

Agonist-, Antagonist-, and Inverse Agonist-Regulated Trafficking of the δ -Opioid Receptor Correlates with, but Does Not Require, G Protein Activation

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ABSTRACT

In this study, we explored the relationship between ligand-induced regulation of surface δ opioid receptors and G protein activation. G protein activation was assessed with [35 S]guanosine-5'-O-(3-thio)triphosphate (GTP γ S) binding assays conducted at both 37 and 0°C. Ligand-independent (constitutive) activity of the δ -receptor was readily observed when the [35 S]GTP γ S binding assay was performed at 37°C. We identified a new class of alkaloid inverse agonists (RTI-5989-1, RTI-5989-23, RTI-5989-25), which are more potent than the previously described peptide inverse agonist ICI-174864 (*N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu). Treatment with these inverse agonists for 18 h caused up-regulation of surface receptors. Eighteen-hour treatment with etorphine resulted in approxi-

mately 90% loss of surface receptor, whereas fentanyl, diprenorphine, and morphine caused between 20 and 50% loss. The abilities of ligands to modulate [35 S]GTP γ S binding at 37°C showed a strong correlation with their abilities to regulate surface receptor number ($r^2 = 0.86$). Interestingly, the ability of fentanyl to activate G proteins was markedly temperature sensitive. Fentanyl showed no stimulation of [35 S]GTP γ S binding at 0°C but was as efficacious as etorphine, morphine, and diprenorphine at 37°C. Neither the ligand-induced receptor increases nor decreases were perturbed by pertussis toxin pretreatment, suggesting that functional G proteins are not required for ligand-regulated δ -opioid receptor trafficking.

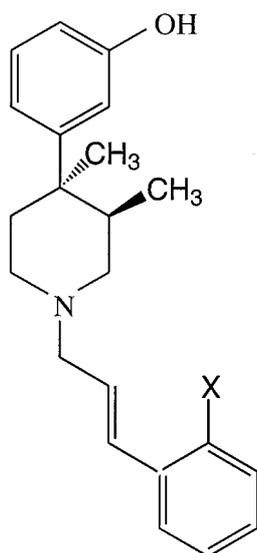
Constitutive activity has become a well described characteristic of many G protein-coupled receptors (GPCRs) and has redefined the concept of how GPCRs function. Ligand-independent activity of GPCRs has been described for a variety of receptors either in their wild-type form or in mutated forms (for reviews, see Milligan et al., 1997; Leurs et al., 1998). With the realization that receptors could be active in the absence of ligand, some ligands have had to be reclassified from being antagonists (ligands that bind to the receptor but do not elicit a response) to being inverse agonists (ligands that elicit a response opposite to that of agonists). One of the first GPCRs to be described as having constitutive activity is the G $_{i/o}$ -coupled δ -opioid receptor. Ligand-independent activity of this receptor was first shown in NG108-15 cells, which endogenously express the murine δ -opioid receptor (Costa and Herz, 1989; Costa et al., 1990). Constitutive activity of the receptor has subsequently been demonstrated in cell lines stably transfected with the δ -receptor from various species (Chiu et al., 1996; Mullaney et

al., 1996; Merkouris et al., 1997; Hosohata et al., 1999; Neilan et al., 1999; Labarre et al., 2000).

The regulation of GPCRs after various ligand treatments has been an active area of research with the majority of studies focusing on the effect of agonist treatment on receptor function. This has been particularly important arena in the opioid field due to the desire to understand the basis of tolerance and dependence to opioids that result from repeated administration of the drug (Nestler and Aghajanian, 1997). Although these adaptational processes that occur in animals are obviously complex, *in vitro* studies on cell lines that express opioid receptors have furthered our understanding of the cellular adaptations that occur after ligand treatment. Generally, opioid receptors have been shown to be phosphorylated and desensitized in response to agonist treatment, although the extent of these processes is dependent on the particular agonist (for review, see Law and Loh, 1999). The extent of opioid receptor internalization is also dependent on the type of agonist. For example, the agonist etorphine is able to cause rapid internalization of μ - and δ -opioid receptors, whereas morphine does not cause this regulatory event (Keith et al., 1996). Opioid receptors have also been

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ABBREVIATIONS: GPCR, G protein-coupled receptor; HEK, human embryonic kidney; GTP γ S, guanosine-5'-O-(3-thio)triphosphate; ICI-174864, *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu; DOR, δ -opioid receptor; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; PTX, pertussis toxin; TIPP, Tyr-Tic-Phe-Phe.



