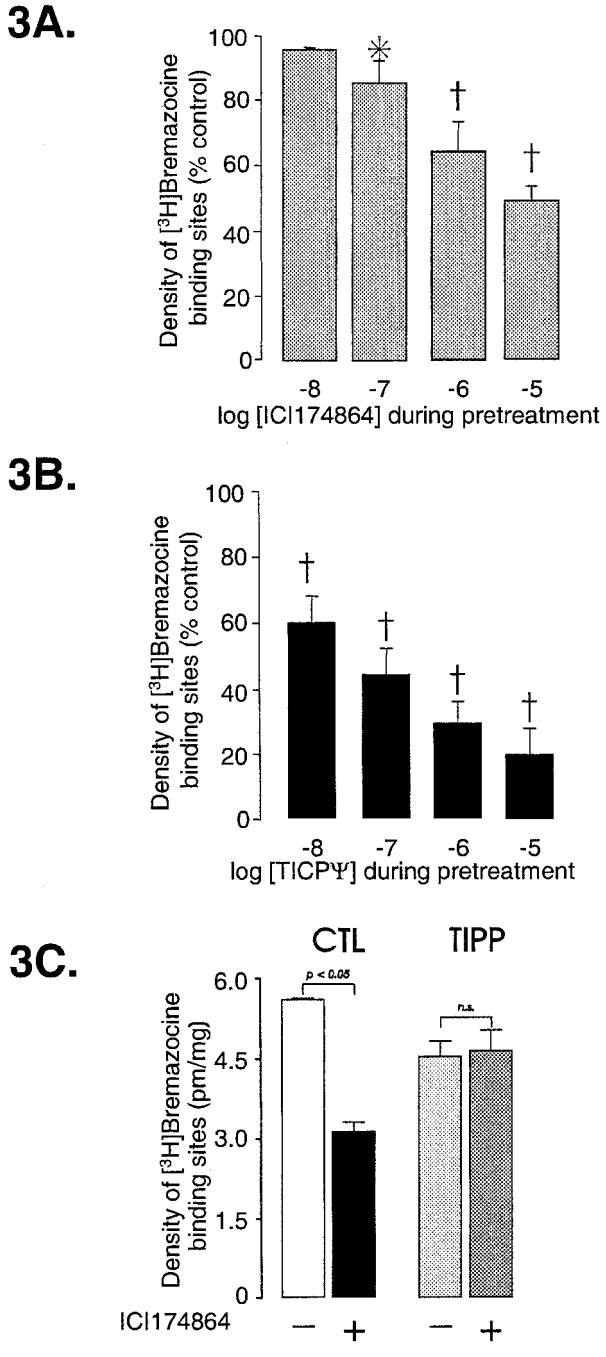

Fig. 3. Effects of increasing concentrations of ICI174864 (A) and TICPΨ (B) on the number of binding sites recognized by the agonist [^{3}H]bremazocine. Cells expressing hδORs (3–6 pmol/mg protein) were incubated at increasing concentrations of drug for 30 min at 37°C and washed three times, and radioligand binding was carried out using a saturating concentration of [^{3}H]bremazocine (15 nM). Data are expressed as the percentage of nontreated control sites ± S.E.M. obtained in four to six experiments performed in triplicate. [^{3}H]Bremazocine binding in nontreated control sites was 8 ± 2 pmol/mg. Statistical significance was determined by one-way ANOVA followed by Student-Newman-Keuls test (*$p < 0.05$; †$p < 0.01$). Calculated values for concentrations of ICI174864 and TICP(Ψ) necessary to reduce [^{3}H]bremazocine binding by 50% were 1 μM and 17 nM, respectively. C, effect of receptor blockade by TIPP on ICI174864-mediated effects on [^{3}H]bremazocine binding. Antagonistic properties of TIPP over ICI174864 were assessed by incubating cells in the presence of 1 μM of each drug added concomitantly. Experiments were performed as described under *Experimental Procedures*. Results correspond to mean ± S.E.M. of five experiments carried out in triplicate and are expressed in pmol/mg protein. Two-tailed, nonpaired *t* tests were used to conduct statistical comparisons.

observation further indicates that the loss of sites recognized by agonist radioligands is not caused by residual amounts of pretreatment drug.

Finally, the reversibility of ICI174864-induced changes on [^{3}H]bremazocine binding was assessed. To do so, binding assays were allowed to proceed for increasing periods of time, and B_{max} values obtained in control cells and after inverse-agonist treatment were compared. Figure 7 shows the recovery of bremazocine-labeled sites expressed as a percentage of the initial maximal loss. Only 28 ± 5% of the lost sites reappeared in the first 6 h. Although increasing assay temperature stimulated the recovery of [^{3}H]bremazocine binding, the process is still too slow to be simply explained by a change in the coupling state of the receptor. Indeed, the idea that slowly interconverting conformations may also occur in basal conditions is suggested by differences in the total number of sites recognized by [^{3}H]TIPP, [^{3}H]TICP, and [^{3}H]DPDPE in the absence of treatment (Table 2).

