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Sorting   of G protein-coupled Receptors
after Endocytosis

by

Patricia I. Tsao

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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in

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of the

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this thesis is dedicated to my parents

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Patricia Tsao
UC San Francisco
401 Parnassus Ave., Box 0984
San Francisco, CA 94143

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Mark von Zastrow, M.D., Ph.D.

Associate Professor

Departments of Psychiatry and Cellular & Molecular Pharmacology

Nina Ireland Laboratory of Developmental Neurobiology

University of California, San Francisco

Sorting of G protein-coupled Receptors after Endocytosis

Patricia I. Tsao

Abstract

Membrane trafficking of G protein-coupled receptors (GPCRs) plays a critical role in regulation of GPCR signal transduction. It is well established that receptor endocytosis and recycling contribute to resensitization, while receptor sorting to lysosomes mediates downregulation in response to prolonged or repeated agonist treatment. This thesis describes an effort to understand how GPCRs are sorted following endocytosis, using epitope-tagged β_2-adrenergic receptors (B2ARs) and δ-opioid receptors (DORs) stably transfected into human embryonic kidney (HEK293) cells as a model system and utilizing pharmacologic, immunofluorescent, flow cytometric, and biochemical techniques. This thesis shows that in marked contrast to the prototypic B2AR which rapidly and efficiently recycles following endocytosis, the DOR is rapidly proteolyzed in lysosomes. The distinct fates of these receptors despite their endocytosis via a common clathrin-mediated mechanism suggest that receptor sorting occurs in the endosome, rather than at the plasma membrane. Further examination of the DOR membrane trafficking suggests that sorting occurs by segregation of receptors into a distinct set of vesicles within 10 minutes after endocytosis, and does not require the continued presence of agonist in the culture medium. This thesis also demonstrates that a truncated mutant opioid receptor which remains unphosphorylated following agonist treatment is still efficiently proteolyzed in lysosomes, indicating that phosphorylation is

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not required for lysosomal targeting. These observations identify critical aspects of the

GPCR lysosomal sorting operation which can be used as a basis for further studies of

the precise membrane mechanisms and specific proteins mediating this process.

Bcccccc 6/16/00

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CHAPTER 1

INTRODUCTION

Regulation of GPCR Signal Transduction: The Role of Receptor

Membrane Trafficking after Endocytosis

G protein-coupled receptors (GPCRs) comprise the largest superfamily of signal transducing receptors and mediate the physiological actions of approximately 80% of all known hormones and neurotransmitters. GPCRs also mediate the actions of diverse agonists including odorants, light, pheromones, and ions. Members of this receptor family are of great clinical importance, as their signaling is disturbed in numerous pathophysiological states.

The function of GPCRs is tightly regulated within the cell by multiple processes. Initial understanding of these regulatory mechanisms arose from studies of the β_2-adrenergic receptor (B2AR) receptor. Subsequent studies of numerous other GPCRs have demonstrated that these mechanisms are highly conserved within the GPCR superfamily.

SECTION I: REGULATION OF GPCR SIGNAL TRANSDUCTION

Classic pharmacological studies of the B2AR have defined three distinct processes of regulation: desensitization, sequestration, and downregulation (reviewed in (1-3)). More recent studies with cloned receptor cDNAs and cell culture model systems indicate that these processes are complex and mediated by multiple, cell biological mechanisms.

Regulation of signaling begins with *desensitization*, which refers to the rapid attenuation of signaling within seconds to minutes after activation. Upon agonist activation, the receptor tail is phosphorylated by two ser/thr kinases, protein kinase A (PKA) and a G protein-coupled receptor kinase (GRK). Phosphorylation of the receptor

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tail impairs interaction with its cognate G protein and GRK-mediated phosphorylation promotes association of a protein called beta-arrestin. Arrestin not only further mediates functional uncoupling of the G protein from the receptor, but also promotes rapid *sequestration* of receptors from the plasma membrane.

Receptor *sequestration* refers to the rapid, agonist-induced loss of receptors accessible to hydrophilic radioligand. For the B2AR in HEK293 and numerous other cell types, this process appears to be primarily mediated by endocytosis into clathrin-coated pits (4-6). However, endocytosis via non-clathrin pathways and caveolae has been reported for other cell lines and certain GPCRs (7-9). The functional effects of endocytosis are dependent on a receptor's trafficking pattern from the endosome. Trafficking of receptors through a rapid recycling pathway promotes functional resensitization of signal transduction (10-12), while stable retention in an endosome has been proposed to prolong receptor desensitization (13). Targeting of receptors to another endocytic compartment, the lysosome, is one mechanism that contributes to the long-term regulatory process called downregulation, which is described below. Some recent studies also suggest a potential role of GPCR endocytosis in activating the MAPK signaling cascade (14,15).

After prolonged or repeated activation (typically over a period of several hours), receptor signaling is modulated by *downregulation*, a process that causes a gradual attenuation of signal transduction. Although much is known about the mechanisms that modulate receptor signaling acutely, considerably less is known about the biochemical mechanisms that regulate GPCRs over a longer time scale. Understanding this process

is of particular interest due to its relevance to the physiological actions of clinically important drugs, which persist in the extracellular milieu and are typically used in a chronic or repeated manner (16).

Mechanisms of GPCR Downregulation

Downregulation of GPCRs is traditionally defined as a reduced number of receptor sites measured using radioligand binding. There is evidence that, in some cases, downregulation of receptors may be mediated by receptor conformational changes without detectable loss of receptor protein (17). However, in many cases downregulation is thought to be associated with a net loss of receptor protein or irreversible conformational changes that necessitate new protein synthesis, as suggested initially by studies demonstrating that recovery from downregulation is dependent on new protein synthesis (18).

Extensive studies of various GPCRs suggest that changes in the rates of both receptor biosynthesis and degradation control the number of receptors present in cultured cells. For example, B2AR mRNA levels are modulated by both transcriptional regulation of the receptor gene (19) and modulation of mRNA stability (20). Studies using subcellular fractionation, biochemical inhibition of lysosomal proteolysis, and immunocytochemical localization of receptors strongly suggest that downregulation of several mammalian GPCRs is associated with translocation of receptors to lysosomes (21-24). Whether this is a consequence of internalization and endocytic sorting to lysosomes or internalization via an alternate pathway has historically been a subject of debate.

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There is also emerging evidence that distinct, non-lysosomal mechanisms can mediate proteolysis leading to downregulation. Previous studies of the V2 vasopressin receptor demonstrated ligand-induced endoproteolytic cleavage by a plasma membrane-associated metalloprotease (25). More recent studies of B2AR downregulation suggest the operation of an alternate proteolytic mechanism in some cell types but not others (26) that is, interestingly enough, insensitive to inhibitors of both lysosomal and proteasome-mediated proteolysis (26).

Membrane trafficking of GPCRs clearly plays an important role in regulation of GPCR signaling. However, due to the relatively recent isolation of cloned receptor DNAs, our understanding of GPCR membrane trafficking is limited relative to our understanding of the trafficking of constitutively internalized receptors and receptor tyrosine kinases (RTKs). The most significant progress to date has been in the understanding of the basic mechanisms of GPCR endocytosis.

SECTION II: GPCR MEMBRANE TRAFFICKING

Mechanisms of Endocytosis

The existence of GPCR endocytosis was suggested by several early observations, including the identification of subcellular fractions enriched in B2AR receptors and depleted of plasma membrane (27-30) and binding studies revealing an agonist-induced reduction in sites for relatively hydrophilic ligands (31). Confocal microscopy studies provided the first direct evidence for internalization by visualizing punctuate B2AR vesicles colocalized with transferrin in HEK293 cells (32). Since then, studies have provided evidence that numerous GPCRs internalize in a variety of

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experimental situations- *in vitro* cell culture systems, cells expressing endogenous

receptor , and *in vivo*.

Recent studies of GPCR trafficking indicate that most of these receptors

undergo ligand-induced endocytosis via clathrin-coated pits, which is promoted by a

highly conserved mechanism mediated by beta-arrestins (33). However, distinct

GPCRs differ significantly in their ability to undergo endocytosis by coated pits, and

there is strong evidence for the existence of receptor-specific and cell type-specific

differences in precise mechanisms of GPCR endocytosis. For example, while the

B2AR is endocytosed by clathrin-coated pits in several cell types (4), morphological

studies suggest that this receptor can endocytose in other cells by membrane

invaginations that resemble caveolae (8,9). CCK receptors have been observed in both

clathrin-coated pits and caveolae in the same cells (7). Endocytosis of several GPCRs

is not detectably inhibited by a dominant-negative mutant form of dynamin, which

blocks endocytosis of both clathrin-coated pits and caveolae (34-36), suggesting that

additional mechanism(s) of GPCR endocytosis may function in some cases (6,37,38).

Fate of GPCRs following endocytosis

The movement of receptors after endocytosis was suggested by the early

observation that surface B2AR ligand binding sites recover after removal of agonist

(31). Direct evidence for translocation of receptor protein came from studies showing

that radiolabeled receptors (39) and antibody-labeled internalized receptors were

capable of efficiently returning to the plasma membrane after removal of agonist (5,32).

Additional studies demonstrated that even if receptors are prestimulated with agonist in

the absence of antibody, subsequently added antibody is also taken up into vesicles,

thus confirming that internalization occurs continuously in the presence of agonist (5).

These results suggested that the pool of internalized B2AR reflects a steady state

amount of receptor rather than a pool of retained receptor. This model of a B2AR

which continuously internalizes and recycles in the presence of agonist has been

confirmed by flow cytometric studies of receptor recycling where return of surface

receptor is detected by cleavability of a protease- sensitive receptor N-terminus tag

(40). Studies described in Chapter 2 of this thesis confirm the continuous recycling of

B2AR in the presence of agonist by another flow cytometric method. They also

demonstrate that amounts of surface B2AR remain stable after agonist treatment for

several hours, a result consistent with continuous cycling of the receptor in the presence

of agonist treatment. Nevertheless, despite the efficient recycling exhibited by

internalized B2AR, the ability of these receptors to undergo downregulation indicates

that a small population of receptors is still capable of being targeted to lysosomes.

Like the B2AR, many GPCRs recycle efficiently after endocytosis and display

relatively low rates of agonist-induced receptor proteolysis (e.g. (3)). However, recent

studies have identified two primary alternate fates for GPCRs after endocytosis. Instead

of recycling back to the plasma membrane, a receptor may be stably retained in an

endocytic compartment, a trafficking pattern recently shown for the V2R (13,41). In

contrast, a receptor may be rapidly degraded after internalization. A well characterized

example of such a receptor is the thrombin receptor, a GPCR which is irreversibly

activated by a proteolytic mechanism (42-44). Studies have demonstrated that rapid

degradation is a primary mechanism of signal attenuation of the thrombin receptor (45),

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thus raising the possibility that rapid agonist-induced degradation is a specific

mechanism evolved to turn off these irreversibly activated receptors. Interestingly,

Chapter 2 of this thesis describes the identification of a nonproteolytically activated

GPCR, the δ subtype of the opioid receptor (DOR), that is also rapidly degraded upon

agonist treatment. These studies suggest that rapid proteolysis may also serve as a

primary mechanism of signal attenuation for receptors activated by nondestructive

mechanisms.

The relatively recent identification of alternate postendocytic fates for distinct

GPCRs provides us with an attractive system for investigating the mechanisms of

GPCR postendocytic trafficking. Previously, the efficient recycling and minimal

lysosomal targeting of receptors like the B2AR limited research in this area. By closely

examining the behavior of the DOR and the B2AR, this thesis begins to address the

following fundamental questions regarding the mechanisms of GPCR postendocytic

trafficking:

    1) What determines the specificity of GPCR trafficking?

    2) What membrane mechanisms mediate GPCR sorting to lysosomes?

    3) Is phosphorylation required for lysosomal targeting of GPCRs?

SECTION III: MECHANISMS OF POSTENDOCYTIC TRAFFICKING FOR GPCRS

Specificity of GPCR Postendocytic Trafficking

An essential step towards understanding the mechanisms of postendocytic

trafficking is to determine what specifies the fate of a receptor. In other words, when is

the decision made regarding a receptor's fate after endocytosis? Does sorting occur in the endosome, in a similar manner to the sorting of lysosomally directed ligands from constitutively internalized receptors? Or does sorting occur at the plasma membrane? Given the multiple endocytic pathways of GPCRs, it is certainly possible that particular modes of endocytosis are conduits to a specific receptor fate.

The observations that ligand-induced sequestration and downregulation of the B2AR occur with significantly different kinetics and that these two processes can be partially distinguished pharmacologically suggested many years ago that mechanistic differences existed between processes of sequestration and downregulation (46,47). Supporting this idea, previous studies identified mutations of the B2AR that differentially affect agonist-induced sequestration and downregulation (e.g., (48)). Furthermore, naturally occurring subtypes of alpha-2 adrenergic receptor downregulate with similar rates (49) despite significant differences in rapid endocytosis (50). Perhaps the most compelling evidence comes from elegant studies indicating that divergent residues located in the carboxyl-terminal cytoplasmic domain specify differences in membrane trafficking of thrombin and substance P receptors between lysosomal and recycling pathways, respectively (51).

