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μ-Opioid Receptor Internalization: Opiate Drugs Have Differential Effects on a Conserved Endocytic Mechanism *In Vitro* and in the Mammalian Brain

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ABSTRACT

μ-Opioid receptors are the pharmacological targets of endogenous opioid peptides and morphine-like alkaloid drugs. Previous studies of transfected cells and peripheral neurons indicate that opioid receptors are rapidly internalized after activation by the alkaloid agonist etorphine but not after activation by morphine. To determine whether opioid receptors in the central nervous system are regulated by a similar process of agonist-selective internalization, μ-opioid receptors were examined in rat brain neurons after treatment of animals with opioid drugs. Internalized μ receptors were observed within 30 min after intraperitoneal injection of the alkaloid agonist etorphine, and this process was blocked by the antagonist naloxone. Colocalization of internalized opioid receptors with transferrin receptors in confocal optical sections indicated that receptor internalization observed *in vivo* is mediated by a membrane trafficking pathway similar to that observed previously *in vitro* using transfected human embryonic kidney 293 cells. Morphine failed to induce detectable rapid internalization of receptors, even when administered to animals at doses far in excess of those required to induce analgesia. To quantify these agonist-selective differences and to analyze an array of opioid ligands for their ability to trigger internalization, we used flow cytometry on stably transfected 293 cells. These studies indicated that the different effects of individual agonists are not correlated with their potencies for receptor activation and that a variety of clinically important agonists differ significantly in their relative abilities to stimulate the rapid internalization of opioid receptors.

Morphine and related opiate drugs are highly effective analgesics that have been used medicinally for many centuries. Opiate drugs mediate their analgesic, euphoriant, and rewarding effects by activating opioid receptors, a class of G protein-coupled receptors that are the targets of endogenously produced opioid peptides, including endorphins, enkephalins, and dynorphins. Based on pharmacology (Goldstein and Naidu, 1989) and molecular cloning (Kieffer, 1995), distinct μ-, δ-, and κ-type opioid receptors have been defined. Of these three receptors, the MOR seems to be the primary target of many clinically used analgesic opiates. Indeed, morphine, a highly effective and widely used analgesic drug, has no detectable analgesic or rewarding properties in mutant mice lacking MORs (Matthes *et al*., 1996). The fact that MORs are activated by both alkaloid drugs and native opioid peptides has led to the notion that opiate drugs act physiologically as molecular mimics of the endogenous peptide ligands. However, it is not clear whether these drugs mimic all aspects of receptor activities induced by native opioid peptides.

The clinical use of opiate drugs is limited in practice by their tendency to cause tolerance and dependence with prolonged or repeated administration. These physiological phenomena are mediated by a complex set of activation-induced regulatory mechanisms, which modulate opioid receptors as well as downstream signaling components. Mechanisms that regulate opioid receptors themselves are of particular interest to the biology of opiate tolerance and dependence because opioid receptors represent the most

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ABBREVIATIONS: MOR, μ-opioid receptor; CNS, central nervous system; HEK, human embryonic kidney; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]-enkephalin.

upstream components in this complex cascade of cellular adaptation.

When activated by various agonist ligands, including endogenously released peptides and exogenously administered drugs, MORs promote guanine nucleotide exchange of heterotrimeric G proteins of the G_i/G_o class. Receptor-mediated activation of these G proteins triggers the acute downstream signaling actions of opioid receptors, including regulation of adenylyl cyclase, mitogen-activated protein kinase, G protein-gated, inwardly rectifying K^+ channels (GIRK1), and voltage-dependent calcium channels (Dhawan *et al.*, 1996; Fukuda *et al.*, 1996). In the continued presence of agonists, these acute actions of receptor activation are followed by regulatory processes, such as desensitization and internalization, that modulate the number and functional activity of opioid receptors present in the plasma membrane. The rapid process of receptor internalization, which occurs within several minutes after MOR or δ receptor activation, has been observed in transfected cells (Keith *et al.*, 1996; Trapaidze *et al.*, 1996) and in myenteric neurons *in vivo* (Sternini *et al.*, 1996). Down-regulation, a much slower process of receptor removal from the cells, can be observed after several hours of continuous exposure to agonists (Law *et al.*, 1982). Both the rapid internalization and slower down-regulation processes are associated with the appearance of opioid receptors in intracellular vesicles (Keith *et al.*, 1996; Trapaidze *et al.*, 1996), whereas down-regulation has been associated with the delivery of receptor/ligand complexes to lysosomes (Law *et al.*, 1984). The rapid internalization of opioid receptors seems to use clathrin-coated pits (Keith *et al.*, 1996) and a population of endocytic vesicles similar or identical to those that mediate the endocytic trafficking of constitutively recycling transferrin receptors (Trowbridge and Omary, 1981). This mechanism is similar to that used by several other classes of G protein-coupled receptor (von Zastrow and Kobilka, 1992; Hoxie *et al.*, 1993; Garland *et al.*, 1994; Mantyh *et al.*, 1995).

