

Ligand-Induced Changes in Surface μ -Opioid Receptor Number: Relationship to G Protein Activation?¹

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ABSTRACT

In this study, we explored the relationship between regulation of surface μ -opioid receptor number, ligand-induced G protein activation [measured by [³⁵S]guanosine-5'-O-(3-thio)triphosphate (GTP γ S) binding] and second messenger signaling (measured by the inhibition of cAMP accumulation). Etorphine and two isomers of *cis*- β -hydroxy-3-methylfentanyl (RTI-1a and RTI-1b), which were full agonists for G protein activation and signaling, caused approximately a 50% loss of surface receptors after 1 h of treatment. Fentanyl and morphine were full agonists for inhibiting cAMP accumulation and partial agonists for stimulating [³⁵S]GTP γ S binding and internalization. Although both agonists were ~80% as efficacious as etorphine in stimulating [³⁵S]GTP γ S binding, fentanyl induced a 35% loss of surface receptors, whereas morphine only caused a 10% loss. Additionally, both long- and short-term treatment with the opi-

oid antagonist naloxone caused increases in surface receptors. Unexpectedly, the weak partial agonists buprenorphine and one isomer of *cis*- β -hydroxy-3-methylfentanyl (RTI-1d) also were found to cause an increase in surface receptors. Treatment with pertussis toxin (PTX) diminished agonist-induced loss of surface receptors. Furthermore, the abilities of morphine and fentanyl to cause internalization were more impaired after PTX treatment than that of etorphine. PTX treatment also significantly enhanced the increase in surface receptor number caused by 18-h treatment with naloxone and buprenorphine. The results of this study suggest that disruption of G protein coupling by PTX treatment affects ligand-regulated μ -receptor trafficking and that partial agonists for signaling can vary greatly in the ability to regulate the number of surface μ -opioid receptors.

The physiological targets of both exogenous and endogenous opioids are three types of 7-transmembrane domain, G protein-coupled receptors (GPCRs): μ -, δ -, and κ -opioid receptors (Dhawan et al., 1996). Opioid receptors act via G proteins to inhibit adenylyl cyclase, increase potassium currents, inhibit calcium channel activity, modulate inositol triphosphate turnover, and activate mitogen-activated protein kinase (Dhawan et al., 1996; Fukuda et al., 1996). These actions culminate in the attenuation of neuronal activity by inhibiting neurotransmitter release and changing neuronal excitability (both pre- and postsynaptically). Of the three opioid receptors, the μ -receptor appears to mediate many of the biological properties of morphine and has a high affinity for many other clinically used opiates (Raynor et al., 1995; Matthes et al., 1996; Sora et al., 1997; Tian et al., 1997; Loh et al., 1998).

Opioid receptors are similar to other GPCRs in that they

undergo adaptations such as desensitization, down-regulation, and internalization in response to agonist treatment (for review, see Bohm et al., 1997). The molecular processes underlying desensitization are thought to include rapid uncoupling of the receptor from its G proteins by phosphorylation of the receptor and/or binding of accessory proteins such as β -arrestins. Receptor internalization (the loss of receptor from the cell surface) has been implicated in the process of dephosphorylation and resensitization of the receptor. After prolonged treatment, there is an eventual loss of receptor protein (down-regulation) that may occur through increased degradation or decreased synthesis of the receptor. Each of these regulatory processes may contribute to the phenomena of tolerance and dependence that undermine the use of opiates as analgesics.

It has been demonstrated that μ -receptors internalize on agonist treatment both *in vitro* and *in vivo* and that this internalization is ligand-specific and reversible by antagonists (Arden et al., 1995; Sternini et al., 1996; Keith et al., 1996, 1998). Although many endogenous opioids and the potent opioid alkaloid etorphine cause the μ -opioid receptor

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ABBREVIATIONS: GPCR, G protein-coupled receptor; HEK, human embryonic kidney; GTP γ S, guanosine-5'-O-(3-thio)triphosphate; PTX, pertussis toxin; MOR, murine μ -opioid receptor; FITC, fluorescein isothiocyanate; RAVE, relative activity versus endocytosis; DAMGO, [D -Ala²,*N*-(MePhe⁴,Gly- o^5)-enkephalin, FBS, fetal bovine serum.

expressed in 293 human embryonic kidney (HEK) cells to internalize within minutes, morphine induces only minimal internalization of the μ -receptor in this cell line (Keith et al., 1996, 1998). Because etorphine is two orders of magnitude more potent than morphine with regard to second messenger signaling, and a number of studies have demonstrated that morphine is a partial agonist (Emmerson et al., 1996; Selley et al., 1997; Kovoor et al., 1998), a pertinent question is whether an agonist's ability to cause μ -receptor internalization is correlated with its potency or efficacy for activating G proteins. Initial studies have implied that there is no clear relationship between an opioid agonist's ability to cause μ -receptor internalization and its signaling ability. It has been observed that the enkephalin analog DAMGO ([D-Ala², N-MePhe⁴, Gly-ol⁵]-enkephalin), which causes μ -receptor internalization to the same extent as etorphine, has a similar potency and efficacy to morphine for signal transduction (Burford et al., 1998; Keith et al., 1998).

These studies have prompted us to further analyze the relationship between μ -opioid receptor internalization and signaling. Herein, we have chosen to study the structurally related alkaloids etorphine, morphine, buprenorphine, and naloxone in addition to fentanyl and four stereoisomers of its congener, *cis*- β -hydroxy-3-methylfentanyl (RTI-1a, RTI-1b, RTI-1c, RTI-1d) (Fig. 1). This series of alkaloids include a number of clinically relevant drugs and exhibit a wide spectrum of in vivo analgesic potencies and efficacies mediated by μ -opioid receptors. For example, etorphine and fentanyl have

high intrinsic efficacy relative to morphine, whereas buprenorphine is a low efficacy agonist (Adams et al., 1990; Duttaroy and Yoburn, 1995; Walker et al., 1998). *cis*- β -Hydroxy-3-methylfentanyl is a derivative of fentanyl comprised of four optically active isomers (isomers RTI-1a, -1b, -1c, and -1d) that are selective for the μ -receptor and that vary dramatically in their in vivo potencies and efficacies. The two isomers that have the highest binding affinity for the μ -receptor (RTI-1a and RTI-1b) cause pseudoirreversible inhibition of μ -receptor binding and also have been shown to have potencies 3,000- to 10,000-fold greater than morphine in various analgesic tests, whereas the other two isomers (RTI-1c and RTI-1d) are weak analgesics (Ni et al., 1993; Brine et al., 1995; Wang et al., 1995).

In this study, we have explored the relationship between a ligand's ability to modulate surface μ -opioid receptor number and its ability to signal as assessed by both stimulation of [³⁵S]guanosine-5'-O-(3-thio)triphosphate (GTP γ S) binding and inhibition of cAMP accumulation in μ -receptor-transfected 293 HEK cells. We also have abolished coupling of the μ -receptor to G_i/G_o proteins by treatment with pertussis toxin (PTX) and analyzed the effects on ligand-induced changes in surface receptor number.