RTI-5989-1: X = H
 RTI-5989-25: X = CH₃
 RTI-5989-23: X = Cl

Fig. 1. Structures of RTI-5989-1, RTI-5989-25, and RTI-5989-23.

shown to be down-regulated in response to chronic agonist treatment both in vivo and in vitro (for review, see Law and Loh, 1999). Unfortunately, the contributions of these various regulatory events to the phenomenon of tolerance and dependence in vivo are still poorly understood.

One area of research that has not been adequately explored is the effect of antagonists and inverse agonists on opioid receptor regulation. Based on the observation that agonists can cause opioid receptor internalization and down-regulation, it would be reasonable to expect that treatment with inverse agonists and perhaps antagonists would result in up-regulation of opioid receptors. Although it has been well documented that treatment with the opioid antagonists causes up-regulation of both μ - and δ -opioid receptors in vivo and in vitro (Barg et al., 1984; Tempel et al., 1984; Yoburn et al., 1990; Belcheva et al., 1991; Zadina et al., 1995; Chen et al., 1997), it was not determined whether these ligands functioned as antagonists or inverse agonists in these various systems. In the present study, we explored the relationship between the ability of a ligand to modulate G protein activation and regulation of δ -opioid surface receptor number in HEK 293 cells stably transfected with a FLAG-tagged murine δ -opioid receptor (293-SF-DOR cells). Additionally, we have identified a new class of inverse agonists for the δ -opioid receptor that are more potent in inhibiting constitutive GTP γ S binding than the well characterized peptide inverse agonist *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI-174864).

Experimental Procedures

Cell Line. 293-SF-DOR cells have been characterized previously (Keith et al., 1996) and were a gift from Dr. Mark von Zastrow (University of California, San Francisco, CA). Briefly, HEK 293 cells were stably transfected with the murine δ -opioid receptor (DOR) cDNA containing the signal FLAG epitope at the amino terminus (293-SF-DOR cells). 293-SF-DOR cells expressed approximately

150,000 receptors/cell, estimated by radioligand binding (Keith et al., 1996). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 0.025 μ g/ml Fungizone.

Flow Cytometric Analysis. FLAG M2 antibody was labeled directly with fluorescein isothiocyanate (FITC) to an F/P ratio of approximately 3.0 as described previously (Keith et al., 1998). For analysis of surface receptors, 293-SF-DOR cells were treated with various drugs for 18 h at 37°C and harvested with 2 mM EDTA/phosphate-buffered saline. Cells were then chilled to 0°C to stop further receptor trafficking and stained with 10 μ g/ml FITC-labeled FLAG for 10 min. Cells were washed once with 2% fetal bovine serum/0.1% NaN₃/phosphate-buffered saline and 5,000 to 10,000 cells/sample were analyzed on a FAC-Scan flow cytometer using CellQuest 3.0.1 for acquisition and analysis (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The mean fluorescence of unstained cells was subtracted from the mean fluorescence of stained cells before calculating the change in surface receptor number after drug treatment.

Membrane Preparation. 293-SF-DOR cells were pelleted, frozen at -70°C for at least 30 min, and then resuspended in ice-cold 50 mM Tris HCl pH 7, 2.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (homogenization buffer). Cells were disrupted in a Dounce homogenizer and centrifuged at 1000g for 10 min at 4°C. The pellet was resuspended in homogenization buffer, rehomogenized, and centrifuged again at 1000g for 10 min at 4°C. Both supernatants were pooled and centrifuged at 13,000g for 45 min at 4°C. The pellet was resuspended in homogenization buffer, rehomogenized, and centrifuged at 13,000g for 45 min at 4°C. The pellet was resuspended in 50 mM Tris HCl pH 7, 0.32 M sucrose and stored at -70°C.

[³⁵S]GTP γ S Binding Assay. [³⁵S]GTP γ S binding was performed as described by Befort et al. (1996), with modifications of temperature and GDP and [³⁵S]GTP γ S concentrations. Briefly, 4 μ g of membrane protein was incubated in 50 mM HEPES pH 7.6, 5 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% BSA, 1 μ M GDP, 0.1 nM [³⁵S]GTP γ S, and various opioid ligands. Membranes were incubated with 10 μ M unlabeled GTP γ S to determine nonspecific binding. The reactions were conducted at either 0°C for 1 h or 15 min at 37°C. The mixtures were harvested with a Brandel M24RS harvester using pre-soaked Whatman GT100 GF/B glass filters and washed with ice-cold 50 mM Tris HCl pH 7.0. Filters were dried and counted in a Beckman LS1600 scintillation counter using Cytoscint ES (ICN, Irvine, CA).