The regulation of opioid receptors by rapid endocytosis has an interesting feature that may be of particular importance for understanding the effects of opiate drugs. *In vitro* studies indicate that although certain alkaloid agonists stimulate the internalization of MORs to a similar extent as native peptide ligands, morphine activates opioid receptors without causing their internalization (von Zastrow *et al.*, 1994; Arden *et al.*, 1995; Keith *et al.*, 1996). Even in the presence of saturating concentrations of morphine, which cause maximal receptor-mediated inhibition of adenylyl cyclase in stably transfected cells, MORs remained in the plasma membrane and were not rapidly internalized. These observations have been reproduced *in vivo* in myenteric neurons, which express native MORs (Sternini *et al.*, 1996). Based on observations of transfected cells and peripheral neurons examined *in vivo*, these studies suggest the possibility that endocytic regulatory mechanisms may play an important role in distinguishing the physiological actions of individual opiate analgesic drugs in the CNS. The agonist specificity of opioid receptor endocytosis could be of particular importance if it can distinguish the actions of individual opiate drugs on neurons, in which opioid receptors mediate neural signals that underlie the cognitive and behavioral components of opiate tolerance and dependence.

In the current study, we investigated whether CNS neurons expressing MORs are internalized after the peripheral injection of opiate agonists. In addition, using flow cytometry on HEK 293 cells stably transfected with the mouse MOR (293-SF-MOR cells), we assessed a series of clinically important opiate drugs for their ability to trigger internalization. The ability to induce internalization was compared with the ability to inhibit cAMP accumulation to determine whether there was a relationship between these receptor functions.

Materials and Methods

Cell lines. 293-SF-MOR cells have been described previously (Keith *et al.*, 1996). Briefly, HEK 293 cells were stably transfected with the urine MOR (mMOR) cDNA containing the signal FLAG epitope (Guan *et al.*, 1992) at the amino terminus. This cell line expresses ≈1.5×10^6 receptors/cell as assessed by [^{3}H]diprenorphine binding. The binding affinity, ligand selectivity, or receptor-mediated inhibition of adenylyl cyclase was not substantially different from that of the native receptor expressed in Chinese hamster ovary cells (Kaufman *et al.*, 1995).
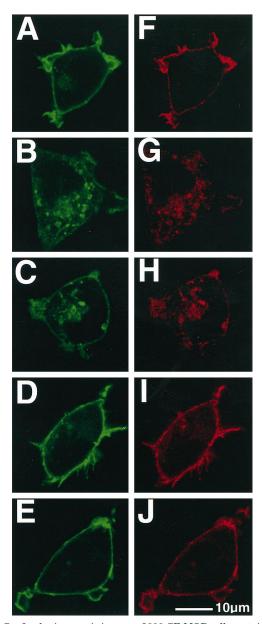
Preparation of antisera. MOR-C12 rabbit polyclonal antiserum was raised against the synthetic peptide LENLEAETAPLP corresponding to the carboxyl-terminal 12 amino acids (387–398) of the rat and mouse MOR (MOR1A). Before use, the antiserum was affinity-purified on an antigen-coupled Sepharose column as described previously (Sternini *et al.*, 1996).

