Chronic Morphine Treatment Inhibits Opioid Receptor Desensitization and Internalization

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Chronic opioid receptor (OR) activation by morphine causes distinct cellular adaptations responsible for the development of tolerance. The present study examines the effect of chronic morphine exposure on the ability of high-efficacy agonists to mediate δ-OR (DOR) and μ-OR (MOR) uncoupling and internalization, two regulatory mechanisms contributing to rapid desensitization of OR function. Chronic morphine treatment (1 μM; 72 hr) of DOR carrying neuroblastoma x glioma (NG108-15) hybrid cells, a prototypical model system frequently used to study cellular aspects of opioid tolerance, completely blocked the capacity of [D-Ala^2, D-Leu^5]enkephalin (DADLE) and etorphine to desensitize opioid-stimulated [^{35}S]GTPγS binding and to mediate DOR internalization. Similar findings were obtained on stably DOR- and MOR-transfected human embryonic kidney (HEK) 293 cells. Chronic morphine treatment also heterologously impaired agonist regulation of non-opioid G-protein-coupled receptors, such as the m_4-muscarinic acetylcholine receptor and the brain-type cannabinoid receptor. As a possible underlying mechanism, we found that chronic morphine treatment completely blocked agonist-induced redistribution of β-arrestin1 in both NG108-15 and stably MOR-transfected HEK293 cells. Moreover, attenuation of β-arrestin1 function appears to depend on persistent stimulation of MAP kinase activity during the course of chronic morphine treatment, because coincubation of the cells together with the MAP kinase blocker PD98059 fully restored β-arrestin1 translocation and receptor internalization. These results demonstrate that chronic morphine treatment produces adaptational changes at the β-arrestin1 level, which in turn attenuates agonist-mediated desensitization and internalization of G-protein-coupled receptors.

Key words: δ-opioid receptor; chronic morphine; receptor desensitization; receptor internalization; β-arrestin; MAP kinase

The cellular mechanisms of opioid tolerance comprise changes at the opioid receptor (OR) level itself as well as on down-stream sites (Taylor and Fleming, 2001). Adaptational changes directly affecting the OR involve their phosphorylation by G-protein-coupled receptor kinases (GRKs) and subsequent binding of β-arrestin, resulting in uncoupling of the receptor from its associated G-proteins (receptor desensitization). Subsequent to receptor uncoupling, cell surface-located receptors may become internalized and dephosphorylated and either recycled back to the cell surface (resensitization) or targeted to lysosomes for degradation (downregulation) (Sibley et al., 1987). Accordingly, uncoupling and internalization effectively contribute to desensitization of OR signaling and, thus, to the phenomenon of opioid tolerance. Interestingly, high-efficacy opioids more effectively trigger these mechanisms than compounds with low intrinsic activity (Sternini et al., 1996; Keith et al., 1998).

A special feature among opioid agonists is exhibited by morphine. Although the development of morphine tolerance requires chronic OR activation (Taylor and Fleming, 2001), this ligand fails to bring about adaptational changes at the receptor level itself, that is, desensitization and internalization (Keith et al., 1996, 1998). Thus, the development of morphine tolerance must involve adaptational changes within receptor-associated signal transduction pathways (Whistler and von Zastrow, 1998). In this respect, chronic morphine treatment has been reported to increase the expression of GRK2, β-arrestin (Terwilliger et al., 1994), dynamin (Noble et al., 2000), protein kinase A (Bernstein and Welch, 1998), and protein kinase C (Li and Roerig, 1999). Because each of these factors may contribute to the mechanism of agonist-induced desensitization of receptor activity, the present study was initiated to investigate whether chronic morphine treatment could possibly affect the regulatory properties of high-efficacy opioids to desensitize OR function. Neuroblastoma x glioma (NG108-15) hybrid cells were used, because they endogenously express high levels of DORs that are better substrates for agonist-induced desensitization than MORs (Koch et al., 1998; Law et al., 2000). In addition, these cells are known to produce cellular correlates of morphine tolerance (Johnson and Fleming, 1989), an essential requirement for the present study. Our results demonstrate that chronic morphine treatment completely blocks the ability of [D-Ala^2, D-Leu^5]enkephalin (DADLE) and etorphine to bring about DOR uncoupling and internalization. Inhibition of receptor desensitization was reproduced in δ-OR (DOR) and μ-OR (MOR) transfected human embryonic kidney (HEK) 293 cells and also extended to other G-protein-coupled receptors (GPCRs), such as the m_4-muscarinic acetylcholine receptor (m_4AChR) and the cannabinoid (CB1) receptor. Further studies revealed that the underlying regulatory mechanism of impaired receptor desensitization is associated with an attenuated β-arrestin function, which might originate from an unimpeded MAP kinase signaling during the state of morphine tolerance.

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MATERIALS AND METHODS

Cell culture and transfections. NG108-15 cells were cultured in DMEM as described by Vachon et al. (1987). HEK293 cells were grown in DMEM under standard conditions (Bot et al., 1997). Cells were stably transfected by the calcium phosphate coprecipitation method with plasmid pcDNA 3.1 (Invitrogen BV, Groningen, The Netherlands) containing full-length cDNAs of the DOR (Yasuda et al., 1993) and MOR (Chen et al., 1993). Stably opioid receptor-expressing cell clones were selected with geneticin (Invitrogen BV). The cell clones used in this study were designated HEK293/δ (1.4 ± 0.2 pmol receptors/mg membrane protein) and HEK293/μ (2.5 ± 0.1 pmol receptors/mg membrane protein). Transient transfections of HEK293/δ and HEK293/μ cells were performed with full-length mouse CB1 cDNA in pcDNA3.1 vector using the calcium phosphate coprecipitation method. Total expression levels of CB1 receptors varied from 0.83 ± 0.1 to 2.8 ± 0.2 pmol/mg membrane protein.

Chronic opioid treatment. Cells were chronically treated with 1 μM morphine for 72 hr. Untreated cells of the same passage served as controls. In some experiments, naloxone (10 μM; Merck, Mannheim, Germany) and pertussis toxin (25 ng/ml; Calbiochem, Bad Soden, Germany) were added. Cells were washed three times with ice-cold PBS, pH 7.4, and resuspended in prewarmed DMEM containing DADLE, [D-Ala^2, *N*-Me-Phe^4, glycinol^5]enkephalin (DAMGO; Bachem, Bubendorf, Switzerland), and etorphine (National Institute on Drug Abuse, Bethesda, MD), respectively, to induce receptor desensitization. For heterologous receptor studies, cells were washed and resuspended in DMEM containing morphine and oxotremorine M (oxo M; Sigma-Aldrich, Deisenhofen, Germany), carbachol (Sigma-Aldrich), or CP55,940 (Tocris, Köln, Germany) as indicated. Subsequently, cells were chilled on ice, washed extensively, and used immediately for experimentation.