Materials. FLAG M2 antibody was purchased from Eastman Kodak (New Haven, CT). [³⁵S]GTP γ S (1250 Ci/mmol) was purchased from PerkinElmer Life Science Products (Boston, MA). FITC, 3-isobutyl-1-methylxanthine, forskolin, and phenylmethylsulfonyl fluoride were purchased from Sigma (St. Louis, MO). PTX was purchased from Sigma (St. Louis, MO) and Calbiochem (La Jolla, CA). Tissue culture supplies were purchased from Omega Scientific (Tarzana, CA). RTI-5989-1, RTI-5989-23, and RTI-5989-25 were synthesized as previously reported (Thomas et al., 1998); all other drugs used in this study were gifts from the National Institute on Drug Abuse (Bethesda, MD).

Results

Ligand-Induced Changes in Surface δ -Opioid Receptors. We have shown previously that in HEK 293 cells transfected with μ -opioid receptors there is a rapid loss of surface receptors in response to etorphine and an up-regulation of surface receptors in response to the partial agonist buprenorphine and the antagonist naloxone (Zaki et al., 2000). We were interested in whether these ligands would have similar effects on the δ -opioid receptor after long-term treatment. We also studied the effects of 18-h treatment of 293-SF-DOR cells with the following alkaloid ligands on surface δ -opioid receptor number: fentanyl; diprenorphine; morphine; naltrindole; naltrexone; and the (+)-3,4-dimethyl-4-(3-hydroxyphenyl)-piperidine derivatives RTI-5989-1, RTI-5989-23, and RTI-5989-25

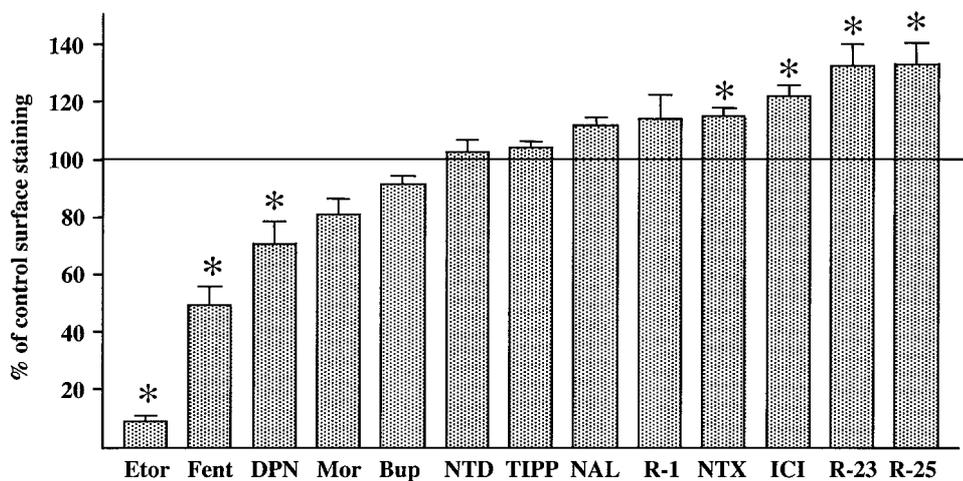


Fig. 2. Effects of long-term ligand treatment on surface δ -receptor number. 293-SF-DOR cells were treated with 10 μ M drug (1 μ M for etorphine) for 18 h at 37°C and then chilled to 0°C to arrest further trafficking. Cells were stained with FITC-labeled FLAG M2 monoclonal antibody and analyzed on a FACS-can flow cytometer. The mean fluorescence of stained cells minus the mean fluorescence of unstained cells was used to calculate the percentage of surface receptor staining. Values are the mean \pm S.E.M. of 6 to 15 experiments. Columns with an asterisk have p values < 0.05 by one-way analysis of variance followed by the post hoc Dunnett's test. Etor, etorphine; Fent, fentanyl; DPN, diprenorphine; Mor, morphine; Bup, buprenorphine; NTD, naltrindole; NAL, naloxone; NTX, naltrexone; ICI, ICI-174864; R-1, RTI-5989-1; R-25, RTI-5989-25; and R-23, RTI-5989-23.