***In vitro* immunofluorescence staining.** After drug treatments of cells on coverslips, cells were washed with ice-cold PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4), fixed with 4% paraformaldehyde/PBS for 15 min on ice, and then permeabilized with 0.2% Tween-20/PBS. After washing, the cells were stained with 2 μg/ml mouse monoclonal Flag M1 (Eastman Kodak, New Haven, CT) and MOR-C12 polyclonal antiserum followed by FITC-conjugated goat anti-mouse IgG and Texas red-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Malvern, PA).

***In vivo* drug treatment and immunofluorescence staining.** Animals were injected intraperitoneally with drug or vehicle (saline) and then killed 30 min later by injection with sodium pentobarbital and transcardial perfusion with 50 ml of PBS at 4° followed by 800 ml of 4% phosphate-buffered paraformaldehyde at 4°. Cold perfusion was performed to eliminate the possibility of the release of endogenous opioid peptides, which might occur as a result of depolarization accompanying death (Maidment *et al.*, 1991). Dissected brains were immersion postfixed for 4 hr in the same fixative at 4° and then cryoprotected in 30% sucrose-PBS. Cryostat sections (40 μm thick) were immunostained free-floating in 10% goat serum, 1% bovine serum albumin, and 0.3% Tween-20, PBS, pH 7.4. Sections were incubated with the primary antibodies (MOR-C12 and mouse monoclonal anti-rat transferrin receptor IgG_{2a}, clone OX 26; Sera-lab, Sussex, England) for 48 hr at 4°, washed three times with PBS, and then incubated with a mixture of Texas Red-conjugated goat anti-mouse IgG_{2a} (1:500) and fluorescein-conjugated goat anti-rabbit IgG (1:300; both from Molecular Probes, Eugene, OR) for 4 hr at room temperature. Sections were washed in PBS and mounted with Prolong (Molecular Probes) for immunofluorescence.

Confocal microscopy. Images for Fig. 1 were acquired and processed on a BioRad (Hercules, CA) MRC-1000 laser scanning confocal microscope using dual excitation and a Zeiss 100× NA1.3 oil-immersion objective. Images for Fig. 2 were acquired on a Zeiss LSM 410 argon laser scanning confocal microscope with a 40× NA1.3 oil-immersion objective and a zoom magnification of 2×. Images for Fig. 3 were acquired on a Leica CLSM confocal microscope using a Leitz 100× NA1.32 oil-immersion objective, and image processing was done with Advanced Visual Systems and Molecular Simulations (Waltham, MA) software running on a Sun workstation.

Flow cytometric analysis. Flag M1 antibody (Eastman Kodak) was labeled directly with FITC to an F/P ratio of 2.95 (Harlow and Lane, 1988). After drug treatment at 37°, cells were chilled to 0° to

cAMP accumulation assay. 293-SF-MOR cells were assayed for inhibition of forskolin-stimulated cAMP accumulation as described previously (Kaufman *et al.*, 1995). Briefly, assays were conducted in polypropylene 96-well plates at 37°. Cells were pretreated with 1 mM 3-isobutyl-1-methylxanthine for 30 min at 37° in Dulbecco's modified Eagle's medium followed by an additional 10-min incubation in 5 μM forskolin and varying amounts of opioid ligands. The samples were then boiled for 3 min and centrifuged at $1000 \times g$, and the supernatants were assayed with a cAMP radioimmunoassay kit (DPC, Los Angeles, CA).

Results

To facilitate the study of MOR internalization in cultured cells, 293-SF-MOR cells were prepared by stably transfecting HEK 293 cells with the mouse MOR containing the signal FLAG epitope at the extracellular amino terminus (Keith *et al.*, 1996). This epitope tag is recognized by the FLAG M1 antibody. To study trafficking of native receptors, we developed an antibody (MOR-C12) that recognizes the intracellular carboxyl terminus of the MOR (Sternini *et al.*, 1996).