[^{35}S]GTPγS binding. Cells were homogenized in ice-cold TM buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4), and a particulate membrane fraction (P2) was prepared according to Vachon et al. (1987). Binding reactions (500 μl total volume) were performed according to Szekeres and Traynor (1997) and consisted of 20 μg protein in incubation buffer, 0.1 nM [^{35}S]GTPγS (1.250 Ci/mmol; NEN Life Science, Zaventem, Belgium), 30 μM GDP, and various concentrations of DADLE, oxo M, and CP55,940 as indicated. Nonspecific binding was determined in the presence of 10 μM GTPγS (Sigma-Aldrich). Binding was at 25°C for 30 min and terminated by rapid filtration through Whatman GF/B glass fiber filters followed by three washes with ice-cold TM buffer. Membrane-associated radioactivity was determined in a Beckman LS-1801 scintillation counter. Each assay was performed in triplicate determination.

Receptor internalization. OR internalization was determined by radioligand binding using intact cells according to Li et al. (1999). Briefly, total receptors were labeled with the lipophilic, membrane-permeable opioid antagonist [^{3}H]diprenorphine (53 Ci/mmol; Amersham Life Science, Buckinghamshire, UK), and cell surface receptors were determined with the peptide agonist DADLE (1 μM). Nonspecific binding was assessed with 10 μM naloxone. Binding reactions (200 μl) included 2×10^4 cells per tube in Tris-HCl buffer (50 mM, pH 7.4), 1 nM [^{3}H]diprenorphine, with or without 10 μM naloxone or 1 μM DADLE. Equilibrium of radioligand binding was established within 2 hr (4°C). Reactions were stopped by rapid filtration through Whatman GF/C glass fiber filters followed by three washes with ice-cold Tris-HCl buffer. Each assay was performed in triplicate determination.

Cell surface m_4AChRs were determined according to Roseberry et al. (2001) using the hydrophilic antagonist [^{3}H]*N*-methyl-scopolamine ([^{3}H]NMS) (83 Ci/mmol; Amersham Life Science) and atropine (Fluka, Deisenhofen, Germany) as the displacer. Reactions (200 μl total volume) included 2×10^4 NG108-15 cells, 10 nM [^{3}H]NMS in 50 mM Tris-HCl buffer, pH 7.4, with or without 1 μM atropine. Reactions were performed for 2 hr at 4°C.

CB1 receptors were determined in particulate membrane preparations (100 μg per reaction) of transiently cannabinoid (CB1) receptor-transfected HEK293/δ and HEK293/μ cells previously subjected to short-term agonist treatment (receptor internalization) or not (control). Total binding was determined by the cannabinoid antagonist [^{3}H]SR141716A (52 Ci/mmol; Amersham Life Science), and nonspecific binding was assessed in the presence of 1 μM SR141716A (National Institute on Drug Abuse). The binding reactions were performed for 1 hr at 25°C.

β-Arrestin translocation. NG108-15 cells were transiently transfected with bovine β-arrestin1 cDNA in pcDNA3.1 using the calcium phosphate coprecipitation technique. The following day, cells were split and kept for another 2 d either in the absence (naive) or presence of morphine to induce tolerance. Cells were chilled on ice, washed three times with ice-cold PBS, and stimulated with various receptor ligands for 10 min at 30°C. Thereafter, cells were harvested in ice-cold TM buffer and broken by 30 passages through a 27 gauge needle, and membranes were prepared as above. Aliquots (10 μg of protein) of the homogenates and P2 pellets were subjected to 10% SDS-polyacrylamide gels (Ammer and Schulz, 2000). Western blotting was done with a β-arrestin1-specific antibody (Transduction Laboratories, Lexington, KY). Immuncomplexes were detected by incubation of the blots with a peroxidase-conjugated donkey anti-rabbit IgG (Promega, Köln, Germany), and the blots were developed using the enhanced chemiluminescence method (Amersham Life Science). Equal protein loading was verified by determination of membrane-associated Gβ subunits using an anti-Gβ1/2 antibody (Ammer and Schulz, 2000).

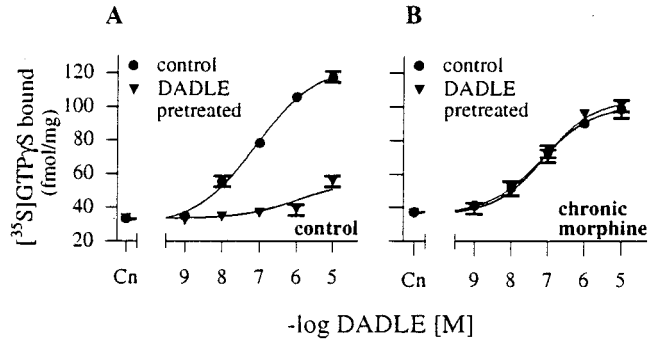

Figure 1. Chronic morphine treatment impairs regulation of DOR activity in NG108-15 cells. *A*, Effect of DADLE on [^{35}S]GTPγS binding. Membranes from untreated (●) and DADLE-pretreated (1 μM; 1 hr; ▼) cells were measured for DADLE-stimulated [^{35}S]GTPγS incorporation. Compared with controls, short-term DADLE treatment substantially decreased agonist-stimulated [^{35}S]GTPγS binding. *B*, DADLE stimulated [^{35}S]GTPγS binding to cell membranes chronically exposed to morphine (1 μM; 72 hr). Chronic morphine treatment blocked DOR desensitization by DADLE. Values are the mean ± SEM of $n = 9$ experiments.

MAP kinase analysis. NG108-15 cells were cultured in DMEM either in the absence or presence of morphine (1 μM) for 72 hr. Before determination of MAP kinase activity, cells were washed and equilibrated in serum-reduced medium (0.1% FCS) with or without morphine (1 μM) for 2 hr. In some experiments, naive cells were washed again after 1 hr of equilibration and subjected to short-term agonist treatment for 1 hr with morphine (1 μM) or DADLE (1 μM). After the medium was removed, cells were washed, and OR-stimulated MAP kinase activity was determined in the presence of DADLE (1 μM) or morphine (1 μM) in the absence and presence of naloxone (10 μM) for 5 min at 37°C. Nontreated cells served as control. Reactions were stopped by removing the medium and solubilizing the cells with Laemmli sample buffer. Extracts were subjected to 10% SDS-PAGE and immunoblotted as described above using phospho-specific or nonspecific extracellular-regulated kinases (ERK1/2)/MAP kinase antibodies as the primary reagents (New England Biolabs, Frankfurt, Germany). Western blots were further processed and developed as above.