Discussion

Results presented in this study show that exposure of the hδOR to an inverse agonist for as little as 30 min induces a decrease in maximal agonist binding and an increase in the total number of receptors recognized by inverse agonists but leaves total receptor protein unchanged. These observations are consistent with the notion that short-term treatment with inverse agonists favors the adoption of receptor conformations that are distinctively recognized by inverse agonists as compared with agonists.

Soon after their initial description (Pert and Snyder, 1973), opiate receptors were proposed to exist in more than one conformation, because of the observation that diverse conditions differently modified agonist and antagonist binding to brain membranes. For instance, sodium ions (Pert et al., 1973) and guanine nucleotides (Blume, 1978) were found to increase the number of antagonist binding sites and to decrease agonist maximal binding capacity. Reciprocally, Mg^{2+} ions (Pasternak et al., 1975) were shown to enhance agonist binding but to reduce the total number of sites recognized by antagonist drugs. The effect of sodium ions and guanine nucleotides on agonist binding was later assessed specifically for the δ isoform. Although purine derivatives decreased the maximal agonist binding capacity (Ott and Costa, 1989), Na^+ ions produced a rightward shift in agonist affinity (Costa et al., 1992). A sodium-induced decrease in agonist binding

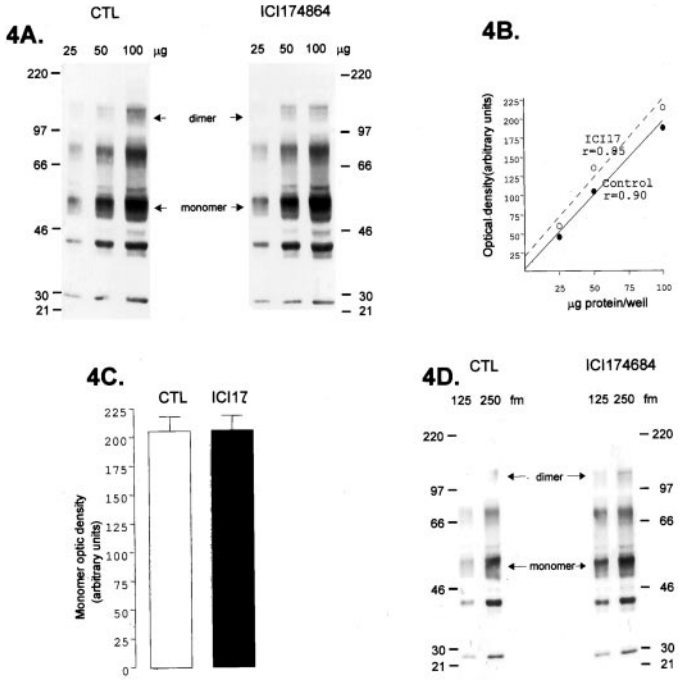

Fig. 4. Effects of ICI174864 treatment on immunoreactive FLAG-tagged hδOR. HEK293s cells expressing hδORs tagged at the carboxyl terminus with FLAG epitope were treated with ICI174864 (1 μM) for 30 min. Western blot analyses using the M2 anti-FLAG antibody were then carried out on crude membrane preparation derived from these cells. A, immunoblot for identical amounts of membrane protein (25–100 μg/well) obtained from control and treated cells. B, densitometric analysis of autoradiogram shown in 5A. C, mean ± S.E.M. of the optical density obtained for the monomeric receptor band obtained in five independent experiments. D, immunoblots for identical number of hδORs labeled by [^{3}H]bremazocine (125–250 fmol/well) obtained from control and treated cells.