Experimental Procedures

Cell Line. 293-SF-MOR cells have been characterized previously (Keith et al., 1996) and were a gift from Dr. Mark von Zastrow

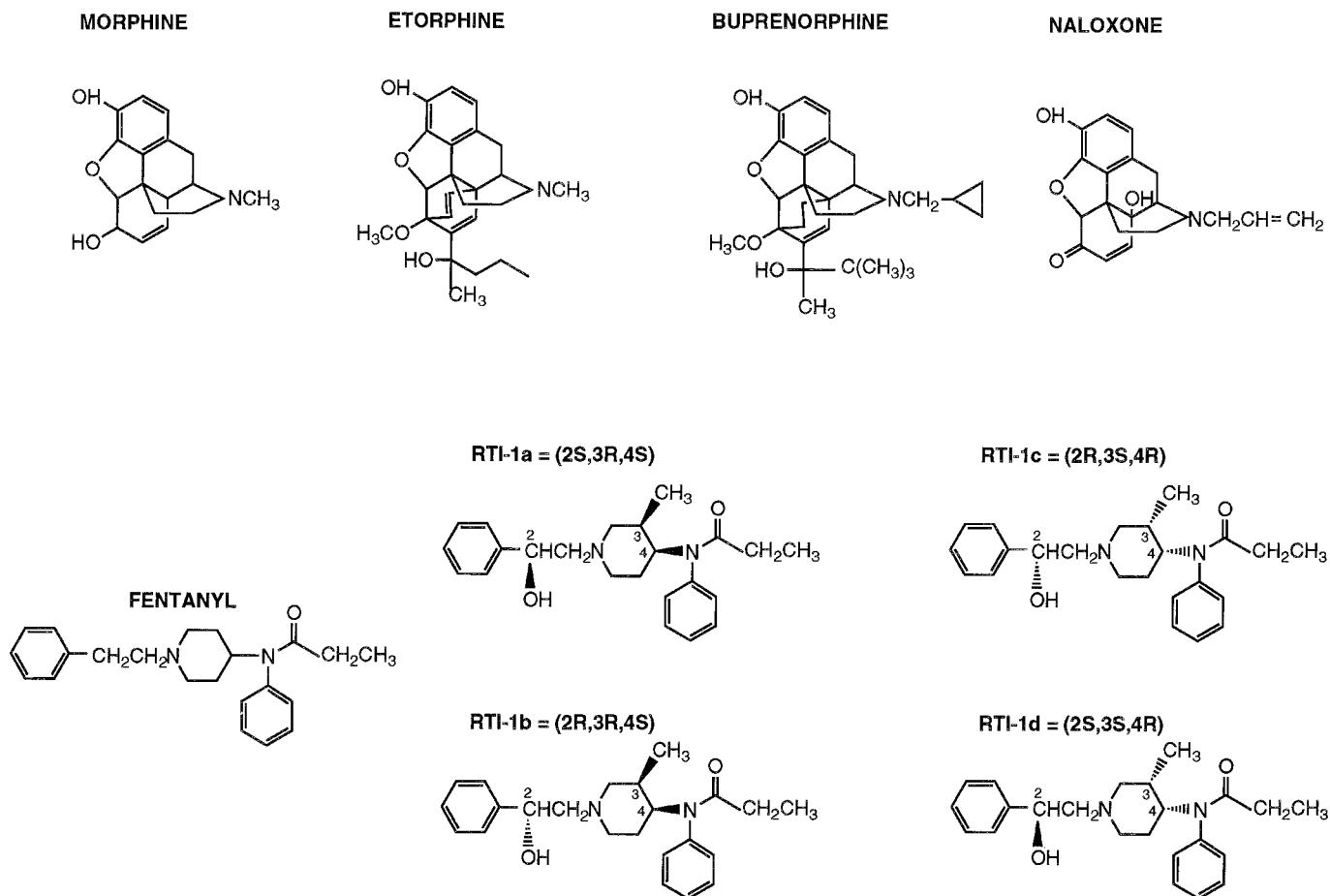


Fig. 1. The structures of the various opioid alkaloids used in this study.

(University of California-San Francisco). Briefly, HEK 293 cells were stably transfected with the murine μ -opioid receptor (MOR) cDNA containing the signal FLAG epitope at the amino terminus. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/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 ~2.95 as described previously (Keith et al., 1998). For internalization experiments, 293-SF-MOR cells were harvested in 2 mM EDTA/PBS then resuspended in culture media and treated with various drugs for either 1 or 18 h at 37°C. Cells were then chilled to 0°C to stop further receptor internalization and stained with 10 μ g/ml FITC-labeled FLAG M2 in 25% FBS for 1 h. Cells were washed two to three times (with 2% FBS/0.1% NaNO₃/PBS) and 5,000 to 10,000 cells/sample were analyzed on a FACScan flow cytometer with CellQuest 3.0.1 for acquisition and analysis (Becton Dickinson, 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.

cAMP Accumulation Assay. 293-SF-MOR cells were harvested and resuspended in PBS and 1 mM 3-isobutyl-1-methylxanthine for 10 min. Cells were then treated with 5 μ M forskolin and various opioid drugs for 15 min in 96-well polypropylene plates. Samples were then sealed and boiled for 5 min, centrifuged at 4000g and supernatants were assayed with an [³H]cAMP assay kit (Diagnostic Products, Los Angeles, CA).

Membrane Preparation. 293-SF-MOR 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, and 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 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 for 2 h at 0°C. Membranes were incubated with 10 μ M unlabeled GTP γ S to determine nonspecific binding. The mixtures were harvested with a Brandel M24RS harvester with presoaked 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 with Cytoscint ES (ICN Pharmaceuticals, Irvine, CA).

Materials. FLAG M2 antibody was purchased from Eastman Kodak (New Haven, CT). [³⁵S]GTP γ S (1250 Ci/mmol) was purchased from NEN (Boston, MA). FITC, 3-isobutyl-1-methylxanthine, forskolin, and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, MO). PTX was purchased from Sigma Chemical Co. (St. Louis, MO) and Calbiochem (La Jolla, CA). Tissue culture supplies were purchased from Omega Scientific (Tazvana, CA). Etorphine, morphine, fentanyl, buprenorphine, and naloxone were gifts from the National Institute on Drug Abuse (Bethesda, MD) and the four stereoisomers of *cis*- β -hydroxy-3-methylfentanyl were obtained from the Research Triangle Institute (Research Triangle Park, NC).

