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Morphine Activates Opioid Receptors without Causing Their Rapid Internalization*

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We have examined the endocytic trafficking of epitope-tagged δ and μ opioid receptors expressed in human embryonic kidney (HEK) 293 cells. These receptors are activated by peptide agonists (enkephalins) as well as by the alkaloid agonist drugs etorphine and morphine. Enkephalins and etorphine cause opioid receptors to internalize rapidly ($t_{1/2}$ ~ 6 min) by a mechanism similar to that utilized by a number of other classes of receptor, as indicated by localization of internalized opioid receptors in transferrin-containing endosomes and inhibition of opioid receptor internalization by hypertonic media. Remarkably, morphine does not stimulate the rapid internalization of either δ or μ opioid receptors, even at high concentrations that strongly inhibit adenylyl cyclase. These data indicate that agonist ligands, which have similar effects on receptor-mediated signaling, can have dramatically different effects on the intracellular trafficking of a G protein-coupled receptor.

Opioid receptors constitute a class of G protein-coupled receptors that mediate the effects of endogenously produced opioid peptides in the central and peripheral nervous systems. An interesting feature of these receptors is that they are activated both by native peptides and by structurally distinct non-peptide alkaloid ligands (1). Following activation, opioid receptors are regulated by multiple mechanisms, which modulate the functional plasticity of the endogenous opioid system and contribute to the development of opiate tolerance and dependence (2–5).

Previous studies have described two distinguishable processes of opioid receptor regulation, termed *desensitization* and *down-regulation* (3, 4, 6–9). Both desensitization and down-regulation of opioid receptors are stimulated by peptide and alkaloid agonists (4, 6). However, individual agonists may have substantially different effects on the rapid regulation of opioid receptors (10–12). We have observed that opioid receptors are regulated by a process of rapid internalization that exhibits a remarkable degree of agonist specificity not observed previously in studies of the intracellular trafficking of other receptors.

EXPERIMENTAL PROCEDURES

Construction and Expression of Epitope-tagged δ and μ Opioid Receptors—cDNAs encoding murine δ (13) and μ (14) opioid receptors were epitope-tagged in the amino-terminal extracellular domain utilizing a shuttle vector containing a signal-FLAG cassette (kindly provided by Drs. Jeff Reagan and Brian Kobilka) (15) and subcloned into pcDNA3 (Invitrogen) for transfection. The structure of each mutant cDNA was confirmed by dideoxy sequencing (Sequenase, U. S. Biochemical Corp.).

Human embryonic kidney (HEK)[1] 293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (University of California San Francisco Cell Culture Facility) and transfected using calcium phosphate coprecipitation (16). Stably transfected cells were isolated following neomycin selection (Geneticin, Life Technologies, Inc.) as described previously (17), and saturation binding of [^{3}H]diprenorphine (see below) was used to estimate the level of receptor expression in individual clones.

Radioligand Binding Assays—Approximately 200,000 cells (determined by counting in a hemocytometer) were placed in polyvinyl chloride microtiter plates (Becton Dickinson) with 0.3–180 nM [^{3}H]diprenorphine (Amersham Corp.) in a total volume of 100 μl as described (11). After incubation on ice for 60 min, the mixture was harvested quickly in a Brandel M24RS harvester using GT100 GF/B glass filters and washed with ice-cold phosphate-buffered saline. After drying, the filters were counted in a Beckman LS1600 scintillation counter using CytoScint (ICN). Nonspecific binding, defined by performing radioligand binding in the presence of 10 μM diprenorphine, represented less than 15% of total binding in all experiments.

cAMP Accumulation Assays—293SFDOR and 293SFMOR cells were incubated with 1 mM isobutyl methylxanthine (Sigma) for 30 min at 37 °C in Dulbecco's modified Eagle's medium followed by a further 5-min incubation in 5 μM forskolin (Sigma) and varying amounts of opioid ligands (13). The samples were assayed with a cAMP radioimmunoassay kit (DPC, Inc., Los Angeles, CA).

Immunocytochemical Staining and Fluorescence Microscopy—Cells grown on glass coverslips were fixed using 4% formaldehyde in phosphate-buffered saline and stained as described (18). For colocalization of opioid receptors with endocytosed transferrin, 293-SFDOR or 293-SFMOR cells were incubated with 25 nM transferrin-Texas Red (Molecular Probes) in serum-free medium for 30 min prior to treatment with various ligands and fixation. Subsequently, receptor localization was visualized by staining with M1 antibody followed by goat anti-mouse FITC conjugate. Localization of both transferrin and opioid receptor immunoreactivity in the same specimens was accomplished using dual color confocal microscopy (Bio-Rad MRC-1000) using dual excitation (to minimize bleed-through) and a Zeiss 100× NA1.3 objective. Imaging of single-labeled specimens confirmed negligible bleed-through between channels.