When untreated 293-SF-MOR cells were labeled with either antibody, immunoreactivity was localized to the plasma membrane (Fig. 1, A and F). After a 30-min treatment with the MOR-selective enkephalin analog DAMGO (Fig. 1, B and G) or the alkaloid agonist etorphine (Fig. 1, C and H), both antibodies detected reduced receptor immunoreactivity in the plasma membrane and the concomitant appearance of brightly stained puncta representing endocytosed receptors located throughout the cytoplasm in intracellular vesicles (Fig. 1). Similar results were observed in cells treated with endogenously expressed opioid peptides (including Met-enkephalin and β-endorphin 1–31, not shown), confirming that native ligands cause rapid internalization of opioid receptors. Internalization was completely blocked by the opiate antagonist naloxone (Fig. 1, D and I), demonstrating pharmacological selectivity. However, the alkaloid agonist morphine (Fig. 1, E and J) failed to stimulate this rapid endocytic process under the same conditions, as reported previously (Keith *et al.*, 1996), indicating that rapid internalization of opioid receptors is induced by a limited subset of agonist ligands.

To determine whether the agonist-selective internalization of MORs observed in the 293-SF-MOR cells also occurred in the CNS, the MOR-C12 antibody was used to visualize native MORs in rat brain sections by confocal fluorescence microscopy. The validation of this affinity-purified antiserum has been described previously (Sternini *et al.*, 1996). The immunostaining using this antiserum was consistent with that described previously (Arvidsson *et al.*, 1995; Mansour *et al.*, 1995; Ding *et al.*, 1996). In the majority of areas, the antiserum stains fiber processes, although in the cortex, striatum, hippocampus, and habenula cell body, immunostaining is readily detected. In the habenular nuclei of saline-treated animals, MOR immunostaining was localized in the plasma membrane of cell bodies and dendritic processes (Fig. 2A). When rats were treated with an analgesic dose (0.4 mg/kg) of etorphine, the distribution of MORs changed dramatically to a pattern in which receptor immunoreactivity was predominantly located in densely staining vesicle-like structures visualized in the cytoplasm of cell bodies and processes (Fig. 2B). Similarly, an intensely staining network of processes and soma was found in the striatum after etorphine treat-

Fig. 1. Confocal microscopic images of 293-SF-MOR cells costained with both receptor antibodies. The cells were stained with the FLAG M1 antibody recognizing the epitope tag sequence (A–E) or MOR-C12 antiserum recognizing the native receptor protein (F–J). Coverslips coated with 293-SF-MOR cells were treated for 30 min with media (A and F), 100 nM etorphine (B and G), 100 nM DAMGO (C and H), 100 nM etorphine plus 10 μM naloxone (D and I), or 20 μM morphine (E and J). After fixation, the cells were stained with FLAG M1 and MOR-C12, followed by FITC-conjugated goat anti-mouse IgG and Texas Red-conjugated goat anti-rabbit IgG. Images are 0.5-μm optical sections imaged through the center of the cell using dual color laser scanning confocal microscopy. *Green* (A–E), FLAG M1 staining. *Red* (F–J), MOR-C12 staining. *Calibration bar*, 10 μm. In control experiments, no staining with either antibody was observed in untransfected HEK 293 cells, and no cross-reactivity was observed between second antibodies. The micrographs illustrate representative results from five experiments.

