δ and κ Opioid Receptors Are Differentially Regulated by Dynamin-dependent Endocytosis When Activated by the Same Alkaloid Agonist*

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Many alkaloid drugs used as analgesics activate multiple opioid receptors. Mechanisms that distinguish the actions of these drugs on the regulation of individual μ-, δ-, and κ receptors are not understood. We have observed that individual cloned opioid receptors differ significantly in their regulation by rapid endocytosis in the presence of alkaloid drug etorphine, a potent agonist of μ-, δ-, and κ opioid receptors. Internalization of epitope-tagged δ opioid receptors from the plasma membrane is detectable within 10 min in the presence of etorphine. In contrast, κ receptors expressed in the same cells remain in the plasma membrane and are not internalized for ≥60 min, even when cells are exposed to saturating concentrations of etorphine. The rapid internalization of δ receptors is specifically inhibited in cells expressing K44E mutant dynamin I, suggesting that type-specific internalization of opioid receptors is mediated by clathrin-coated pits. Examination of a series of chimeric mutant ω/δ receptors indicates that at least two receptor domains, including the highly divergent carboxyl-terminal cytoplasmic tail, determine the type specificity of this endocytic mechanism. We conclude that structurally homologous opioid receptors are differentially sorted by clathrin-mediated endocytosis following activation by the same agonist ligand. These studies identify a fundamental mechanism of receptor regulation mediating type-specific effects of analgesic drugs that activate more than one type of opioid receptor.

Structurally homologous μ-, δ-, and κ-type opioid receptors are conserved in mammals and signal via similar heterotrimERIC G proteins (1–3). These receptors are activated both by endogenously expressed opioid peptides and by structurally distinct alkaloid agonists, which are clinically important analgesics and drugs of abuse (4). Although many alkaloid analgesics activate more than one type of opioid receptor at clinically relevant concentrations, significant differences in the regulation of δ, μ, and κ receptor-mediated processes by individual agonists have been observed (5–7). Molecular mechanisms underlying this functional specificity, even in the presence of relatively nonselective opiate drugs, are not understood. We have identified a mechanism of receptor regulation that mediates type-specific regulation of opioid receptors following activation by the same alkaloid agonist.

Previous studies have identified multiple processes that regulate opioid receptors. Ligand-dependent phosphorylation of cytoplasmic residues is thought to modulate the functional activity of opioid receptors within minutes after activation (8, 9). Agonist-induced down-regulation, which is associated with a gradual translocation of receptors from the plasma membrane to lysosomes, occurs over a more prolonged time course (10–12). A distinguishable rapid endocytic process, which occurs within several minutes after receptor activation and is not associated with degradation of receptors, has been shown recently to regulate δ and μ opioid receptors (13–15). Individual analgesic drugs differ significantly in their ability to stimulate this process of rapid internalization (13, 14), suggesting that opiate drugs can have agonist-selective effects on the physiological regulation of receptors (16).

Here we show that, in addition to the agonist selectivity of this endocytic mechanism, structurally homologous opioid receptors are differentially internalized in a type-specific manner following activation by the same alkaloid agonist. We have established this by focusing on the effects of etorphine, a potent and relatively nonselective opiate agonist (17), on the membrane trafficking of epitope-tagged mutant δ and κ opioid receptors expressed in transfected cells. These observations identify a fundamental mechanism of receptor regulation that mediates type-specific effects of relatively nonselective opiate drugs that activate multiple types of opioid receptor in vivo.

EXPERIMENTAL PROCEDURES
Receptor Mutagenesis and Expression Constructs—Construction of FLAG-tagged δ opioid receptors was reported previously (13). The cloned murine κ opioid receptor (5) was provided by Dr. Terry Reisine (University of Pennsylvania) and was tagged in the amino-terminal extracellular domain in the same manner. For experiments involving co-expression of δ and κ receptors in the same cells, the murine δ opioid receptor was tagged in the amino-terminal extracellular domain with the HA1 epitope (YPYDVPDYA), exactly as described previously in studies of adrenergic receptors (18, 19). Chimeric mutant δκ opioid receptors were constructed as follows. Exchanges in the carboxy-terminal cytoplasmic domain were accomplished by engineering an EcoRI site immediately distal to a conserved cysteine residue in the δ and κ receptors (residues 328 and 340, respectively) using synthetic oligonucleotides and polymerase chain reaction (Vent polymerase, New England Biolabs) under standard conditions. The κ and δ sequences distal to this cysteine residue were amplified by polymerase chain reaction and fused into this site to exchange the divergent portions of this domain, creating Crs. 1 and 2. Cr. 3 was constructed by exchange