Immunofluorescence Flow Cytometry—Epitope-tagged SF-DOR and SF-MOR cells were treated in Dulbecco's modified Eagle's medium alone or with opioid ligand for the times indicated. Cells were then chilled and stained on ice with anti-FLAG M1 monoclonal (10 μg/ml) in 50% fetal bovine serum/phosphate-buffered saline, then with FITC conjugated goat anti-mouse IgG (10 μg/ml), and analyzed on a FACScan

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[1] The abbreviations used are: HEK, human embryonic kidney; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DAMGO, [D-Ala^2,*N-methyl*-Phe^4,Gly^5-ol]enkephalin; FITC, fluorescein isothiocyanate.

TABLE I
Radioligand binding and inhibition of adenylyl cyclase by epitope-tagged opioid receptors

A	293-SF-DOR	293-SF-MOR
K_d of diprenorphine (nM)	8.49	21.9
B_{max} (picomoles of [^{3}H]DPN bound per 10^6 cells)	0.246	2.50
Number of receptors/cell	148,000	1,500,000

B Drug[a]	IC_{50} for cyclase	
	293-SF-DOR	293-SF-MOR
DADLE	8.72 nM	7.70 nM
Etorphine	1.76 nM	0.37 nM
Morphine	9.2 μM	16.47 nM
DPDPE	4.22 nM	ND[b]
DAMGO	ND	4.81 nM

[a] DPDPE, [D-penicillamine 2,5]enkephalin.
[b] ND, not determined.

flow cytometer (Becton Dickinson Immunocytometry Systems, Inc.). Live cells were gated by light scatter or exclusion of propidium iodide, and 10,000 cells were acquired for each time point. Mean fluorescence of all live cells, minus mean fluorescence of cells stained only with FITC-conjugated second antibody, was used for calculations (19). Kinetic parameters were estimated by fitting the agonist-induced reduction in surface receptor fluorescence to a single exponential function utilizing a linear least-squares algorithm. Each time point was collected in triplicate in each experiment, and kinetic parameters were calculated by averaging the results between five separate experiments.

RESULTS AND DISCUSSION

An Experimental System for Studying Opioid Receptor Cell Biology—Transiently and stably transfected HEK293 cells were used as a model system to study the functional properties and intracellular trafficking of cloned opioid receptors. Murine δ and μ opioid receptors were examined, because both receptors bind and are activated by opioid peptides (enkephalins) as well as by the alkaloid agonists morphine and etorphine. In contrast, κ and ORL-1 receptors exhibit negligible affinity for morphine and bind enkephalins with extremely low affinity (20, 21). Stably transfected HEK293 cells expressing tagged δ or μ receptors exhibited readily detectable specific binding of [^{3}H]diprenorphine, while untransfected cells exhibited no detectable specific binding of this radioligand. Expression levels in transfected HEK293 cells typically ranged from 100,000 to 2,000,000 receptors/cell, as determined by saturation binding analysis. Clones of stably transfected cells expressing δ (293SFDOR) or μ (293SFMOR) receptors with B_{max} values of 148,000 and 1,500,000 receptors/cell, respectively (Table IA), were selected for further characterization of the functional properties of epitope-tagged opioid receptors over this range of expression levels.

Etorphine, morphine, and enkephalin analogs (but not the opiate antagonist naloxone) caused opioid receptor-mediated inhibition of adenylyl cyclase in 293SFDOR and 293SFMOR cells, while no inhibition was observed in nontransfected cells. (Table IB). The different potency of morphine at the δ, compared with the μ, receptor is consistent with the pharmacology of the wild-type opioid receptors (13, 14, 20, 22).