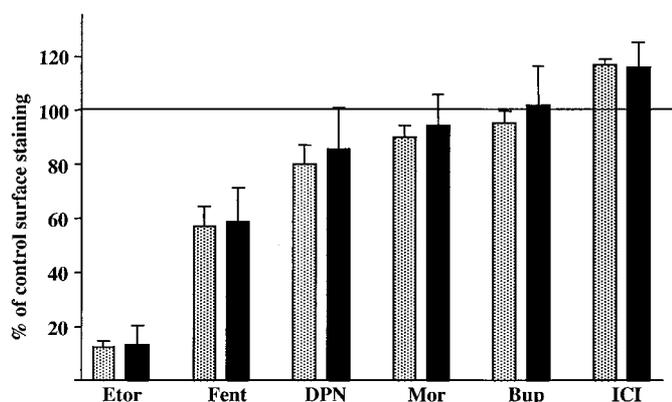


Fig. 3. Effect of PTX treatment on ligand-induced changes in surface δ -opioid receptor number. 293-SF-DOR cells were pretreated overnight with 100 ng/ml PTX. The percentage of surface receptor staining after 18-h drug treatment (1 μ M for etorphine and 10 μ M all other drugs) was calculated by dividing the mean fluorescence of the cells in each drug treatment by the mean fluorescence of nondrug-treated control and PTX-treated cells. Values are the mean \pm S.E.M. of three to nine separate experiments. \square , control cells; \blacksquare , PTX-treated cells. For drug abbreviations, see legend for Fig. 2.

(Fig. 1). We also treated cells with the peptides Tyr-Tic-Phe-Phe (TIPP) and ICI-174864. Eighteen-hour etorphine treatment decreased the amount of δ -opioid surface receptor staining by approximately 90%, whereas fentanyl and diprenorphine induced between a 25 and 50% loss of surface staining (Fig. 2). Buprenorphine and morphine induced small decreases in surface receptor number that were not significantly different from untreated cells. The classical δ -antagonists TIPP and naltrindole had negligible effects on surface receptor staining, and both naloxone and RTI-5989-1 showed a tendency for increasing surface receptor number that did not reach statistical significance (Fig. 2). In contrast, naltrexone, ICI-174864, RTI-5989-23, and RTI-5989-25 caused a significant increase in surface staining ranging from approximately 15 to 30% (Fig. 2).

Effect of PTX on Ligand-Induced Changes in Surface δ -Opioid Receptors. 293-SF-DOR cells were treated for 18 h with 100 ng/ml PTX, which abolished etorphine-induced stimulation of [35 S]GTP γ S binding when measured at both 37°C (106 \pm 4%, S.E.M., $n = 5$) and at 0°C (99 \pm 10%, S.E.M., $n = 5$). However, PTX cotreatment had no effect on either ligand-induced increases or decreases in surface δ -opioid receptor number after 18-h drug treatment (Fig. 3).

Constitutive Activity of δ -Opioid Receptor Is Evident at 37°C in [35 S]GTP γ S Binding Assay.

Measurement of [35 S]GTP γ S binding has been widely used to assess G protein activation and constitutive activity. We found in this study that constitutive activity of the receptor was strongly evident when the [35 S]GTP γ S binding assay was conducted at 37°C as opposed to 0°C. First, basal [35 S]GTP γ S binding was 49 \pm 7% higher at 37°C compared with 0°C (S.E.M., $n = 5$). The specific basal binding for a typical experiment was 3510 dpm [35 S]GTP γ S/10 μ g of membrane protein at 37°C. Second, PTX treatment was able to decrease basal [35 S]GTP γ S binding by 41 \pm 5% at 37°C (S.E.M., $n = 5$), but by only 12 \pm 5% at 0°C (S.E.M., $n = 5$). Finally, as shown in Fig. 4, the previously described inverse agonist ICI-174864 was able to decrease basal [35 S]GTP γ S binding by 24 \pm 3% at 37°C (S.E.M., $n = 8$), while only inhibiting basal binding by 7 \pm 4% at 0°C (S.E.M., $n = 8$).

Efficacies of Various Ligands for Modulating [35 S]GTP γ S Binding. The [35 S]GTP γ S binding assay was performed at both 37 and 0°C to assess the efficacies of various ligands for activating G proteins (Fig. 4). Etorphine (1 μ M) caused significantly more stimulation when the assay was performed at 37°C than at 0°C (172 \pm 10% stimulation over basal versus 142 \pm 4% stimulation over basal; S.E.M., $n = 8$; paired Student's t test, $p < 0.05$). The opioid ligands diprenorphine, morphine, and fentanyl were similarly efficacious at 37°C (approximately 130% stimulation over basal), which was significantly less efficacious than etorphine ($n = 8$; paired Student's t test, $p < 0.05$). Interestingly, both diprenorphine and morphine were as efficient at stimulating [35 S]GTP γ S binding at 37°C as at 0°C, while fentanyl was significantly less efficacious at 0°C than at 37°C. Buprenorphine and naltrindole had no significant activity at either 37 or 0°C. As mentioned above, ICI-174864 exhibited inverse agonist activity at 37°C as evidenced by the 26 \pm 3% decrease in [35 S]GTP γ S binding; naltrindole (10 μ M) was able to block the effects of ICI-174864 (percentage of stimulation over control with 10 μ M ICI-174864 = 70 \pm 4 versus 105 \pm 4% with 10 μ M ICI-174864 and 10 μ M naltrindole; $n = 4$, S.E.M.). RTI-5989-1, RTI-5989-23, and RTI-5989-25 were also found to be inverse agonists (see below).