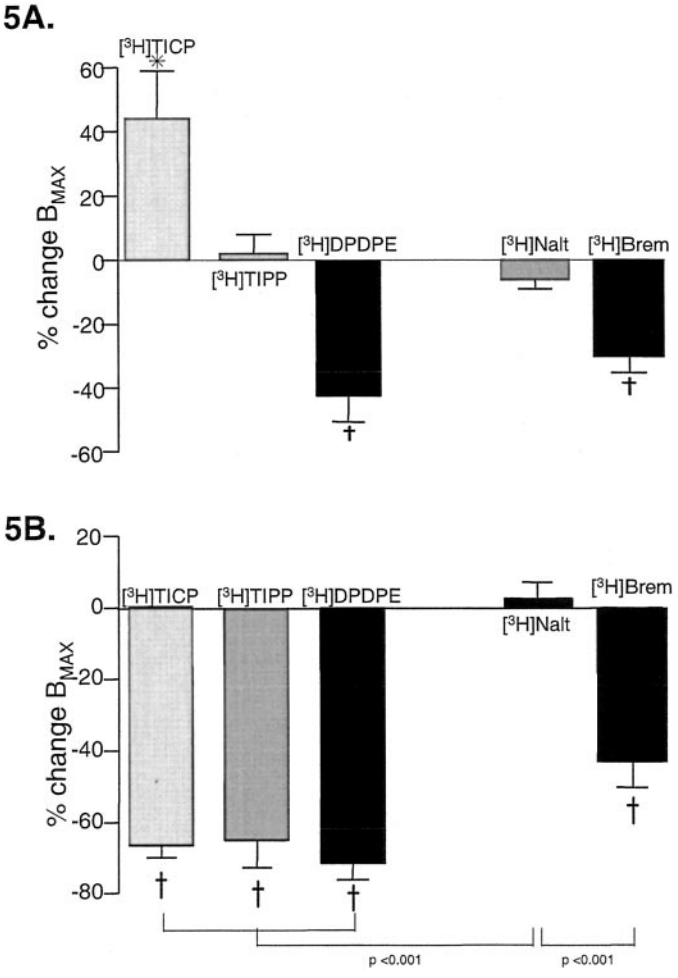

Fig. 5. Effect of ICI174864 (A) or SNC (B) pretreatment on [^{3}H]TICPψ (control B_{max}, 490 ± 62 fmol/mg), [^{3}H]TIPPψ (control B_{max}, 952 ± 130 fmol/mg), [^{3}H]DPDPE (control B_{max}, 201 ± 48 fmol/mg), [^{3}H]naltrindole (control B_{max}, 3.2 ± 0.4 pmol/mg), and [^{3}H]bremazocine (control B_{max}, 2.1 ± 0.4 pmol/mg) binding. HEK293s cells stably expressing the hδOR were incubated with ICI174864 or SNC for 30 min at 37°C and washed three times with PBS, and then saturation binding was obtained. Data are expressed as the mean ± S.E.M. of 4 to 16 experiments and represent the percentage change in B_{max} value with respect to nontreated cells. Statistical significance of the effect of pretreatment on each radioligand was determined using Student's t test to compare control versus treated cells (*$p < 0.01$; †$p < 0.001$).

capacity for the δOR was only observed after pretreatment with PTX (Wüster et al., 1984). All these type of changes have been interpreted previously in terms of the ternary complex model (De Léan et al., 1980) and more recently by means of its extended versions (Samama et al., 1994; Weiss et al., 1996). In general, such models propose the existence of a spontaneous equilibrium between the G protein (G), the inactive receptor (R), and its active conformation (R*) on the one hand and a heterodimeric complex (R*G) on the other. Agonists are believed to promote the stabilization of the active ternary complex (AR*G), whereas drugs that favor the inactive, uncoupled state of the receptor are currently known as inverse agonists (e.g., ICI174864 and TICPΨ). A decrease in the spontaneous interaction between R and G (decrease in M; see Fig. 8 in *Appendix*) is the proposed mechanistic explanation for the effect of ions and guanine nucleotides on agonist binding (De Léan et al., 1980; Wregget and De Léan, 1984). Intuitively, this rationale could explain the observed decrease in [^{3}H]bremazocine and [^{3}H]DPDPE binding (Fig. 3) and the concomitant increase in that of [^{3}H]TICP (Fig. 6A) by arguing a reduction in coupling ability between receptor and G protein after ICI174864 treatment. This hypothesis is also supported by the observation that the effect of ICI174864 on [^{3}H]bremazocine binding could be mimicked, but not augmented, by its combination with PTX-GppNHp treatment (Fig. 6). However, a reduction in agonist maximal binding such as the one observed in the present study or that reported for nonhydrolizable GTP analogs (Lee et al., 1986; Bouaboula et al., 1997; Ohtaki et al., 1998; Ott and Costa, 1989), cannot be quantitatively accounted for by changes in R*G stability. Indeed, although ternary complex formation may adequately represent efficacy-related changes in drug affinity, none of its theoretical constructs predict changes in maximal binding capacity resulting from modification in G protein coupling (Lee et al., 1986). Figure 9 in the *Appendix* shows that changes in receptor-G protein precoupling (simulated with the cubic ternary complex model) do not result in modification of ligand binding capacity, but rather of K_d values.

Despite the obvious similarities between the effect of nonhydrolizable GTP analogs and inverse-agonist pretreatment on agonist binding capacity, an important distinction should be made. Although radioligand binding studies with guanine