RESULTS

Chronic morphine treatment blocks DOR desensitization in NG108-15 cells

Prolonged DOR activation in NG108-15 cells by morphine brings about tolerance without any effects at the receptor level (Loh et al., 1988). In contrast, short-term exposure of the cells to the high-efficacy agonist DADLE produces strong degrees of receptor desensitization. As shown in Figure 1*A*, DADLE treatment (1 μM; 1 hr) abolished [^{35}S]GTPγS binding by a second DADLE stimulus, indicating complete uncoupling of DORs from their associated G-proteins. In contrast, chronic morphine treatment of NG108-15 cells had no effect on receptor/G-protein interaction (Fig. 1*B*). In chronically morphine-treated cells, calculated EC_{50}

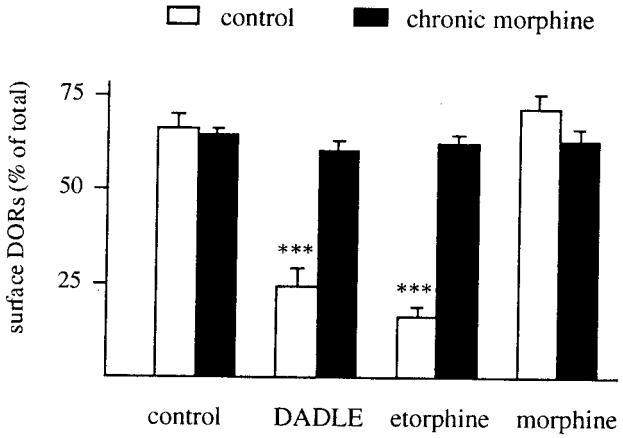

Figure 2. Agonist-induced DOR internalization is blocked in morphine-tolerant NG108-15 cells. Cell-surface DORs were determined by [^{3}H]diprenorphine binding. In naive cells (*open columns*), short-term exposure to DADLE (1 μM) and etorphine (100 nM) but not to morphine (1 μM) resulted in substantial DOR internalization. Although chronic morphine treatment (1 μM; 72 hr) (*filled columns*) has no effect on cell-surface DOR density (685.4 fmol/mg membrane protein), it prevents internalization by acute exposure to DADLE (1 μM; 1 hr) or etorphine (100 nM; 1 hr). Each *column* represents the mean ± SEM of three independent experiments. Statistical differences were determined by ANOVA. $^{***}p < 0.001$.

and E_{max} values of DADLE-stimulated [^{35}S]GTPγS binding were identical to those obtained from naive cells, regardless of a previous DADLE stimulus. These results suggest that chronic morphine treatment prevents DOR desensitization by short-term DADLE treatment.

Blockade of DOR internalization by chronic morphine treatment

We next assessed whether DADLE is able to produce DOR internalization in NG108-15 hybrid cells. In control cells, a constant fraction of 66.6 ± 3.8% ($n = 9$) of total receptor is localized to the plasma membrane. Short-term exposure to DADLE (1 hr; 1 μM) significantly reduced the fraction of cell surface receptors to 25.7 ± 4.4% (Fig. 2), without affecting total DOR capacity. A similar effect was observed with 100 nM etorphine. Thus, short-term treatment of NG108-15 cells with high-efficacy agonists brings about DOR internalization. In contrast, both short-term (1 hr) and long-term (72 hr) treatment of the cells with morphine (1 μM) failed to induce DOR internalization (Fig. 2). Although chronic morphine treatment had no effect on overall DOR abundance (0.68 ± 0.03 vs 0.69 ± 0.02 pmol receptors/mg membrane protein in control and chronically morphine-treated cells, respectively), it completely blocked subsequent DOR internalization by an acute challenge with DADLE (1 μM; 1 hr) or etorphine (100 nM; 1 hr) (Fig. 2).

Pharmacology of chronic morphine-induced blockade of DOR internalization

Pretreatment of NG108-15 cells with 1 μM morphine time-dependently inhibited DADLE-induced DOR internalization (Fig. 3*A*). Receptor internalization was half-maximally reduced after 20 hr and totally blocked after 72 hr of chronic morphine exposure. DADLE-induced internalization was further tested in the presence of increasing morphine concentrations, revealing a half-maximal effect at 60 nM morphine. Internalization completely failed at 1 μM morphine (Fig. 3*B*). To determine whether

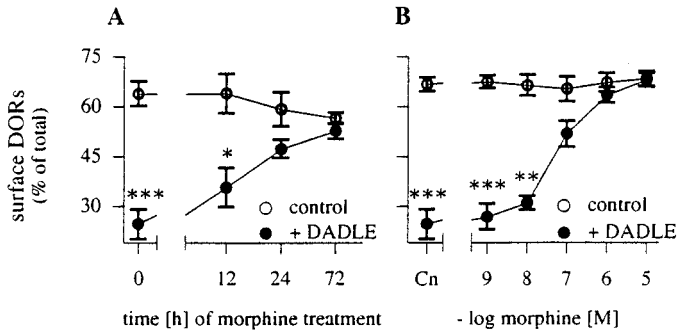

Figure 3. Morphine-induced blockade of DOR internalization. *A*, Time course. NG108-15 cells were chronically treated with 1 μM morphine for 12, 24, and 72 hr and washed, and DOR internalization was determined by acute exposure to DADLE (1 μM; 1 hr, ●). *B*, Dose–response relationship. NG108-15 cells were chronically treated with increasing concentrations of morphine before receptor internalization was determined. Data are the mean ± SEM of $n = 9$ experiments. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 1. Effect of PTX, naloxone, and opioid withdrawal on morphine-induced blockade of DOR internalization