Results

Agonist-Selective Internalization of μ -Opioid Receptors. 293-SF-MOR cells were treated with various ligands for 1 h at 37°C, chilled on ice, and then stained with FITC-

labeled FLAG M2 antibody to quantitate the number of surface receptors by flow cytometric analysis. Figure 2A shows the effects of etorphine, fentanyl, and morphine on surface receptor staining. Etorphine induced a substantial loss of surface receptor staining (~50%) with low nanomolar potency, whereas morphine only caused a relatively small loss (~10%) of surface receptor staining at the highest concentration tested. Fentanyl triggered a 35% loss of surface receptors (an amount of internalization that was ~65% of the maximal internalization caused by etorphine) with an EC₅₀ one order of magnitude greater than that of etorphine. Figure 2B shows the effects of the four stereoisomers of the fentanyl congener *cis*- β -hydroxy-3-methylfentanyl on surface receptor staining. Only two isomers of *cis*- β -hydroxy-3-methylfentanyl, RTI-1a and RTI-1b, induced μ -receptor internalization. The poten-

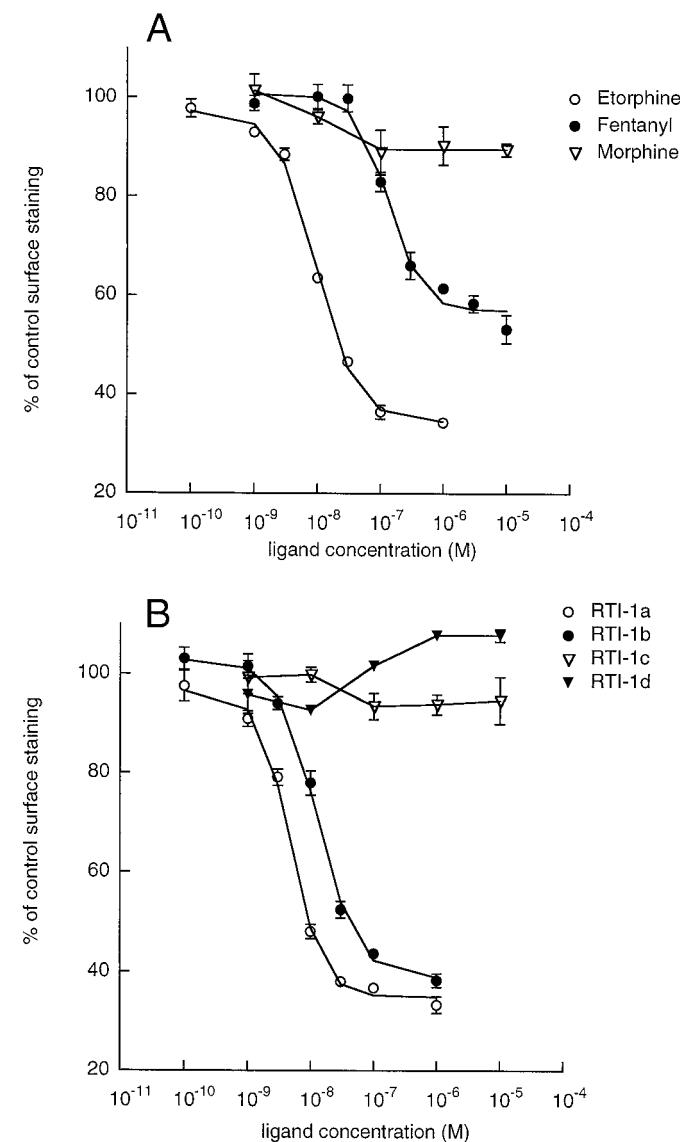


Fig. 2. Flow cytometric analysis of μ -opioid receptor internalization. A and B, 293-SF-MOR cells were treated with various concentrations of drugs for 1 h at 37°C and then chilled to 0°C to arrest further trafficking. Cells were stained with FITC-labeled FLAG M2 monoclonal antibody and analyzed on a FACScan flow cytometer. The mean fluorescence of 10,000 cells minus the mean fluorescence of unstained cells was used to calculate the percentage of surface receptor staining. Values are the means \pm S.D. of triplicate determinations in a representative experiment.

cies and efficacies of these two compounds for causing internalization were similar to that of etorphine (Table 1).

Ligand-Selective Up-Regulation of Surface μ -Opioid Receptors. Table 1 summarizes the effects of the alkaloid ligands on surface receptor staining. Whereas etorphine, RTI-1a, RTI-1b, fentanyl, and morphine induced measurable internalization, the opiate antagonist naloxone induced a 16% increase in surface μ -receptors. Furthermore, RTI-1d and buprenorphine also caused a significant increase in surface receptor staining. RTI-1c showed a tendency to increase surface receptors but this did not reach statistical significance. Longer treatment (18 h) of the cells with buprenorphine or naloxone resulted in a greater increase in surface receptor number than treatment for 1 h (Fig. 3B).

Potencies and Efficacies of Various Opioids for Second Messenger Modulation. The potencies and efficacies of the alkaloid ligands to inhibit cAMP accumulation in forskolin-stimulated 293-SF-MOR cells are shown in Table 2. Etorphine, RTI-1a, and RTI-1b, which all induced maximal μ -receptor internalization, inhibited cAMP accumulation to the same extent and had subnanomolar potencies. Fentanyl and morphine also were full agonists in this assay and had similar low nanomolar potencies, whereas RTI-1c, RTI-1d, and buprenorphine were partial agonists in this assay. RTI-1c and buprenorphine had similar efficacies (~80% of maximal inhibition), although buprenorphine was two orders of magnitude more potent than RTI-1c. Naloxone showed no inverse agonist activity in this assay (data not shown).

Potencies and Efficacies of Various Opioids for Activating G Proteins. It has been shown that only a small fraction of receptors need to be activated to achieve maximal inhibition of adenylyl cyclase activity (Fantozzi et al., 1981). Therefore, it was considered that the [35 S]GTP γ S binding assay might more realistically reflect the "intrinsic efficacy" of agonists because this assay gives a measure of agonist efficacy at the first level of signal transduction: activation of the G protein. As seen in Table 3, etorphine, RTI-1a, and RTI-1b all stimulated [35 S]GTP γ S binding to the same extent with low nanomolar potencies. Morphine and fentanyl were

TABLE 1

Potencies and efficacies of various ligands for causing a loss or increase in surface μ opioid receptor number

Change in surface receptor staining was determined as described in *Experimental Procedures*. The percentage of maximal internalization was calculated to be the amount of internalization observed relative to that caused by etorphine in each experiment. Results for morphine, RTI-1c, RTI-1d, buprenorphine, and naloxone were based on a 1-h treatment with 10 μ M of each drug. Curves were fitted to a standard four-parametric logistic equation with SigmaPlot (SPSS Inc., Chicago, IL). Values are means \pm S.E.

| Drug | n | EC ₅₀ | Surface Receptor Staining | % Maximal Internalization |
|---------------|----|------------------|---------------------------|---------------------------|
| nM | | | | |
| Etorphine | 4 | 8.7 \pm 1.7 | 47 \pm 8 | 100 |
| Morphine | 4 | ND | 91 \pm 2 | 17 \pm 2 ^a |
| Fentanyl | 4 | 113 \pm 30 | 65 \pm 7 | 66 \pm 4 ^a |
| RTI-1a | 4 | 3.3 \pm 1.5 | 49 \pm 7 | 99 \pm 6 |
| RTI-1b | 4 | 7.8 \pm 2.2 | 48 \pm 8 | 99 \pm 3 |
| RTI-1c | 10 | NA | 103 \pm 4 | NA |
| RTI-1d | 10 | NA | 108 \pm 3 ^b | NA |
| Buprenorphine | 9 | NA | 110 \pm 3 ^b | NA |
| Naloxone | 15 | NA | 116 \pm 2 ^b | NA |

ND, not determined; NA, not applicable.

^a Significantly less than etorphine-induced internalization, $P < .05$ by Student's *t* test.

^b Significantly more than control surface staining, $P < .05$ by Student's *t* test.