Potencies of Inverse Agonists in [35 S]GTP γ S Binding Assay. The potencies of the inverse agonists RTI-5989-1, RTI-5989-23, and RTI-5989-25 were determined in the

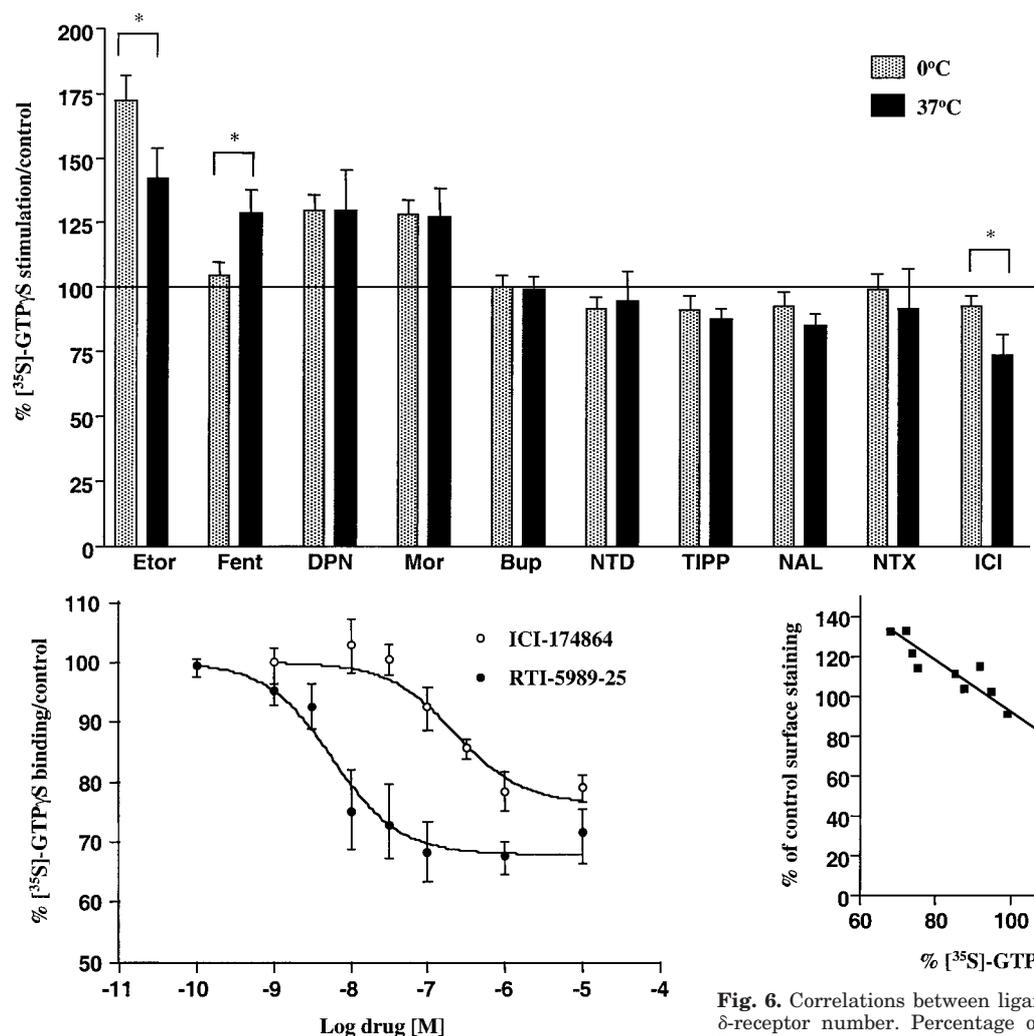


Fig. 5. Dose-response curves of RTI-5989-25 and ICI-174864 for the inhibiting basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding. 293-SF-DOR membranes were incubated in 50 mM HEPES pH 7.6, 5 mM MgCl_2 , 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% BSA, 1 μM GDP, 0.1 nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$, and various concentrations of RTI-5989-25 and ICI-174864 for 15 min at 37°C. The mixtures were harvested and washed with ice-cold 50 mM Tris HCl pH 7.0. Values are the mean \pm S.E.M. of four to five experiments. ●, RTI-5989-25; ○, ICI-174864.