	Surface DORs in NG108-15 cells (% of total)	
Pretreatment (72 hr)	Control	DADLE challenge (1 μM; 1 hr)
None	66.6 ± 3.8	25.7 ± 4.4 (−61.4%)
Morphine	56.5 ± 1.8	54.3 ± 2.3 (−3.9%)
Morphine + PTX	56.7 ± 1.5	24.7 ± 2.8 (−56.4%)
Morphine + naloxone	63.2 ± 2.4	23.7 ± 3.2 (−62.5%)
Morphine +		
Withdrawal for:		
15 min	57.3 ± 2.7	58.4 ± 3.1 (+1.9%)
30 min	61.5 ± 4.3	48.2 ± 0.6 (−21.6%)
45 min	60.5 ± 3.0	31.5 ± 1.8 (−47.9%)
60 min	62.7 ± 2.7	30.9 ± 1 (−50.7%)

NG108-15 cells were treated for 72 hr with morphine (1 μM) alone, morphine and PTX (24 ng/ml), or morphine and naloxone (10 μM). Chronically morphine-treated cells (1 μM; 72 hr) were washed extensively to induce opioid withdrawal and were cultured for 15, 30, 45, and 60 min in the absence of ligand. The effect of short-term DADLE treatment (1 μM; 1 hr) on cell-surface DORs was determined by [^{3}H]diprenorphine binding. Data are the mean ± SEM of $n = 3$ experiments.

chronic morphine-induced blockade of DOR internalization recovers during morphine withdrawal, NG108-15 cells were treated with morphine (1 μM) for 72 hr, washed, and kept in the absence of agonist for 15, 30, 45, and 60 min. The ability of DADLE (1 μM; 1 hr) to mediate DOR internalization is gradually restored over 60 min (Table 1). First evidence for receptor internalization is observed after morphine washout for 30 min, but it takes 45–60 min until DOR internalization resembles the effect of DADLE on nonpretreated cells. The effect of chronic morphine treatment was also blocked by coincubation of the cells with the opioid antagonist naloxone (10 μM; 72 hr), indicating a specifically DOR-mediated effect (Table 1). We also examined whether persistent activation of inhibitory G-proteins is required for the generation of this chronic morphine effect. Pertussis toxin is known to inhibit $G_{i/o}$ protein activation and does not affect DADLE-promoted DOR internalization (Chakrabarti et al., 1997). Concomitant exposure of the cells to morphine (1 μM; 72 hr) and pertussis toxin (25 ng/ml; 72 hr) retained the ability of

Table 2. Chronic morphine impairs DOR internalization in HEK293/δ cells

	Surface DORs (% of total)	
	Control	Morphine pretreated
Control	60.1 ± 2.5	51.1 ± 3.3
DADLE (1 hr; 1 μM)	36.1 ± 0.4 (−39.9%)	48.3 ± 3.5 (−5.4%)
Etorphine (1 hr; 100 nM)	28.9 ± 0.7 (−51.9%)	46.9 ± 4.0 (−8.2%)

HEK293/δ cells were chronically treated with morphine (1 μM; 72 hr) before the effect of DADLE (1 μM; 1 hr) and etorphine (100 nM; 1 hr) on cell surface receptors was determined by [^{3}H]diprenorphine binding. Results are the mean ± SEM from three separate experiments.

DADLE to induce DOR internalization (Table 1). Thus, morphine-induced blockade of receptor internalization requires an intact DOR/G-protein interaction, implicating persistent G-protein stimulation.

Chronic morphine regulation of DOR sensitivity in HEK293 cells

HEK293 cells have been widely used during the past to study receptor internalization (Krueger et al., 1997; Koch et al., 1998). We thus examined whether attenuation of DOR internalization can be reproduced in morphine-treated HEK293 cells stably expressing the mouse DOR. As found for NG108-15 cells, chronic morphine treatment (1 μM; 72 hr) had no effect on overall DOR abundance (1.4 ± 0.2 vs 1.2 ± 0.1 pmol receptors/mg membrane protein in naive and chronically morphine-treated cells, respectively) or on the fraction of cell surface receptors in this cell line, but significantly reduced the ability of DADLE and etorphine (100 nM) to internalize the DOR (Table 2). The results clearly document that the action of morphine to affect DOR internalization is not restricted solely to NG108-15 cells but also occurs in heterologous cell systems.

Chronic morphine exposure affects MOR internalization

To test whether chronic morphine inhibition of receptor internalization is restricted to the DOR, similar experiments were performed in HEK293 cells stably transfected to express the rat MOR (HEK293/μ). As with the DOR, incubation of HEK293/μ cells with the μ-selective peptide agonist DAMGO (1 μM) as well as with etorphine (100 nM) reduced the fraction of cell surface-located MORs (Fig. 4). In contrast, both short-term (1 hr) and long-term (72 hr) treatment of cells with morphine (1 μM) had no effect on cell surface receptors or on overall receptor abundance (data not shown). In chronically morphine-treated (1 μM; 72 hr) cells, the ability of DAMGO and etorphine to induce MOR internalization in HEK293/μ cells was strongly impaired. Thus, impaired regulation of opioid receptor responsiveness by chronic morphine occurred to both DORs and MORs and is independent of the opioid used for second receptor activation.

Chronic morphine treatment blocks internalization of heterologous GPCRs

We next examined whether chronic morphine treatment would also affect the regulation of non-opioid GPCRs. For this, endogenous m_4AChRs were studied in NG108-15 cells (Lazareno et al., 1990). In general, oxo M given to membranes of nontreated NG108-15 cells resulted in stimulation of [^{35}S]GTPγS binding (Fig. 5*A*). Pretreatment of the cells with oxo M (1 μM; 1 hr) desensitized these receptors as demonstrated by a loss of [^{35}S]GTPγS incorporation. Chronic exposure of the cells to morphine (1 μM; 72 hr) had no effect on oxo M-stimulated [^{35}S]GTPγS binding, but clearly blocked m_4AChR desensitization by oxo M (1 μM; 1 hr) (Fig. 5*A*).

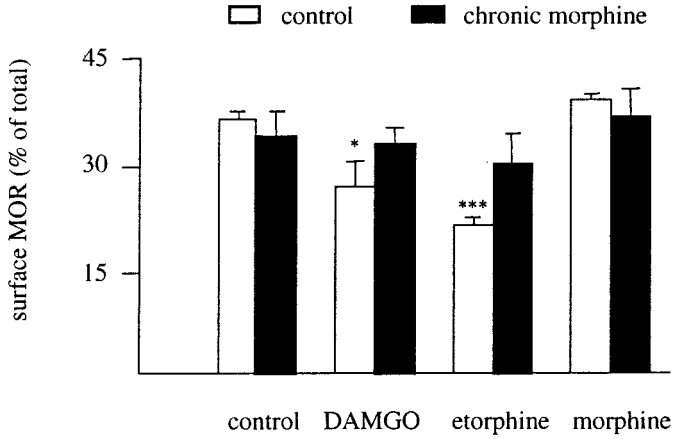

Figure 4. Chronic morphine treatment impairs MOR internalization in HEK293/μ cells. Control (*open column*) and morphine-pretreated (*filled column*) HEK293/μ cells were challenged with DAMGO (1 μM), etorphine (100 nM), and morphine (1 μM) for 1 hr. Surface MORs were determined by radioligand binding. Data are the mean ± SEM of $n = 9$ experiments. $^*p < 0.05$; $^{***}p < 0.001$.