arrest further trafficking and stained with 10 μg/ml FITC-labeled FLAG M1 in 50% fetal bovine serum. After washing, the cells were analyzed on a FACScan flow cytometer using LYSYS II software for acquisition and CellQuest 3.0 for analysis (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Live cells were gated by light scatter or exclusion of propidium iodide. The mean fluorescence of 10,000 live cells minus the mean fluorescence of unstained cells was used to calculate percent internalization.
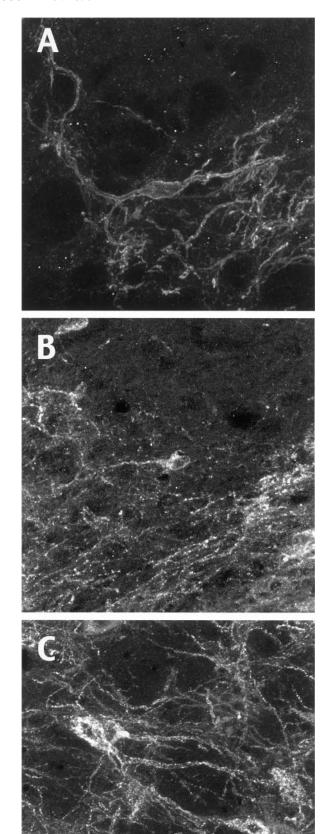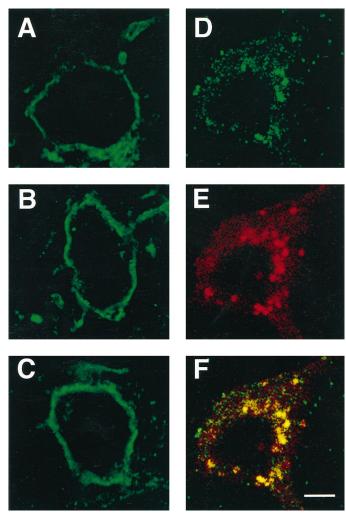
Fig. 3. Confocal microscopic sections through cells from layer II of parietal cortex. Animals were injected with saline (A), 0.4 mg/kg etorphine (D-F), etorphine plus 10 mg/kg naloxone (B), or 40 mg/kg morphine (C). After 30 min, the rats were killed, and sections were prepared for staining. Tissue was processed as described above and then stained with both MOR-C12 rabbit polyclonal serum and mouse monoclonal anti-rat transferrin receptor followed by FITC-labeled goat anti-rabbit Ig (*green*) and Texas Red-conjugated goat anti-mouse IgG2a (*red*). Colocalization of MOR-C12 (D) and transferrin receptor (E) is shown in *yellow* (F). All images represent 0.3-μm optical sections through the center of cell bodies. *Calibration bar*, 5 μm.

ment (Fig. 3C), which is in contrast to the prominent fiber staining in control animals.

The etorphine-induced redistribution of opioid receptors as observed in all brain areas examined, including layer II of the parietal cortex (Fig. 3, A and D). These etorphine-induced changes occurred within 30 min of intraperitoneal injection and were abolished by coadministration of naloxone (Fig. 3B). The receptor redistribution could be observed in animals injected with etorphine at doses as low as 0.01 mg/kg (data
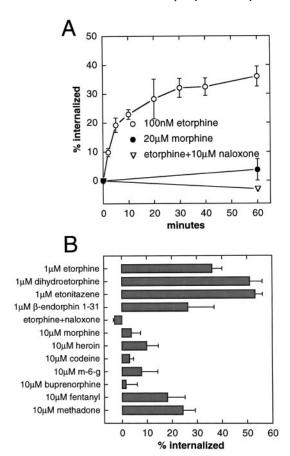
Fig. 2. Confocal three-dimensional projections of MOR immunoreactivity in the rat habenula and striatum. Adult male Sprague-Dawley rats (250–300 g) were injected intraperitoneally with saline (A) or 0.4 mg/kg etorphine (B and C) 30 min before death. Tissue sections (40 μm) were stained with MOR-C12 rabbit polyclonal serum followed by FITC-labeled goat anti-rabbit Ig. Each image is a three-dimension projection of 11 optical confocal microscopic images (0.8 mm thick) at 1-mm intervals throughout the habenular nuclei (A and B) and striatum (C). *Calibration bar*, 10 μm.

not shown), which are well within the typical analgesic dose range for this drug. Receptor internalization also was observed in animals injected with the clinically used etorphine derivative dihydroetorphine (not shown), which has similar analgesic potency as etorphine but is a more μ-selective agonist. In contrast to etorphine, but in agreement with the results obtained in cultured cells, no receptor internalization was observed in rats treated with morphine, even at high doses (40 mg/kg) in excess of those required to cause analgesic effects (Fig. 3C). Furthermore, no internalization of opioid receptors was detected in animals treated with 200 mg/kg morphine, a near-lethal dose (data not shown).

In rare cortical neurons expressing both MORs and transferrin receptors in sufficient quantities to facilitate localization of both receptors in the same cells, extensive colocalization in the same intracellular vesicles was observed by dual-label confocal microscopy in etorphine-injected animals (Fig. 3, D–F). This colocalization with transferrin receptors, which mark recycling (early) endosomes, confirmed that opioid receptors are internalized via similar endocytic membranes in CNS neurons, as observed previously in transfected HEK 293 cells (Keith *et al.*, 1996). We observed this colocalization in other cell types *in vitro*, including transfected neuroblastoma (Neuro2A) and lymphoid (Raji lymphoma) cells (S. R. Murray and M. von Zastrow, unpublished observations), suggesting that opioid receptors are internalized in CNS neurons *in vivo* by a highly conserved endocytic pathway that operates in a wide variety of cell types.