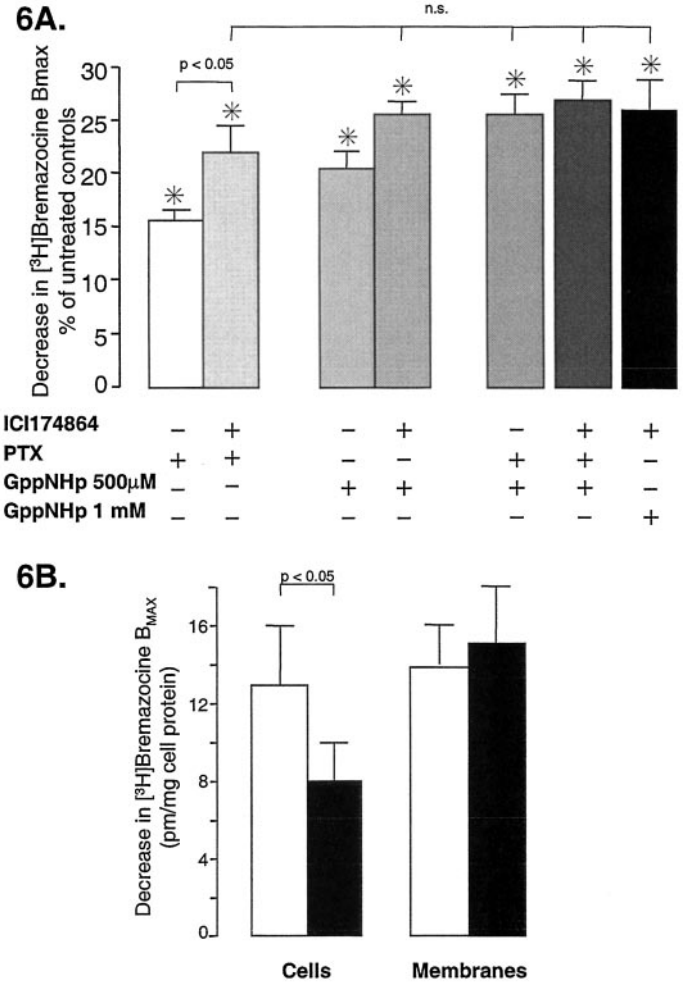

Fig. 6. A, effect of PTX, GppNHp, and ICI174864 on [^{3}H]bremazocine binding. HEK293s expressing the hδOR were incubated or not for 16 h with PTX (50 ng/ml) and were then treated with ICI174864 or vehicle (1 μl of 50% dimethyl sulfoxide and 50% H_2O) for 30 min at 37°C. Membranes prepared from these cells were then used for [^{3}H]bremazocine binding assays performed in the presence or absence of GppNHp (500 μM or 1 mM). Changes in [^{3}H]bremazocine maximal binding are expressed as a percentage ± S.E.M. of untreated control cells (7 ± 2 pmol/mg). Statistical significance of the observed changes was assessed using one-way ANOVA followed by Student-Newman-Keuls test to compare different treatments; the asterisk indicates statistical difference with respect to nontreated control cells ($p < 0.04$; $n = 6$). B, differential effect produced by cell or membrane exposure to ICI174864 (1 μM; 30 min) on [^{3}H]bremazocine binding sites. [^{3}H]Bremazocine binding was assessed as described under *Experimental Procedures*. Shown are the results (mean ± S.E.M.) obtained from five experiments. In each experiment, cells were either directly exposed to ICI174864 or were lysed, and the membranes were prepared and then treated with the inverse agonist, before [^{3}H]bremazocine binding was assessed. Two-tailed, nonpaired t tests were used to conduct statistical comparisons.

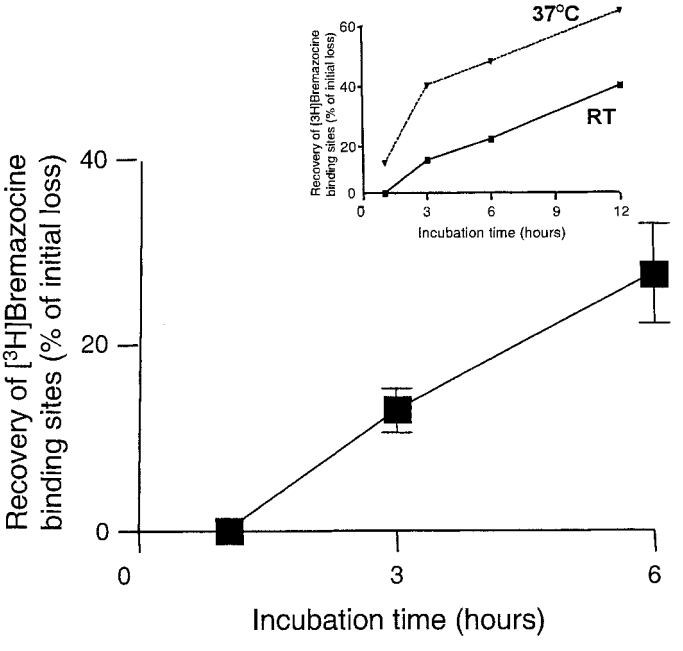

Fig. 7. Time course for the recovery from ICI174864-induced loss in [^{3}H]bremazocine B_{max} binding. HEK293s cells expressing hδOR (2.5 ± 0.4 pmol/mg) were incubated with ICI174864 for 30 min at 37°C. Treatment was stopped by dilution in PBS followed by centrifugation. [^{3}H]Bremazocine binding was assessed at different incubation times after the wash at room temperature. Results represent the percentage of recovered [^{3}H]bremazocine sites, where 100% corresponds to the maximal loss (0.9 ± 0.1 pmol/mg). Inset: effect of temperature on the recovery from ICI174864-induced loss in [^{3}H]bremazocine B_{max} binding.