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between Crs. 1 and 2 using a conserved BglII site located in the third extracellular domain of the δ and κ receptors. Crs. 4 and 5 were constructed from the wild-type δ and κ receptors using this BglII site, and Cr. 6 was constructed from the δ receptor and Cr. 2 using the same approach. Mutant sequences produced at each step were verified by dideoxynucleotide sequencing (Sequenase, U. S. Biochemical Corp.). Homology and completed cDNAs were cloned into pcDNA3 (Invitrogen) for expression in mammalian cells. cDNAs encoding HA-tagged wild-type and K44E mutant dynamin I (20) were provided by Dr. Richard Vallee (Worcester Institute, Shrewsbury, MA) and were cloned into pcDNA3 for expression.

**Cell Culture and Transfection**—Human 293 cells (American Type Culture Collection) were maintained and passaged in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (University of California at San Francisco Cell Culture Facility). Cells were transfected by calcium phosphate precipitation. Clones of stably transfected cells were selected using 0.5 mg/ml geneticin (Life Technologies, Inc.). Relative levels of receptor expression were compared between cell clones by fluorescence flow cytometry, as described previously (13). Experiments using transiently transfected cells were conducted 48–72 h after transfection. Experiments with stably transfected cells were conducted on individual clones uniformly expressing epitope-tagged receptors, as determined by immunofluorescence microscopy and flow cytometry.

**Measurement of Receptor-mediated Inhibition of Adenylyl Cyclase**—The functional integrity of mutant opioid receptors was tested by examining their ability to mediate etorphine-dependent inhibition adenyl cyclase in intact cells. Stably transfected cells grown in 24-well dishes were preincubated for 15 min with 1 mM isobutylmethylxanthine, and then 5 μM forskolin was added to stimulate adenyl cyclase in the absence or the presence of 5 μM etorphine to activate opioid receptors. Cells were extracted with 70% ethanol after 10 min, and cAMP accumulation was quantitated in the extracts using a protein binding radioassay kit (Diagnostic Products Corporation, Los Angeles, CA), as described previously (13).

**Immunocytochemical Staining**—For immunocytochemical localization experiments, cells were grown on glass coverslips, fixed in 4% formaldehyde in phosphate-buffered saline (PBS), and permeabilized using 0.2% Triton X-100 (Sigma). Indirect immunofluorescence staining of FLAG-tagged and HA-tagged receptors was performed using M1 anti-FLAG antibody (3 μg/ml, Kodak IBI, New Haven, CT) and 12CA5 antibody (2 μg/ml, BAbCO, Berkeley, CA), respectively, using the same methods and fluorochrome-labeled secondary antibodies as described previously (13, 18). Dual localization of FLAG-tagged κ and HA-tagged δ receptors in the same cells was performed by first staining permeabilized cells with 12CA5 antibody (2 μg/ml) to detect HA-tagged δ receptors, washing in PBS, and detecting bound 12CA5 antibody with Texas Red-conjugated goat anti-mouse IgG (5 μg/ml, Jackson Immunoresearch, Malvern, PA). Stained cells were then washed in PBS and incubated in 10% normal mouse serum to block residual anti-mouse binding sites, and FLAG-tagged κ receptors were detected by incubating cells with the M1 anti-FLAG antibody that had been directly labeled with fluorescein isothiocyanate (Molecular Probes, Eugene, OR) by standard methods. A similar staining protocol was used to separately detect FLAG-tagged opioid receptors and HA-tagged dynamin constructs co-expressed in the same cells. Antibody uptake experiments were conducted by a modification of a procedure described previously (13, 21). M1 anti-FLAG antibody was added to the culture medium (4 μg/ml), cells were then incubated for 30 min in the presence or the absence of agonist, and then cells were fixed in 4% formaldehyde dissolved in PBS. Endocytosed antibody was detected by staining permeabilized coverslips with fluorescein-conjugated goat anti-mouse antibody (5 μg/ml, Jackson Immunoresearch). Stained specimens were examined by conventional epifluorescence microscopy using a Nikon Diaphot microscope equipped with a 60× NA1.4 objective and standard fluorescein/Texas Red dichroic filter sets. Confocal fluorescence microscopy was performed using a Bio-Rad MRC1000 confocal microscope equipped with a Zeiss 100× NA1.3 objective.