Rapid Internalization of Opioid Receptors Stimulated by Opioid Peptide and the Alkaloid Agonist Etorphine but Not by the Agonist Alkaloid Morphine—Epitope-tagged δ receptors were localized primarily in the plasma membrane of untreated cells (Fig. 1*a*). Within 10 min after the addition of the enkephalin analog DADLE, δ receptors redistributed from the plasma membrane to a population of intracellular vesicles. Surprisingly, in contrast to the effects of these agonists on promoting

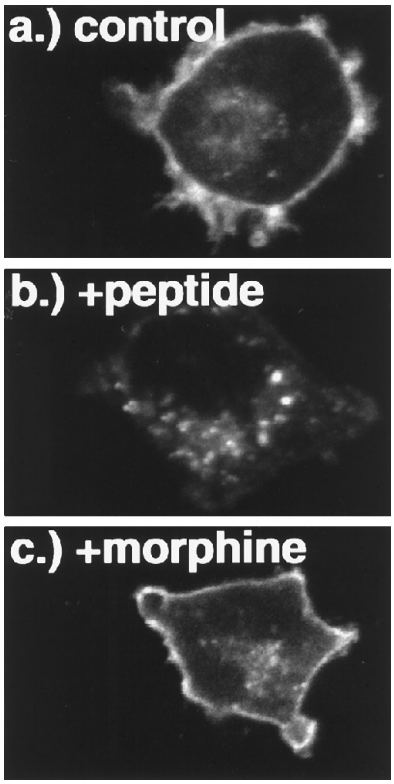

FIG. 1. **Immunofluorescence localization of epitope-tagged δ opioid receptors.** 293SFDOR cells were incubated in the absence of ligand (*a*), presence of 100 nM DADLE (*b*), or presence of 10 μM morphine (*c*) for 10 min prior to fixation, permeabilization, and immunofluorescence staining of receptors using monoclonal antibody recognizing the epitope tag sequence engineered into the receptor protein. Specimens were imaged by confocal fluorescence microscopy, using a plane of focus adjusted 3–5 μM above the surface of the coverslip (to produce a cross-section through the center of the cell). Bright staining of the plasma membrane of cells is apparent in *a* and *c*, while prominent staining of intracellular vesicles distinct from the plasma membrane (which appear as bright punctate accumulations of staining present within the cytoplasm of cells) is apparent in *b*. All images were collected using the same instrument settings and are displayed at the same magnification.

the rapid internalization of opioid receptors, the alkaloid agonist morphine failed to induce detectable redistribution of δ receptors from the plasma membrane (Fig. 1*c*).

Because morphine binds to the δ receptor with relatively low affinity compared with etorphine and enkephalins, and morphine has been suggested to have partial agonist activity at the δ receptor (6, 20), we questioned whether the failure of morphine to stimulate rapid internalization of opioid receptors could be explained solely by these pharmacological differences. Morphine binds to the μ receptor with equal or higher affinity than etorphine or enkephalin (14, 20, 23), and morphine has similar intrinsic efficacy as the other agonist ligands tested (24). Epitope-tagged μ opioid receptors were localized in the plasma membrane of untreated 293SFMOR cells (Fig. 2*a*) and internalized rapidly following activation by the enkephalin analog DAMGO or alkaloid agonist etorphine (Fig. 2, *b* and *c*, respectively). However, as observed with δ receptors, no internalization of μ receptors was observed under the same conditions in morphine-treated cells (Fig. 2*d*). Morphine failed to cause detectable rapid internalization of μ receptors at all concentrations tested even up to 100 μM, a dose nearly 10,000-fold higher than required to cause maximal inhibition of adenylyl cyclase (Table I) and 100,000-fold higher than the K_d for binding to the μ receptor (20).

Fluorescence flow cytometry was used to measure ligand-de-

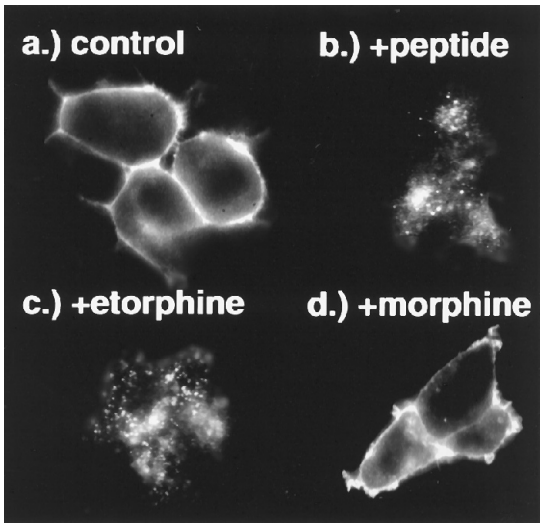

FIG. 2. **Immunofluorescence localization of epitope-tagged μ opioid receptors.** 293SFMOR cells were incubated in the absence of ligand (*a*) or presence of 500 nM DAMGO (*b*), 500 nM etorphine (*c*), or 10 μM morphine (*d*) for 10 min prior to fixation and immunofluorescence staining of receptors using monoclonal antibody recognizing the epitope tag sequence. Specimens were examined by epifluorescence microscopy using a plane of focus centered 3–5 μM above the surface of the coverslip. Bright staining of the plasma membrane is apparent in *a* and *d*, while prominent staining of intracellular vesicles (which appear as punctate accumulations visualized within the cytoplasm) is observed in *b* and *c*. All photomicrographs were collected using the same magnification and camera settings.

