Phosphorylation Is Not Required for Dynamin-dependent Endocytosis of a Truncated Mutant Opioid Receptor*

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Opioid receptors are regulated within minutes after activation by G protein-coupled receptor kinase-mediated phosphorylation and dynamin-dependent endocytosis. We addressed the question of whether phosphorylation is required for opioid receptor endocytosis by examining a functional, truncated mutant δ opioid receptor (DOR344T), which is missing phosphorylation sites located in the carboxyl-terminal cytoplasmic domain. DOR344T receptors expressed in Chinese hamster ovary cells remained predominantly in the plasma membrane, even in the presence of saturating concentrations of agonist, consistent with previous studies demonstrating strongly inhibited endocytosis of truncated receptors in this cell type. In marked contrast, DOR344T receptors expressed at similar levels in human embryonal kidney (HEK) 293 cells exhibited rapid, ligand-induced internalization either in the presence of peptide (DADLE) or alkaloid (etorphine) agonist. Quantitative assays using ELISA and flow cytometric techniques indicated that DOR344T receptors were endocytosed in HEK293 cells with similarly rapid kinetics as full-length DOR ($t_{1/2} < 10$ min), and both full-length DOR and DOR344T mutant receptors were endocytosed by a dynamin-dependent mechanism involving clathrin-coated pits. Nevertheless, DOR344T receptors failed to undergo any detectable constitutive or agonist-induced phosphorylation in the same cells in which dynamin-dependent endocytosis was observed. These findings establish the first example of a G protein-coupled receptor that does not require phosphorylation to undergo dynamin-dependent endocytosis, and they suggest that significant cell type-specific differences exist in the biochemical requirements for ligand-induced concentration of opioid receptors in clathrin-coated pits.

Opioid receptors are G protein-coupled receptors (GPCRs)$^1$ activated both by endogenously produced opioid peptides and by alkaloid analgesic drugs (1–4). Opioid receptors are regulated by multiple mechanisms, which contribute to the physiological plasticity of the endogenous opioid system and function in the development of tolerance and dependence to opiate drugs (5–9). Within minutes after activation by peptide or alkaloid agonists, opioid receptors expressed in HEK293 cells are phosphorylated by G protein-coupled receptor kinases (GRKs) (10) and are endocytosed in a dynamin-dependent manner by clathrin-coated pits (11–13). Despite the fundamental importance of these mechanisms to opiate drug action and addiction, the precise role of phosphorylation in the mechanism of opioid receptor endocytosis is not understood.

Previous studies of other GPCRs suggest that mechanisms of receptor phosphorylation and endocytosis are closely related. For example, GRK-mediated phosphorylation of the carboxyl-terminal cytoplasmic domain of the β2-adrenergic receptor promotes receptor interaction with β-arrestin, which thereby promotes the association of activated receptors with clathrin-coated pits (14–17). In the case of the DOR, truncation and point mutations that remove candidate phosphorylation sites located in the carboxyl-terminal cytoplasmic domain strongly inhibit agonist-induced internalization observed in CHO (12) and NG108 (18) cells, suggesting that a similar model may apply to opioid receptors. Consistent with this hypothesis, overexpression of either GRKs or arrestins is sufficient to enhance agonist-induced endocytosis of opioid receptors (19, 20). Interestingly, however, studies of truncated mutant δ opioid receptors suggest that phosphorylation sites located in the carboxyl-terminal cytoplasmic domain play distinct roles both in promoting endocytosis of activated receptors and in preventing endocytosis in the absence of agonist (21). While these observations suggest a more complex function of phosphorylation of the carboxyl-terminal cytoplasmic domain in modulating the membrane trafficking of opioid receptors, they also raise the more fundamental question of whether or not phosphorylation is actually required for agonist-induced endocytosis of opioid receptors.

We have addressed this question by examining a truncated mutant δ opioid receptor (DOR344T) that is functional to mediate ligand-dependent signal transduction but is not phosphorylated either in the absence or presence of agonist. Surprisingly, while DOR344T mutant receptors remain predominantly in the plasma membrane of CHO cells, we have observed that these receptors undergo rapid, agonist-induced endocytosis when expressed at similar levels in HEK293 cells. Furthermore, endocytosis of DOR344T mutant receptors occurs with similarly high efficiency as full-length DOR and is mediated by a dynamin-dependent mechanism involving clathrin-coated pits. To our knowledge, this study is the first to demonstrate phosphorylation-independent endocytosis of any GPCR by clathrin-coated pits. In addition, these observations suggest that significant cell type-specific differences exist in the bio-

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1 The abbreviations used are: GPCR(s), G protein-coupled receptor(s); GRK, G protein-coupled receptor kinase; DOR, δ opioid receptor; CHO, Chinese hamster ovary; HA, hemagglutinin; HEK, human embryonal kidney; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay.
chemical requirements for the entry of activated opioid receptors into a dynamin-dependent endocytic pathway.