**Fluorescence Flow Cytometry**—After appropriate treatments of cells grown on plastic dishes at 37 °C, dishes were equilibrated to 4 °C, cells were lifted from the dishes by incubation in ice-cold calcium-free PBS containing 1 mM EDTA, and suspended cells were incubated for 30 min in isotonc medium containing 10% fetal bovine serum for 60 min to label epitope-tagged receptors present in the plasma membrane. Cells were washed in PBS to remove residual unbound antibody, and receptor staining intensity was measured by fluorescence flow cytometry using a FACScan instrument (Becton Dickinson, Palo Alto, CA). Gating of live cells by propidium iodide and light scatter and data analysis to quantitate changes in mean surface receptor fluorescence values were performed using CellQuest software (Becton Dickinson).

**RESULTS**

Functional expression of epitope-tagged murine δ and μ opioid receptors was reported previously (13). Epitope-tagged κ receptors were constructed in the same manner. The functional activity of tagged κ receptors was confirmed by measuring etorphine-dependent inhibition of adenyl cyclase in transfected human 293 cells. Epitope-tagged κ receptors strongly inhibited forskolin-stimulated cAMP accumulation in transfected cells when exposed to saturating concentrations of etorphine (5 μM), whereas minimal etorphine-induced inhibition was observed in untransfected cells (Table I, middle column). Examination of the concentration dependence of this inhibition indicated that etorphine activates epitope-tagged κ receptors with high potency. The IC50 for etorphine-induced inhibition of adenyl cyclase measured in cells expressing FLAG-tagged κ receptors was 4.9 μM (Table I, right column). This high potency is similar to that reported previously for etorphine-induced activation of wild-type opioid receptors examined both in naturally expressing and transfected cell types (10, 11, 17). In addition, this potency is similar to that for causing inhibition of adenyl cyclase mediated by epitope-tagged δ receptors expressed in 293 cells (Table I, right column, and Ref. 13). These observations indicate that epitope-tagged κ receptors are functional opioid receptors that retain their ability to be activated with high potency by etorphine. Because of its comparable potency for activation of both epitope-tagged δ and κ receptors, etorphine was used in subsequent studies comparing the membrane trafficking properties of δ, κ, and chimeric mutant receptors.

The subcellular distribution of epitope-tagged opioid receptors was examined by immunofluorescence microscopy. δ receptors tagged with monoclonal antibody remained in the plasma

| Receptor construct | Inhibition of cAMP accumulation | IC50 μM |
|---------------------|---------------------------------|--------|
| None (untransfected) | 8.1 ± 1.5 (n = 7)               | 57.6   |
| FLAG-δ              | 62 ± 3.9 (n = 6)                | 1.8    |
| FLAG-κ              | 53 ± 8.6 (n = 7)                | 4.9    |
| Cr. 1               | 52 ± 7.3 (n = 6)                | 4.7    |
| Cr. 2               | 66 ± 4.7 (n = 6)                | 4.7    |
| Cr. 3               | 69 ± 7.5 (n = 6)                | 4.7    |
| Cr. 5               | 19 ± 6.8 (n = 10)               | 7.3    |
| Cr. 6               | 77 ± 4.1 (n = 6)                | 4.7    |

The table represents the mean value derived from analysis five separate concentration-inhibition response assays, in which cAMP accumulation at each concentration of etorphine was assayed in triplicate wells.
membrane of untreated cells (Fig. 1a) and were internalized within several minutes in the presence of etorphine, as indicated by translocation of antibody-tagged receptors to endosomal vesicles located throughout the cytoplasm (Fig. 1b). δ receptors examined under the same conditions were also observed in the plasma membrane of untreated cells (Fig. 1c). However, in marked contrast to the readily detectable internalization of δ receptors, κ receptors failed to internalize even after 30 min in the presence of etorphine (Fig. 1d). No internalization was observed even in the presence of 5 μM etorphine, a saturating concentration approximately 1000-fold greater than that required to cause receptor-mediated inhibition of adenylyl cyclase (Table I).