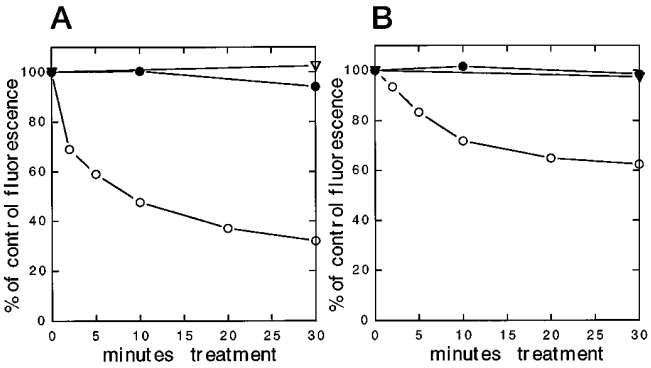

FIG. 3. **Flow cytometric analysis of opioid receptor internalization.** The relative number of opioid receptors present in the plasma membrane of 293SFDOR (*A*) or 293SFMOR (*B*) cells was determined using flow cytometric analysis of surface-stained cells after treatment with 500 nM etorphine (*open circles*), 100 μM (δ) or 10 μM (μ) morphine (*closed circles*), or etorphine plus 10 μM of the opiate antagonist naloxone (*open triangles*) for the indicated time periods, as described under "Experimental Procedures."

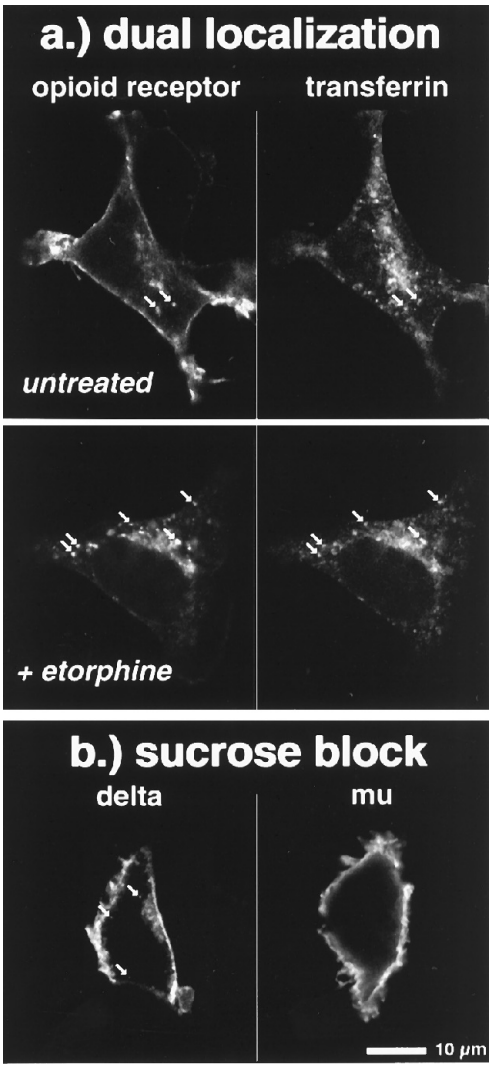

FIG. 4. **Colocalization of internalized opioid receptors in transferrin-containing endosomes and inhibition of receptor internalization by mild hypertonicity.** *a*, in untreated 293SFDOR cells, δ opioid receptors are localized predominantly in the plasma membrane (*top left*) and are not observed in endosomes containing transferrin-Texas Red (*top right*). In DADLE-treated cells, opioid receptors are observed in numerous intracellular vesicles (*bottom left*), many of which can be identified as endosomes by colocalization with transferrin (*bottom right*). *Bar*, 10 μm. *b*, 293SFDOR (*left*) or 293SFMOR (*right*) cells were preincubated in culture medium supplemented with 0.35 M sucrose for 30 min prior to addition of 25 nM transferrin-Texas Red, then adding 500 nM etorphine to activate opioid receptors for an additional 15 min. Cells were then fixed and processed for dual color confocal microscopy. Visualization of transferrin fluorescence confirmed that sucrose-containing medium completely blocked clathrin-mediated internalization of transferrin receptors as expected (not shown). The localization of δ (*left*) and μ (*right*) opioid receptor immunoreactivity in in the plasma membrane indicates that etorphine-induced internalization of the opioid receptors was also strongly inhibited under these conditions. Rare intracellular vesicles containing opioid receptors were observed in some cells (*arrows*).