nucleotides take place in the presence of the uncoupling agent, the loss of agonist-labeled sites observed in the present report persists in the absence of any physical agent preventing the agonist from reverting the system to the pretreatment distribution of receptor conformations. For this observation to be possible, conversion from low- to high-affinity agonist binding states should be slow enough not to occur within the time frame of the experiment. This kinetic interpretation is consistent with the long time required to recover [^{3}H]bremazocine-binding capacity upon removal of ICI174869 (Fig. 7) and explains efficacy-related changes in binding capacity by proposing that agonist or inverse-agonist ligands undergo equilibrium binding to different receptor subpopulations that do not readily interconvert within the time frame of the experiment. The effect of inverse-agonist pretreatment could therefore be considered as the establishment of a state of "hemi-equilibrium" (Robertson et al., 1994; Lew et al., 2000), originating from a change in the isomerization rate between different receptor conformations. A change in isomerization kinetics is also supported by the acceleration in the recovery of [^{3}H]bremazocine binding sites after an increase in assay temperature.

Changes in isomerization rate as a mechanism contributing to the loss of agonist binding sites observed after pretreatment with an inverse agonist does not exclude a concomitant role for receptor-G protein uncoupling in generating the response. However, failure of ICI174864 to reduce [^{3}H]bremazocine binding capacity when directly incubated with cell membranes (Fig. 6B) indicates that the latter is not sufficient. The requirement of cell integrity suggests that the effect of inverse agonists on the regulation of isomerization could be caused by turning off downstream signaling events that are elicited by constitutive receptor activity. This interpretation is consistent with functional effects in which prolonged inverse-agonist treatment induced 5-hydroxytryptamine-2C receptor sensitization (Berg et al., 1999). Several candidates such as changes in receptor phosphorylation, cytoskeleton-mediated translocation of the receptor into membrane microdomains, oligomerization, or interaction with accessory proteins could be the target of such regulation.

Slow transition among different receptor states has also been reported for other peptide-binding GPCRs, including NK_1, NK_3, and pituitary adenylyl cyclase-activating protein (Hastrup and Schwartz, 1996; Hashimoto et al., 1997; Krause et al., 1997; Sagan et al., 1997). In the case of these receptors, the observation that led to the proposal of a slow isomerization rate among different conformations was the inability of pairs of drugs with documented high affinity, as measured by direct radioligand binding, to compete in displacement assays (Maggi and Schwartz, 1997). Similarly, impairing interconversion between different conformations of the NK_1 or κ-opioid receptor by mutation causes a decrease

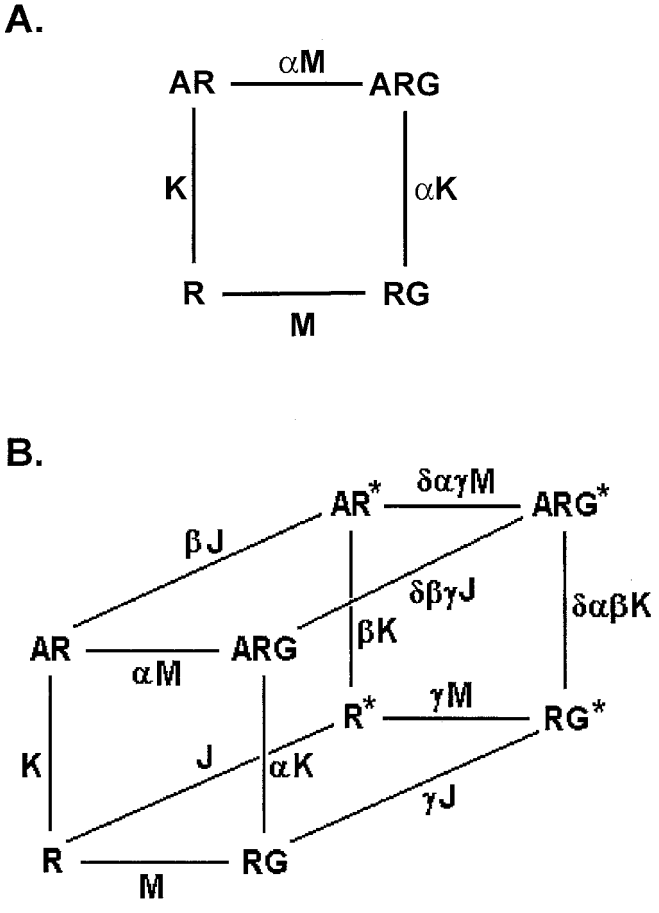

Fig. 8. Receptor species and equilibrium constants in cubic ternary complex model. A, simple form of ternary complex model. B, fully extended version of ternary complex model, cubic model.