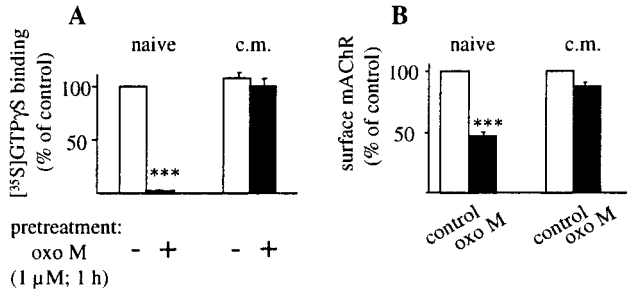

Figure 5. Chronic morphine exposure blocks agonist-induced internalization of m_4AChRs in NG108-15 cells. *A*, Activation of m_4AChRs by oxo M (1 μM; 30 min) results in threefold stimulation of basal [^{35}S]GTPγS binding (25.5 fmol/mg protein), which was set to 100%. Short-term oxo M pretreatment (1 μM; 1 hr) abolished agonist-induced GTPγS binding. In contrast, chronic morphine (*c.m.*) pretreatment (1 μM; 72 hr) prevents m_4AChR desensitization and sustained [^{35}S]GTPγS binding by oxo M. *B*, Effect of morphine pretreatment on m_4AChR internalization. In naive cells, oxo M (1 μM; 1 hr) substantially decreased cell surface [^{3}H]NMS binding. Although chronic morphine (*c.m.*) has no effect on m_4AChR receptor density, it significantly blocks subsequent m_4AChR internalization. The data shown are the mean ± SEM from four independent experiments. $^{***}p < 0.001$.

The effect of chronic morphine treatment on m_4AChR internalization in NG108-15 cells was investigated next. The membrane-impermeable radioligand [^{3}H]NMS was used to quantify the levels of cell-surface mAChRs in intact cells. As demonstrated in Figure 5*B*, acute exposure of the cells to oxo M (1 μM; 1 hr) resulted in a significant loss of cell-surface m_4AChRs by 48.8 ± 3.4%. This agonist-induced m_4AChR internalization was blocked by chronic morphine pretreatment (1 μM; 72 hr).

Similar results were obtained in experiments using HEK293/δ cells transiently expressing the cannabinoid CB1 receptor. Exposure of these cells to the CB1 agonist CP55,940 (1 μM; 1 hr) desensitized CB1 receptors, as demonstrated by the loss of agonist-induced [^{35}S]GTPγS binding and the downregulation of [^{3}H]SR141716A binding sites in isolated plasma membranes (Ta-

Table 3. CB1 receptor desensitization is abolished in morphine-treated HEK293/δ cells

	CB1 receptors in HEK293/δ cells				
	CB1-stimulated [^{35}S]GTPγS binding (%)		[^{3}H]SR141716A binding (%)		
	Control	Morphine pretreated	Control	Morphine pretreated	DADLE pretreated
Control	100	98.1 ± 3.1	100	98.3 ± 3.7	97.4 ± 2.4
CP55,940	3.8 ± 4.5 (−96.2%)	97.7 ± 2.3 (−0.4%)	48.9 ± 2.7 (−51.1%)	91.4 ± 3.9 (−6.9%)	46.0 ± 2.2 (−55.4%)

Naive and morphine- (1 μM) or DADLE- (1 μM) treated HEK293/δ cells transiently expressing the CB1 receptor were challenged with CP55,940 (1 μM; 60 min) to desensitize and internalize the CB1 receptor. Membranes were prepared and assayed for CP55,940 (1 μM; 30 min) stimulated [^{35}S]GTPγS incorporation and receptor density using [^{3}H]SR141716A as the radioligand. Because transient expression of CB1 receptors varied in each experiment, data were set to 100% (untreated controls). Values are the mean ± SEM from three to four separate experiments.

ble 3). Again, chronic morphine pretreatment impaired CB1 receptor desensitization and internalization by CP55,940 (Table 3). Chronic MOR activation by morphine showed very similar effects on CB1 regulation. Although activation by CP55,940 induced a loss of surface CB1 receptors by 36.2 ± 8.6% in naive HEK293/μ cells, no internalization could be observed when cells were rendered tolerant to morphine for 72 hr (−3.3 ± 8.4%).

To verify that this heterologous effect is typical for morphine, we also assessed the effect of prolonged DADLE exposure on CB1 receptor internalization in HEK293/δ cells. Long-term treatment with DADLE (1 μM; 72 hr) brought about a complete loss of total [^{3}H]diprenorphine binding sites (2.1 ± 0.24 vs 0.05 ± 0.03 pmol receptors/mg membrane protein for naive and DADLE-treated cells, respectively), which is indicative of DOR downregulation. In contrast to chronic morphine, the ability of CP55,940 to internalize the CB1 receptor remained unaffected by this chronic DADLE treatment regimen (Table 3).

Cellular mechanism of morphine-induced blockade of GPCR regulation

The fraction of cell-surface receptors represents the steady state between receptors undergoing endocytosis and recycling. After endocytosis, receptors are dephosphorylated and recycled to the cell surface (Krueger et al., 1997). Thus, attenuation of DOR uncoupling and internalization during the state of morphine tolerance could be caused by an enhanced receptor recycling or an attenuated receptor desensitization. To discriminate between these possibilities, we investigated DOR internalization in the presence of monensin (50 μM; 2 hr), an inhibitor of receptor recycling (Basu et al., 1981). Although monensin treatment slightly enhanced DADLE-induced DOR internalization in naive cells (−80% of cell surface DORs), it failed to restore DOR internalization in NG108-15 cells chronically treated with morphine (Fig. 6). Thus, chronic morphine treatment appears to block receptor desensitization and internalization rather than accelerate receptor recycling.