There is evidence for endocytosis of both thrombin (44) and substance P receptors (3) via clathrin-coated pits. However, endocytic mechanisms exhibit considerable cell type specificity and therefore it is not known whether these receptors or other GPCRs are capable of being sorted to distinct fates in the same cells. For example, D1 and D2 dopamine receptors co-expressed in the same cell line are

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observed in separate vesicles immediately after internalization yet exhibit similar rates of proteolytic degradation (38).

Based on studies of constitutively internalized receptors and their ligands, there is certainly ample evidence for sorting events occurring in the endosome (52). Studies with fluorescently labeled transferrin have demonstrated that lumenal contents of endocytic vesicles are delivered to lysosomes, while constitutively endocytosed receptors are efficiently returned to the plasma membrane by an iterative mechanism that recycles membrane components by default (53) or preferential exit into recycling vesicles (54) .

Moreover, recent studies suggest that GPCRs can be sorted to distinct destinations after endocytosis by the same membrane mechanism. Elegant studies demonstrating that a dominant-negative mutant form of dynamin inhibits both agonist-induced sequestration and downregulation of the B2AR in HEK293 cells suggest that endocytosis of receptors by clathrin-coated pits is an obligate first step common to membrane pathways leading to recycling endosomes and lysosomes (55). However, since the rate of B2AR downregulation is typically very slow relative to the rate of endocytosis by clathrin-coated pits, it is possible that the degradation responsible for B2AR downregulation is simply a consequence of "leakage" from endosomes rather than an active sorting event. Therefore, it still remains to be determined how receptors with completely opposite fates are sorted.

In Chapter 2 of this thesis, I present data demonstrating that two GPCRs, the B2AR and DOR, have different fates after endocytosis. These receptors have been

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previously shown to internalize via the same mechanism in HEK293 cells, thus allowing us to conclude that sorting occurs in the endosome.

Molecular Mechanisms of GPCR Sorting to Lysosomes

In addition to understanding what determines the specificity of GPCR trafficking, the functional significance of GPCRs makes it imperative to understand the precise molecular mechanisms directing receptors towards lysosomes and recycling endosomes. A recent study of B2AR trafficking in mammalian cells has proposed an endocytic sorting mechanism that promotes receptor recycling. This mechanism requires a PDZ-domain mediated interaction of the receptor with NHERF/EBP50 family proteins as well as additional protein interactions with the cortical actin cytoskeleton (56). However, to our knowledge, no previous studies have elucidated the membrane mechanisms by which GPCRs are targeted to lysosomes.

Considerable study of the membrane trafficking of epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK) that is rapidly degraded after activation, and lysosomally directed ligands provide some ideas as to how GPCRs may be targeted to lysosomes. Studies of constitutively recycling nutrient receptors indicate that lysosomally directed ligands accumulate in endocytic vesicles after constitutive endocytosis. These pre-lysosomal compartments are proposed to arise either via gradual maturation of the early endosome or by formation of a distinct membrane compartment (52,57). Studies of the EGFR indicate that it is physically retained in a maturing multivesicular body (MVB) membrane compartment after internalization (58). In fact, there is some suggestion that lysosomal trafficking of GPCRs involves the

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formation of multivesicular endocytic intermediates similar to those involved in targeting the EGFR to lysosomes. Antibody-labeled B2AR have been observed in lumenal membranes of MVBs (8). Furthermore, analogous endocytic carrier vesicles have been proposed to function in the trafficking of Ste2p, a yeast pheromone GPCR that trafficks to the vacuole, the yeast equivalent of a lysosome (59).

Chapter 2 of this thesis provides additional insights into the precise steps of the sorting process to lysosomes. Our studies suggest that, in contrast to a model where lysosomally directed receptors are retained in a MVB (i.e. the EGFR) that remains accessible to endocytosed transferrin, internalized DOR is rapidly segregated into a distinct population of transferrin-inaccessible endocytic membranes. Interestingly, it appears that DOR is stably retained in these inaccessible vesicles for a prolonged period of time before targeting to lysosomes.

Role of Phosphorylation in Lysosomal Targeting of GPCRs

Phosphorylation has been previously established to regulate other GPCR trafficking events, in particular, in the early and recycling endocytic pathway. Rapid endocytosis of GPCRs is promoted by phosphorylation of agonist-activated receptors and an arrestin-dependent mechanism that links activated receptors to clathrin-coated pits (60,61). Studies of the B2AR and DOR using phosphatase inhibitors conclude that receptor dephosphorylation is required for recycling (10,62). Two recent studies identify putative mechanisms for this requirement. Persistent phosphorylation of specific residues located in the cytoplasmic tail of the V2 vasopressin receptor appears to retain internalized receptors in endocytic membranes by preventing dissociation of

receptor-arrestin complexes (13,41), while study of the B2AR has identified a kinase-regulatable PDZ domain-mediated interaction that promotes recycling (56). In contrast to the well established functions of phosphorylation in promoting endocytosis and inhibiting recycling of GPCRs, the possible role of phosphorylation in controlling sorting of internalized receptors to lysosomes is not well understood.

The most compelling, albeit indirect, evidence for phosphorylation playing a role in lysosomal targeting of GPCRs comes from studies of downregulation. Several early studies of B2AR signaling have examined the potential role of phosphorylation in downregulation. Stimulation of receptor downregulation kinetics in the presence of cAMP analogs first suggested that PKA phosphorylation promotes receptor degradation (47). This idea was supported by evidence that mutation of PKA sites inhibits downregulation (47). However, phosphorylation does not appear to be required for downregulation, as a receptor lacking agonist-induced phosphorylation still downregulates normally (48). Similar conclusions were obtained from study of a receptor lacking all PKA and GRK sites (63) and PKA-deficient cells (64).

Although the precise mechanisms of opioid receptor regulation are likely to differ in some aspects from the B2AR, the results presented in Chapter 3 suggest that, at least with regards to the requirement for receptor phosphorylation during downregulation, a similar story applies. We find that a truncated mutant DOR which remains unphosphorylated following agonist treatment nevertheless is still able to undergo downregulation via lysosomal targeting.

Summary

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In summary, this thesis attempts to elucidate the mechanisms by which GPCRs are sorted after endocytosis. Specifically, I focus on understanding how receptors are sorted to lysosomes. All studies were conducted with HEK293 cells stably transfected with epitope-tagged receptor. Chapter 2 describes the identification of a GPCR, the DOR, which is rapidly proteolyzed after endocytosis and details aspects of the mechanism by which these receptors are sorted to lysosomes. It also serves as an introduction to several quantitative techniques developed by our laboratory to examine the trafficking of GPCRs. Chapter 3 describes a receptor which is not phosphorylated in response to agonist treatment, yet is still rapidly targeted to lysosomes. The Appendix contains a review of past and current research on the multiple mechanisms contributing to receptor downregulation.

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CHAPTER 2

Type-specific Sorting of G Protein-coupled Receptors after

Endocytosis

Type-specific Sorting of G Protein-coupled Receptors after Endocytosis*

Patricia I. Tsao‡ and Mark von Zastrow§

From the Program in Cell Biology, Departments of Biochemistry and Biophysics, Psychiatry,
and Cellular and Molecular Pharmacology, University of California, San Francisco, California 94143-0984

The β_2-adrenergic receptor (B2AR) and δ-opioid receptor (DOR) are structurally distinct G protein-coupled receptors (GPCRs) that undergo rapid, agonist-induced internalization by clathrin-coated pits. We have observed that these receptors differ substantially in their membrane trafficking after endocytosis. B2AR expressed in stably transfected HEK293 cells exhibits negligible (<10%) down-regulation after continuous incubation of cells with agonist for 3 h, as assessed both by radioligand binding (to detect functional receptors) and immunoblotting (to detect total receptor protein). In contrast, DOR exhibits substantial (≥50%) agonist-induced down-regulation when examined by similar means. Degradation of internalized DOR is sensitive to inhibitors of lysosomal proteolysis. Flow cytometric and surface biotinylation assays indicate that differential sorting of B2AR and DOR between distinct recycling and non-recycling pathways (respectively) can be detected within ~10 min after endocytosis, significantly before the onset of detectable proteolytic degradation of receptors (~60 min after endocytosis). Studies using pulsatile application of agonist suggest that after this sorting event occurs, later steps of membrane transport leading to lysosomal degradation of receptors do not require the continued presence of agonist in the culture medium. These observations establish that distinct GPCRs differ significantly in endocytic membrane trafficking after internalization by the same membrane mechanism, and they suggest a mechanism by which brief application of agonist can induce substantial down-regulation of receptors.

Agonist-induced endocytosis of G protein-coupled receptors (GPCRs)[1] plays multiple roles in the physiological regulation of signal transduction. Endocytosis of receptors is a process that can contribute to functional resensitization of signal transduction by promoting dephosphorylation and recycling of receptors to the plasma membrane (1, 2). Endocytosis also contributes to down-regulation of receptors, a process that leads to functional

[1] The abbreviations used are: GPCR, G protein-coupled receptor; B2AR, β_2-adrenergic receptor; DOR, δ-opioid receptor; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; PNGase, peptide N-glycosidase; HA, hemagglutinin; HEK, human embryonic kidney; DADLE, [D-Ala², D-Leu⁵]enkephalin.

desensitization of signal transduction by reducing the number of receptors present in the plasma membrane and promoting degradation of receptors in lysosomes (3–6). These processes of receptor regulation are thought to involve membrane trafficking of receptors via distinct recycling or degradative pathways and can mediate opposite effects on the regulation of functional signal transduction (1, 2, 7). Consequently, understanding mechanisms that direct GPCRs to distinct membrane pathways is of fundamental physiological importance. Although a great deal has been learned about the mechanism mediating the initial endocytosis of certain GPCRs from the plasma membrane, relatively little is known about mechanisms that determine the specificity of GPCR trafficking after endocytosis.

Sequestration and down-regulation of the B2AR are differentially affected by pharmacological manipulations and selectively perturbed by receptor mutation, suggesting that these processes are mediated by separate endocytic mechanisms (8–10). Indeed, previous studies provide evidence for endocytosis of receptors by various membrane structures, including clathrin-coated pits (11, 12), noncoated membrane invaginations (6, 13), and caveolae (14). However, the ability of dominant-negative mutant dynamin to inhibit down-regulation of the B2AR suggests that the endocytic pathway mediating rapid internalization and recycling of receptors may also contribute to a slower process of receptor down-regulation (15).

It has also been shown that structurally distinct GPCRs can differ significantly in their endocytic trafficking. For example, substance P and thrombin receptors are differentially targeted to distinct recycling and degradative pathways, respectively (16, 17). However, in this case it is not known whether these GPCRs are endocytosed by the same or different membrane mechanism(s). Indeed, structurally homologous receptors can be endocytosed by distinguishable membrane mechanisms (18–21) and packaged into distinct primary endocytic vesicles (20).

Thus fundamental questions remain about the relationship between the membrane pathways that target GPCRs to distinct recycling or degradative fates. First, in addition to its role in degradation of a limited fraction of receptors, can rapid endocytosis of GPCRs via clathrin-coated pits serve as a major pathway targeting certain GPCRs to lysosomes? Second, if this is true, are distinct GPCRs sorted to different membrane pathways after endocytosis by the same endocytic vesicles? Third, does lysosomal degradation of internalized receptors require the continued presence of agonist in the culture medium?

We have addressed these questions by comparing the endocytic membrane trafficking of epitope-tagged B2AR and δ-opioid receptors (DOR). Both of these receptors undergo rapid, agonist-induced endocytosis by a conserved, β-arrestin and dynamin-dependent mechanism mediated by clathrin-coated pits (11, 12, 22–25). Here we demonstrate that, despite the similar-

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ity in their mechanism of endocytosis, B2AR and DOR differ significantly in membrane trafficking after internalization.

EXPERIMENTAL PROCEDURES
Cell Culture and Transfection

Human embryonic kidney cells (HEK293) cells were maintained and passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin (University of California, San Francisco Cell Culture Facility). cDNAs encoding wild type human B2AR (26) and murine DOR (27) possessing a FLAG or HA epitope in the amino-terminal extracellular domain (28, 29) were ligated into pcDNA3.0 (Invitrogen) and introduced into HEK293 cells (American Type Culture Collection) by calcium phosphate coprecipitation (28–30). FLAGB2AR (31), FLAGDOR (32), and HADOR (22) cDNAs, constructed as described previously, were subcloned into pcDNA3.0 (Invitrogen). Stably transfected cells were selected in 500 μg/ml geneticin (Life Technologies, Inc.), and clones expressing similar numbers of FLAG-tagged B2AR (B2AR 3) or FLAG-tagged DOR (DOR 5) were identified by fluorescence flow cytometry (23) and used for further study. B2AR 3 expressed the FLAG-tagged B2AR at 2.3 pmol/mg protein, as estimated by radioligand binding using [³H]dihydroalprenolol. DOR 5 expressed FLAG-tagged DOR at 0.8 pmol/mg protein, as estimated by radioligand binding using [³H]diprenorphine (see below for methods) A stably transfected clone of HEK293 cells expressing both the FLAG-tagged B2AR and HA-tagged DOR (B2DOR 1) was generated by cotransfecting cells with both constructs and selecting as above for expression of both receptors. These cells express FLAG-tagged B2AR at 0.7 pmol/mg and HA-tagged DOR at 0.2 pmol/mg.