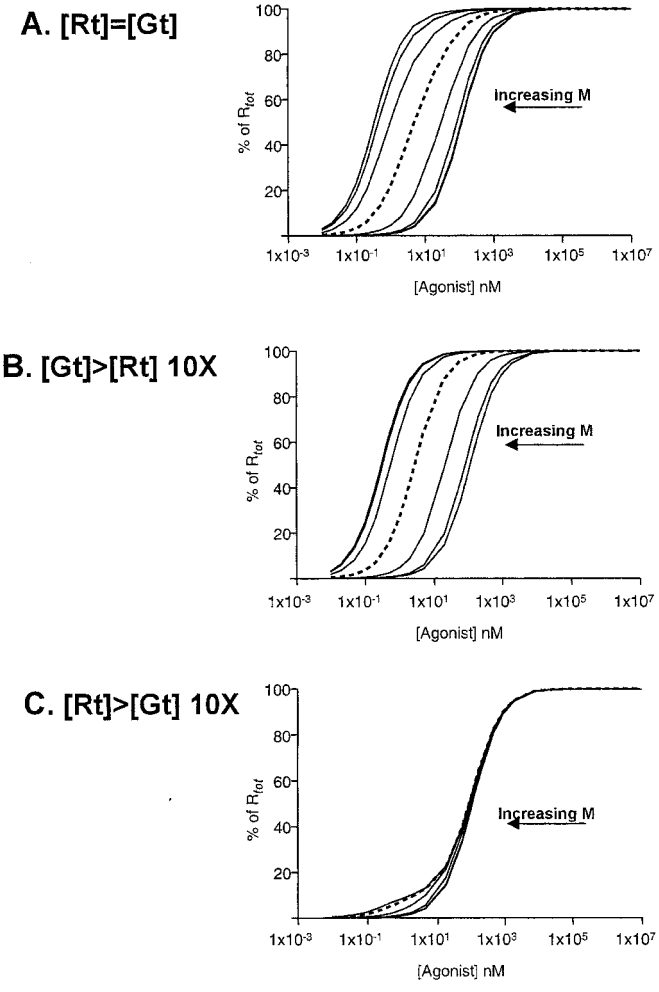

Fig. 9. Effect of reductions of M on agonist binding capacity. The simulated ligand is a full agonist (α = 75; β = δ = 6).

in competitive potency of ligands that otherwise display high binding affinity in direct-saturation experiments (Rosenkilde et al., 1994; Hjorth et al., 1997). Moreover, apart from regulating binding properties, the isomerization rate between receptor states has also been suggested to control the specificity of receptor-G protein interaction (Riitano et al., 1997). These results, together with those presented in the present study, emphasize the extent to which the isomerization rate between different conformers may determine the pharmacological response elicited by a multistate receptor population.

Further support for the existence of multiple receptor states is provided by the fact that both ICI174864 as well as TICPΨ displayed inverted "coupling efficiency ratios" (K_d/EC_{50}) for cAMP accumulation compared with reduction in agonist binding (ICI174864: cAMP accumulation ratio = 1000, reduction in agonist binding ratio = 0.07; TICPΨ: cAMP accumulation ratio = 9, reduction in agonist binding ratio = 0.003). A shift in EC_{50} values to the right of K_d values has been previously described not only for the δOR (Costa et al., 1985) but also for the closely related nociceptin receptor (Albrecht et al., 1998), the β_2-adrenergic receptor (Seifert et al., 1999), and the human formyl peptide receptor (Gether et al., 1995). Such a mismatch has consistently been interpreted as an indication of the effect of the drug in question being produced via an ultralow affinity state. Extended to our study this interpretation would imply the coexistence of at least two distinct receptor states: a high-affinity site that regulates cyclase activity, and a very low-affinity site mediating the observed changes in binding capacity. Furthermore, the fact that 20% of the receptors that recognize [^{3}H]TIPP are exclusively labeled by this ligand is also consistent with the existence of a third, slowly isomerizing form of the hδOR that does not readily recognize drugs with agonist or inverse-agonist efficacy.