Uncoupling and internalization of GPCRs requires their binding to cytosolic arrestins. Therefore, the effect of chronic morphine treatment on receptor-stimulated β-arrestin1 translocation was examined. First, naive NG108-15 cells overexpressing β-arrestin1 were stimulated with DADLE for 10 min, and membrane translocation of β-arrestin1 was followed by Western blot analysis. Clearly, DOR activation induced β-arrestin1 redistribution to the plasma membrane, an effect that was antagonized by naloxone (Fig. 7). Activation of m_4AChRs by carbachol also increased the abundance of β-arrestin1 in the plasma membrane of NG108-15 cells. These results are consistent with findings that activation of both DOR and m_4AChR results in the recruitment of β-arrestin1 to the membrane (Vogler et al., 1999; Zhang et al., 1999). In contrast to high-efficacy agonists, both short- and long-term DOR stimulation by morphine failed to induce β-arrestin1 translocation. When membranes from morphine-tolerant cells were analyzed, neither DADLE nor carbachol was able to redistribute β-arrestin1 to the plasma membrane (Fig. 7). These results indicate that chronic morphine treatment interacts with β-arrestin function.

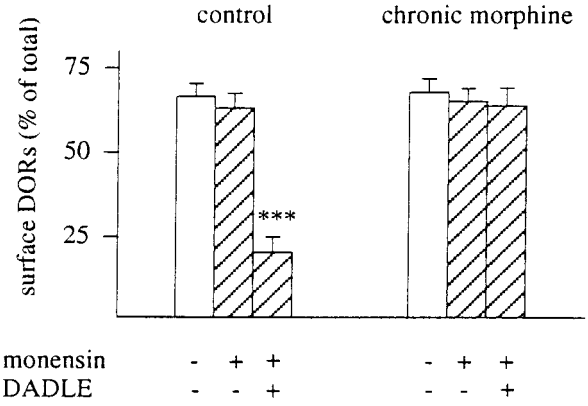

Figure 6. Monensin fails to reconstitute receptor internalization in morphine-pretreated cells. Monensin pretreatment has no effect on cell-surface receptor density or on DADLE-induced receptor internalization in NG108-15 cells. Monensin also fails to restore DOR internalization in morphine-exposed (1 μM; 72 hr) cells. Data are the mean ± SEM of three independent experiments. ***$p < 0.001$.

Chronic morphine regulation of MAP kinase activity

β-Arrestin1 is inactivated by MAP kinase-mediated phosphorylation (Lin et al., 1997). We therefore investigated whether chronic morphine treatment would result in persistent MAP kinase activation, which in turn could affect β-arrestin1 function. MAP kinase itself is activated by phosphorylation, which is easily detected by immunoblotting using a phospho-specific ERK1/2 antibody. We first examined the effect of acute DOR activation on MAP kinase phosphorylation. As shown in Figure 8 (*top panel*), both DADLE and morphine strongly stimulated MAP kinase phosphorylation without changing overall MAP kinase abundance (*bottom panel*). DADLE-stimulated MAP kinase phosphorylation is attenuated mostly by naloxone, indicating a DOR-mediated effect. In analogy to receptor desensitization, short-term DADLE treatment (1 μM; 1 hr) completely abolished subsequent MAP kinase activation by a second DADLE stimulus (Fig. 8). In contrast, the ability of DADLE to stimulate MAP kinase phosphorylation remained unaffected after pretreatment of the cells with morphine (1 μM; 1 hr). Even more pronounced results were obtained in cells chronically exposed to DADLE (1 μM) and mor-

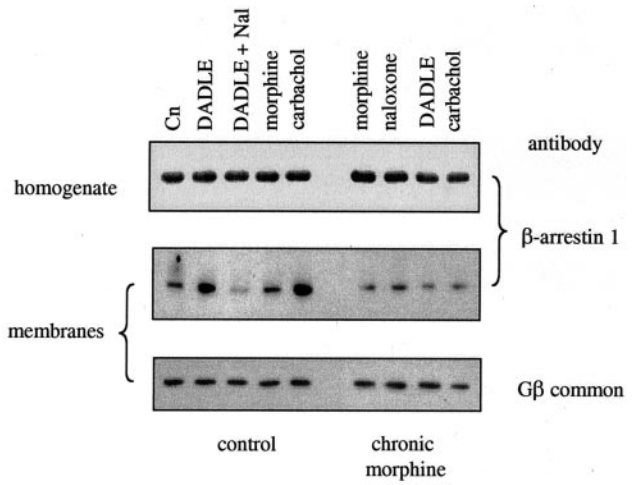

Figure 7. Effect of chronic morphine treatment on β-arrestin1 translocation. Transiently β-arrestin1-transfected NG108-15 cells were stimulated with distinct agonists for 10 min to initiate translocation of cytosolic β-arrestin to the plasma membrane. Cells were homogenized, membranes were prepared, and β-arrestin1 was detected by Western blot determination (*top* and *middle panels*). Although high-efficacy agonists (*DADLE, carbachol*) increased β-arrestin1 immunoreactivity in membranes from naive cells, chronic morphine prevents this effect. Changes of β-arrestin expression were not detected (*top panel).* Control of equal membrane protein loading was performed by using Gβ common antibody (*bottom panel*).

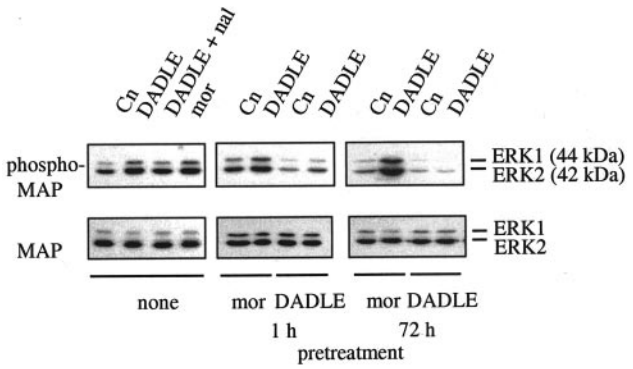

Figure 8. MAP kinase phosphorylation in morphine-treated NG108-15 cells. NG108-15 cells were kept in either the absence or presence of chronic treatment with DADLE and morphine for 1 and 72 hr. Receptor-mediated phosphorylation of MAP kinase was detected by Western blot experiments using a phospho-specific ERK1/2 antibody (*top panel*). Overall MAP kinase abundance was evaluated with a phospho-insensitive ERK1/2 antibody (*bottom panel*). After equilibration in serum-free medium, cells were washed and MAP kinase was determined for 5 min at 37°C in the absence (C*n)* or presence of the indicated ligand. Note that DADLE is still able to stimulate MAP kinase phosphorylation in chronically morphine but not DADLE pretreated cells.

phine (1 μM) for 72 hr (Fig. 8). These results indicate that MAP kinase is under persistent DOR control in chronically morphine-treated NG108-15 cells.