This type-specific difference in the regulated internalization of δ and κ receptors was confirmed in heterogeneous populations of transfected cells using an antibody uptake assay similar to that developed previously for the study of adrenergic receptors (22). In control experiments, anti-FLAG antibody did not induce or block internalization of receptors (not shown). No endocytosis of antibody was observed in untransfected cells or in cells expressing receptors tagged with a different epitope (HA) not recognized by the anti-FLAG antibody (Fig. 2a, HA-δ bars), indicating that antibody uptake observed in this assay was mediated specifically by receptor internalization. δ receptors tagged with the appropriate FLAG epitope mediated little endocytosis of monomolecular antibody in the absence of agonist, whereas agonist-induced internalization of these receptors was indicated by a large and rapid increase in the number of antibody-positive endosomes in cells exposed to etorphine (Fig. 2a, FLAG-δ bars). In contrast, cells expressing κ receptors tagged with the same epitope tag exhibited little antibody uptake when incubated for 30 min either in the absence or the presence of etorphine, δ receptors were observed predominantly in endocytic vesicles located throughout the cytoplasm (b), whereas κ receptors remained in the plasma membrane (d).

![Type-specific internalization of opioid receptors visualized by fluorescence microscopy](image1)

**Fig. 1.** Type-specific internalization of opioid receptors visualized by fluorescence microscopy. Transfected 293 cells expressing FLAG-tagged δ (a and b) or κ (c and d) opioid receptors were incubated with M1 monoclonal antibody in the presence or the absence of 5 μM etorphine for 30 min and then fixed and processed for immunofluorescence microscopy as described under “Experimental Procedures.” Representative micrographs of untreated (a and c) and etorphine-treated cells (b and d) are shown. Antibody-labeled receptors were visualized predominantly in the plasma membrane of untreated cells expressing both δ (a) and κ (c) receptors. In the presence of etorphine, δ receptors were observed predominantly in endocytic vesicles located throughout the cytoplasm (b), whereas κ receptors remained in the plasma membrane (d).

![Quantitation of type-specific differences in opioid receptor internalization in transiently and stably transfected cells](image2)

**Fig. 2.** Quantitation of type-specific differences in opioid receptor internalization in transiently and stably transfected cells. a, receptor internalization was assayed in transiently transfected cells by visualizing receptor-mediated endocytosis of M1 monoclonal antibody, which recognizes the extracellular epitope tag sequence of mutant δ and κ opioid receptors, into endocytic vesicles visualized by fluorescence microscopy. Nonspecific uptake of monoclonal antibody from the culture medium was negligible (not shown), and the specificity of uptake was further confirmed by the lack of antibody uptake in cells expressing HA-tagged δ receptors that are not recognized by the M1 antibody (a, HA-δ bars). A small number of endocytic vesicles contained endocytosed M1 antibody in untreated cells expressing appropriately FLAG-tagged δ receptors, and a large increase in antibody uptake was induced by etorphine (5 μM × 30 min), consistent with a large amount of etorphine-induced internalization of δ receptors (FLAG-δ bars). In contrast, FLAG-tagged κ receptors mediated little endocytosis of monoclonal antibody, either in untreated or etorphine-treated cells, consistent with the failure of κ opioid receptors to undergo rapid internalization in the presence of etorphine (FLAG-κ bars). The bars represent the mean number of antibody-positive vesicles (± S.E.) observed in 25 randomly selected cells expressing epitope-tagged receptors from a population of transiently transfected cells. δ, receptor internalization was quantitated in stably transfected cells using fluorescence flow cytometry to measure the etorphine-dependent removal of epitope-tagged receptors from the plasma membrane. Stably transfected cells expressing similar amounts of FLAG-tagged δ (closed circles) or κ receptors (closed squares) were incubated at 37 °C for the indicated times with 10 μM etorphine, and then receptors present in the plasma membrane were labeled with fluorescein-conjugated M1 antibody and quantitated by flow cytometry, as described under “Experimental Procedures.” Points represent mean fluorescence values (± S.D.) for triplicate determinations made at each time point. Etorphine-induced internalization of δ receptors is indicated by the rapid, time-dependent reduction in antibody-labeled receptors present in the plasma membrane of etorphine-treated cells, whereas the failure of κ receptors to internalize is indicated by the constant amount of receptor immunoreactivity measure in the plasma membrane under the same conditions.
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Fig. 3. Type-specific differences in the membrane trafficking of δ and κ receptors. Human 293 cells transiently transfected with FLAG-tagged κ and HA-tagged δ receptors were incubated in the absence (a–c) or the presence (d–f) of 10 μM etorphine for 30 min and then fixed, permeabilized, and processed for dual-label confocal immunofluorescence microscopy as described under “Experimental Procedures.” Both δ (a) and κ opioid receptors (b) were visualized in the plasma membrane of untreated cells, as indicated by yellow staining caused by superimposition of green (δ) and red (κ) immunoreactivity in the merged image (c). In etorphine-treated cells, δ receptors were redistributed to endocytic vesicles (d), whereas κ receptors remained in the plasma membrane (e), as indicated by the separation of green and red immunoreactivity in the merged image (f).