TABLE 1

Potencies and efficacies of inverse agonists in the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assay

Measurement of the ability of various ligands to inhibit $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding was performed as described under *Experimental Procedures*. Curves were fitted to a sigmoidal dose response curve with the Hill slope = -1 (GraphPad Prism; GraphPad, San Diego, CA). Values are the mean \pm S.E.M. of three to five experiments.

Ligand	IC ₅₀ nM	% Binding over Control
ICI-174864	176 \pm 44	75 \pm 2
RTI-5989-1	13.6 \pm 3.8	69 \pm 3
RTI-5989-23	8.2 \pm 3.7	65 \pm 2
RTI-5989-25	6.6 \pm 3.3	66 \pm 4

$[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assay at 37°C, and were 13, 21, and 27 times more potent, respectively, than the previously described inverse agonist ICI-174864 (Fig. 5; Table 1).

Correlations between Ligand Signaling and Alteration in Surface δ -Receptor Number. Figure 6 is a plot of percentage of control δ -receptor surface staining after 18-h ligand treat-

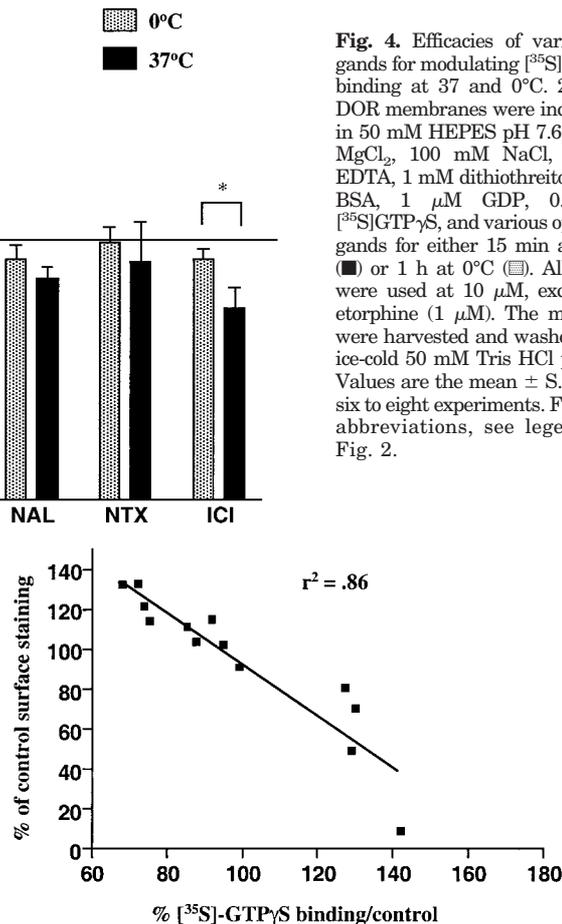


Fig. 6. Correlations between ligand signaling and alteration in surface δ -receptor number. Percentage of control surface staining after 18-h ligand treatment is plotted on the x-axis versus percent stimulation $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding over control at 37°C on the y-axis, using all 13 ligands described in this study.

ment versus percentage of stimulation $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding over control at 37°C. The correlation coefficient (r^2) was 0.86.

Discussion

Constitutive activity of GPCRs has become a widely studied phenomenon and has been extensively described for the δ -opioid receptor. The peptide opioid ligand ICI-174864 was shown to inhibit basal GTP hydrolysis in a high-affinity GTPase assay in membranes of the neuroblastoma-glioma NG108-15 cells (Costa and Herz, 1989; Costa et al., 1990) and is now thought of as the prototypical inverse agonist for the δ -receptor. Another peptide inverse agonist, (2S,3R)TMT-L-TIC-OH, has been recently described (Hosohata et al., 2000). Treatment of NG108-15 cells with PTX, which abolishes coupling of GPCRs to their cognate $G_{i/o}$ proteins, lowered basal GTPase activity and basal $\text{GTP}\gamma\text{S}$ binding (Costa et al., 1990; Szekeres and Traynor, 1997) and is further evidence that the δ -receptor is constitutively active. Cloning of the δ -receptor made it possible to exogenously express the receptor in cell lines and determine whether the ligand-independent activity of the receptor is simply a function of the cellular environment of the NG108-15 cells or whether the receptor has an intrinsic constitutive activity that is observable in other cel-