The effects induced by short-term exposure to an inverse agonist (30 min) observed herein are different from those obtained after longer treatments (12–48 h). Sustained exposure of various GPCRs to different inverse agonists is known to increase the total number of receptors (MacEwan and Milligan, 1996a,b; Smit et al., 1996; Lee et al., 1997; McLean et al., 1999). In contrast, short-term treatment did not modify the amount of receptor protein measured by neutral antagonist radioligand binding (Fig. 5A) or by immunoblot (Fig. 4). The nature of the effect of short-term inverse-agonist treatment should also be distinguished from that induced by agonist exposure. Unaltered [^{3}H]naltrindole binding after a 30-min treatment with the agonist SNC-80 (Fig. 5B) indicates that, as for the treatment with inverse agonist, the total amount of receptor protein is conserved. However, the B_{max} value for the membrane-impermeable [^{3}H]TIPP remained unchanged after treatment with the inverse agonist ICI174864 but is decreased after treatment with SNC-80, implying that only the latter induces receptors to enter a compartment that is inaccessible to peptide ligands. Thus, although changes in [^{3}H]TICPΨ and [^{3}H]DPDPE labeling induced by ICI174864 are consistent with changes in isomerization rate, those after SNC-80 are most likely a result of receptor redistribution from the cell surface to intracellular compartments. Such redistribution may conceal any change in the way surface-bound receptors recognize ligands with different efficacies. The agonist-promoted loss of [^{3}H]bremazocine binding cannot be attributed to redistribution of receptors because this ligand has access to both cell surface and intracellular sites. However, uncoupling from G protein as a consequence of receptor phosphorylation (Lohse et al., 1990; Pals-Rylaarsdam et al., 1995; Diviani et al., 1996) may account for the observed decrease in B_{max} value. A loss of agonist binding as a consequence of uncoupling is also consistent with the observed reduction in [^{3}H]bremazocine binding observed upon addition of GppNHp (Fig. 6A). If these desensitizing events took place at the same time as changes in the isomerization rate, they may have masked any increase in agonist binding resulting from kinetic changes. None of these results rule out a possible effect of agonist treatment on receptor isomerization. However, the multifactorial nature of the response elicited by agonist drugs hindered the possibility of establishing reciprocity between the effects induced by agonist and inverse-agonist exposure.

In summary, the data presented in this study are consistent with a model of multiple receptor states capable of discriminating among ligands of different efficacies and shows that by modifying isomerization rates among them, short-term treatment with an inverse agonist alters the relative frequency of the different conformers. In addition to questioning our current views on models describing receptor binding and activation, these results have practical implications for the interpretation given to B_{max} values obtained in radioligand binding assays using ligands with different efficacies.

Appendix

The adequacy of ternary complex formation to reflect changes in apparent ligand binding capacity, as those observed in this study, was explored using the fully developed cubic version of the model (Weiss et al., 1996). In its simple form (De Léan et al., 1980), the model was described in terms of 1) ligand binding to the free receptor [AR] (K_{min}), 2) ligand binding to the coupled receptor [ARG] (K_{max}), 3) interaction (M) between the receptor [R] and a membrane regulatory component [RG], and 4) the factor α, characterizing the extent to which agonist binding to the receptor promotes receptor-G protein interaction (Fig. 8A). In this early version of the model, the only active receptor species, [ARG], is promoted by the agonist. In its fully extended version, the model incorporates not only the ability of the receptor to spontaneously isomerize (J) to an active state [R*], which preferentially binds the agonist over [R] by a factor of β but also the precoupling of the inactive state of the receptor to the G protein [RG]. The higher tendency of the active receptor [R*] to form the [R*G] complex is given 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. 8B). The corrected coupling constant $M' = M(1+\gamma J/1+J)$ accounts for the higher tendency of the activated receptor [R*] to interact with the G protein [G]. The coupling efficiency factor α is also modified to incorporate the ability of the agonist to promote coupling of the activated state of the receptor, yielding the following equivalence: $\alpha' = \alpha(1 + J / 1 + \beta J)(1 + \delta B\gamma J / 1 + \gamma J)$.

Using this model, total ligand binding may be simulated by calculating the amount (at equilibrium) of the various ligand-bound states of the receptor as a function of $[A]_{tot}$, $[R]_{tot}$,

$[G]_{tot}$; the drug constants K, α, β, and δ; and the receptor constants J, M, and γ (Weiss et al., 1996): Bound = AR + ARG + AR* + AR*G. Figure 9 shows that for a full agonist ($\alpha = 75$; $\beta = \delta = 6$), the maximal amount of radioligand bound remains unchanged as M is varied across 8 orders of magnitude ($1 \times 10^{-8} - 1 \times 10^{-1}$) at different $[R_{tot}]/[G_{tot}]$ ratios. Similar simulations of changes in M for a partial agonist ($\alpha = 32.5$; $\beta = \delta = 2$) or for a full agonist at higher levels of spontaneous activity (J100x or γ100x) also yielded changes in K_d value with no modification in the apparent B_{max} value for the drug. Based on this simulation, it is possible to conclude that a model of heterotropic cooperativity such as ternary complex formation does not account for the effect induced by short-term inverse agonist treatment on agonist (or inverse agonist) binding capacity, as observed in the present study.

Notwithstanding, a phenomenological representation may be obtained by using a multisite model of noninteracting, noninterconverting sites. This choice is valid only if interconversion among the multiple states is assumed negligible within the time frame of the binding assay, an assumption consistent with slow recovery of agonist binding sites after inverse-agonist treatment (Fig. 7). In fact, differences in B_{max} values for [^{3}H]TIPP, [^{3}H]TICPΨ, and [^{3}H]DPDPE (Table 2) indicate that at least three noninterconverting sites with a distinct affinity for drugs with different levels of efficacy exist even before pretreatment. From this observation, simulations were made using a three-site model. (Reciprocal changes in agonist and inverse-agonist B_{max} values may also be simulated with a two-site population; nonetheless, three sites provided a better representation of the data.)