TABLE 2

Potencies and efficacies of various ligands for inhibiting cAMP accumulation

Measurement of the ability of various ligands to inhibit cAMP accumulation was performed as described in *Experimental Procedures*. The maximal amount of inhibition caused by each ligand is expressed as a percentage of the maximal amount of inhibition caused by etorphine. Curves were fitted to a standard four-parametric logistic equation with SigmaPlot (SPSS Inc., Chicago, IL). Values are mean \pm S.E.

| Drug | n | IC ₅₀ | % Maximal Inhibition |
|---------------|---|-------------------|-------------------------|
| nM | | | |
| Etorphine | 7 | 0.135 \pm 0.029 | 100 |
| Morphine | 3 | 4.92 \pm 0.76 | 101 \pm 9 |
| Fentanyl | 6 | 2.06 \pm 0.63 | 99 \pm 2 |
| RTI-1a | 4 | 0.128 \pm 0.024 | 103 \pm 4 |
| RTI-1b | 4 | 0.247 \pm 0.077 | 104 \pm 4 |
| RTI-1c | 5 | 170 \pm 20 | 79 \pm 3 ^a |
| RTI-1d | 4 | 49.2 \pm 14.9 | 64 \pm 2 ^a |
| Buprenorphine | 4 | 1.55 \pm 0.36 | 79 \pm 4 ^a |

^a Significantly less than etorphine-induced inhibition, $P < .05$ by Student's *t* test.

TABLE 3

Potencies and efficacies of various ligands for stimulating [35 S]GTP γ S binding

Measurement of the ability of various ligands to stimulate [35 S]GTP γ S binding was performed as described in *Experimental Procedures*. The maximal amount of stimulation caused by each ligand is expressed as a percentage of the maximal amount of stimulation caused by etorphine. Curves were fitted to a standard four-parametric logistic equation with SigmaPlot (SPSS Inc., Chicago, IL). Results for RTI-1c, RTI-1d, and buprenorphine were based on treatment with 10 μ M of each drug. Values are means \pm S.E.

| Drug | n | EC ₅₀ | % Maximal Stimulation |
|---------------|----|-------------------|-------------------------|
| nM | | | |
| Etorphine | 11 | 0.973 \pm 0.195 | 100 |
| Morphine | 6 | 36.3 \pm 7.1 | 82 \pm 2 ^a |
| Fentanyl | 8 | 59.7 \pm 11.1 | 77 \pm 2 ^a |
| RTI-1a | 4 | 1.01 \pm 0.25 | 103 \pm 6 |
| RTI-1b | 5 | 4.46 \pm 0.49 | 105 \pm 7 |
| RTI-1c | 8 | ND | 13 \pm 4 ^a |
| RTI-1d | 8 | ND | 8 \pm 2 ^a |
| Buprenorphine | 8 | ND | 21 \pm 3 ^a |

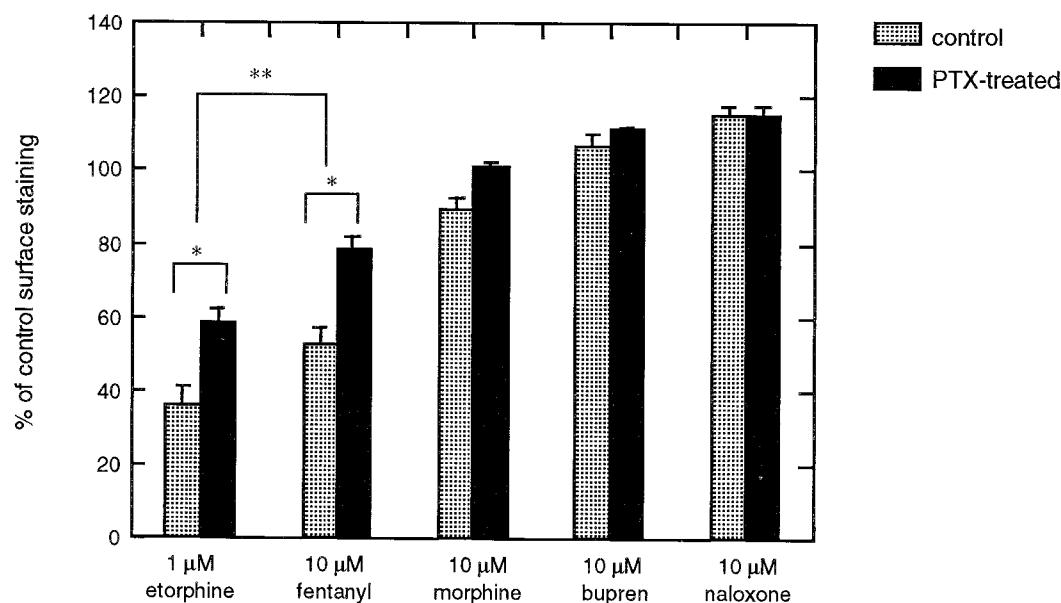
ND, not determined.

^a Significantly less than etorphine-induced stimulation, $P < .05$ by Student's *t* test.

partial agonists in this assay, with similar efficacies and potencies. RTI-1c, RTI-1d, and buprenorphine only stimulated between 7 and 21% of maximal [35 S]GTP γ S, which is consistent with the fact that they were partial agonists for inhibiting cAMP accumulation. Naloxone was a neutral antagonist in this assay (data not shown).

Effect of PTX on Ligand-Induced Changes in Surface μ -Receptor Number. PTX has been shown to ADP-ribosylate inhibitory G proteins and thereby uncouple GPCRs from their cognate G_{i/o} proteins (Kurose et al., 1983). Cells were treated overnight with 100 ng/ml PTX, which blocked stimulation of [35 S]GTP γ S binding by etorphine (176 \pm 14% increase in stimulation over basal versus a 9 \pm 10% increase in PTX-treated cells; standard deviation, $n = 2$). PTX treatment also decreased maximal inhibition of cAMP accumulation by etorphine from 82 \pm 3% (standard error, $n = 9$) to 12 \pm 3% (standard error, $n = 5$). Figure 3A shows the change of surface receptor staining induced by a 1-h treatment with etorphine, fentanyl, morphine, buprenorphine, and naloxone in both PTX-treated and untreated cells. PTX treatment alone did not result in a significant change in surface receptor staining compared with untreated cells (data not shown).