Fig. 4. Efficacies of various ligands for modulating $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding at 37 and 0°C. 293-SF-DOR membranes were incubated in 50 mM HEPES pH 7.6, 5 mM MgCl_2 , 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% BSA, 1 μM GDP, 0.1 nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$, and various opioid ligands for either 15 min at 37°C (■) or 1 h at 0°C (▨). All drugs were used at 10 μM , except for etorphine (1 μM). The mixtures were harvested and washed with ice-cold 50 mM Tris HCl pH 7.0. Values are the mean \pm S.E.M. of six to eight experiments. For drug abbreviations, see legend for Fig. 2.

lular backgrounds. The murine, rat, and human δ -opioid receptors have been heterologously expressed in a variety of cell lines and constitutive activity of the receptor has been demonstrated (Chiu et al., 1996; Mullaney et al., 1996; Merkuris et al., 1997; Hosohata et al., 1999; Neilan et al., 1999; Labarre et al., 2000).

In the current study, we have also shown that the δ -opioid receptor is constitutively active in HEK 293 cells stably transfected with a FLAG-tagged murine δ -opioid receptor (293-SF-DOR cells). The inverse agonist ICI-174864 was able to inhibit basal [35 S]GTP γ S binding by $25 \pm 2\%$ when the [35 S]GTP γ S binding assay was conducted at 37°C. Additionally, PTX treatment was able to decrease basal [35 S]GTP γ S binding by $41 \pm 5\%$ at 37°C. These results are in agreement with the numerous studies cited above.

A novel finding of this study is the description of a new class of potent δ -opioid alkaloid inverse agonists, *trans*-cinnamyl *N*-substituted (+)-3,4-dimethyl-4-(3-hydroxyphenyl) piperidines, RTI-5989-1, RTI-5989-23, and RTI-5989-25. These compounds have recently been described as some of the most potent and selective μ -opioid receptor antagonists available, although they still retain a relatively high affinity for the δ - and κ -opioid receptors (Thomas et al., 1998). These compounds were able to inhibit basal [35 S]GTP γ S binding with IC₅₀ values of approximately 10 nM, making these alkaloids considerably more potent than the peptide inverse agonist ICI-174864 (IC₅₀ = 155 nM). Although RTI-5989-1, RTI-5989-23, and RTI-5989-25 have very high affinities for the μ -receptor, they did not inhibit basal [35 S]GTP γ S binding at either 37 or 0°C in membranes from 293 cells expressing μ -receptors (personal observation). Recently, two groups have identified additional nonpeptide inverse agonists for the δ -opioid receptor (Neilan et al., 1999; Labarre et al., 2000). These compounds, as well as the RTI series described in this study, should facilitate the development of selective and efficacious compounds to investigate the role of constitutively active δ -receptors in vivo.

Although inverse agonism in 293-SF-DOR cells was clearly detected at 37°C, when the [35 S]GTP γ S binding assay was conducted at 0°C, a temperature at which agonist activity can readily be measured, ICI-174864 inhibition of basal [35 S]GTP γ S binding was negligible. Interestingly, G protein activation profiles of alkaloid agonists were also very different between 37 and 0°C. The most striking difference in activity between the two temperatures was observed with fentanyl and etorphine (Fig. 4). Although the percentage of stimulation caused by etorphine in the [35 S]GTP γ S binding assay was significantly larger at 0°C than at 37°C, the reverse was observed for fentanyl. Indeed, fentanyl did not cause any stimulation of [35 S]GTP γ S binding at 0°C, but at 37°C was as efficacious as morphine and diprenorphine. Morphine and diprenorphine were similarly efficacious at both temperatures. One hypothesis is that the receptor can assume a range of conformations for G protein activation, and the ability of each drug to achieve an active conformation may be temperature-dependent. Thus, in the case of fentanyl the unique conformational change in the δ -receptor that is required to stimulate [35 S]GTP γ S binding may not be achievable at 0°C. This may also be true for the conformation required to generate constitutive activity, given that constitutive activity is greatly reduced at 0°C.