Radioligand Binding Assay

Analysis of Receptor Number in a Crude Membrane Fraction—Cell monolayers were lifted with PBS supplemented with 2 mM EDTA, washed twice with PBS by centrifugation (200 × g for 5 min), and lysed in 10 mM Tris-Cl, 2 mM EDTA, pH 7.4, containing a protease inhibitor mixture (leupeptin, aprotinin, pepstatin, and phenylmethylsulfonyl fluoride) followed by four passes using a tight-fitting Dounce homogenizer. Large particulates and nuclear material were removed by centrifugation at 500 × g for 5 min, and a crude membrane and cytosol fraction was isolated. Binding assays were conducted in 120 μl of 25 mM Tris-Cl, 1 mM EDTA, pH 7.4 Assay tubes contained 50–100 μg of the crude membrane preparation (determined by the method of Bradford *et al.* (33) using reagents from Bio-Rad) and 2 nM [³H]diprenorphine (opioid binding) or 10 nM [³H]alprenolol (adrenergic binding) and were incubated for 30 min at room temperature. Incubations were terminated by vacuum filtration through glass fiber filters (Packard Instruments) and repeated washes with ice-cold Tris-buffered saline, pH 7.4. Bound radioactivity was determined by scintillation counting (Scintiverse, Fisher) using a Beckman LS 6500 instrument. Bound counts represented ≤10% of input radioligand. Nonspecific binding, defined by assays conducted in the presence of 10 μM naloxone (opioid binding) or alprenolol (adrenergic binding), was ≤10% of total counts isolated on filters. All assays were conducted in triplicate with similar results. Results are expressed as mean picomoles of radioligand specifically bound per mg of crude membrane preparation assayed.

Assay of Receptor Down-regulation in Intact Cells—Agonist-induced down-regulation of receptors was assayed in intact cells using a previously described method (34). Briefly, monolayers of cells expressing FLAG-tagged B2AR (B2AR 3) or DOR (DOR 5) were incubated for 3 h at 37 °C in the absence or presence of 10 μM isoproterenol or 10 μM DADLE (Research Biochemicals), respectively. To ensure a saturating concentration of peptide agonist over the incubation period, monolayers incubated with DADLE were supplemented with fresh peptide every hour during the incubation. At the end of the incubation, cells were lifted with PBS supplemented with EDTA and washed four times by centrifugation with 10 ml of warm (37 °C) PBS. Then cells were washed once by centrifugation in 10 ml of Krebs-Ringer HEPES buffer (KHRB: 110 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, pH 7.3). Radioligand binding was carried out in 120 μl of KHRB containing equal amounts of washed cells (50–100 μg of protein) and ligand concentrations as above. Incubations were carried out for 30 min at room temperature, and cells were harvested and washed using vacuum filtration on glass fiber filters as above. For all determinations, bound radioligand represented ≤10% of total radioligand present in the incubation, and nonspecific binding (defined as above) was ≤10% of counts isolated on glass fiber filters.

Immunoblotting

Monolayers of cells expressing FLAG-tagged B2AR (B2AR 3) or DOR (DOR 5) were incubated in the absence or presence of the appropriate agonist (as indicated in the text). For experiments using inhibitors of lysosomal proteolysis, monolayers were preincubated for 1 h at 37 °C with 100 μg/μl leupeptin (Calbiochem), 200 μM chloroquine (Sigma), or 50 mM ammonium chloride (Sigma) before agonist addition, and these reagents were present in the medium during agonist incubation. Equal amounts of cell lysate (prepared by extracting monolayers with 0.1% Triton X-100 (Sigma)) were resolved by SDS-PAGE (10% acrylamide), transferred to nitrocellulose membranes, and blotted for FLAG-tagged receptor using M1 monoclonal antibody (Sigma) followed by detection using horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) and ECL (Amersham Pharmacia Biotech). Immunoblots were quantitated by densitometric scanning of films exposed in the linear range.

Biochemical Analysis of Receptor Degradation Using Noncleavable Biotin

Proteolytic degradation of surface-biotinylated receptors was estimated using a previously described protocol (20). Briefly, stably transfected cells expressing FLAG-tagged B2AR or DOR were surface-biotinylated by incubation at 4 °C with 30 mg/ml sulfo-NHS-biotin (Pierce), rinsed with Tris-buffered saline to quench the biotinylation reaction, warmed to 37 °C, and incubated under various conditions as described in the text, and then chilled on ice to stop further membrane trafficking. Cells were extracted and receptor immunoprecipitates were prepared, and biotinylated receptor protein recovered in immunoprecipitates was detected by streptavidin overlay. Enzymatic deglycosylation was performed by incubating receptor immunoprecipitates in the presence of PNGase F (Roche Molecular Biochemicals) for 60 min at 37 °C Receptor degradation was indicated by a loss of biotinylated protein recovered in immunoprecipitates and was quantitated by densitometric scanning of streptavidin overlays exposed in the linear range. Samples representing equal numbers of cells were loaded in each lane.

Quantitation of Receptor Recycling by Fluorescence Flow Cytometry

Surface Recovery Assay—Recycling of epitope-tagged receptors back to the plasma membrane was estimated by assaying the recovery of immunoreactive receptors accessible at the cell surface to monoclonal antibody recognizing the extracellular epitope tag. This assay is a variant of a previously described flow cytometric assay for estimating receptor internalization (35). Briefly, cell monolayers expressing FLAG-tagged B2AR or DOR were incubated in the presence of 10 μM of the appropriate agonist (isoproterenol or etorphine, respectively) for 30 min at 37 °C to drive agonist-induced internalization to steady-state levels (23, 29), then rinsed with ice-cold PBS, and subsequently incubated at 37 °C in the presence of the appropriate antagonist (10 μM alprenolol or naloxone (Research Biochemicals)) to block additional endocytosis of receptors. At the indicated time points, monolayers were chilled on ice to stop membrane trafficking, and cells were lifted with a protease-free Cell Dissociation Buffer (Life Technologies, Inc.). Resuspended cells were then incubated at 4 °C for 60 min in the presence of 10 μg/ml M1 anti-FLAG antibody (Eastman Kodak Co.) that had been conjugated with fluorescein isothiocyanate (Molecular Probes) using standard methods, and receptor immunoreactivity was quantitated by fluorescence flow cytometry (FACScan, Becton Dickinson, Palo Alto, CA). Fluorescence intensity of 10,000 cells was collected for each sample. Cellquest software (Becton Dickinson) was used to calculate the mean fluorescence intensity of single cells in each population. All experiments were conducted ≥3 times with similar results The mean values for each experiment were averaged to obtain the overall mean fluorescence intensity and S.E. reported in the figure.

Loss of Internal Receptor Assay—Recycling of antibody-labeled receptors from the endocytic pathway was estimated using an alternate flow cytometric assay. FLAG-tagged B2AR present in the plasma membrane of stably transfected cells were specifically labeled with fluorescein-conjugated M1 anti-FLAG antibody (5 μg/ml), which binds the FLAG epitope in a calcium-dependent manner. Cell monolayers were incubated with 10 μM isoproterenol for 30 min at 37 °C to stimulate receptor internalization and then rinsed three times with calcium, magnesium-free PBS supplemented with 0.4% EDTA in order to elute antibody bound to residual receptors remaining in the plasma membrane and selectively label endocytosed receptors. At this point, different protocols were followed to measure recycling under different conditions. To measure recycling in the presence of antagonist, samples were incubated at

37 °C in DMEM in the presence of 10 μM alprenolol (to block additional endocytosis) and then chilled at the indicated time point to stop membrane trafficking. Cells were again rinsed three times at 4 °C with EDTA-supplemented PBS to elute antibody from antibody-labeled receptors that recycled to the cell surface during the incubation with antagonist. To measure recycling in the presence of agonist, samples were incubated at 37 °C in EDTA-supplemented PBS containing 10 μM isoproterenol. Under these conditions, antibody bound to receptors that recycle back to the plasma membrane was immediately eluted and was therefore not re-endocytosed in the presence of isoproterenol. Control experiments indicated that >95% of surface receptors were eluted within 1 min under these conditions. At the indicated time point, monolayers were again chilled to 4 °C, lifted, washed, and analyzed by flow cytometry (as above) to detect antibody bound to internalized receptors remaining within the cell.

Loss of Internal Receptor Measured Using Cleavable Biotin

Receptor recycling was measured biochemically by the loss of internalized receptor protein specifically labeled with disulfide-linked (cleavable) biotin. The assay is a variant of a previously described method using cleavable biotin to detect internalization (35). Briefly, stably transfected cells expressing B2AR or DOR were surface-biotinylated with 30 mg/ml sulfo-NHS-S-S biotin (Pierce) for 30 min at 4 °C and quenched by three washes with ice-cold TBS. At this point, samples were saved on ice to measure total surface-biotinylated receptor. The remaining samples were incubated with media containing the appropriate agonist (10 μM isoproterenol or etorphine) for either 30 or 10 min and then immediately chilled on ice to stop internalization. Samples used to measure internalization of receptor at this time point were set aside at 4 °C. Samples used to assay recycling were rinsed with PBS to remove residual agonist, rewarmed to 37 °C for 30 min in media containing the appropriate antagonist (10 μM alprenolol or naloxone), and then chilled to 4 °C to stop membrane trafficking. In order to estimate the amount of residual biotinylated receptor remaining in the endocytic pathway, monolayers were treated for 15 min at 4 °C with glutathione strip solution (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 10% fetal bovine serum in water) to cleave biotin groups from receptors accessible at the cell surface. Cells were then washed for 20 min at 4 °C with iodoacetamide buffer (50 mM iodoacetamide, 1% BSA in PBS, pH 7.4) to quench residual glutathione, extracted, immunoprecipitated, and processed for streptavidin overlay.

Immunocytochemical Staining and Fluorescence Microscopy

Dual Staining of Permeabilized Cells—Colocalization of FLAG-tagged B2AR and HA-tagged DOR expressed in a single cell line was examined using a modification of a previously described protocol for dual fluorescence immunohistochemical staining (20). Briefly, cells grown on glass coverslips (Corning) were treated with 10 μM isoproterenol and 10 μM etorphine (Research Biochemicals) for 60 min, washed, fixed with a 4% formaldehyde solution in PBS, and permeabilized in 0.1% Triton X-100 in Blotto (3% dry milk in TBS + 1 mM CaCl₂). Specimens were incubated with anti-FLAG M1 antibody (IgG2b, 5 μg/ml) and mouse monoclonal anti-HA antibody (HA.11, IgG1, 5 μg/ml, Berkeley Antibody Co.) for 30 min, washed, incubated with subtype-specific rabbit anti-mouse IgG2b antibody (0.5 μg/ml, Zymed Laboratories Inc.) to label the M1 antibody, washed again, and treated with 0.1% Triton X-100 in Blotto. Finally, the B2AR (labeled with M1 monoclonal and rabbit anti-mouse IgG2b) and the DOR (labeled with anti-HA mouse monoclonal) were visualized by incubating with Texas Red donkey anti-rabbit (5 μg/ml, Jackson ImmunoResearch) and fluorescein isothiocyanate subtype-specific anti-mouse IgG1 (2 μg/ml, Roche Molecular Biochemicals). 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 microscopy was performed using a Bio-Rad MRC1000 confocal microscope equipped with a Zeiss 100× NA1.3 objective. Negligible bleed through was confirmed in dual labeling experiments by imaging single-labeled control specimens. The estimated depth of optical sections under the confocal imaging conditions used was 0.5–1 μm.

Pulse-Chase of Receptors with Endocytosed Transferrin—An immunocytochemical "pulse-chase" assay was developed to estimate the degree to which a "pulse" of internalized B2AR or DOR was accessible to a subsequent "chase" of endocytosed transferrin. Briefly, stably transfected cells expressing either FLAG-tagged B2AR or DOR (grown on glass coverslips) were preincubated at 37 °C in serum-free DMEM; receptors were surface-labeled with 5 μg/ml M1 antibody, and cells

were incubated with 10 μM isoproterenol or etorphine for 30 min to drive endocytosis of antibody-labeled receptors. Next, cells were chilled on ice, rinsed with EDTA-supplemented PBS to elute antibody bound to residual receptors remaining in the plasma membrane, rewarmed to 37 °C for 15 min in serum-free media lacking agonist but containing Texas Red-conjugated diferric transferrin (50 μg/ml, Molecular Probes), and coverslips fixed with 4% formaldehyde in PBS. Cells were permeabilized using 0.1% Triton X-100 in Blotto (3% dry milk in TBS + 1 mM CaCl₂), and antibody-labeled B2AR or DOR was detected using fluorescein-conjugated donkey anti-mouse antibody (2 μg/ml, Jackson ImmunoResearch). Dual-label fluorescence microscopy was performed as described above.