A



B

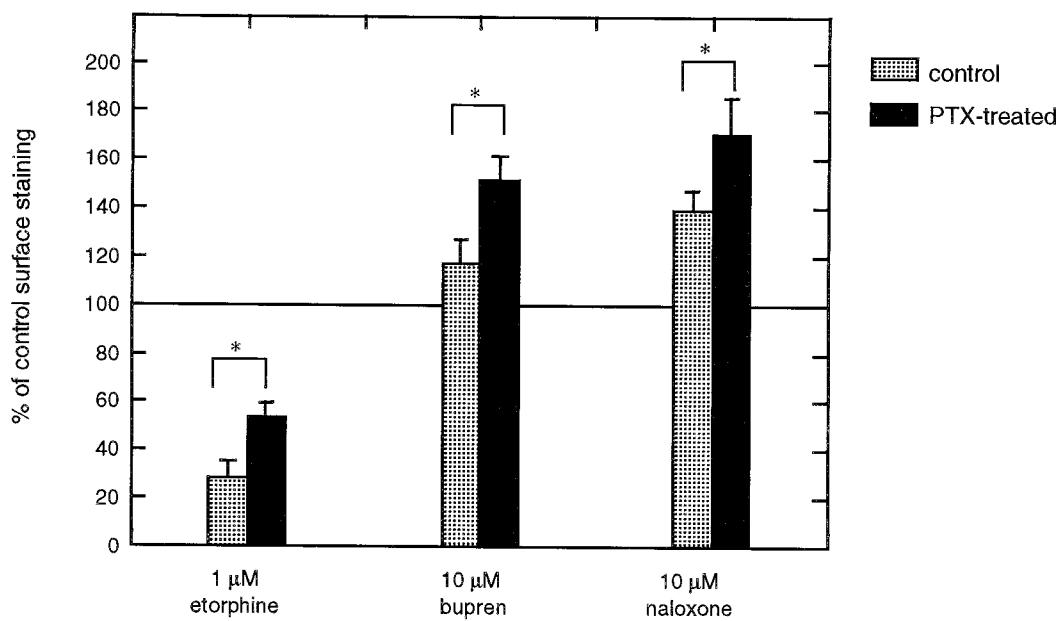


Fig. 3. Effect of PTX treatment on ligand-induced changes in surface μ -opioid receptor number. 293-SF-MOR cells were pretreated overnight with 100 ng/ml PTX. A and B, percentage of surface receptor staining after 1 h of drug treatment (A) and 18 h of drug treatment (B) was calculated by dividing the mean fluorescence of the cells in each drug treatment by the mean fluorescence of nondrug-treated control and PTX-treated cells. Values are the means \pm S.E. of three to nine separate experiments. A single asterisk denotes that the effect of drug treatment on PTX-treated cells is significantly different from that of control cells that were not treated with PTX (Student's *t* test, $P < .05$). The double asterisk denotes that PTX treatment caused a greater impairment of the ability of fentanyl to cause internalization compared with that of etorphine (Student's *t* test, $P < .05$).

Etorphine (1 μ M) induced a $42 \pm 3\%$ loss of surface receptor in PTX-treated cells, whereas the same concentration of drug caused a $63 \pm 4\%$ loss of surface receptor in untreated cells (standard error, $n = 6$). Hence, treatment with PTX inhibited the ability of 1 μ M etorphine to cause μ -receptor internalization by 33%. Fentanyl (10 μ M) induced a $22 \pm 3\%$ loss of surface receptor in PTX-treated cells but a $46 \pm 3\%$ loss of

surface receptor in untreated cells (standard error, $n = 6$). Therefore, PTX treatment impaired the ability of fentanyl to cause internalization by 49%, which was significantly greater than the inhibition of etorphine-induced internalization (Student's *t* test, $P < .05$). The EC₅₀ values of both etorphine and fentanyl were not significantly different after PTX treatment (data not shown). PTX also attenuated the ability of etor-

phine to cause a loss of surface receptor after 18 h ($72 \pm 7\%$ loss of surface receptor in control cells versus a $47 \pm 6\%$ loss of surface receptor in PTX-treated cells; standard error, $n = 5$, Student's *t* test, $P < .05$) (Fig. 3B).

Treatment with PTX completely abolished the slight internalization caused by 10 μM morphine; surface staining after 1 h of 10 μM morphine treatment was $90 \pm 3\%$ of control surface staining in untreated cells and $101 \pm 1\%$ of control surface staining in PTX-treated cells (standard error, $n = 6$) (Fig. 3A). Furthermore, the increases in surface receptor staining caused by 1-h treatment with either 10 μM naloxone and 10 μM buprenorphine were not significantly affected by PTX (Fig. 3A). Treatment with naloxone induced a $15 \pm 2\%$ increase in surface staining in control cells and a $15 \pm 2\%$ increase in surface staining in PTX-treated cells (standard error, $n = 9$). Buprenorphine treatment caused a $7 \pm 3\%$ increase in control cells and an $11 \pm 0.4\%$ increase in PTX-treated cells (standard error, $n = 3$). Interestingly, PTX treatment augmented the robust increase in surface receptor staining caused by 18 h of naloxone from a $39 \pm 8\%$ increase in surface staining in control cells to a $70 \pm 16\%$ increase in PTX-treated cells (standard error, $n = 5$, Student's *t* test, $P < .05$) (Fig. 3B). PTX-treatment also increased the effect of 18 h of buprenorphine treatment ($17 \pm 10\%$ increase in control cells versus $51 \pm 10\%$ increase in PTX-treated cells; standard error, $n = 5$, Student's *t* test, $P < .05$) (Fig. 3B).

Blocking of Agonist-Induced Internalization. We next determined whether morphine, which caused a slight amount of internalization, and the ligands that did not induce internalization (naloxone, RTI-1c, RTI-1d, buprenorphine) were able to block agonist-induced internalization. Table 4 shows the "EC₅₀ values" for these ligands to block internalization induced by 10 nM etorphine (a concentration that is close to etorphine's EC₅₀ for inducing internalization). These ligands blocked etorphine-induced internalization with the following rank order potencies: buprenorphine > naloxone \gg RTI-1d \gg RTI-1c = morphine. These potencies generally parallel their rank order of binding affinities to the μ -receptor (Brine et al., 1995; Raynor et al., 1995; P.A.Z., unpublished data).

Discussion

The relationship between G protein activation and receptor internalization in GPCRs is unclear and appears to vary among receptors. Receptors are generally internalized in response to agonist binding (Bohm et al., 1997), although antagonists or antibodies are able to trigger internalization of some receptors (Roettger et al., 1997; Bhowmick et al., 1998; Tolbert and Lameh, 1998; Willins et al., 1999). Studies on β_2 -adrenergic and muscarinic receptors have demonstrated that partial agonists cause less internalization than full agonists and the amount of receptor internalization caused by an agonist generally correlates with coupling efficiency (Toews and Perkins, 1984; Thompson and Fisher, 1990; January et al., 1997; Szekeres et al., 1998).

The availability of a variety of ligands for the μ -opioid receptor facilitates the study of the relationship between G protein coupling and receptor internalization. As shown in this study, alkaloid ligands that are full agonists in 293-SF-MOR cells as assessed by [³⁵S]GTP γ S binding and cAMP

TABLE 4

Blocking of 10 nM etorphine-induced μ receptor internalization

Surface receptor staining was determined as described in *Experimental Procedures*. Curves were fitted to a standard four-parametric logistic equation with SigmaPlot (SPSS Inc., Chicago, IL). EC₅₀ values represent the concentration of drug that produced half its maximal effect on surface receptor staining. Values are mean \pm S.E.