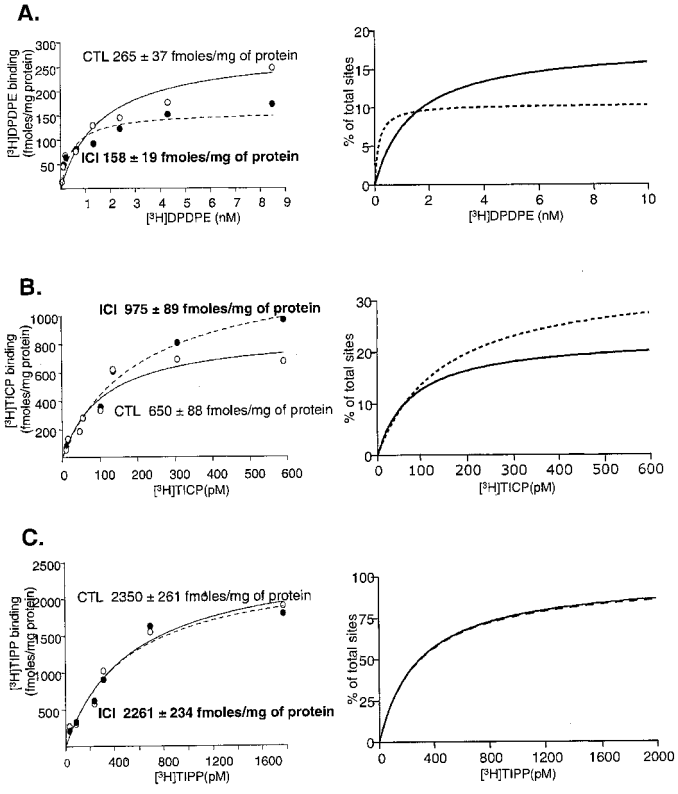

Fig. 10. Simulated effect of pretreatment with ICI174864. Left, results from a representative experiment. Right, the corresponding simulations. Solid lines, control cells; dashed lines, treated cells.

In the simulation shown in Fig. 10, drug affinity for each of the three sites were set to obtain K_{app} values equivalent to those experimentally observed. In the experimental setting, variability did not allow discrimination between single and multiple sites, requiring default fitting to a one-site hyperbola to obtain K_{app} values shown in Table 1. Thus, for the curves simulated in Fig. 10, data were generated with a multisite equation, Bound = $B_{max_1} \times [A] / (K_{d_1} + [A]) + B_{max_2} \times [A] / (K_{d_2} + [A]) + B_{max_3} \times [A] / (K_{d_3} + [A])$, but were fitted to a single site. Simulated K_{app} values corresponded to the actual values when the agonist was allowed to recognize site 1 better than site 2, and site 2 better than site 3 (K_{d1} = 0.8 nM; K_{d2} = 80 nM; K_{d3} = 8 μM). Conversely, the inverse agonist was set to recognize site 2 better than site 1, and site 1 better than site 3 (K_{d2} = 1 pM; K_{d1} = 20 pM; K_{d3} = 8 nM). The affinity of the three sites for the antagonist was kept constant ($K_{d1} = K_{d2} = K_{d3}$ = 145 pM).

To represent the effect of inverse-agonist pretreatment on agonist and inverse-agonist binding capacity, the total number of sites was kept constant, but the relative frequency of each subtype was allowed to change. The magnitude of the change in relative frequency was settled to account for a greater increase in observed inverse-agonist B_{max} value than the decrease in agonist capacity. The increase in [^{3}H]TICP binding was 323 fmol/mg, whereas the reduction in [^{3}H]DPDPE binding was 133 fmol/mg (Fig. 10, left). Therefore, redistribution was represented by an increase in the ratio of inverse agonists preferring site 2 at the expense of both site 1 (agonist-preferring site) and site 3 (antagonist binding site). The initial proportion for each site was calculated from control [^{3}H]DPDPE, [^{3}H]TICP, and [^{3}H]TIPP B_{max} values and represent the percentage of total [^{3}H]TIPP sites specifically recognized by each ligand. A 44% decrease in apparent [^{3}H]DPDPE maximal binding and a concomitant 47% increase in [^{3}H]TICP apparent B_{max} values could be simulated by changing the site proportion from site 1 (15%), site 2 (22%), and site 3 (67%) in control condition to site 1 (8%), site 2 (34%), and site 3 (58%) after ICI174864 treatment. Congruence of experimental and simulated results should not be taken as definite proof of the adequacy of any given model. However, it constitutes additional evidence supporting the idea that changes in the relative frequencies of distinct receptor states may account for the changes in ligand binding capacity observed in the present study.

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