Internalization of MORs next was measured quantitatively using the 293-SF-MOR cell *in vitro* system, which allowed the effects of individual ligands to be examined in the absence of ligand-specific differences in bioavailability and pharmacokinetics that are intrinsic to *in vivo* studies. Flow cytometry was used to quantify the internalization of epitope-tagged MORs in intact cells detected by immunostaining with the FLAG M1 antibody. Consistent with quantitative experiments reported previously (Keith *et al.*, 1996), etorphine triggered a rapid, naloxone-reversible loss of opioid receptors from the plasma membrane (Fig. 4A). The alkaloid agonists dihydroetorphine and etonitazene also were observed to strongly stimulate the rapid internalization of MORs (Fig. 4B). The amount of receptor internalization caused by these alkaloid agonists was similar to that caused by natively expressed and synthetic derivatives of opioid peptides, such as β-endorphin 1–31 (Fig. 4B) and DAMGO (Fig. 5B). Although this survey of agonists was conducted primarily using saturating concentrations of ligands, further studies indicated that drug-induced internalization of opioid receptors also was induced by much lower drug concentrations, which are in the same range as plasma concentrations produced by clinically relevant analgesic doses of these drugs (see below).

Morphine failed to cause any detectable rapid internalization of MORs under the same conditions (Fig. 4A). Several alkaloid agonists were identified that, like morphine, caused little or no internalization of MORs even when administered at saturating concentrations (Fig. 4B); these agonists included codeine, heroin, buprenorphine, and morphine-6-glucuronide (an active metabolite of morphine). The alkaloid agonists fentanyl and methadone caused partial internalization and only when present at high concentrations (≥10 μM). However, when tested at lower concentrations (1–50 nM),
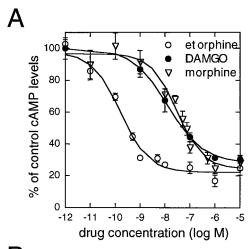

Fig. 4. Flow cytometric analysis of MOR internalization. A, 293-SF-MOR cells were treated for the times shown with 100 nM etorphine, 20 μM morphine, or 100 nM etorphine plus 10 μM naloxone. B, 293-SF-MOR cells were treated with the drugs shown for 60 min. After drug treatment, the cells were chilled to 0° to arrest further trafficking and stained with FITC-labeled FLAG M1 monoclonal antibody. The cells then were analyzed on a FACScan flow cytometer, and the mean fluorescence of 10,000 live cells minus the mean fluorescence of unstained cells was used to calculate percent internalization. Values are the mean ± standard error of three to eight separate experiments. *m-6-g*, morphine-6-glucuronide.

which activate receptor signaling and are more comparable to plasma concentrations produced by clinically relevant analgesic doses, fentanyl and methadone, like morphine, failed to induce detectable internalization of MORs (not shown).

The *in vitro* assay system was used further to compare the dose dependence of three representative agonists (etorphine, DAMGO, and morphine) for activating receptor signaling (Fig. 5A) and promoting receptor internalization (Fig. 5B). All three agonists caused similar maximal levels of inhibition of adenylyl cyclase, although morphine and DAMGO were ≈100 times less potent than etorphine (Fig. 5A). DAMGO also was ≈100 times less potent than etorphine for promoting receptor internalization but caused the same maximal level of internalization. In contrast, morphine was essentially incapable of inducing any internalization, even at 500 μM, ≈10,000 times the concentration required for receptor signaling in these cells (Fig. 5, A and B).