In addition to identifying whether the δ -opioid receptor was

constitutively active in our system, we were interested in determining whether there was a relationship between the ability of a ligand to modulate G protein activation and its ability to alter surface receptor number. We found that 18-h treatment of 293 SF-DOR cells with the high-efficacy agonist etorphine caused a dramatic loss of surface receptor (>90%), as assessed by flow cytometry. Fentanyl caused a moderate decrease in surface receptor staining, while diprenorphine and morphine caused smaller decreases. Buprenorphine, naltrindole, and TIPP, which did not significantly change [35 S]GTP γ S binding, did not cause an appreciable change in δ -surface receptor. We found that a significant correlation exists between a ligand's ability to modulate G protein activation (when measured at 37°C) and alter δ -surface receptor number after chronic ligand treatment ($r^2 = 0.86$) (Fig. 6). There was a weaker correlation when G protein activation was assessed at 0°C ($r^2 = 0.74$) because no inverse agonist activity was apparent and fentanyl did not stimulate [35 S]GTP γ S binding but did stimulate loss of surface receptors (data not shown).

The inverse agonists ICI-174864, RTI-5989-23, and RTI-5989-25 as well as naltrexone caused a small but significant up-regulation of surface receptor number and RTI-5989-1, and naloxone showed a tendency for up-regulation that did not reach statistical significance. The neutral antagonists TIPP and naltrindole caused no change in surface receptor number. This is the first demonstration that ligand treatment is able to increase δ -opioid cell surface receptor. Antagonist treatment has been shown to up-regulate the number of cell surface A1 adenosine receptors (Ciruela et al., 1997) and inverse agonists can up-regulate histamine H2 receptors (Smit et al., 1996; Alewijnse et al., 1998) and cannabinoid receptors (Rinaldi-Carmona et al., 1998; Bouaboula et al., 1999). Various wild-type dopamine receptors have been shown to be up-regulated in response to both agonist and antagonist treatment (Filtz et al., 1994; Zhang et al., 1994; Cox et al., 1995; Ng et al., 1997; Geurts et al., 1999). Finally, it should be noted that treatment with inverse agonists does not always lead to up-regulation of GPCRs. For instance, treatment of 5-hydroxytryptamine_{2c} receptors with inverse agonists, but not agonists or antagonists, results in a decrease in receptor binding sites (Barker et al., 1994; Labrecque et al., 1995; Millan et al., 1999).

μ -Opioid receptors are also up-regulated in response to antagonist treatment both in vitro and in vivo (Zadina et al., 1995). In contrast to the δ -receptor, the μ -receptor shows dramatically greater up-regulation in HEK 293 cells (Zaki et al., 2000). Another significant difference between the μ - and δ -opioid receptor is that, in addition to antagonists, partial agonists such as buprenorphine up-regulate the number of surface μ -receptors, whereas inverse agonists and only some antagonists caused an increase in the number of surface δ -receptors. Additionally, partial agonists such as morphine and diprenorphine caused a loss of surface δ -receptors.

We have also shown that overnight treatment with PTX did not alter any of the ligand-induced changes in surface δ -opioid receptor number (Fig. 3). This is in contrast to the μ -receptor, where PTX treatment attenuates the decrease in surface receptor caused by long-term treatment and augments the increase caused by partial agonists and antagonists (Zaki et al., 2000). These findings agree with previous studies that have shown that PTX inhibits agonist-induced internalization and down-regulation of the μ -, but not the

δ -opioid receptor (Chakrabarti et al., 1997; Yabaluri and Medzihradsky, 1997; Remmers et al., 1998).

This study provides some insights into ligand-induced regulatory mechanisms of δ -receptors and highlights the individuality of different drugs with regard to receptor trafficking and G protein activation. The observation that ligand-induced actions can be differentially sensitive to temperature is important because measurement of ligand efficacies is often performed at reduced temperatures. This study also contrasts ligand-induced regulatory mechanisms of δ -receptors with those of μ -opioid receptors (Keith et al., 1996; Zaki et al., 2000). Agonist-induced loss of surface receptors of δ -receptors is more extensive than that of μ -receptors, whereas antagonist-induced up-regulation of surface μ -receptors is more extensive than that of δ -receptors. In addition, all partial agonists tested tended to decrease surface δ -receptors, but μ -receptors are up-regulated by a number of weak partial agonists. Finally, we have identified a new series of inverse agonists, which if modified to increase selectivity for δ -receptors, could help determine potential functions of δ -receptor constitutive activity in vivo. Recent data suggest that in addition to modulating pain and gut transit, δ -receptors may also regulate mood, and a potential role for constitutive activity in these functions is intriguing (Filliol et al., 2000).

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Flow cytometric analyses were performed in the Janis V. Giorgi Flow Cytometry Laboratory at UCLA.

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