RESULTS

B2AR and DOR Differ in Agonist-induced Down-regulation—As an initial step toward comparing the effects of agonist on numbers of B2AR and DOR present in cells, we used an established radioligand binding assay (34) to measure down-regulation of receptor binding activity following incubation of cells in the presence of agonist for 3 h. This time point was chosen because, whereas both B2AR and DOR have been previously shown to undergo substantial down-regulation after incubation of cells for 18–24 h in the continuous presence of agonist, down-regulation has also been observed for certain GPCRs at much shorter times (e.g. Refs. 5, 16, and 17). Consistent with previous studies (36), incubation of stably transfected cells expressing B2AR for 3 h with a saturating concentration (10 μM) of the adrenergic agonist isoproterenol caused little (<10%) down-regulation of receptor sites measured in whole cells (Fig. 1A). In contrast, incubation of cells expressing the FLAG-tagged DOR for 3 h in the presence of the opioid agonist DADLE revealed a substantial (greater than 50%) decrease in diprenorphine-binding sites detected under these conditions (Fig. 1A). The diprenorphine concentration used in this assay was near saturation, suggesting that this decrease reflects a change in the number of DOR-binding sites (34). Saturation binding analysis confirmed that DADLE pretreatment caused a profound decrease in B_{max} (Fig. 1B). A similar amount of down-regulation was observed with radioligand binding on membrane preparations of DOR-expressing cells treated with [D-Pen²,⁵]enkephalin.[2] Substantial down-regulation of functional DOR has been observed previously under similar conditions in studies conducted in neuroblastoma cells, where this process has been associated with proteolytic degradation of receptors in lysosomes (4, 37).

To determine whether the down-regulation of DOR detected by radioligand-binding sites was associated with proteolytic degradation of the receptor, whole-cell extracts were resolved by SDS-PAGE and immunoblotted using anti-FLAG monoclonal antibody recognizing the epitope-tagged receptor. Both the B2AR and DOR were specifically detected as strong immunoreactive bands in stably transfected cells (Fig. 1C, 1st and 3rd lanes). The specificity of this detection was confirmed by the negligible background immunoreactivity detected in untransfected cells not expressing epitope-tagged receptors (Fig. 1C, 2nd lane). Whereas both B2AR and DOR primarily resolved in these reducing gels at an apparent molecular mass consistent with a monomeric complex-glycosylated species (Fig. 1C), as described previously (29, 38), additional immunoreactive species were also observed in some experiments at higher apparent molecular mass, consistent with previously described oligomers of B2AR and DOR (39, 40). Incubation of cells with isoproterenol for 3 h caused little or no detectable change in amounts of B2AR protein detected by immunoblotting (Fig. 1D, 1st and 2nd lanes). Consistent with the down-regulation observed by radioligand binding, there was also a pronounced

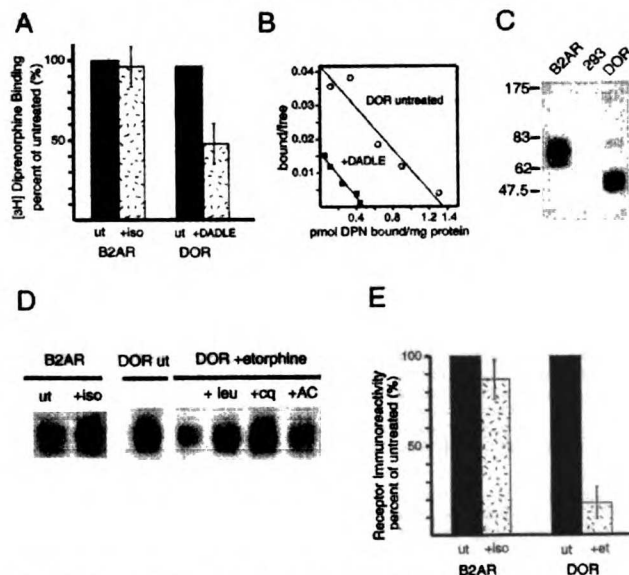[2] G. Pineyro, personal communication.

Fig. 1. **Agonist promotes substantial loss of DOR functional ligand-binding sites and total receptor protein.** Loss of ligand-binding sites and total receptor protein was measured by intact cell ligand binding assays and immunoblotting, respectively, performed on stably transfected cells expressing FLAG-tagged B2AR or DOR as described under "Experimental Procedures." *A*, cells were incubated in the absence (untreated, *ut*) or presence of 10 μM agonist (isoproterenol, *iso*, or DADLE) for 3 h. Ligand-binding sites were measured as bound tritium counts and expressed as picomoles of ligand specifically bound/mg of total cell protein. Results are plotted as percentage of total bound specific radioactivity in untreated cells. *Error bars* represent S.E. ($n = 3$ assays performed in triplicate). *B*, Scatchard analysis of saturation ligand binding to DOR-expressing cells incubated in the absence (*open circles*) or presence (*filled squares*) of DADLE for 3 h. Each data point represents the mean of triplicate determinations. *C*, immunoreactive bands resolving at the appropriate molecular mass of the complex-glycosylated B2AR and DOR were detectable in extracts from cells expressing epitope-tagged receptors but not from untransfected cells. The mobility of molecular mass standards (in kDa) is indicated to the *left*. *D*, receptor protein was detected on immunoblots loaded with equal amounts of extract. Cells were incubated in the absence (untreated, *ut*) or presence of 10 μM agonist (isoproterenol, *iso*, or etorphine, *et*) for 3 h. Studies using lysosomal protease inhibitors (leupeptin (*leu*), chloroquine (*cq*), or ammonium chloride (*AC*)) were performed by preincubation with inhibitors for 1 h at 37 °C and then treatment with agonist for 3 h in the continued presence of inhibitors. *E*, immunoblots were quantitated by densitometric scanning from multiple experiments ($n = 3$). Results are plotted as percentage of total receptor in untreated cells. *Error bars* represent S.D. of three experiments.

decrease in the amount of immunoreactive DOR protein following DADLE treatment (Fig. 1*D*, *3rd* and *4th lanes*). Interestingly, quantitation of multiple experiments by densitometric scanning indicates that the loss of immunoreactive DOR protein was even greater than the down-regulation of functional receptors detected by radioligand binding (Fig. 1*E*). This may reflect the existence of proteolytic receptor intermediates at this time point which bind ligand yet lack the FLAG epitope. Significant agonist-induced reduction in immunoreactive DOR was observed for the major band corresponding to the monomeric receptor protein (Fig. 1*D*), as well as for minor species of detectable DOR (not shown). Importantly, these differences between agonist-induced reduction in immunoreactive receptor protein were observed in cells expressing closely similar receptor numbers, including cells expressing DOR at lower numbers than B2AR (see "Experimental Procedures"). Further studies indicated that the agonist-induced reduction in immunoreactive DOR protein was highly sensitive to inhibitors of lysosomal proteolysis (Fig. 1*D*), confirming that this reduction represents proteolytic degradation of DOR and supporting pharmacological and immunocytochemical studies suggesting that internalized DOR traffic to lysosomes in neuroblastoma and neuro2a cells (34, 37). Taken together, these observations strongly suggest that internalized DOR are selectively targeted for relatively rapid degradation in lysosomes in HEK293 cells, in contrast to the much slower rate at which internalized B2AR are targeted to lysosomes in this cell type (3, 41).

B2AR and DOR Differ in Subcellular Localization after En-

docytosis—If internalized B2AR and DOR do indeed differ in endocytic trafficking, one would expect significant differences in the subcellular localization of receptors at some point after internalization. As an initial step toward testing this hypothesis, fluorescence microscopy was used to compare the subcellular localization of B2AR and DOR when coexpressed in the same stably transfected HEK293 cells (clone B2DOR 1, see "Experimental Procedures"). Previous studies have established that both B2AR and DOR internalize in HEK293 cells with a $t_{1/2}$ <10 min (23, 29). In addition, both receptors colocalize extensively with internalized transferrin receptors immediately (within 10–15 min) after endocytosis (12, 21–24). To compare receptor localization after more prolonged incubation with agonist, we performed confocal microscopy on cotransfected cells (B2DOR 1, see "Experimental Procedures") fixed after incubation with both agonists for 60 min. This time point was chosen because it is relatively long compared with the rate of receptor endocytosis and corresponds to the time at which proteolytic degradation of DOR is first detected biochemically (see below and Fig. 3*E*). Under these conditions, substantial differences were observed in the subcellular distribution of immunoreactive B2AR and DOR (Fig. 2, *A* and *B*, respectively). Whereas vesicles containing comparable amounts of immunoreactive B2AR and DOR were still observed at this time point (examples of colocalized structures are indicated by *solid arrows* in Fig. 2), we also observed numerous endocytic vesicles that were selectively enriched in DOR and contained little or no detectable B2AR (*e.g.* Fig. 2, *open arrows*). These differences are empha-
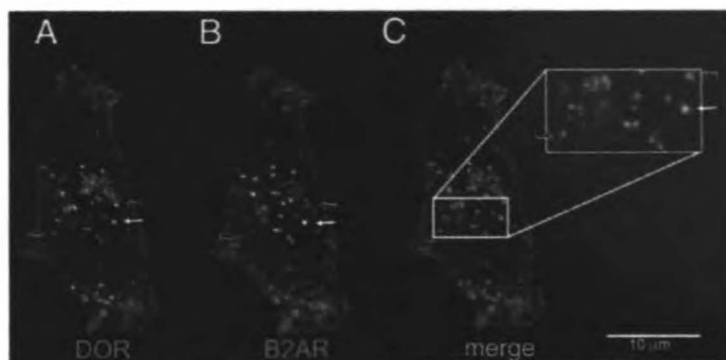
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FIG. 2. **Dual localization of B2AR and DOR by confocal microscopy.** Stably transfected cells coexpressing FLAG-tagged B2AR and HA-tagged DOR were treated with 10 μM isoproterenol and 10 μM etorphine for 60 min, fixed, and processed for dual localization of receptor immunoreactivity by confocal fluorescence microscopy as described under "Experimental Procedures." DOR immunoreactivity is displayed in *A*. B2AR immunoreactivity is displayed in *B*. Colocalization of DOR (*green*) and B2AR (*red*) is indicated in the merged image (*C*) by *yellow* staining. The *inset* represents a 2-fold magnification of the *boxed region*. *Solid arrows* indicate examples of vesicles containing comparable amounts of B2AR and DOR immunoreactivity. *Open arrows* indicate examples of vesicles relatively enriched in DOR immunoreactivity. *Bar*, 10 μm.

sized in the merged color image (Fig. 2C), in which colocalized structures appear *yellow* and membranes selectively enriched in DOR appear *green*.

B2AR and DOR Differ in Their Rate of Degradation after Agonist-induced Endocytosis—Our studies thus far address the effects of agonists on the total complement of B2AR and DOR detected in cells, including receptors present in the plasma membrane as well as various intracellular membranes. To examine specifically the trafficking of receptors from the plasma membrane, we used a biochemical method to label selectively receptors present on the cell surface. Intact cells were reacted with a membrane-impermeant biotinylation reagent (sulfo-NHS-biotin), which labels proteins exposed on the cell surface but not proteins present in intracellular membranes. After various manipulations, surface-labeled receptors were detected in receptor immunoprecipitates (prepared from whole-cell extracts) using streptavidin overlay. Whereas no detectable biotinylated receptor signal was observed in control immunoprecipitates prepared from untransfected cells, surface-biotinylated B2AR and DOR were readily detected in transfected cells (Fig. 3A). Both receptors resolved as a heterogeneous protein band by SDS-PAGE, consistent with the predominant receptor species detected by immunoblotting of whole-cell extracts. This heterogeneity resulted from complex N-linked glycosylation, as indicated by digestion to single band with the N-linked endoglycosidase PNGase F (Fig. 3B).

The fate of B2AR and DOR present initially in the plasma membrane was examined by determining the amount of biotinylated receptor protein recovered from surface-biotinylated cells after incubation under various conditions. Immunoprecipitations were conducted under conditions of antibody excess to ensure that the amount of biotinylated receptor protein isolated in immunoprecipitates provided a reliable measure of the relative amount of surface-labeled receptor protein present in the cell extracts. In the absence of agonist, surface-biotinylated B2AR exhibited little or no degradation for prolonged periods of time, as indicated by the uniformly high recovery of biotinylated B2AR at all time points examined. Moreover, B2AR was recovered efficiently from cells incubated in the prolonged presence of saturating concentrations of isoproterenol (Fig. 3B, *upper panels*). Two additional pieces of evidence support the remarkable biochemical stability of the B2AR in the presence of agonist. First, enzymatic cleavage of N-linked glycans using PNGase F (which is expected to provide a more

sensitive assay for small changes in the electrophoretic mobility of receptors that could result from partial proteolysis) failed to reveal any evidence for B2AR proteolysis, even after 3 h in the continuous presence of isoproterenol (Fig. 3B, *lower panels*). Second, immunoprecipitation of B2AR using an antibody recognizing the distal carboxyl terminus (29, 38) (rather than the proximal amino-terminal epitope tag sequence) also revealed no evidence for agonist-induced proteolysis of the B2AR (not shown).

The same experiments conducted on the DOR yielded markedly different results. Surface-biotinylated DOR, like B2AR, was highly stable in the absence of agonist. However, in the presence of the opiate agonist etorphine, the amount of biotinylated DOR isolated from cells was rapidly and dramatically reduced. Proteolytic degradation of labeled receptors was nearly complete after 3 h and readily detectable even without enzymatic deglycosylation of receptors (Fig. 3C). Quantitation of these results by scanning densitometry of streptavidin overlays confirmed that surface-labeled B2AR and DOR differ significantly in their biochemical stability (Fig. 3, *D* and *E*). These observations were also confirmed in cells coexpressing both B2AR and DOR (tagged selectively with HA and FLAG epitopes, respectively, not shown), further confirming that these differences reflect receptor-specific differences in proteolytic degradation following endocytosis. In addition, similar results for the DOR were obtained when cells were incubated with the the peptide agonist DADLE for 3 h (not shown).