PD98059 reverses chronic morphine-induced blockade of DOR internalization

When persistent MAP kinase stimulation accounts for impaired receptor regulation by phosphorylation and inactivation of β-arrestin1, the MAP kinase blocker PD98059 should reverse the chronic morphine effect on receptor internalization. We first tested the effect of PD98059 (20 μM) on cell-surface DORs.

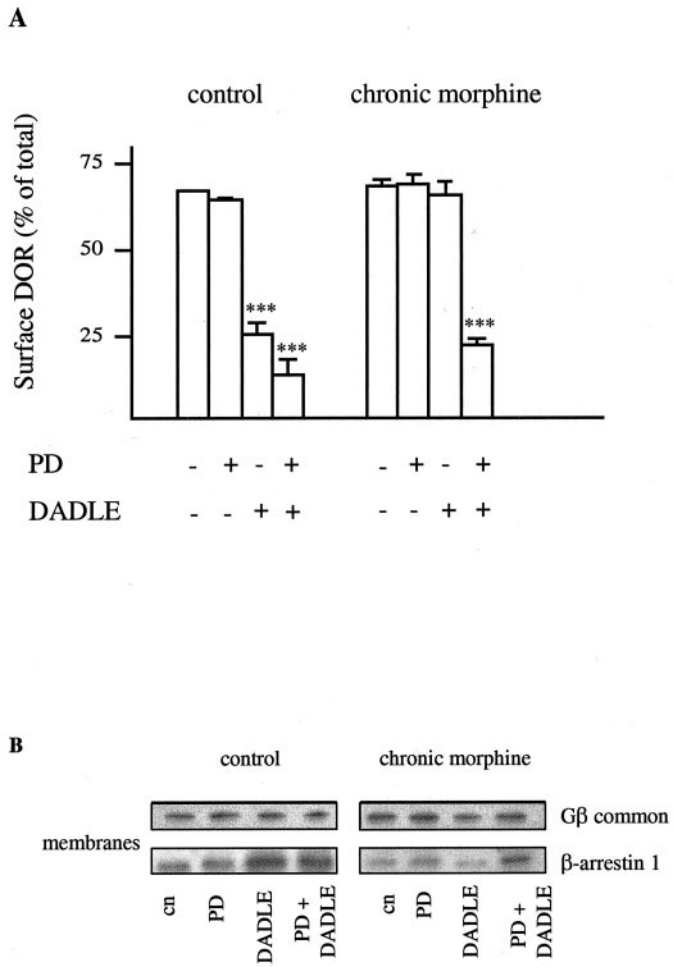

Figure 9. PD98059 restores β-arrestin translocation and DOR internalization in morphine-pretreated NG108-15 cells. *A*, Cell surface DORs of control and morphine-pretreated NG108-15 cells were determined after incubation with PD98059 (20 μM; 60 min; *PD*), DADLE (1 μM; 60 min; *DADLE*) alone, and a combination of DADLE and PD (*PD + DADLE*). Note that PD98059 fails to affect DADLE-induced DOR abundance and internalization in control cells. However, PD98058 restores the ability of DADLE to mediate DOR internalization in morphine-pretreated cells. The data shown are the mean ± SEM from three independent experiments. $^{**}p < 0.01$. *B*, Effect of PD98059 (20 μM; *PD*) on β-arrestin1 redistribution in control (*control*) and morphine-pretreated (*chronic morphine*) NG108-15 cells. In the presence of PD98059, the ability of DADLE (1 μM; *PD + DADLE*) to target β-arrestin1 to the plasma membrane is restored in both naive and morphine-tolerant cells.

Exposure of naive NG108-15 cells to this compound for 1 hr had no detectable effect on the amount of cell-surface DORs (Fig. 9*A*). It also failed to affect DADLE-induced receptor internalization when applied simultaneously with the opioid to NG108-15 cells (Fig. 9*A*). Next, NG108-15 cells exposed to morphine (1 μM; 72 hr) were washed and exposed to PD98059 for 1 hr. [^{3}H]diprenorphine binding revealed no effect on the number of cell-surface DORs by this treatment. However, when cells chronically exposed to morphine were washed extensively and subsequently exposed to PD98059 in the presence of 1 μM DADLE for 1 hr, a significant loss of cell-surface DOR could be observed (Fig. 9*A*). Thus, blockade of MAP kinase activity reconstitutes the ability of high-efficacy agonists at least partly to induce DOR internalization in morphine-treated cells. Similarly, PD98059 also restored etorphine-promoted MOR internalization in chronically

morphine-treated (1 μM; 72 hr) HEK293/μ cells, observable by the loss of surface MORs by 46.8 ± 2.3%.

To examine whether PD98059-induced reconstitution of opioid receptor internalization is associated with β-arrestin1 translocation, morphine-treated NG108-15 cells were challenged to PD98059 and DADLE and homogenized, and membrane-associated β-arrestin1 was examined by Western blot analysis. Incubation of naive cells with PD98059 had no effect on basal levels of membrane-bound β-arrestin1 or on DADLE-stimulated β-arrestin1 translocation. Although in chronically morphine-treated cells termination of persistent MAP kinase activation by the MEK inhibitor PD98059 had only little effect on the fraction of membrane-associated β-arrestin1, it fully restored the ability of DADLE to trigger β-arrestin1 redistribution (Fig. 9*B*). These findings demonstrate that in morphine-pretreated cells, inhibition of MAP kinase activity results in functional reconstitution of β-arrestin1-mediated receptor internalization.

DISCUSSION

The present study examined the effect of chronic morphine treatment on agonist-induced receptor desensitization in NG108-15 hybrid cells (endogenous DOR) as well as on cell systems stably transfected with the DOR and MOR (HEK293 cells). Our results demonstrate that chronic morphine treatment impairs the ability of high-efficacy opioids to mediate receptor uncoupling and internalization. Inhibition of receptor desensitization requires persistent intracellular signal transduction during the course of chronic morphine treatment and heterologously extends to other GPCRs, such as the m_4AChR and CB1 receptor. These results indicate that chronic morphine treatment blocks the intracellular machinery involved in agonist-induced regulation of receptor sensitivity.