The concentrations required to achieve 50% of maximal response (EC_{50}) were calculated for both internalization and inhibition of cAMP accumulation (Table 1). When the ratios of these EC_{50} values are compared, etorphine and DAMGO have comparable ratios (Table 1), even though they differ ≈100-fold in absolute potency. A ≈40-fold higher concentra-
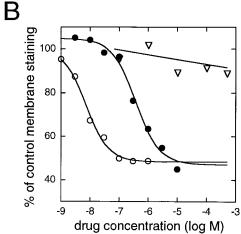
A

B

Fig. 5. Comparison of potencies for inhibition of forskolin-stimulated cAMP accumulation and receptor internalization. A, 293-SF-MOR cells were assayed for inhibition of cAMP accumulation using etorphine, DAMGO, and morphine. Results are from a representative experiment assayed in triplicate. B, 293-SF-MOR cells were assayed for internalization using flow cytometry to measure surface receptor staining as described in the legend to Fig. 3. Results are from a representative experiment with each point calculated from the mean of 10,000 cells.

TABLE 1
Comparison of potencies for endocytosis and inhibition of cAMP accumulation

Data from Fig. 4, A and B, were fitted to a four-parameter logistic function to yield the EC_{50} values shown here, and the ratio of these two values is given.

Drug	EC_{50}		
	For endocytosis	For cyclase	Ratio
Etorphine	7.28 ± 1.25 nM	0.16 ± 1.17 nM	45.5
DAMGO	547 ± 69.0 nM	13.0 ± 2.6 nM	42.0
Morphine	>1 mM	30.2 ± 9.0 nM	>30,000

tion of either agonist is required to achieve the EC_{50} value for receptor internalization than is required to achieve the EC_{50} value for inhibition of cAMP accumulation. In contrast, the internalization/signaling potency ratio for morphine is at least 30,000; thus morphine, which is similar in potency to DAMGO for receptor signaling, is unable to trigger receptor endocytosis, even at 4000 times the EC_{50} value for inhibition of cAMP accumulation. This result suggests that the relative efficacy of individual agonists for promoting receptor internalization is not correlated with signaling potency or efficacy

but seems to represent a distinct functional property that distinguishes individual alkaloid analgesic drugs.

Discussion

We have shown that agonist-induced MOR internalization can be observed not only in transfected cell lines (Arden *et al.*, 1995; Keith *et al.*, 1996) and gut neurons (Sternini *et al.*, 1996) but also in CNS neurons after intraperitoneal administration of etorphine. Colocalization of the internalized receptor with the transferrin receptor demonstrates that the endocytic pathway used by the MOR in cortical neurons is similar to that observed *in vitro* (Keith *et al.*, 1996). Many G protein-coupled receptors are internalized via this conserved endocytic pathway, and our results confirm that similar trafficking pathways are used by MORs in brain neurons. Although we focused on the effects of opiate alkaloid drugs in the *in vivo* studies, we found that a variety of peptide agonists (including endogenously produced opioid peptides) trigger MOR internalization in cultured cells. This strongly suggests that CNS opioid receptors also would be endocytosed after activation by endogenously released peptide ligands. In support of this hypothesis, structurally similar neurokinin receptors, which are endocytosed in cultured cells by a similar ligand-dependent endocytic pathway as opioid receptors (Hoxie *et al.*, 1993), also exhibit rapid endocytosis *in vivo* after activation by endogenously released substance P (Mantyh *et al.*, 1995).

Interestingly, in contrast to opioid peptides and certain alkaloid agonists (etorphine, dihydroetorphine, etonitazene), many clinically used opiate agonists either do not induce receptor internalization at all (morphine and buprenorphine) or do so only at high concentrations (methadone and fentanyl). By comparing the potencies of three agonists for internalization with the potencies for inhibition of cAMP accumulation, we attempted to determine whether these differences among agonists to stimulate internalization corresponded to their potencies for receptor signaling. Such a correspondence was observed for etorphine and DAMGO, both of which stimulated receptor internalization with potencies that were ≈40-fold lower than their potencies for activating receptor signaling (Table 1). In contrast, morphine and DAMGO exhibited similar potencies for cyclase inhibition yet differed enormously (>1000-fold) in their abilities to promote internalization. Thus, the markedly different effects of morphine on receptor internalization could not be accounted for by differences in agonist potency.