Confirmation That Internalized B2ARs Recycle Efficiently to the Plasma Membrane in the Continuous Presence of Agonist— The dramatically different rates of agonist-induced degradation of surface-labeled B2AR and DOR, together with the effects of inhibitors of lysosomal proteolysis, suggest that receptors differ significantly in trafficking between recycling and lysosomal pathways after endocytosis. To examine this hypothesis in greater detail, we devised several assays to measure specifically the recycling of receptors to the plasma membrane after agonist-induced internalization to steady-state levels (*i.e.* 30 min agonist treatment (23, 29)).

Recycling of internalized B2AR was first estimated using fluorescence flow cytometry to measure the recovery of surface receptors in the plasma membrane after removal of agonist from the culture medium. Consistent with previous immunocytochemical and pharmacological studies (42–45), this "surface recovery" assay indicated that internalized B2AR under-
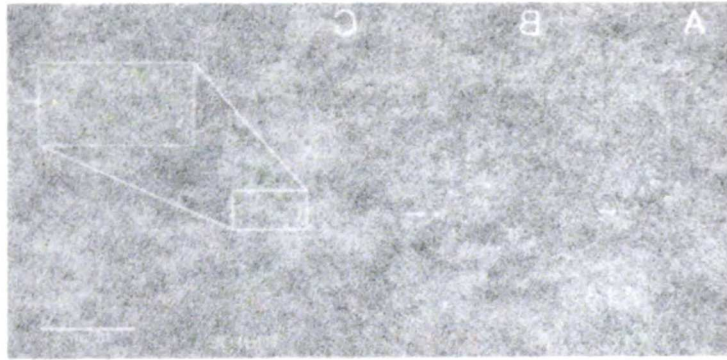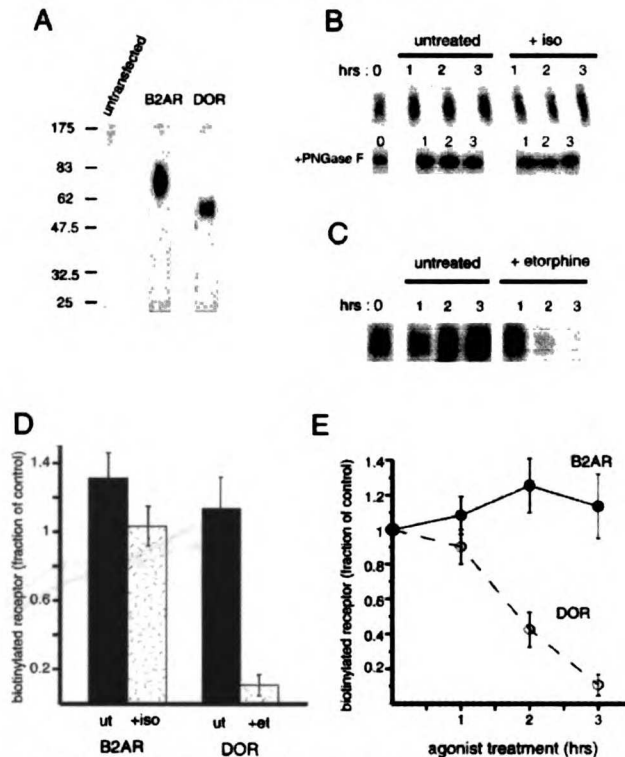
FIG. 3. **Agonist promotes rapid degradation of surface DOR but not B2AR.** Proteolytic degradation of surface-biotinylated receptors was measured as described under "Experimental Procedures." *A*, biotinylated protein bands resolving at the appropriate molecular mass of the complex-glycosylated B2AR and DOR were specifically recovered in immunoprecipitates prepared from cells expressing the epitope-tagged receptors but not from untransfected cells. The mobility of molecular mass standards (in kDa) is indicated to the *left*. *B* and *C* display biotinylated protein recovered from equal amounts of cells prepared after incubation of cells in the absence (untreated) or presence of the appropriate agonist for the indicated time. *B*, under all conditions examined, B2AR was recovered in similar amounts as present in cells lysed immediately after biotinylation (*hrs*). Comparable amounts of B2AR were also observed after enzymatic deglycosylation (PNGase F, *lower lanes*). *C*, in cells incubated in the absence of agonist, DOR was recovered in similar amounts as present in cells lysed immediately after biotinylation (*hrs: 0*). In cells incubated in the presence of agonist, a significant reduction in the amount of biotinylated receptor protein was observed. *D* and *E*, biotinylated receptor protein was quantitated by densitometric scanning of streptavidin overlays from multiple experiments ($n \geq 3$). Results are plotted as the mean recovery of biotinylated receptor protein (relative to that isolated at $t = 0$). *Error bars* represent S.D. of three experiments. *D* represents relative amount of biotinylated B2AR and DOR recovered after incubation of cells for 3 h in the absence (*solid bar*) or presence (*stippled bar*) of the agonist. *E*, displays a time course of the amounts of biotinylated receptor recovered from agonist-treated cells.

goes rapid recycling to the plasma membrane following removal of agonist (Fig. 4A). After a brief lag, approximately 50% of internalized B2AR recycled within 15 min, and nearly complete recycling was observed within 30 min. Rapid recovery of surface B2AR was observed even in cells incubated in the presence of the protein synthesis inhibitor cycloheximide (200 μM), indicating that this recovery resulted from receptor recycling rather than from the biosynthesis of new receptor protein.

The biochemical stability of B2AR in the continuous presence of saturating concentrations of agonist (Figs. 1 and 3) as well as previous studies by others (42–44) predict that the B2AR recycles efficiently to the plasma under these conditions. To confirm this, we assayed the efflux of antibody-labeled receptors from endocytic vesicles in cells incubated in calcium-depleted medium. Antibody attached to receptors accessible at the cell surface is efficiently dissociated within seconds in this medium, allowing recycling of receptors pre-labeled with the fluorochrome-conjugated antibody to be monitored directly using fluorescence flow cytometry. In the absence of agonist, recycling of antibody-labeled B2AR was readily observed (Fig. 4B, *open*

circles and *solid line*) and occurred with similarly rapid kinetics as recycling of receptors estimated by the surface recovery assay (Fig. 4A). Moreover, antibody-labeled receptors returned to the plasma membrane at a similarly rapid rate in the continuous presence of isoproterenol (Fig. 4B, *closed triangle* and *dashed line*), further validating the flow cytometric assay and directly confirming the ability of internalized B2AR to recycle rapidly even in the continuous presence of agonist.

Internalized DOR Is Selectively Retained at an Early Stage in the Endocytic Pathway—In contrast to the rapid rate and complete extent of recycling of the B2AR (Fig. 5A, *closed circles* and *dotted line*), the surface recovery assay indicated that internalized DOR returned to the plasma membrane to a significantly smaller extent (<50%), even after incubation of cells in the presence of antagonist for 45 min (Fig. 5A, *open circles* and *solid line*). We typically conducted these experiments by adding the antagonist naloxone to the culture medium after agonist washout. This was done to block possible effects of residual agonist that may remain associated with cells after agonist washout (46). We do not believe that the failure of DOR to
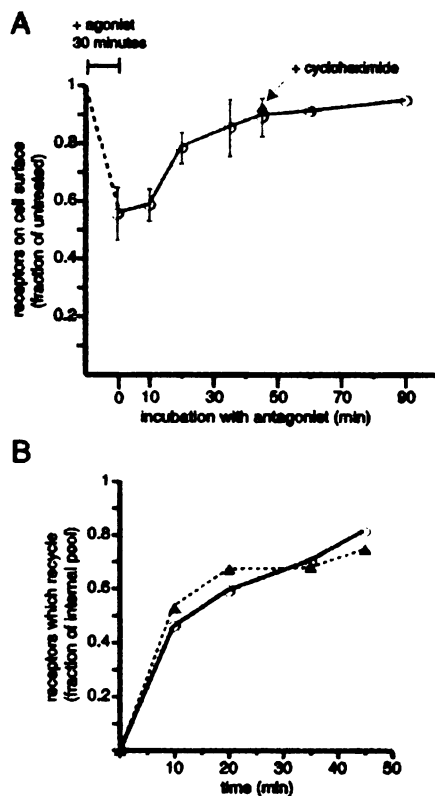
FIG. 4. **Internalized B2AR recycles rapidly and efficiently to the plasma membrane.** Two fluorescence flow cytometric assays were used to estimate recycling of internalized B2AR under various conditions. *A*, represents the surface recovery assay. Cells were incubated with 10 μM isoproterenol for 30 min (indicated by *dotted line*), washed, and then incubated with 10 μM alprenolol for the indicated times to stop further endocytosis. Receptors present in the plasma membrane at each time point were labeled with fluorescein-conjugated M1 antibody and quantitated by flow cytometry, as described under "Experimental Procedures." Data (*open circles*) indicate the mean surface receptor fluorescence of cells (relative to surface fluorescence of cells not exposed to agonist). *Error bars* represent S.E. of mean fluorescence data collected from multiple experiments ($n \geq 4$). Some experiments were performed in the presence of 200 μM cycloheximide to exclude the possible contribution of new receptor synthesis (*closed triangle*). *B*, represents the "loss of internal receptor" assay. Internalized B2AR was specifically labeled with monoclonal antibody and the loss of cell-associated antibody (which indicates recycling of the receptor protein) was measured by flow cytometry, as described under "Experimental Procedures." *Closed triangles* indicate efflux of internalized receptors observed from cells incubated in the absence of agonist, and *open circles* represent receptor efflux in cells incubated in the presence of 10 μM isoproterenol.

FIG. 5. **Inefficient recycling and selective endocytic retention of internalized DOR.** *A*, the surface recovery assay was used to estimate recycling of receptors following agonist-induced internalization. *Open circles* (*solid line*) represent recovery of immunoreactive DOR in the plasma membrane. *Error bars* represent S.E. calculated from the mean fluorescence of cells in multiple experiments ($n = 3$). *Closed circles* (*dashed line*) represent surface recovery of B2AR measured by the same assay (see Fig. 4A). *B–D*, receptor recycling was measured biochemically by the loss of internalized receptor protein specifically labeled with disulfide-linked (cleavable) biotin, as described under "Experimental Procedures." Control experiments (*B*) display surface-biotinylated B2AR or DOR detected by streptavidin overlay (*lanes 1* and *3*). Surface-biotinylated receptors were completely cleaved by glutathione (*lanes 2* and *4*), confirming that any glutathione-resistant signal represents internalized receptor protein that is inaccessible to added glutathione. *C*, a strong signal of internalized B2AR was observed in cells incubated with 10 μM isoproterenol for 30 min (*iso = 30*). Additional incubation of agonist-treated cells for 30 min in the presence of 10 μM alprenolol (*iso → alp*) caused the complete disappearance of internalized B2AR. *D*, the identical experiment performed on cells expressing DOR (*left lanes*) indicated that a significant fraction of biotinylated DOR remained internalized after incubation for 30 min with 10 μM naloxone. Significant retention of internalized DOR was observed even in cells incubated with etorphine for only 10 min and subsequently incubated in the presence of naloxone for 30 min (*right lanes*). The results shown are representative of three independently conducted experiments.

recycle is due to the presence of antagonist binding as naloxone induces neither detectable endocytosis (not shown) nor proteolytic degradation (see below and Fig. 6B, *lane e*) of DOR.