The development of opioid tolerance may be accompanied by adaptations at the receptor level (Taylor and Fleming, 2001), such as receptor uncoupling from its cognate G-proteins and receptor internalization. Here, we confirm that in NG108-15 cells, short-term treatment with the high-efficacy opioids DADLE and etorphine leads to rapid uncoupling and sequestration of the DOR from the cell surface (Willets and Kelly, 2001). In contrast, both short- and long-term treatment of the cells with morphine had no effect on DOR sensitivity and abundance. The failure of morphine to induce receptor desensitization and internalization was also observed for stably DOR- and MOR-transfected HEK293 cells, confirming previous data obtained *in vitro* (Keith et al., 1996; Whistler et al., 1998) and *in* vivo (Keith et al., 1998). Although the reason for this unique property of morphine is still elusive, it does not appear to depend on its chemical structure or affinity (Bot et al., 1997). Instead, the finding that morphine fails to induce GRK-mediated receptor phosphorylation and β-arrestin translocation (Zhang et al., 1999) led to the suggestion that, although it mediates activation of receptor-associated G-proteins, morphine is unable to induce a distinct receptor conformation required for GRK-mediated phosphorylation (Keith et al., 1996).

Given the fact that high-efficacy agonists are still able to activate OR-associated G-proteins in chronically morphine-pretreated cells, the present study strongly supports the idea that the state of morphine tolerance is characterized by functional intact OR signaling (Whistler and von Zastrow, 1998). However, our results also confirm that persistent intracellular opioid signaling represents an essential requirement for the induction of chronic morphine adaptations at the level of receptor-associated signal transduction pathways. In this respect, long-term treatment of stably DOR-transfected HEK293 cells with DADLE primarily resulted in desensitization and downregulation of the binding site, but failed to influence agonist-induced desensitization of cotransfected CB1 receptors.

The present study demonstrates that the inhibitory effect of chronic morphine treatment on agonist-induced receptor desensitization develops in a time- and dose-dependent manner and is reversible during morphine withdrawal. The kinetics shown in Figure 3*A* and Table 1 are similar to those described previously for other chronic morphine effects, including an enhanced PKC activity (Li and Roerig, 1999), the induction of adenylyl cyclase supersensitivity (Avidor-Reiss et al., 1996), and downregulation of high-affinity PGE_1 and β_2-adrenergic receptors (Ammer and Schulz, 1996, 2000). Because chronically morphine-treated HEK293 cells also develop cellular correlates of tolerance and dependence (Blake et al., 1997; Bot et al., 1997), the present finding of chronic morphine-induced inhibition of DOR and MOR desensitization is suggested to represent another example for chronic morphine-induced adaptations on post-receptor levels associated with the development of tolerance.

The investigation of agonist-induced regulation of m_4AChRs and CB1 receptors in morphine-treated cells provided valuable insight into the underlying regulatory mechanism. Chronic exposure of NG108-15 cells to morphine heterologously impaired both desensitization and internalization of the m_4AChR after stimulation with the high-efficacy agonist oxo M. Likewise, agonist-induced regulation of another non-opioid GPCR, the brain-type cannabinoid CB1 receptor, was also affected in both chronically morphine-treated DOR- and MOR-transfected HEK293 cells. This cross-inhibition of receptor regulation is indicative of a biochemical mechanism other than direct alteration of the chronically activated opioid receptor itself. Instead, a more common mechanism involved in the regulation of multiple GPCRs may be affected.

One such candidate mechanism might involve β-arrestin, which represents an essential regulatory factor in the mechanism of receptor uncoupling and sequestration (Ferguson et al., 1996) and plays a critical role in the development of tolerance to morphine both *in vitro* (Chakrabarti et al., 2001) and *in vivo* (Terwilliger et al., 1994; Bohn et al., 1999). Although high-efficacy opioids (Cheng et al., 1998), muscarinic acetylcholine receptor agonists (Vogler et al., 1999), and cannabinoids (Jin et al., 1999) are known to induce β-arrestin translocation from the cytosol to the plasma membrane, chronic morphine treatment completely blocked the ability of each of these agonists to redistribute β-arrestin1 in transiently transfected NG108-15 hybrid cells. Thus, the failure of β-arrestin1 to translocate to the plasma membrane during receptor activation might represent a plausible mechanism underlying inhibition of GPCR desensitization.

There are two possibilities by which chronic morphine treatment could block β-arrestin translocation, i.e., (1) an altered phosphorylation of agonist-occupied receptors and (2) an altered activation of cytosolic β-arrestin. Although we are currently not able to discriminate between both mechanisms, our results clearly indicate a critical role for MAP kinase in the failure of β-arrestin to translocate to the plasma membrane. Previous studies have demonstrated that both GRK2 (Pitcher et al., 1999) and β-arrestin 1 (Lin et al., 1999) are substrates for direct phosphorylation and inactivation by MAP kinase. In addition, morphine tolerance is associated with an enhanced MAP kinase activity *in vivo* (Berhow et al., 1996; Ma et al., 2001). It is thus tempting to

speculate whether persistent stimulation of MAP kinase activity during the course of chronic morphine treatment as demonstrated in the present study would result in blockade of receptor desensitization. Regardless of the mechanism involved, the ability of the MAP kinase blocker PD98058 to fully restore agonist-induced β-arrestin1 redistribution as well as receptor internalization strongly supports the involvement of MAP kinase in chronic morphine-induced blockade of receptor desensitization.

Taken together, the present results demonstrate that the cellular machinery involved in agonist-induced desensitization of opioid receptor activity is a target of chronic morphine action. Although identified in isolated cell systems, blockade of receptor sensitivity changes *in vivo* would provide a plausible explanation for the phenomenon of long-term sensitization to the effects of high-efficacy opioids and non-opioids in response to chronic morphine treatment. These phenomena include sensitization to the hyperalgesic effects of the opioid peptide [D-Ala^2]deltorphin II (Melchiorri et al., 1992) as well as supersensitivity of 5-HT$_{1A}$ autoreceptors and α_2-adrenoceptors (Sastre-Coll et al., 2002) and behavioral sensitization to cannabinoids (Pontieri et al., 2001) in morphine-tolerant rats. In this respect, it would be interesting to determine whether at least some of the physical and behavioral signs of opioid withdrawal might be attributed to an impaired attenuation to excitatory stimuli. In addition, blockade of receptor desensitization by morphine could provide the basis for novel treatment strategies to prevent the development of tolerance to the analgesic effect of high-efficacy opioids after repeated application.

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