In the case of the β_2-adrenergic receptor, internalization is induced by both full and partial agonists (Morrison *et al.*, 1996). In contrast, morphine and buprenorphine have been reported to act as a partial agonists in some assays (Mello and Mendelson, 1980; Sim *et al.*, 1996), but both drugs completely failed to stimulate the internalization of opioid receptors in the 293-SF-MOR cells, even at extremely high concentrations. Although morphine caused maximal inhibition of cAMP accumulation in this study (Fig. 4A), this may not be an optimal assay for the detection of partial agonists because only partial receptor occupancy may be required for maximal inhibition. Because of the high expression level in this cell line (≈1.5×10^6 receptors/cell), a large number of receptors must internalize to be detected in our assay; ≈75,000 internalized receptors represent only 5% of the total. A compari-

son of Fig. 4, A and B, shows that for etorphine and DAMGO, there is a small amount of internalization occurring at concentrations that are nearly maximal for signaling. It is possible that internalization is occurring at lower concentrations but that it is too small a fraction to be detected by our assay.

In future studies, it will be interesting to examine the relationship between the ability of individual agonists to trigger endocytosis with their intrinsic efficacies (rather than potencies) for receptor activation. The weak ability of fentanyl to stimulate opioid receptor endocytosis, despite its reportedly high agonist potency and efficacy (Duttaroy and Yoburn, 1995), suggests that the ability of an agonist to stimulate endocytosis of opioid receptors is not a function of its intrinsic efficacy for activation of receptor-mediated signaling. Therefore, it seems that endocytosis is not related directly to either potency or intrinsic efficacy, and it is possible that receptor internalization may identify a new functional property of opioid agonists, in addition to potency and pharmacokinetic parameters, that may be important in distinguishing the physiological actions of individual analgesics.

There seem to be several physiological roles served by ligand-dependent endocytosis of G protein-coupled receptors, and these roles can differ for various receptors. In addition to rapidly removing receptors from the cell surface, endocytosis seems to play a longer term role in down-regulating receptor signaling by delivering receptors to lysosomes for degradation. For example, thrombin receptors are endocytosed after activation via clathrin coated pits and then seem to undergo degradation in lysosomes (Hoxie *et al.*, 1993). Resensitization of cellular responsiveness to thrombin requires new protein synthesis (Hoxie *et al.*, 1993) or the delivery of uncleaved receptors to the plasma membrane from intracellular reserves (Hein *et al.*, 1994). Internalization of other receptors clearly is not required for desensitization of signaling (Lefkowitz *et al.*, 1993; Garland *et al.*, 1996). Instead, rapid internalization of these receptors may play a role in mediating functional recovery, or resensitization, of receptor-mediated signaling by promoting receptor/ligand dissociation (Grady *et al.*, 1995) and dephosphorylation of receptors that have been desensitized by regulatory phosphorylation (Yu *et al.*, 1993; Pippig *et al.*, 1995). Yet another role for internalization is suggested by studies of muscarinic acetylcholine receptors, in which rapid endocytosis of receptors seems to delay the functional resensitization of receptor signaling (Bogatkewitsch *et al.*, 1996).

Although opiates remain among the most effective analgesics known, the clinical use of these drugs is limited by their potential to cause tolerance, dependence, and addiction. Each opiate drug has a unique profile for analgesic efficacy and capacity to induce these side effects. These different profiles may be due to different activities at the MOR itself as well as other variables, such as differences in pharmacokinetics, selective accessibility to anatomically distinct MORs, and differences in the relative pharmacological selectivity of individual drugs for μ, δ, and κ receptors. A number of regulatory processes have been proposed to be involved in these clinically undesirable side effects, and chronic administration of morphine is associated with a wide variety of physiological adaptations, including changes in the abundance of mRNAs encoding diverse molecules that function in other signaling systems (Nestler *et al.*, 1993).

Although etonitazene and etorphine are not used clinically,

dihydroetorphine is used as an analgesic in China and reportedly causes less physiological dependence than morphine in both clinical (Qin *et al.*, 1994) and animal (Huang *et al.*, 1994; Tokuyama *et al.*, 1994) studies. It is important to not oversimplify the complex biology associated with the acute and chronic effects of opiates; however, receptor internalization may play a role in the pharmacological actions of this diverse class of drugs and thus prove to be a useful parameter in the design of better therapeutic opiates.

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