To determine whether the failure of internalized DOR to recycle reflects a *bona fide* sorting event or is simply a consequence of proteolytic degradation of the receptor, we devised a biochemical assay to analyze DOR retained in cells after agonist-induced endocytosis. Cells expressing B2AR or DOR were surface-biotinylated at 4 °C using "cleavable" sulfo-NHS-S-S-biotin, and endocytosis of receptors was induced by incubating cells with the appropriate agonist at 37 °C for 30 min. Cells
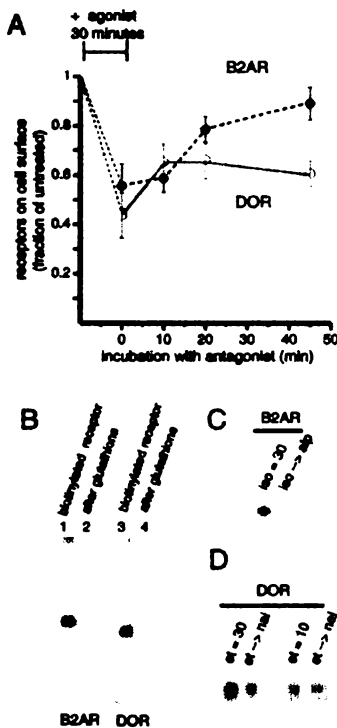
were then washed at 4 °C in the absence of agonist and subsequently incubated at 37 °C in the presence of the appropriate antagonist for 30 min, in order to block additional endocytosis of surface-biotinylated receptors and to allow sufficient time for "maximal" recycling of receptors to occur (Fig. 5A). Following this incubation, cells were incubated at 4 °C in the presence of a membrane-impermeant reducing agent that cleaves biotinylated receptors present in the plasma membrane (Fig. 5B, *lanes 1–4*). Under these conditions, only those receptors that were internalized from the cell surface and failed to recycle in the
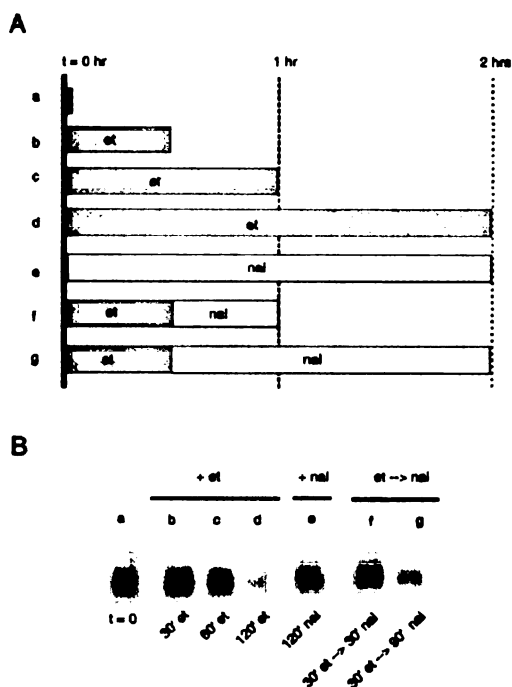
A

B

FIG. 6. **Degradation of the retained pool of DOR does not require the continuous presence of agonist.** Degradation of biotinylated receptors was examined under various conditions to determine the ligand dependence of this process. *A* outlines the experimental protocol. *B* displays biotinylated DOR detected by streptavidin overlay under each of the following conditions: *a*, untreated; *b*, treated with 10 μM etorphine (*et*) for 30 min; *c*, treated with etorphine for 1 h; *d*, treated with 10 μM etorphine for 2 h; *e*, treated with 10 μM naloxone (*nal*) for 2 h; *f*, treated with 10 μM etorphine for 30 min, washed, and then treated with 10 μM naloxone for 30 min; *g*, treated with 10 μM etorphine for 30 min, washed, and then treated with 10 μM naloxone for 1.5 h.

presence of antagonist remained biotinylated. Essentially no residual biotinylated B2AR was detected in the endocytic pathway under these conditions (Fig. 5C) whereas, in marked contrast, a substantial amount of biotinylated DOR failed to recycle (Fig. 5D, *1st* and *2nd lanes*). Furthermore, residual biotinylated DOR detected under these conditions resolved with an electrophoretic mobility indistinguishable from that of the full-length receptor protein, suggesting that internalized DOR is retained in endocytic pathway without any detectable proteolytic degradation. Moreover, although it was difficult to quantitate precisely the fraction of retained DOR using this complex assay, a significant amount of internalized DOR was reproducibly retained within the endocytic pathway, even after preincubation of cells for as little as 10 min with agonist (Fig. 5D, *3rd* and *4th lanes*). These observations, which were confirmed in three separate experiments, strongly suggest that intracellular retention of rapidly internalized DOR can be distinguished from, and significantly precedes, the proteolytic degradation of receptors observed after more prolonged incubation of cells with agonist.

Later Stages of Membrane Trafficking Leading to Proteolytic Degradation of Retained DOR Do Not Require the Continuous Presence of Agonist—We next examined the fate of internalized DOR after removal of agonist from the culture medium. Based on recent studies of the V2 vasopressin receptor (47, 48), one

might expect internalized DOR to remain in the endocytic pathway for a prolonged period of time without proteolytic degradation. However, since the DOR is rapidly proteolyzed in the continuous presence of agonist, it is possible that internalized DOR is delivered to lysosomes and degraded, even after removal of agonist. To distinguish between these possibilities, we used a pulse-chase protocol (Fig. 6A) in which surface-biotinylated cells were incubated for 30 min with agonist (pulse) to induce substantial internalization of biotinylated DOR without causing detectable proteolysis (Fig. 3) and then incubated for an additional 90 min under various conditions (chase). Degradation of DOR was estimated by the recovery of biotinylated receptor protein in immunoprecipitates (Fig. 6B).

In cells incubated in the continuous presence of etorphine (pulse and chase), pronounced degradation of DOR occurred over the 2-h time course (Fig. 6B, *lanes a–d*), fully consistent with the agonist-induced degradation of receptors observed previously (Fig. 3). In contrast, no detectable degradation of DOR was observed after incubation of cells for this time in the continuous presence of the opiate antagonist naloxone (Fig. 6B, *lane e*). However, in cells pulsed with etorphine for 30 min followed by agonist washout and chase incubation in the presence of naloxone (which completely blocks receptor-mediated inhibition of adenylyl cyclase in intact cells, not shown), significant proteolytic degradation of biotinylated DOR was observed after an initial lag period of approximately 30 min (Fig. 6B, *lanes f* and *g*). These results further confirm that internalized DOR fail to recycle to the plasma membrane following agonist removal, and they indicate that, in marked contrast to the stability of internalized V2 receptors shown previously (47, 48), internalized DOR undergo substantial proteolytic degradation even after removal of agonist from the culture medium.

Visualization of the Endocytic Trafficking of Surface-labeled Receptors by Fluorescence Microscopy—Internalized DOR could fail to recycle because they remain physically retained in the same early endocytic compartment through which other membrane proteins rapidly recycle, as has been suggested to occur for internalized epidermal growth factor receptors (49). Alternatively, it is possible that internalized DOR are rapidly segregated out of the clathrin-mediated early endocytic pathway and delivered to a distinct population of endocytic vesicles that do not recycle. To begin to examine these hypotheses, we used an immunocytochemical pulse-chase assay to estimate the degree to which internalized B2AR or DOR remains associated with endocytic vesicles that can be labeled with Texas Red-labeled transferrin, a well established marker of early and recycling endosomes that mediate rapid recycling (50, 51).

FLAG-tagged B2AR or DOR present in the plasma membrane was specifically labeled by incubating intact cells with monoclonal antibody, and then agonist was added to cells to initiate a 30-min pulse of endocytosis. Cells were chilled to 4 °C, and antibodies bound to receptors remaining in the plasma membrane were eluted, in order to label selectively the newly internalized receptors. Washed cells were then warmed to 37 °C in the absence of agonist and chased for an additional 15 min in the presence of labeled transferrin, conditions which label both early and recycling endosomes (50). The extent of colocalization between the pulse of internalized B2AR or DOR and the endocytic tracer was examined using dual-label confocal microscopy.

Many vesicles containing internalized B2AR colocalized with endocytosed transferrin, (Fig. 7A, colocalization is indicated by the numerous *yellow* structures observed in the *merge panel*), consistent with the rapid recycling of internalized B2AR via early and recycling endosomes (29). However, the same experiment conducted with internalized DOR yielded significantly

FIG. 7. **Pulse-chase analysis of internalized B2AR and DOR relative to endocytosed transferrin.** Confocal fluorescence microscopy of double-labeled cells stably expressing B2AR (*A*) or DOR (*B*) was used to examine colocalization between a 30-min pulse of antibody-labeled receptor (*green*) followed by a 15-min chase with transferrin (*red*) in the absence of agonist, as described under "Experimental Procedures." A representative region of labeled cells is indicated in each panel. Extensive overlap between an internalized pulse of B2AR (*A*) with the transferrin chase is indicated by the numerous *yellow* vesicular structures visualized in the merged color image (*A*), whereas vesicles containing internalized B2AR without detectable labeled transferrin were observed relatively rarely (*e.g. arrow*). In contrast, in the same experiment conducted using DOR-expressing cells (*B*), we observed numerous DOR-enriched vesicles that contained no detectable labeled transferrin (*arrows*). These observations are quantitated in *C*, which indicates the mean fraction of receptor-containing endocytic vesicles that also contained labeled transferrin. *Error bars* represent the S.D. of these data collected from multiple cells (*n* = 6) examined in coded specimens. *Bar*, 5 μm.
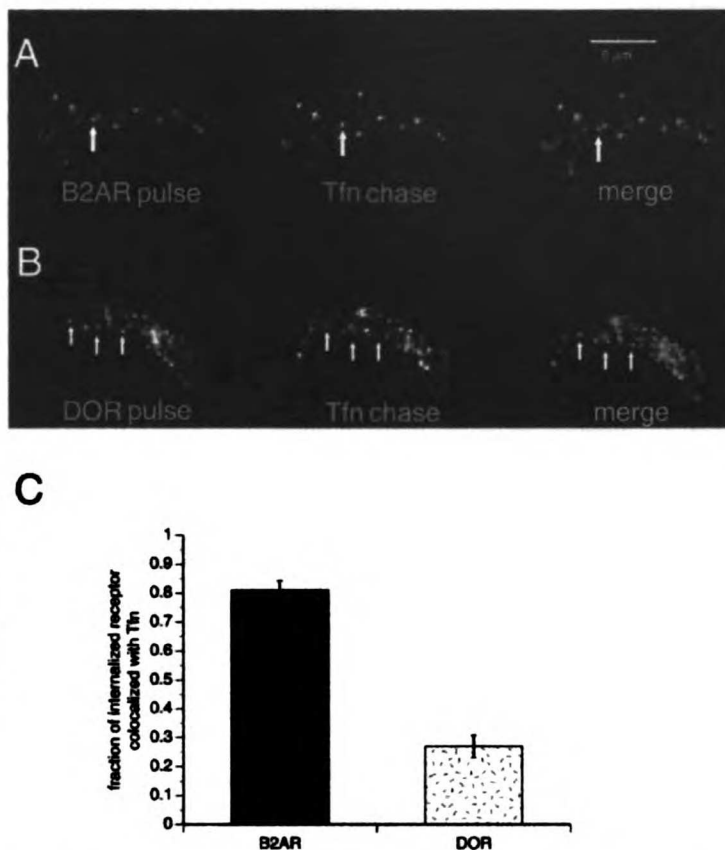
different results. In this case we observed a large number of endocytic vesicles containing DOR but no detectable transferrin (Fig. 7*B*, *arrows* indicate examples of such vesicles, which appear *green* in the *merge panel*). This difference in the endocytic trafficking of surface-labeled DOR was confirmed by quantitation of vesicular colocalization observed in multiple cells examined in coded specimens (Fig. 7*C*). These observations support the idea that the failure of internalized DOR to recycle rapidly is mediated, at least in part, by sorting of internalized receptors to a population of endocytic vesicles distinct from those that constitute the conserved recycling pathway marked by transferrin.
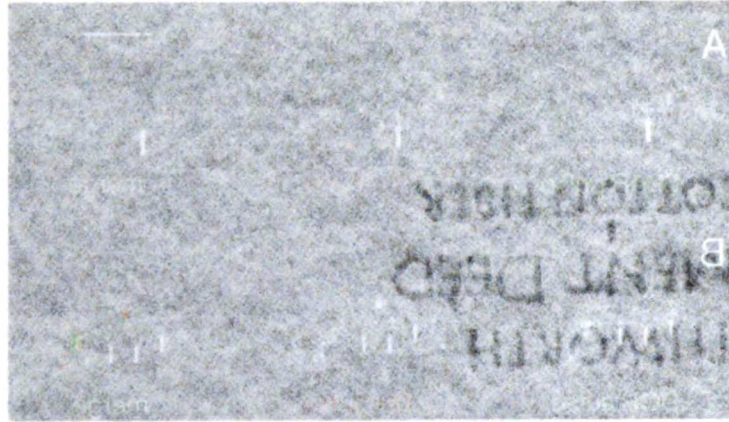
DISCUSSION

In the present study we have shown that distinct GPCRs are differentially sorted between distinct recycling and degradative pathways after undergoing endocytosis by the same membrane mechanism. We accomplished this by examining the endocytic membrane trafficking of the B2AR and DOR expressed in HEK293 cells, a previously established model system in which both receptors undergo agonist-induced endocytosis by a highly conserved, β-arrestin-dependent mechanism mediated by clathrin-coated pits (11, 25). B2AR remains stable in cells incubated for several hours in either the absence or presence of agonist. DOR is similarly stable in the absence of agonist but, in marked contrast to B2AR, is degraded rapidly in the presence of agonist. Importantly, these observations were made in

cells expressing B2AR or DOR at similar levels and were confirmed in cells coexpressing both receptors, indicating that these structurally distinct GPCRs differ significantly in membrane trafficking after endocytosis.

Previous studies have demonstrated differences in the endocytic membrane trafficking of distinct GPCRs (16–21). However, in these cases either the receptors are not endocytosed by the same mechanism (18–21) or their endocytic mechanism is unknown (16, 17). Conversely, other studies have established that the same GPCR (*e.g.* the B2AR (15)) can be targeted to both recycling endosomes and lysosomes via the same endocytic mechanism. However, it has not been established whether *distinct* GPCRs endocytosed by this mechanism can be sorted differentially between these pathways. Thus, to our knowledge, the present results provide the first direct evidence that structurally distinct GPCRs are differentially sorted between recycling and degradative pathways after endocytosis by the same membrane mechanism.