| Drug | <i>n</i> | EC ₅₀ | Maximal Surface |
|---------------|----------|------------------|--------------------------------|
| | | | Receptor Staining % control |
| Morphine | 5 | 4900 \pm 1500 | 95 \pm 4 |
| RTI-1c | 5 | 3000 \pm 700 | 102 \pm 7 |
| RTI-1d | 5 | 421 \pm 123 | 110 \pm 4 |
| Buprenorphine | 4 | 13 \pm 2 | 112 \pm 6 |
| Naloxone | 5 | 28 \pm 6 | 114 \pm 6 |

assays (etorphine, RTI-1a, RTI-1b) induce maximal μ -receptor internalization. We also show that morphine and fentanyl, which are partial agonists for stimulating [³⁵S]GTP γ S binding, cause significantly less internalization than the full agonist etorphine. Although fentanyl and morphine differ considerably in their ability to induce internalization (66 versus 17% of maximal internalization, respectively), they are similarly efficacious in stimulating [³⁵S]GTP γ S binding (~80% that of etorphine). Selley et al. (1997) also have shown that morphine and fentanyl have comparable efficacies in stimulating [³⁵S]GTP γ S binding through μ -receptors, although two other groups have found that fentanyl is more efficacious than morphine in stimulating [³⁵S]GTP γ S binding (Traynor and Nahorski, 1995; Emmerson et al., 1996). These discrepancies may result from differences in cell lines, levels of receptor expression, and incubation temperatures. It is of interest to note that the introduction of a methyl and a hydroxyl group (RTI-1a and RTI-1b) can confer full agonist properties to fentanyl both in [³⁵S]GTP γ S binding and internalization assays.

A novel finding in this study is that weak partial agonists (buprenorphine and RTI-1d) are able to cause a significant increase in the number of surface receptors, similar to the classical opioid antagonist naloxone. Increases in μ -receptor binding following chronic exposure to naloxone have previously been described in cell lines and in vivo (Zadina et al., 1995; Unterwald et al., 1995; Koch et al., 1998), whereas decreases in μ -receptor binding have been shown to result after chronic buprenorphine treatment in vivo (Belcheva et al., 1993). However, this decrease might represent the failure to dissociate buprenorphine from μ -receptors (Boas and Villiger, 1985). That buprenorphine and RTI-1d, which have low efficacies for activating G proteins, are unable to trigger μ -receptor internalization suggests that the ability of a ligand to activate a certain level of G proteins is a prerequisite for initiating detectable receptor internalization. Furthermore, we have shown that ligands such as buprenorphine can act as agonists with regard to one function (i.e., signaling) and as antagonists for another (i.e., receptor internalization).

In addition to studying the effects of ligands with varying intrinsic activities on surface receptor number, we assessed the effects of blocking G protein function with PTX that ADP-ribosylates G_i/G_o proteins and thus interferes with the ability of these G proteins to be activated by the receptor (Kurose et al., 1983). Other studies that have looked at the effect of PTX on GPCR internalization find either no effect on agonist-induced

internalization (Hsieh et al., 1999; Li et al., 1999) or a reduction in the rate or extent of internalization (Van Koppen et al., 1994; Koenig et al., 1997). A previous immunocytochemical study showed that the μ -receptors expressed in HEK cells still exhibit agonist-induced internalization after PTX treatment (Segredo et al., 1997), whereas another group showed internalization of μ -receptors expressed in Neuro_{2A} cells was blocked after PTX treatment (Chakrabarti et al., 1997). One limitation of immunocytochemical studies is that they are not readily quantifiable. Flow cytometry allows for quantification of changes in cell surface receptors and can reveal even subtle differences in surface receptor number. In this study, we found that PTX treatment impairs the ability of agonists to induce μ -receptor internalization. Furthermore, the ability of fentanyl to cause internalization is significantly more affected by PTX treatment than that of etorphine (49% reduction versus 33% reduction, respectively) and the slight internalization caused by morphine is completely abolished by PTX treatment. Thus, ligands that are extremely efficacious for inducing receptor internalization and causing maximal G protein activation appear to be more resistant to the effects of PTX treatment than less efficacious ligands. The PTX results parallel previous findings that have shown that PTX treatment did not greatly affect the ability of full agonists to cause down-regulation of the μ -receptor (total loss of receptor protein as assessed by radioligand binding), whereas the ability of partial agonists to cause down-regulation was greatly impaired (Yabaluri and Medzihradsky, 1997). Similar to results from other studies (Selley et al., 1998), PTX did not completely block opioid-mediated inhibition of adenylyl cyclase and residual signaling activity may explain the inability of PTX to completely block the internalization caused by etorphine.

The ability of buprenorphine and naloxone to cause up-regulation of surface receptors after 1 h does not appear to be affected by PTX treatment. However, PTX treatment causes a considerable augmentation of the increase in surface receptor number induced by 18-h treatment with buprenorphine or naloxone. Thus, PTX clearly modulates the ability of ligands to regulate surface receptors, impairing the internalization mechanism while potentiating the ability of antagonists and low-efficacy partial agonists to increase surface μ -receptors. This is consistent with studies showing that heterotrimeric G proteins are involved in protein trafficking (Helms, 1995). Additionally, ADP-ribosylation of G proteins may alter the association of G proteins with their cognate receptors and thus modify the accessibility of proteins involved in trafficking.

Finally, the ability of various ligands to block etorphine-induced receptor internalization was assessed. Ligands block internalization with potencies that parallel their binding affinities for the μ -receptor (buprenorphine > naloxone \gg RTI-1d \gg RTI-1c = morphine). Morphine has been reported to induce enkephalin release in vivo and its ability to block internalization may contribute to its physiological actions (Olive and Maidment, 1998). Perhaps more importantly, the observation that buprenorphine also can block internalization with a high potency and potentially up-regulate μ -receptors in vivo may aid in explaining its utility as a treatment for drug addiction.

Overall, this study provides a number of insights into the relationship between G protein activation and regulation of μ -opioid receptor number on the cell surface. Although there is no strict correlation within groups, full agonists for G

protein activation induce maximal internalization, whereas high-efficacy partial agonists for G protein activation (morphine and fentanyl) induce partial internalization. The data suggest that strong agonists are able to put the receptor in a conformation that is favorable for both activating G proteins and entering the endocytic route. Agonists such as etorphine and DAMGO, which efficiently trigger internalization, have been shown to cause more μ -receptor phosphorylation than morphine and might allow for a state of the receptor wherein adaptor proteins (such as β -arrestins) bind and subsequently direct the receptor into the clathrin-mediated endocytic pathway (Yu et al., 1997; Ferguson et al., 1998). Two articles have demonstrated that morphine is able to efficiently induce internalization of the μ -receptor if G protein-coupled receptor kinase or β -arrestin is overexpressed (Whistler and von Zastrow, 1998; Zhang et al., 1998). Ligands that are not able to elicit a threshold activation state (i.e., antagonists and weak partial agonists) may put the receptor in a conformational state that is unfavorable for entering the endocytic pathway. Indeed, we have shown that the partial agonists buprenorphine and RTI-1d, as well as the antagonist naloxone, not only fail to induce internalization but also cause an up-regulation of surface μ -receptors.