pendent changes in the number of receptors present in the plasma membrane of 293SFDOR and 293SFMOR cells. Etorphine caused rapid internalization of opioid receptors as indicated by a ligand-induced reduction in the fluorescence intensity of antibody-labeled cells. Both δ and μ receptors were internalized with similar kinetics (Fig. 3, *A* and *B*, respectively, *open circles*), and the agonist-induced internalization of opioid receptors was blocked by the opiate antagonist naloxone (*open triangles*). The $t_{1/2}$ for opioid receptor internalization calculated by fitting these data to a first order process was 5.9 ± 2.2 min in 293-SFDOR cells and 6.0 ± 1.7 min in 293-SFMOR cells (mean ± S.E., n = 5). No internalization of either δ or μ receptors was observed in the presence of saturating concentrations of morphine (*closed circles*).

Characterization of the Intracellular Pathway Mediating Rapid Internalization of Opioid Receptors—To determine whether opioid receptors activated by etorphine or enkephalin are endocytosed via a similar pathway as certain other G-protein-coupled receptors (17, 25, 26), the subcellular localization of opioid receptors was compared with that of endocytosed transferrin by dual label fluorescence microscopy. In cells treated with etorphine (or DADLE, not shown), dramatically reduced receptor staining was observed in the plasma membrane, and opioid receptors were observed in numerous intra-

cellular vesicles, many of which could be identified as endosomes by colocalization with endocytosed transferrin (Fig. 4*a*, *lower panels*). Ligand-dependent internalization of receptors was examined in culture medium supplemented with 0.35 M sucrose to produce mildly hypertonic conditions, which inhibits clathrin-mediated endocytosis (27). Internalization of δ and μ opioid receptors was strongly inhibited under these conditions (Fig. 4*b*, *left* and *right panels*, respectively; compare with *lower left panel* in Fig. 4*a* for representative internalization in normal medium), further suggesting that the mechanism of opioid receptor endocytosis is similar to that mediating rapid internalization of certain other receptors. To compare the process of rapid internalization described in this study with the previously described process of receptor down-regulation, ligand-induced down-regulation of opioid receptors was measured by a saturation radioligand binding assay using [^{3}H]diprenorphine in cell lysates (3). Essentially no down-regulation of opioid receptors (<5% reduction in specific binding of diprenorphine) was observed in 293SFDOR or 293SFMOR cells treated with 500 nM etorphine for 30 min, while ≥40% of opioid receptors are internalized with a $t_{1/2}$ of ~6 min under the same conditions (Fig. 3). In chronically treated cells (18 h), however, both etorphine and morphine caused significant down-regulation of both δ and μ receptors (41 ± 14% and 25 ± 11%, respectively, $n = 4$).

In conclusion, we have shown for the first time that that both δ and μ opioid receptors are internalized within several minutes following activation by certain agonists, including native peptide ligands (enkephalins) and the alkaloid agonist drug etorphine, while morphine is an agonist ligand which has the remarkable ability to strongly activate opioid receptor signaling without stimulating the process of rapid internalization. Morphine has been observed to be a full agonist of μ receptors in membranes prepared from cultured neuroblastoma cells (24), although there is not general consensus that morphine is a full μ agonist in all studies (28). Other G protein-coupled receptors have been observed to internalize following activation by both full and partial agonists (29). The present findings suggest that different agonists can induce different activated states of receptor that similarly activate heterotrimeric G proteins but differ significantly in their intracellular trafficking. This possibility is consistent with mutational studies indicating, conversely, that subtle changes in opioid receptor structure differentially affect the binding of individual agonist ligands (30–33). The different effects of agonists, which are closely similar in structure (*i.e.* morphine and etorphine), suggest that conformational differences required to mediate the observed differences in rapid internalization might be quite subtle.

The present data are of general significance to the cell biology of G protein-coupled receptors and and may have important implications for understanding molecular mechanisms of opiate drug action and addiction.

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