Pathways mediating the endocytic membrane trafficking of GPCRs are generally thought to be similar to those traversed by constitutively internalized nutrient receptors and their ligands (52). However, recent studies indicate an unexpected level of diversity in the fate of GPCRs after endocytosis by clathrin-coated pits. Although B2AR recycles rapidly following endocytosis in HEK293 cells (29, 42, 44), V2R expressed in these cells is stably retained in intracellular vesicles for pro-

longed periods after agonist-induced endocytosis (47, 48). The present results identify a third fate of GPCR membrane trafficking after endocytosis by coated pits. Internalized DOR is selectively retained in the endocytic pathway and subsequently degraded by lysosomes, in contrast to internalized V2R that is retained in endocytic vesicles without detectable down-regulation over a similar time course. In this sense, the trafficking of DOR may be similar to that of the epidermal growth factor receptor, which is retained via specific protein interactions in a maturing endocytic compartment (multivesicular body) after ligand-induced internalization and subsequently delivered to lysosomes via endocytic carrier vesicles (49). Although recent studies suggest a protein interaction that promotes highly efficient recycling of the B2AR (53), in general, recycling of membrane proteins can occur by default (54). Therefore, molecular mechanisms that specifically promote lysosomal targeting of DOR remain to be elucidated.

Native DOR expressed in intact brain tissue and in cultured neuroblastoma cells exhibit significant agonist-induced down-regulation measured by radioligand binding (55). Furthermore, previous studies clearly establish that down-regulation of native as well as transfected opioid receptors in neuroblastoma cells is associated with the accumulation of internalized receptors in lysosomes (4, 37). Thus the sorting event described in the present studies, which selectively sorts internalized DOR to a membrane pathway leading to lysosomes, may be of considerable physiological relevance to receptor down-regulation observed *in vivo*. However, as previous studies indicate that multiple mechanisms contribute to receptor down-regulation observed physiologically (56–59), further studies will be necessary to determine the precise role of specific mechanisms in mediating physiological down-regulation observed in various cell types.

An interesting feature of the sorting operation described in the present study is that it causes substantial proteolytic degradation of receptors even after a relatively brief application of agonist. This observation suggests the possibility that, once receptors are internalized and sorted in the early endocytic pathway, subsequent stages of intracellular transport leading to lysosomes are independent of agonist. However, at present we cannot rule out the possibility that ligand interactions with the *internalized* receptor may influence trafficking. Precedent for this comes from studies with certain receptor tyrosine kinases, where continued occupation of internalized receptors by peptide ligands promotes receptor trafficking to lysosomes (60). Indeed, internalized opioid receptors and their ligands have been detected in similar vesicles (4, 61).

The ability of internalized DOR to undergo relatively rapid proteolytic degradation in cells after brief periods of agonist exposure may have important physiological implications. To our knowledge, other processes that contribute to receptor down-regulation (*e.g.* control of receptor gene transcription and mRNA stability) have been shown to function only in the prolonged presence of agonists, as might be expected because these processes are regulated by downstream G protein-coupled effectors (*e.g.* adenylyl cyclase) (58). However, whereas certain agonist drugs persist in the extracellular milieu for prolonged periods, activation of many GPCRs by physiological agonists (particularly biochemically labile ligands such as opioid peptides) is thought to occur in a much more intermittent or pulsatile manner. In this case, one might expect significant down-regulation of receptors to occur only via mechanism(s) that do not require the prolonged presence of agonist in the extracellular milieu. Thus we anticipate that the endocytic sorting process described in the present study, in addition to its potential importance to understanding the long term actions of

agonist drugs, may have particular relevance to the physiological regulation of certain GPCRs by native ligands.

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CHAPTER 3

Phosphorylation is Not Required for Sorting of a

Truncated Mutant Opioid Receptor

SUMMARY

Phosphorylation is well established to regulate the rapid endocytosis and recycling of many G protein-coupled receptors (GPCRs) via a highly conserved membrane pathway mediated by clathrin-coated pits. Sorting of certain GPCRs, such as the delta opioid receptor (DOR), from this pathway to lysosomes has emerged as an important mechanism mediating the distinct process of GPCR downregulation. While there is considerable evidence that phosphorylation influences GPCR downregulation, it is not known whether phosphorylation is required for downregulation mediated specifically by sorting of internalized receptors to lysosomes. We have addressed this question by examining the endocytic membrane trafficking of a truncated mutant delta opioid receptor (DOR344T), which is not phosphorylated in HEK293 cells yet undergoes rapid, agonist-induced endocytosis by clathrin-coated pits. Here we demonstrate that this phosphorylation-defective mutant opioid receptor exhibits agonist-induced downregulation and is sorted to lysosomes after endocytosis with similarly rapid kinetics as the full length DOR. These observations establish that phosphorylation is not required for the efficient sorting of certain GPCRs to lysosomes, thereby suggesting the existence of phosphorylation-independent mechanisms that control proteolytic downregulation of GPCRs.

INTRODUCTION

G protein-coupled receptors (GPCRs) are regulated by multiple processes. Many GPCRs undergo a process of rapid and reversible desensitization within seconds to minutes after ligand-induced activation. This process is mediated by ligand-

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dependent phosphorylation of receptors followed by the association of phosphorylated

receptors with arrestins, causing functional uncoupling of receptors from heterotrimeric

G proteins and promoting endocytosis of certain receptors by clathrin-coated pits

(reviewed in (1-3)). Endocytosis of GPCRs by clathrin-coated pits can target receptors

to a rapid recycling pathway that contributes to functional resensitization of signal

transduction. A distinct, slower process of downregulation causes a prolonged

attenuation of signal transduction by reducing the total number of receptors present in

cells (4,5). Downregulation of GPCRs is of great interest because it is associated with

certain pathological states and can be influenced by clinically relevant drugs (6).

Multiple mechanisms can contribute to GPCR downregulation (4,5,7). Recent studies

emphasize the importance of regulated proteolysis in mediating downregulation of

several GPCRs.

One mechanism mediating proteolysis of opioid receptors involves

Opioid receptors comprise a subfamily of GPCRs that undergo desensitization,

endocytosis, and downregulation following ligand-induced activation (8-11). Rapid

desensitization and endocytosis of opioid receptors are mediated by a highly conserved

mechanism promoted by receptor phosphorylation (8,12-15). Previous studies suggest

that phosphorylation may also be required for downregulation of opioid receptors (16-

18). However, this may not be true in all cases (19), and no previous studies have

defined the role of phosphorylation in specific mechanisms of downregulation. In

particular, it is not known whether phosphorylation is required for regulated proteolysis

of opioid receptors.

One mechanism mediating proteolysis of opioid receptors involves

endocytosis of receptors and delivery to lysosomes (11,20,21). Recent studies have

established that opioid receptors are targeted to lysosomes after endocytosis by clathrin-coated pits, a mechanism that can also deliver receptors to a distinct recycling pathway involved in receptor resensitization (22,23). The critical mechanism controlling opioid receptor downregulation is a molecular sorting operation, which occurs within several minutes after endocytosis and specifically targets the delta opioid receptor (DOR) to lysosomes (21). While phosphorylation is well known to promote endocytosis and inhibit recycling of certain GPCRs (reviewed in (1-3)), including opioid receptors (8,13,24), it is not known whether phosphorylation of any GPCR is specifically required for sorting of internalized receptors to lysosomes.

This question has been difficult to address because, in many systems, manipulations or mutations that block phosphorylation of receptors also strongly inhibit endocytosis by clathrin-coated pits, thereby precluding study of later step(s) in the pathway mediating receptor trafficking to lysosomes. In the present study we have circumvented this complication by examining a previously described truncated mutant delta opioid receptor (DOR344T) which is unable to undergo any detectable constitutive or ligand-induced phosphorylation yet, when expressed in HEK293 cells, exhibits rapid agonist-induced endocytosis by clathrin-coated pits (25). Here we demonstrate that phosphorylation is not required for efficient sorting of this mutant receptor to lysosomes.

RESULTS AND DISCUSSION

To begin to address whether phosphorylation is necessary for lysosomal targeting of opioid receptors, we utilized radioligand binding to compare the ability of

full length DOR and phosphorylation-defective DOR344T mutant receptor to undergo agonist-induced downregulation. Consistent with previous studies, ~50% downregulation of the total number of full length DOR was detected in cells within 3 hours after the addition of peptide (DADLE) or alkaloid (etorphine) agonist to the culture medium. The DOR344T mutant receptor exhibited a similar amount of downregulation under the same conditions (Figure 1 A). Saturation binding analysis confirmed that this decrease in radioligand binding truly reflected reduced receptor number, and was not simply a consequence of reduced ligand binding affinity or residual agonist (Figure 1 B). This analysis also confirmed that the DOR344T mutant receptor was expressed at closely similar levels as the full length DOR. When expressed at this level, the B2AR is rapidly recycled to the plasma membrane without detectable proteolysis (21), confirming that the DOR344T mutant receptor is downregulated in a specific manner following agonist-induced activation.

The ability of agonist to promote downregulation of DOR344T suggested that the phosphorylated receptor may undergo agonist-induced proteolysis. To examine this possibility directly, we utilized immunoblotting to detect DOR and DOR344T receptor protein present in cells. As shown previously (21), full length DOR resolved predominantly at an apparent molecular mass corresponding to the complex-glycosylated monomeric receptor protein (Figure 2 A, left arrow), although minor species possibly reflecting biosynthetic intermediates or receptor oligomers were also resolved. A prominent band of immunoreactive DOR344T mutant receptor resolved at an apparent molecular mass consistent with glycosylated monomeric truncated receptor (Figure 2 A, right arrow). In addition, several minor species of immunoreactive

DOR344T resolved at higher apparent molecular mass (Figure 2 A, right lane). We focused specifically on these predominant forms of DOR and DOR344T, as they corresponded to the species of receptor protein present in the plasma membrane (see below).

Both full length DOR and DOR344T mutant receptors exhibited extensive proteolysis within three hours after the addition of either alkaloid or peptide agonist to the culture medium (Figure 2 B, top and bottom panels, respectively). Quantitation of multiple experiments by densitometric scanning confirmed that the DOR344T mutant receptor was proteolyzed to a closely similar extent as full length DOR under these conditions (Figure 2 C). Furthermore, proteolysis of the DOR344T mutant receptor, like that of the full length DOR, was sensitive to inhibitors of lysosomal proteolysis (Figure 2 D). This observation indicates that the phosphorylation-defective DOR344T mutant receptor, like the wild type DOR, undergoes extensive agonist-induced proteolysis in lysosomes. The DOR344T mutant receptor is missing all phosphorylatable residues implicated in previous studies (9,26) and exhibits no detectable phosphorylation in HEK293 cells (25). Point mutation of the only potentially phosphorylatable residues remaining in the cytoplasmic tail of DOR344T (Thr 335 and Ser 344) to alanine did not inhibit agonist-induced proteolysis (not shown), further confirming that phosphorylation is not required for lysosomal proteolysis of this receptor.

To specifically examine the fate of receptors present in the plasma membrane, we utilized a previously described assay involving surface biotinylation (21). SDS-PAGE analysis resolved the surface-biotinylated DOR344T mutant receptor as a single

band corresponding to the major species detected in whole-cell extracts by immunoblotting (Figure 3 A). To determine whether the DOR344T mutant receptors present in the plasma membrane undergo ligand-dependent proteolysis, we examined the effect of agonist incubation on the amount of surface-biotinylated receptor detected in cell extracts. In the absence of agonist, there was little or no proteolysis of biotinylated DOR344T receptor, as indicated by the uniformly high recovery of receptor from immunoprecipitates (Figure 3 A). In contrast, biotinylated receptors were nearly undetectable after agonist incubation of cells for 3 hours, indicating that the entire pool of DOR344T present initially in the plasma membrane can be extensively proteolyzed within this time period. Quantitation of multiple experiments by densitometric scanning confirmed that biotinylated DOR344T mutant receptors were proteolyzed to a closely similar extent as full length DOR expressed at comparable levels (Figure 3 B). Furthermore, examination of the time course of agonist-induced proteolysis indicated that DOR and DOR344T were proteolyzed with indistinguishable rates (Figure 3 C).