Fig. 4. Inhibition of δ opioid receptor internalization by K44E mutant dynamin. Stably transfected 293 cells expressing FLAG-tagged δ opioid receptors were transiently transfected with HA-tagged K44E mutant (a and b) or wild-type (WT; c and d) dynamin. 48 h after transfection, cells plated on glass coverslips were incubated in the absence (a and c) or the presence (b and d) of 10 μM etorphine for 30 min and then fixed, permeabilized, and processed for dual-label immunofluorescence microscopy. In cells expressing K44E mutant dynamin (as confirmed by bright staining throughout the plasma membrane and cytoplasm of cells by anti-HA antibody, not shown), FLAG-tagged δ receptors remained in the plasma membrane of both untreated and etorphine-treated cells (a and b, respectively), indicating that etorphine-induced internalization was significantly inhibited (e.g., compare with Fig. 1, a and b). In contrast, etorphine-induced internalization of δ receptors was readily observed in cells expressing HA-tagged wild-type dynamin, as indicated by an etorphine-dependent redistribution of FLAG-tagged receptors from the plasma membrane to endocytic vesicles (c and d).

 unearthly transfected with FLAG-tagged κ and HA-tagged δ receptors were incubated in the absence (a–c) or the presence (d–f) of 10 μM etorphine for 30 min and then fixed, permeabilized, and processed for dual-label confocal immunofluorescence microscopy as described under “Experimental Procedures.” Both δ (a) and κ opioid receptors (b) were visualized in the plasma membrane of untreated cells, as indicated by yellow staining caused by superimposition of green (δ) and red (κ) immunoreactivity in the merged image (c). In etorphine-treated cells, δ receptors were redistributed to endocytic vesicles (d), whereas κ receptors remained in the plasma membrane (e), as indicated by the separation of green and red immunoreactivity in the merged image (f).

Type-specific differences in opioid receptor internalization were further confirmed in clonal populations of stably transfected cells using fluorescence flow cytometry to quantitate etorphine-induced removal of receptors from the plasma membrane. Etorphine caused a rapid loss of epitope-tagged δ receptors from the plasma membrane, which could be detected within several minutes and occurred with a t1/2 < 10 min (Fig. 2b, closed circles). In contrast, etorphine-activated κ receptors examined under the same conditions remained in the plasma membrane without any detectable internalization for >30 min (Fig. 2b, closed squares). Identical results were observed in multiple clones of stably transfected cells, including those expressing epitope-tagged κ receptors at levels equal to or less than δ receptors (as estimated by comparing receptor staining intensities measured by flow cytometry). These observations suggest that type-specific differences in opioid receptor internalization are not caused by differences in levels of receptor expression but instead reflect intrinsic differences in the endocytic sorting of structurally homologous receptor proteins.