EXPERIMENTAL PROCEDURES

cDNA Constructs—The DOR344T truncated mutant receptor was constructed by engineering a stop codon following residue 344 in the coding sequence of the FLAG-tagged murine DOR described previously (11) using oligonucleotide site-directed mutagenesis mediated by the polymerase chain reaction (Vent polymerase, New England Biolabs). Receptor cDNAs were cloned into pcDNA3 (Invitrogen) and verified by dyeoxyribonucleotide sequencing (Sequenase, U. S. Biochemical Corp.). cDNAs encoding HA-tagged wild type and dominant negative (K44E) mutant dynamin I (22) were kindly provided by Dr. Richard Vallee (Worcester Foundation for Biomedical Research, Shrewsbury, MA) and were cloned into pcDNA3 for expression.

Cell Culture and Transfection—CHO cells and HEK293 cells (American Type Culture Collection) were maintained and passaged in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (University of California, San Francisco Cell Culture Facility). Cell monolayers grown in 6-cm dishes were transfected with cDNA constructs cloned into pcDNA3 using calcium phosphate (23). Transiently transfected cells were examined 48–72 h after transfection. Stably transfected cell clones derived from HEK293 cells were selected using 0.5 mg/ml G418 (Geneticin, Life Technologies, Inc.). Relative levels of expression and uniformity of expression were evaluated using flow cytometry and immunofluorescence microscopy as described previously (11).

Confirmation of the Functional Integrity of DOR344T Mutant Opioid Receptors—The functional integrity of the DOR344T mutant receptor was confirmed by an assay of receptor-mediated inhibition of adenyl cyclase (11) in stably transfected HEK293 cells (clone DOR344T/4#) expressing truncated mutant receptors at 1.8 × 10⁵ receptors/cell. DOR344T mutant receptors mediated agonist-dependent inhibition of adenyl cyclase activity in these cells (57 ± 12% inhibition in the presence of 5 μM etorphine; 61 ± 14% inhibition in the presence of 5 μM DADLE, n = 3), indicating that DOR344T mutant receptors are functional.

Receptor Internalization—Stably transfected HEK293 cells expressing either full-length DOR or DOR344T mutant receptors at similar levels were plated in 10-cm dishes containing phosphate-free DMEM supplemented with 2% dialyzed fetal bovine serum (University of California, San Francisco Cell Culture Facility) and metabolically labeled for 3 h with 0.5 μCi/ml [3H]Pitrophosphate (6000 Ci/mmol, New England Nuclear Products, Inc.). Cells were then incubated in the absence or presence of 10 μM DADLE or etorphine for the indicated periods, then transferred to ice and immediately extracted in 1 ml/dish ice-cold extraction buffer (25 mM NaHEPES, pH 7.4, 50 mM NaCl, 0.1% Triton X-100, 1 mM CaCl₂, 50 mM NaF, 80 mM β-glycerol phosphate, 0.1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 2 μg/ml phenylmethylsulfonyl fluoride), and receptors were immunoprecipitated from extracts using M1 anti-FLAG monoclonal antibody (3 μg/ml, Kodak Scientific Imaging Systems) and protein A-Sepharose (Amersham Pharmacia Biotech). Immunoprecipitates were washed extensively by centrifugation in wash buffer (25 mM NaHEPES, pH 7.4, 50 mM NaCl, 0.1% Triton X-100, 1 mM CaCl₂), then immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis. Gels were either dried and exposed directly to a PhosphorImage plate (Molecular Dynamics, Inc.) for autoradiography or were electrotransferred to nitrocellulose membranes (24) for autoradiography followed by immunoblotting of the same membrane using biotinylated M1 anti-FLAG antibody, horseradish peroxidase-streptavidin, and chemiluminescence (ECL, Amersham Pharmacia Biotech) to detect receptor protein. Immunoblots were quantitated by densitometric scanning of film (Kodak BioMax) exposed in the linear range.

Immunocytochemical Localization and Fluorescence Microscopy—Immunocytochemical localization of epitope-tagged opioid receptors in fixed specimens was performed using M1 anti-FLAG monoclonal antibody (3 μg/ml) as described previously (11). Specific visualization of opioid receptor endocytosis by translocation of antibody-labeled receptors from the plasma membrane to intracellular membranes was performed using a previously established protocol (13). Dual localization of FLAG-tagged receptors and HA-tagged dynamin in the same cells was performed as described (13). Conventional fluorescence microscopy was performed using a Nikon inverted microscope equipped with a 60× NA1.4 objective and standard epifluorescence optics. Confocal fluorescence microscopy was performed using a Bio-Rad MRC1000 confocal imaging system interfaced to a Zeiss inverted microscope equipped with a 100× NA1.3 objective.