In a recent article by Whistler et al. (1999), it was suggested that the relative activity versus endocytosis (RAVE) value of a drug is predictive of the ability of that drug to induce tolerance and/or dependence (Roth and Willins, 1999; Whistler et al., 1999). Drugs with a high RAVE value (e.g., morphine) cause more tolerance in certain dosing paradigms than drugs that have RAVE values much lower than morphine (e.g., etorphine and methadone). Although this is an intriguing and provocative theory, drugs with high specificity for the μ -opioid receptor should be tested to further support this hypothesis. For instance, etorphine has a very high affinity for the δ - and κ -opioid receptors in addition to the μ -receptor (Raynor et al., 1994) and methadone can act as a noncompetitive antagonist at N-methyl-D-aspartate receptors, a property shown to block opioid tolerance (Davis and Inturrisi, 1999). Although there are no data available concerning the tolerance and dependence liabilities of the isomers of cis- β -hydroxy-3-methylfentanyl, these compounds will undoubtedly prove to be excellent tools with which to explore the relationship between μ -receptor internalization and tolerance and dependence to different opioid drugs given their high μ -receptor selectivity (>15,000 times more selective for μ -receptors than δ - and κ -receptors for RTI-1a and RTI-1b) (Brine et al., 1995; Wang et al., 1995). Although the precise role of ligand-regulated trafficking of μ -receptors in adaptational processes after acute and chronic opioid treatment remains to be determined, differences in trafficking will surely be found to contribute to the unique pharmacological profiles of the different opiate drugs.

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References

- Adams JU, Paronis CA and Holtzman SG (1990) Assessment of relative intrinsic activity of μ opioid analgesics in vivo by using β -funaltrexamine. *J Pharmacol Exp Ther* **255**:1027–1032.