Supporting this hypothesis, δ and κ receptors were observed to differ in membrane trafficking even when co-expressed in the same cells. κ and δ receptors were tagged with FLAG and HA epitope tags, respectively, and then visualized in the same transfected cells by dual color confocal microscopy. In the absence of agonist, both δ and κ receptors were observed in the plasma membrane (Fig. 3, a and b, respectively). Colocalization of both receptors in the same cells is indicated by yellow staining in the merged image, produced by overlapping green and red fluorescence (Fig. 3c). In the presence of etorphine, δ receptors were internalized, whereas κ receptors examined in the same cells remained in the plasma membrane and were not translocated to endosomes (Fig. 3, d and e), as confirmed by the separation of green and red fluorescence signals observed in the merged image (Fig. 3f).

Previous studies indicate that the internalization of δ and μ opioid receptors is sensitive to biochemical inhibitors of clathrin function (13, 15), suggesting the possibility that type-specific internalization of opioid receptors may be mediated by clathrin-coated pits. Supporting this hypothesis, etorphine-induced internalization of δ receptors was strongly inhibited in cells expressing K44E mutant dynamin I, which specifically blocks endocytosis mediated by clathrin-coated pits (20, 23, 24). Little etorphine-dependent redistribution of δ receptors from the plasma membrane to endosomal vesicles was observed in cells expressing an HA-tagged version of K44E mutant dynamin (Fig. 4, a and b, compare with Fig. 1, a and b), whereas internalization of receptors was readily observed under the same conditions in cells expressing wild-type dynamin I (Fig. 4, c and d). These observations were quantitated using the antibody uptake assay, confirming a specific inhibition of δ receptor internalization in cells expressing K44E mutant dynamin compared with cells not transfected with mutant dynamin or wild-type dynamin I (Fig. 5). In contrast, overexpression of wild-type
or mutant dynamin had no effect on κ opioid receptors, which remained in the plasma membrane both in untreated and etorphine-treated cells (not shown). These results confirm the hypothesis that δ receptors are internalized by clathrin-coated pits, and they suggest that structurally homologous opioid receptors can be differentially sorted into clathrin-coated pits following activation by the same agonist.

To identify receptor domains that determine the type specificity of opioid receptor sorting, we examined a series of chimeric mutant opioid receptors constructed by exchanging corresponding sequences between κ and δ receptors. These studies were feasible because δ and κ receptors share extensive sequence homology, facilitating the construction of functional chimeras that bind relatively nonselective alkaloid ligands such as etorphine (25, 26). Indeed, chimeric mutant receptors examined in these studies bound etorphine and were functional, as indicated by etorphine-dependent inhibition of forskolin-stimulated adenylyl cyclase in stably transfected cells (Table I). The only exception was Cr. 5, which was significantly impaired in its ability to mediate etorphine-dependent signaling but was included in the analysis because it exhibited very pronounced etorphine-induced internalization (see below). Examination of etorphine-dependent internalization of chimeric mutant receptors in transiently transfected cells (using the vesicle counting assay) and stably transfected cells (by flow cytometry) yielded similar results (Fig. 6, a and b, respectively). These results are summarized in Fig. 7, which shows representative fluorescence micrographs of etorphine-treated cells and ranks the etorphine-induced internalization of mutant receptors measured using the quantitative assays.

The effect of exchanging the carboxyl-terminal cytoplasmic tail was examined first because this cytoplasmic domain diverges greatly between individual opioid receptors, and truncation and point mutations within this domain have been reported to influence receptor internalization and down-regulation (15, 27). Replacing the carboxyl tail of the δ receptor with the corresponding sequence from the κ receptor created a chimeric mutant receptor (Cr. 1, Fig. 7), which was functional as determined by etorphine-dependent inhibition of adenylyl cyclase (Table I) but which exhibited significantly reduced agonist-induced internalization compared with the δ receptor. This inhibitory effect was readily apparent by fluorescence microscopy and was confirmed both in transiently and stably transfected cells using the antibody endocytosis assay. In transiently transfected 293 cells, this inhibitory effect was readily apparent by fluorescence microscopy and was confirmed both in transiently and stably transfected cells using the antibody endocytosis assay (Figs. 6 and 7). Interestingly, the converse mutation (Cr. 2) failed to cause etorphine-induced internalization of the κ receptor, even though this chimera was also functional (Table I). Similarly, replacing other portions of κ receptor with the corresponding δ sequence created functional chimeric mutant receptors but failed to promote significant etorphine-in-
Type-specific Endocytosis of Opioid Receptors