RESULTS AND DISCUSSION

Full-length DOR and a truncated mutant receptor (DOR344T) missing candidate phosphorylation sites located in the carboxy-terminal cytoplasmic domain (12, 18, 26) were localized by fluorescence microscopy in transfected CHO and HEK293 cells. When expressed in CHO cells, DOR344T mutant receptors remained predominantly in the plasma membrane over an approximately 10-fold range of expression levels, as estimated by immunocytochemical staining intensity measured using a digital cooled charge-coupled device camera (not shown).

The ability of DOR344T mutant receptors to undergo rapid,
agonist-induced endocytosis in HEK293 cells was confirmed in stably transfected cells using quantitative assays of receptor internalization. A cell surface ELISA assay (25) indicated that both full-length and DOR344T mutant receptors expressed in several different clones of HEK293 cells exhibited >40% internalization after 30 min in the presence of 5 μM etorphine (B and E), or in the presence of 5 μM DADLE (C and F). Confocal microscopy was then used to visualize the redistribution of antibody-labeled receptors, as in Fig. 1. In the absence of agonist, both wild type and truncated receptors were associated predominantly with the plasma membrane (A and D, respectively). In the presence of 5 μM etorphine (B and E) or 5 μM DADLE (C and F), both full-length and DOR344T mutant receptors redistributed within 30 min from the plasma membrane into numerous intracellular vesicles. Fluorescence flow cytometry was used to measure the internalization of receptors induced by 5 μM etorphine (G) or 5 μM DADLE (H). In these experiments, receptors were labeled with antibody after incubating cells with ligands and chilling cells to 4°C to stop membrane trafficking (see “Experimental Procedures”), to confirm that receptor internalization observed was not caused by antibody. Each data point represents the mean ± S.D. of receptor internalization, determined by loss of receptor immunoreactivity from the cell surface, determined in an analysis of 5000 cells performed in triplicate. A least squares fit of these data to a single exponential function estimated that both full-length and DOR344T mutant receptors are internalized with similarly rapid kinetics in response to etorphine (t½ ~ 4.4 and 5.1 min, respectively) or DADLE (t½ ~ 10.0 and 5.9 min, respectively).

**Fig. 2.** Rapid internalization of both full-length DOR and DOR344T mutant receptors in HEK293 cells. Transiently transfected HEK293 cells expressing full-length DOR (A–C) or DOR344T mutant receptors (D–F) were labeled with M1 antibody and incubated for 30 min in the absence of agonist (A and D), in the presence of 5 μM etorphine (B and E), or in the presence of 5 μM DADLE (C and F). Confocal microscopy was then used to visualize the redistribution of antibody-labeled receptors, as in Fig. 1. In the absence of agonist, both wild type and truncated receptors were associated predominantly with the plasma membrane (A and D, respectively). In the presence of 5 μM etorphine (B and E) or 5 μM DADLE (C and F), both full-length and DOR344T mutant receptors redistributed within 30 min from the plasma membrane into numerous intracellular vesicles. Fluorescence flow cytometry was used to measure the internalization of receptors induced by 5 μM etorphine (G) or 5 μM DADLE (H). In these experiments, receptors were labeled with antibody after incubating cells with ligands and chilling cells to 4°C to stop membrane trafficking (see “Experimental Procedures”), to confirm that receptor internalization observed was not caused by antibody. Each data point represents the mean ± S.D. of receptor internalization, determined by loss of receptor immunoreactivity from the cell surface, determined in an analysis of 5000 cells performed in triplicate. A least squares fit of these data to a single exponential function estimated that both full-length and DOR344T mutant receptors are internalized with similarly rapid kinetics in response to etorphine (t½ ~ 4.4 and 5.1 min, respectively) or DADLE (t½ ~ 10.0 and 5.9 min, respectively).
Phosphorylation-independent Endocytosis of Opioid Receptors