- Arden JR, Segredo V, Wang Z, Lameh J and Sadee W (1995) Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged μ opioid receptor expressed in HEK 293 cells. *J Neurochem* **65**:1636–1645.
- Befort K, Tabbara L and Kieffer BL (1996) [35 S]GTP γ S binding: A tool to evaluate functional activity of a cloned opioid receptor transiently expressed in COS cells. *Neurochem Res* **21**:1301–1307.
- Belcheva MM, Barg J, McHale RJ, Dawn S, Ho MT, Ignatova E and Coscia CJ (1993) Differential down- and up-regulation of rat brain opioid receptor types and subtypes by buprenorphine. *Mol Pharmacol* **44**:173–179.
- Bhowmick N, Narayan P and Puett D (1998) The endothelin subtype A receptor undergoes agonist- and antagonist-mediated internalization in the absence of signaling. *Endocrinology* **139**:3185–3192.
- Boas RA and Villiger JW (1985) Clinical actions of fentanyl and buprenorphine. The significance of receptor binding. *Br J Anaesth* **57**:192–196.
- Bohm SK, Grady EF and Bunnett NW (1997) Regulatory mechanisms that modulate signaling by G-protein-coupled receptors. *Biochem J* **322**:1–18.
- Brine GA, Stark PA, Liu Y, Carroll FI, Singh P, Xu H and Rothman RB (1995) Enantiomers of diastereomeric *cis*-N-[1-(2-hydroxy-2-phenylethyl)-3-methyl-4-piperidyl]-N-phenylpropanamides: Synthesis, X-ray analysis, and biological activities. *J Med Chem* **38**:1547–1557.
- Burford NT, Tolbert LM and Sadee W (1998) Specific G-protein activation and μ opioid receptor internalization caused by morphine, DAMGO and endomorphin I. *Eur J Pharmacol* **342**:123–126.
- Chakrabarti S, Yang W, Law PY and Loh HH (1997) The μ opioid receptor down-regulates differently from the δ opioid receptor: Requirement of a high affinity receptor/G protein complex formation. *Mol Pharmacol* **52**:105–113.
- Davis AM and Inturrisi CE (1999) d-Methadone blocks morphine tolerance and *N*-methyl-d-aspartate-induced hyperalgesia. *J Pharmacol Exp Ther* **289**:1048–1053.
- Dhawan BN, Cesselin F, Raghuram R, Reisine T, Bradley PB, Portoghese PS and Hamon M (1996) International Union of Pharmacology. XII. Classification of opioid receptors. *Pharmacol Rev* **48**:567–592.
- Duttaroy A and Yoburn BC (1995) The effect of intrinsic efficacy on opioid tolerance. *Anesthesiology* **82**:1226–1236.
- Emmerson PJ, Clark MJ, Mansour A, Akil H, Woods JH and Medzihradsky F (1996) Characterization of opioid agonist efficacy in a C6 glioma cell line expressing the μ opioid receptor. *J Pharmacol Exp Ther* **278**:1121–1127.
- Fantozzi R, Mullikin Kilpatrick D and Blume AJ (1981) Irreversible inactivation of the opiate receptors in the neuroblastoma x glioma hybrid NG108-15 by chloral-trexamine. *Mol Pharmacol* **20**:8–15.
- Ferguson SS, Zhang J, Barak LS and Caron MG (1998) Molecular mechanisms of G protein-coupled receptor desensitization and resensitization. *Life Sci* **62**:1561–1565.
- Fukuda K, Kata S, Morikawa H, Shoda T and Mori K (1996) Functional coupling of the δ -, μ -, and κ -opioid receptors to mitogen-activated protein kinase and arachidonate release in Chinese hamster ovary cells. *J Neurochem* **67**:1309–1316.
- Helms JB (1995) Role of heterotrimeric GTP binding proteins in vesicular protein transport: Indications for both classical and alternative G protein cycles. *FEBS Lett* **369**:84–88.
- Hsieh C, Brown S, Derleth C and Mackie K (1999) Internalization and recycling of the CB1 cannabinoid receptor. *J Neurochem* **73**:493–501.
- January B, Seibold A, Whaley B, Hipkin RW, Lin D, Schonbrunn A, Barber R and Clark RB (1997) β_2 -adrenergic receptor desensitization, internalization, and phosphorylation in response to full and partial agonists. *J Biol Chem* **272**:23871–23879.
- Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet-Agius G, Stewart PL, Evans CJ and Zastrow M (1998) μ opioid receptor internalization: Opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Mol Pharmacol* **53**:377–384.
- Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ and von Zastrow M (1996) Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem* **271**:19021–19024.
- Koch T, Schulz S, Schroder H, Wolf R, Raulf E and Holt V (1998) Carboxyl-terminal splicing of the rat μ opioid receptor modulates agonist-mediated internalization and receptor resensitization. *J Biol Chem* **273**:13652–13657.
- Koenig JA, Edwardson JM and Humphrey PP (1997) Somatostatin receptors in Neuro2A neuroblastoma cells: Ligand internalization. *Br J Pharmacol* **120**:52–59.
- Kovoor A, Celver JP, Wu A and Chavkin C (1998) Agonist induced homologous desensitization of μ -opioid receptors mediated by G protein-coupled receptor kinases is dependent on agonist efficacy. *Mol Pharmacol* **54**:704–711.
- Kurose H, Katada T, Amano T and Ueda M (1983) Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via α -adrenergic, cholinergic, and opiate receptors in neuroblastoma x glioma hybrid cells. *J Biol Chem* **258**:4870–4875.
- Li JG, Luo LY, Krupnick JG, Benovic JL and Liu-Chen LY (1999) U50,488H-induced internalization of the human κ opioid receptor involves β -arrestin- and dynamin-dependent mechanism. κ Receptor internalization is not required for mitogen-activated protein kinase activation. *J Biol Chem* **274**:12087–12094.
- Loh HH, Liu HC, Cavalli A, Yang W, Chen YF and Wei LN (1998) μ Opioid receptor knockout in mice: Effects on ligand-induced analgesia and morphine lethality. *Brain Res Mol Brain Res* **54**:321–326.
- Matthes HWD, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Lemeur M, Dolle P, Tzavara E, Hanoune J, Roques BP and Kieffer BL (1996) Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ opioid-receptor gene. *Nature (Lond)* **383**:819–823.
- Ni Q, Xu H, Partilla JS, Stark PA, Carroll FI, Brine GA and Rothman RB (1993) Stereoselective requirements for pseudoirreversible inhibition of opioid μ receptor binding by the 3-methylfentanyl congeners, RTI-46144 and its enantiomers: Evidence for different binding domains. *Synapse* **15**:296–306.
- Olive MF and Maidment NT (1998) Opioid regulation of pallidal enkephalin release: Bimodal effects of locally administered μ and δ opioid agonists in freely moving rats. *J Pharmacol Exp Ther* **285**:1310–1316.
- Raynor K, Kong H, Chen Y, Yasuda K, Yu L, Bell GI and Reisine T (1994) Pharmacological characterization of the cloned κ , δ -, and μ -opioid receptors. *Mol Pharmacol* **45**:330–334.
- Raynor K, Kong H, Mestek A, Bye LS, Tian M, Liu J, Yu L and Reisine T (1995) Characterization of the cloned human μ opioid receptor. *J Pharmacol Exp Ther* **272**:423–428.
- Roettger BF, Ghanekar D, Rao R, Toledo C, Yingling J, Pinon D and Miller LJ (1997) Agonist-stimulated internalization of the G protein-coupled cholecystokinin receptor. *Mol Pharmacol* **51**:357–362.
- Roth BL and Willins DL (1999) What's all the RAVE about receptor internalization? *Neuron* **23**:629–631.
- Segredo V, Burford NT, Lameh J and Sadee W (1997) A constitutively internalizing and recycling mutant of the μ -opioid receptor. *J Neurochem* **68**:2395–2404.
- Selley DE, Breivogel CS and Childers SR (1998) Opioid inhibition of adenylyl cyclase in membranes from pertussis toxin-treated NG108-15 cells. *J Recept Signal Transduct Res* **18**:25–49.
- Selley DE, Sim LJ, Xiao R, Liu Q and Childers SR (1997) μ Opioid receptor-stimulated guanosine-5'-O-(γ -thio)triphosphate binding in rat thalamus and cultured cell lines: Signal transduction mechanisms underlying agonist efficacy. *Mol Pharmacol* **51**:87–96.
- Sora I, Takahashi N, Funada M, Ujike H, Revay RS, Donovan DM, Miner LL and Uhl GR (1997) Opiate receptor knockout mice define μ receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc Natl Acad Sci USA* **94**:1544–1549.
- Sternini C, Spann M, Anton B, Keith DE Jr, Bunnett NW, von Zastrow M, Evans C and Brecha NC (1996) Agonist-selective endocytosis of μ opioid receptor by neurons in vivo. *Proc Natl Acad Sci USA* **93**:9241–9246.
- Szekeres PG, Koenig JA and Edwardson JM (1998) The relationship between agonist intrinsic activity and the rate of endocytosis of muscarinic receptors in a human neuroblastoma cell line. *Mol Pharmacol* **53**:759–765.
- Thompson AK and Fisher SK (1990) Relationship between agonist-induced muscarinic receptor loss and desensitization of stimulated phosphoinositide turnover in two neuroblastomas: Methodological considerations. *J Pharmacol Exp Ther* **252**:744–752.
- Tian M, Broxmeyer HE, Fan Y, Lai Z, Zhang S, Aronica S, Cooper S, Biggs RM, Steinmetz R, Engle SJ, Mestek A, Pollock JD, Lehman MN, Jansen HT, Ying M, Stambrook PJ, Tischfield JA and Yu L (1997) Altered hematopoiesis, behavior, and sexual function in μ opioid receptor-deficient mice. *J Exp Med* **185**:1517–1522.
- Toews ML and Perkins JP (1984) Agonist-induced changes in β -adrenergic receptors on intact cells. *J Biol Chem* **259**:2227–2235.
- Tolbert LM and Lameh J (1998) Antibody to epitope tag induces internalization of human muscarinic subtype 1 receptor. *J Neurochem* **70**:113–119.
- Traynor JR and Nahorski SR (1995) Modulation by μ -opioid agonists of guanosine-5'-O-(3- 35 S)thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol Pharmacol* **47**:848–854.
- Unterwald EM, Rubenfeld JM, Imai Y, Wang JB, Uhl GR and Kreek MJ (1995) Chronic opioid antagonist administration upregulates μ opioid receptor binding without altering μ opioid receptor mRNA levels. *Brain Res* **33**:351–355.
- Van Koppen CJ, Sell A, Lenz W and Jakobs KH (1994) Deletion analysis of the m4 muscarinic acetylcholine receptor. Molecular determinants for activation of but not coupling to the Gi guanine-nucleotide-binding regulatory protein regulate receptor internalization. *Eur J Biochem* **222**:525–531.
- Walker EA, Zernig G and Young AM (1998) In vivo apparent affinity and efficacy estimates for μ opiates in a rat tail-withdrawal assay. *Psychopharmacology* **136**:15–23.
- Wang ZX, Zhu YC, Jin WQ, Chen XJ, Chen J, Ji RY and Chi ZQ (1995) Stereoisomers of N-[1-hydroxy-(2-phenylethyl)-3-methyl-4-piperidyl]-N-phenylpropanamide: synthesis, stereochemistry, analgesic activity, and opioid receptor binding characteristics. *J Med Chem* **38**:3652–3659.
- Whistler JL, Chuang HH, Chu P, Jan LY and von Zastrow M (1999) Functional dissociation of mu opioid receptor signaling and endocytosis: Implications for the biology of opiate tolerance and addiction. *Neuron* **23**:737–746.
- Whistler JL and von Zastrow M (1998) Morphine-activated opioid receptors elude desensitization by β -arrestin. *Proc Natl Acad Sci USA* **95**:9914–9919.
- Willins DL, Berry SA, Alsayegh L, Backstrom JR, Sanders-Bush E, Friedman L and Roth BL (1999) Clozapine and other 5-hydroxytryptamine-2A receptor antagonists alter the subcellular distribution of 5-hydroxytryptamine-2A receptors in vitro and in vivo. *Neuroscience* **91**:599–606.
- Yabaliru N and Medzihradsky F (1997) Down-regulation of μ opioid receptor by full but not partial agonists is independent of G protein coupling. *Mol Pharmacol* **52**:896–902.
- Yu Y, Zhang L, Yin X, Sun H, Uhl GR and Wang JB (1997) μ Opioid receptor phosphorylation, desensitization, and ligand efficacy. *J Biol Chem* **272**:28869–28874.
- Zadina JE, Kastin AJ, Harrison LM, Ge LJ and Chang SL (1995) Opiate receptor changes after chronic exposure to agonists and antagonists. *Ann NY Acad Sci* **757**:353–361.
- Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY and Caron MG (1998) Role for G protein-coupled receptor kinase in agonist-specific regulation of μ opioid receptor responsiveness. *Proc Natl Acad Sci USA* **95**:7157–7162.

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