We have shown that δ and κ opioid receptors are differentially regulated by rapid endocytosis, even when co-expressed in the same cells and activated by the same agonist. δ receptors were internalized within several minutes in the presence of etorphine, in agreement with previous studies (13, 15). In contrast, κ receptors remained in the plasma membrane and were not internalized when examined under the same conditions and in the same cells, even though both δ and κ receptors mediate etorphine-dependent inhibition of adenyl cyclase by coupling to similar heterotrimeric G proteins (28). Significant differences in the membrane trafficking of δ and κ receptors were observed over a wide range of etorphine concentrations, including extremely high concentrations, even though etorphine is a relatively nonspecific alkaloid agonist that activates δ, μ, and κ receptors with comparably high potencies and has been shown previously to strongly stimulate the rapid internalization of δ and κ receptors in the same cells (13). This observation distinguishes the type specificity of opioid receptor internalization demonstrated in this study from the agonist selectivity of internalization reported in previous studies.

The specific inhibition of this internalization process by a dominant-negative mutant dynamin supports the hypothesis that opioid receptors are endocytosed by clathrin-coated pits. This idea, proposed initially from studies using biochemical inhibitors of clathrin function (13, 15), suggests that opioid receptor internalization is mediated by a highly conserved mechanism similar to that utilized by adrenergic receptors (21, 29, 30). The present results also indicate that individual, structurally homologous opioid receptors can be sorted into coated pits in a type-specific manner following activation. These results reveal an additional level of complexity in the regulation of opioid receptors by clathrin-mediated endocytosis (Fig. 8). Although structurally homologous adrenergic and muscarinic receptors have been shown previously to differ in ligand-induced internalization (19, 31), opioid receptors constitute the first class of G protein-coupled receptors whose membrane trafficking has been shown to be regulated by dynamin-dependent endocytosis in both an agonist-selective and type-specific manner.

An analysis of chimeric mutant κ/δ receptors indicates that the carboxyl-terminal cytoplasmic domain of the receptor protein, which is conserved across species but diverges greatly between individual types of opioid receptor, plays an important role in mediating type-specific differences in receptor internalization. Replacing this domain in the δ receptor with the corresponding κ sequence strongly inhibits etorphine-induced internalization, and this domain from the δ receptor is required for etorphine-induced internalization of all chimeric κ/δ opioid receptors examined. These findings are consistent with a pre-

**Fig. 7. Summary of internalization data for the mutant κ/δ opioid receptors.** The structure of FLAG-tagged δ (SFDOR) and κ (SFKOR) receptors and each chimeric mutant opioid receptor is diagrammed, together with a representative fluorescence micrograph of receptor internalization in cells treated with 5 μM etorphine for 30 min. The amount of receptor internalization measured using the quantitative assays in Fig. 6 is ranked from − (<10% internalization by flow cytometry), to + (10–30% internalization), to ++ (>30% internalization). Internalization of the δ receptor was exchanged in combination with the carboxyl-terminal tail (Cr. 5). This chimeric mutant receptor exhibited etorphine-induced internalization to a similar or even greater extent as the wild-type δ receptor, both in transiently and stably transfected cells (Figs. 6 and 7). This effect on receptor internalization was not unique to this domain. Significant agonist-induced internalization was also observed with a chimeric mutant receptor in which a distinct, nonoverlapping domain of the δ receptor was exchanged in combination with the carboxyl-terminal tail (Cr. 6). Taken together, these results indicate that the carboxyl tail is necessary but not completely sufficient to mediate subtype-specific differences in the regulated internalization of opioid receptors. Additional domain(s) from the δ receptor appear to be necessary, in combination with the carboxyl-terminal cytoplasmic tail, to promote etorphine-induced internalization of chimeric mutant opioid receptors.