Stably transfected HEK293 cells expressing wild-type (DOR) or truncated mutant (DOR344T) δ opioid receptors were metabolically labeled with [32P]orthophosphate, and receptor phosphorylation was determined in receptor immunoprecipitates prepared from equal numbers of cells, as described under “Experimental Procedures.” Minimal basal phosphorylation of full-length δ opioid receptors (DOR) was observed in receptor immunoprecipitates prepared from cells incubated in the absence of agonist (A, lane 2) or presence of the antagonist naloxone (not shown), which was not significantly greater than nonspecific background levels observed in control immunoprecipitates prepared from untransfected HEK293 cells (A, lane 1). Phosphorylation of a protein band corresponding to the mature receptor (solid arrow) was induced both by etorphine and DADLE (A, lanes 3 and 4). In contrast, no phosphorylation of a protein band corresponding to DOR344T truncated mutant receptors (open arrow) was observed, either in the absence of ligand (A, lane 5) or presence of etorphine or DADLE (lanes 6 and 7, respectively). The failure of DOR344T mutant receptors to undergo phosphorylation in HEK293 cells either in the absence of presence of etorphine was confirmed by autoradiography (B, upper strip) and immunoblotting (B, lower strip) of identical specimens to confirm equal recovery of DOR and DOR344T mutant receptors in immunoprecipitates.

The ability of DOR344T mutant opioid receptors to undergo rapid, dynamin-dependent endocytosis in HEK293 cells raised the question of whether these receptors may be phosphorylated in this cell type. To address this question, we assayed in vivo phosphorylation of opioid receptors in the same stably trans-
fectected cell clones in which receptor endocytosis was examined. HEK293 cells expressing similar levels of full-length (clone DOR#5) or mutant (clone DOR344T#4) receptors were metabolically labeled with [32P]orthophosphate, incubated in the presence or absence of various agonists, then receptors were isolated from cell lysates by immunoprecipitation and analyzed by SDS-polyacrylamide gel electrophoresis autoradiography (Fig. 4A). Nonspecific signal in these experiments was negligible (lane 1), while a small amount of constitutive phosphorylation of full-length δ opioid receptors was observed in cells incubated in the absence of agonist (lane 2). When incubated in the presence of either alkaloid (etorphine) or peptide (DADLE) agonist, phosphorylation of full-length δ opioid receptors was strongly stimulated, as indicated by the appearance of a heavily phosphorylated band of immunopurified receptor (lanes 3 and 4) corresponding to the mature form of the receptor glycoprotein (solid arrow). In contrast, DOR344T mutant receptors expressed at similar levels and analyzed in the same manner exhibited no detectable phosphorylation, either in the absence of agonist or in the presence of saturating concentrations of etorphine or DADLE (Fig. 4A, lanes 5–7; open arrow indicates position in the gel of the mature truncated mutant receptor protein). These results were replicated in experiments in which identical samples were analyzed by autoradiography (Fig. 4B, upper panel) and immunoblotting (Fig. 4B, lower panel) to confirm that essentially identical amounts of receptor protein were present in each sample. Quantitation by PhosphorImager scanning indicated that agonist-induced phosphorylation of DOR344T mutant opioid receptors was completely (>95%) blocked at all time points examined, from 5 to 30 min after the addition of agonist.

In conclusion, the present studies demonstrate that phosphorylation is not required for dynamin-dependent endocytosis of a truncated mutant opioid receptor. Although phosphorylation is not required for endocytosis of certain other GPCRs (28, 29), it has not been determined whether these receptors are endocytosed by clathrin-coated pits or by an alternate, dynamin-independent mechanism that does not require GRK-mediated phosphorylation (30, 31). Thus, to our knowledge, the present results are the first to establish phosphorylation-independent endocytosis of any GPCR by clathrin-coated pits. Previous studies indicate that phosphorylation of the carboxyl-terminal cytoplasmic domain is required for agonist-induced endocytosis of opioid receptors in CHO and NG108 cells (12, 18). Our studies of receptor localization in CHO cells are consistent with this conclusion, suggesting that significant differences exist among individual cell types in biochemical requirements for the regulated endocytosis of opioid receptors. Interestingly, while overexpression of GRKs or arrestins is sufficient to enhance endocytosis of opioid receptors (19, 20), these proteins are expressed endogenously in CHO and HEK293 cells at closely similar levels and β2-adrenergic receptors internalize in both cell types with similarly high efficiency (16). Thus we speculate that additional cellular protein(s), distinct from GRKs and arrestins, may be differentially expressed among these cell types and mediate the observed cell type-specific differences in phosphorylation-independent endocytosis of receptors. The identification of these proteins and the mechanism by which they function is an important goal for future studies. The present studies make a significant step in this direction by establishing, for the first time, that dynamin-dependent endocytosis of opioid receptors can occur in the absence of receptor phosphorylation and that significant cell type-specific differences exist in the biochemical requirements for agonist-induced association of opioid receptors with clathrin-coated pits.

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