**DISCUSSION**

We have shown that δ and κ opioid receptors are differentially regulated by rapid endocytosis, even when co-expressed in the same cells and activated by the same agonist. δ receptors were internalized within several minutes in the presence of etorphine, in agreement with previous studies (13, 15). In contrast, κ receptors remained in the plasma membrane and were not internalized when examined under the same conditions and in the same cells, even though both δ and κ receptors mediate etorphine-dependent inhibition of adenyl cyclase by coupling to similar heterotrimeric G proteins (28). Significant differences in the membrane trafficking of δ and κ receptors were observed over a wide range of etorphine concentrations, including extremely high concentrations, even though etorphine is a relatively nonspecific alkaloid agonist that activates δ, μ, and κ receptors with comparably high potencies and has been shown previously to strongly stimulate the rapid internalization of δ and κ receptors in the same cells (13). This observation distinguishes the type specificity of opioid receptor internalization demonstrated in this study from the agonist selectivity of internalization reported in previous studies. The specific inhibition of this internalization process by a dominant-negative mutant dynamin supports the hypothesis that opioid receptors are endocytosed by clathrin-coated pits. This idea, proposed initially from studies using biochemical inhibitors of clathrin function (13, 15), suggests that opioid receptor internalization is mediated by a highly conserved mechanism similar to that utilized by adrenergic receptors (21, 29, 30). The present results also indicate that individual, structurally homologous opioid receptors can be sorted into coated pits in a type-specific manner following activation. These results reveal an additional level of complexity in the regulation of opioid receptors by clathrin-mediated endocytosis (Fig. 8). Although structurally homologous adrenergic and muscarinic receptors have been shown previously to differ in ligand-induced internalization (19, 31), opioid receptors constitute the first class of G protein-coupled receptors whose membrane trafficking has been shown to be regulated by dynamin-dependent endocytosis in both an agonist-selective and type-specific manner.

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**Fig. 8. Model for type-specific internalization of opioid receptors.** In the presence of etorphine, δ opioid receptors activate heterotrimeric G proteins and are endocytosed by a clathrin and dynamin-dependent mechanism. κ opioid receptors mediate etorphine-dependent signaling via similar G proteins but do not enter clathrin-coated pits, causing segregation of δ and κ opioid receptors in different membrane compartments.
vious study showing that truncations and point mutations within this domain influence receptor trafficking (15, 27). Interestingly, this domain from the δ receptor cannot by itself cause etorphine-induced internalization of a functional mutant κ receptor (Cr. 2). These observations indicate that exchanging only the carboxy-terminal cytoplasmic domain between receptors is sufficient to cause a “loss of function” mutation but not a converse “gain of function” of etorphine-induced internalization of the corresponding chimeras. Thus the regulated membrane trafficking of opioid receptors cannot be fully explained by a single contiguous sequence motif in the receptor protein. Our results suggest that additional divergent domain(s) are required in combination with the carboxy-terminal cytoplasmic domain to fully determine type-specific differences in the regulated endocytosis of opioid receptors. These observations suggest that the membrane trafficking of opioid receptors may be regulated by the interaction of multiple cellular proteins with different receptor domains or by the interaction of a single protein with a complex structure comprised of more than one receptor domain. Interestingly, the chimeric mutant receptor that exhibited the largest amount of etorphine-induced internalization in our studies (Cr. 5) appeared to be significantly impaired in signaling via adenylyl cyclase. This observation suggests that structural features of opioid receptors required for regulated endocytosis are distinguishable from those required for ligand-dependent activation of heterotrimeric G proteins.

Studies of other G protein-coupled receptors indicate that ligand-dependent internalization can play multiple, important roles in the modulation of cell signaling (32–35). The remarkable selectivity of opioid receptor internalization demonstrated in the present study may be of great importance for understanding type-specific differences in the physiological regulation of opioid receptors following activation by relatively nonselective opiate analgesic drugs. Further studies will be required to elucidate precisely how type-specific endocytosis of opioid receptors influences the selectivity of opioid signaling in vivo. The present studies make a significant contribution toward this goal by identifying, for the first time, a mechanism of receptor endocytosis that can differentially regulate structurally homologous opioid receptors activated by the same alkaloid agonist.

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