We conclude that phosphorylation is not required for a specific mechanism mediating proteolytic downregulation of opioid receptors. While phosphorylation has been shown previously to play an important role in regulating rapid endocytosis and recycling of GPCRs (24,27-29), to our knowledge the present study is the first to establish that phosphorylation is not necessary for the efficient sorting of internalized receptors to lysosomes. These observations may provide new insight into how certain agonists and receptor mutations can differentially affect rapid internalization and slower downregulation of opioid receptors (9,30) and certain other GPCRs that are

internalized by clathrin-coated pits (e.g. (31)). Recent studies have shown that persistently phosphorylated V2 vasopressin receptors are unable to recycle to the plasma membrane after endocytosis yet do not undergo detectable downregulation (32,33). Thus it is possible that phosphorylation of certain GPCRs is neither necessary nor sufficient for endocytic sorting to lysosomes. Nevertheless, it is clear that distinct GPCRs can differ greatly in their membrane trafficking after endocytosis by clathrin-coated pits (21). Taken together, these observations suggest the existence of phosphorylation-independent mechanisms controlling GPCR sorting in the endocytic pathway. Elucidating these mechanisms and their physiological consequences is an important goal for future studies.
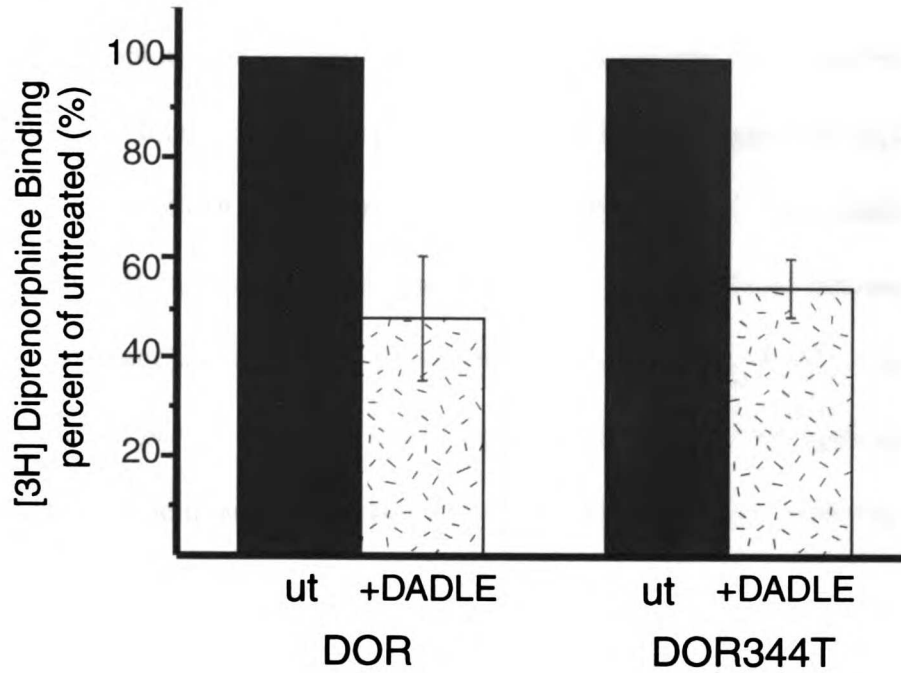
FIGURE LEGENDS

Figure 1 Agonist-induced downregulation of DOR344T

Radioligand binding assays using [3H] diprenorphine were performed on stably

transfected cells expressing full length or truncated mutant DOR (DOR344T) as

described in *Experimental Procedures*. A. Cells were incubated in the absence

(untreated, ut, solid bar) or presence of 10 µM peptide agonist DADLE (+DADLE,

stippled bar) for 3 hours. Ligand binding sites were measured as bound tritium counts

and expressed as picomoles of ligand specifically bound/ mg of total cell protein.

Results are plotted as the percentage of total bound specific radioactivity in untreated

cells. Error bars represent S.E. (n=3 assays performed in triplicate). B. Scatchard

analysis of saturation ligand binding to DOR344T-expressing cells incubated in the

absence (open circles) or presence (filled circles) of DADLE for 3 hours. Each data

point represents the mean of triplicate determinations.

A

[3H] Diprenorphine Binding percent of untreated (%)

DOR: ut, +DADLE

DOR344T: ut, +DADLE

B

bound/free vs pmol DPN bound/mg protein
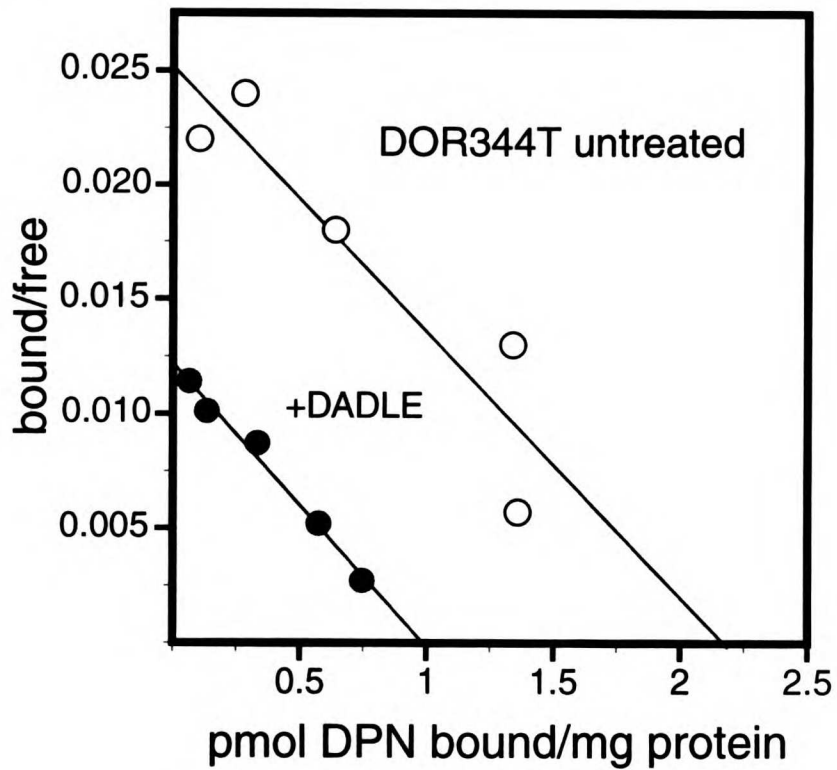
DOR344T untreated

+DADLE

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Figure 2 Agonist-induced proteolysis of DOR344T in lysosomes

Total immunoreactive DOR and DOR344T receptor protein was measured by

immunoblotting using anti-FLAG antibody as described in *Experimental Procedures.*

Panel A: Immunoreactive bands resolving at the appropriate molecular mass (arrows)

of full length DOR (left lane) and DOR344T (right lane) were detectable in extracts

from cells expressing epitope-tagged receptors, but not from untransfected cells (293,

middle lane). The mobility of molecular mass standards (in kD) is indicated to the left.

Receptor protein was detected on immunoblots loaded with equal amounts of extract

(20 μg protein/lane). Panel B: Cells expressing full-length DOR (top panel) or

DOR344T (bottom panel) were incubated in the absence (untreated, ut) or presence of

10 μM agonist (DADLE or etorphine, et) for 3 hours. Panel C: Immunoblots for full-

length DOR and DOR344T were quantitated by densitometric scanning from multiple

experiments (n = 3). Results are plotted as percentage of total receptor in untreated

cells. Error bars represent S.D. of 3 experiments. Panel E: The effect of leupeptin (leu),

ammonium chloride (AC), or chloroquine (cq) on proteolysis of DOR344T was

determined by preincubation of cells with inhibitors for 1 hour at 37°C and then

treatment with 10 μM etorphine for 3 hours in the continued presence of inhibitors.

A

	DOR	293	DOR344T
175 —			
83 —			
62 —			
47.5 —			

B

ut	+et	+DADLE

DOR

DOR344T

C

Receptor immunoreactivity
percent of untreated (%)

ut +et +DADLE   ut +et +DADLE
DOR   DOR344T

D

+etorphine

ut   +leu +AC +cq
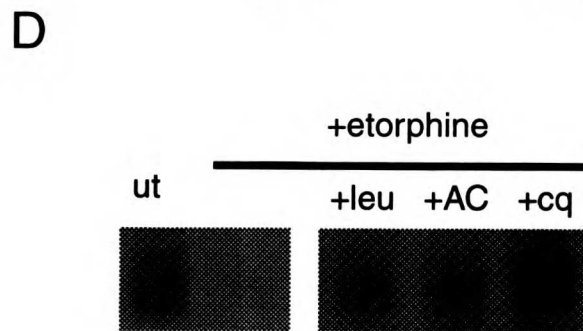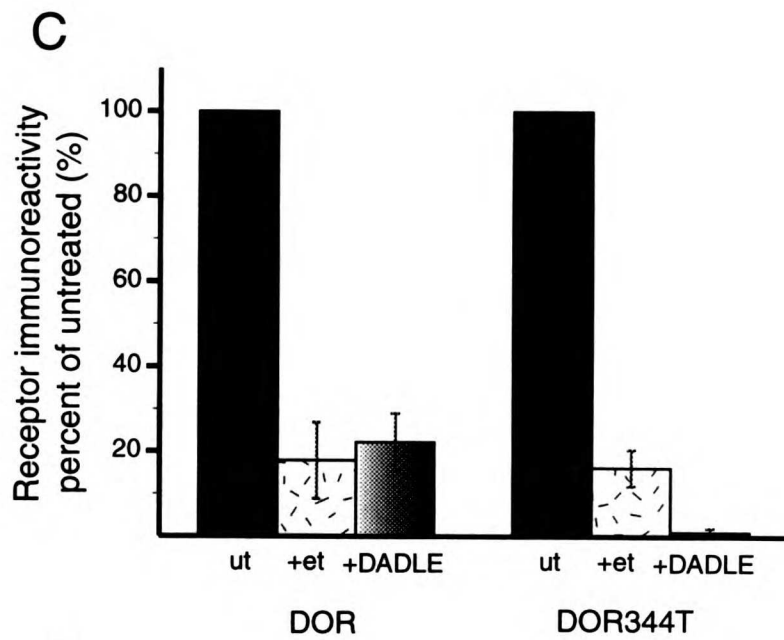
Figure 3 Surface DOR344T is targeted for proteolysis with similar kinetics as full length DOR

Proteolysis of surface-biotinylated receptors was measured as described in *Experimental Procedures*. Panel A: Biotinylated protein bands resolving at the appropriate molecular mass of DOR344T (arrow) were specifically recovered in immunoprecipitates prepared from cells expressing the epitope-tagged receptors, but not from untransfected cells (293). The mobility of molecular mass standards (in kD) is indicated to the left. Lanes on the right display biotinylated protein recovered from equal amou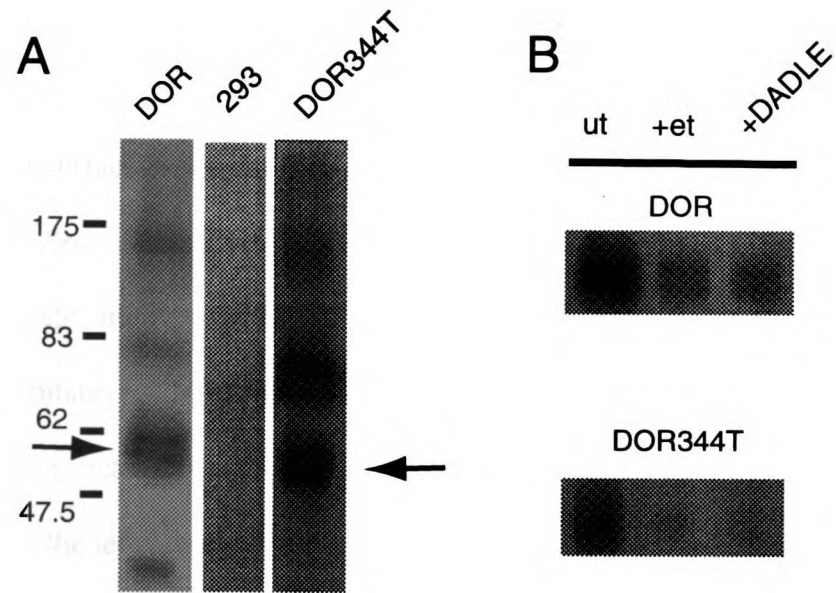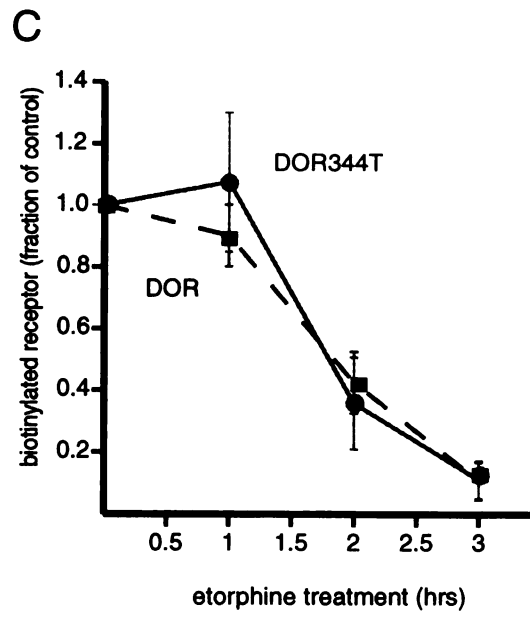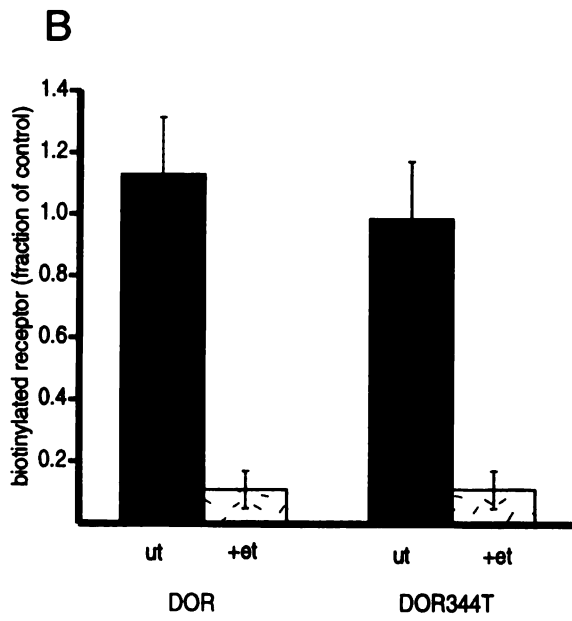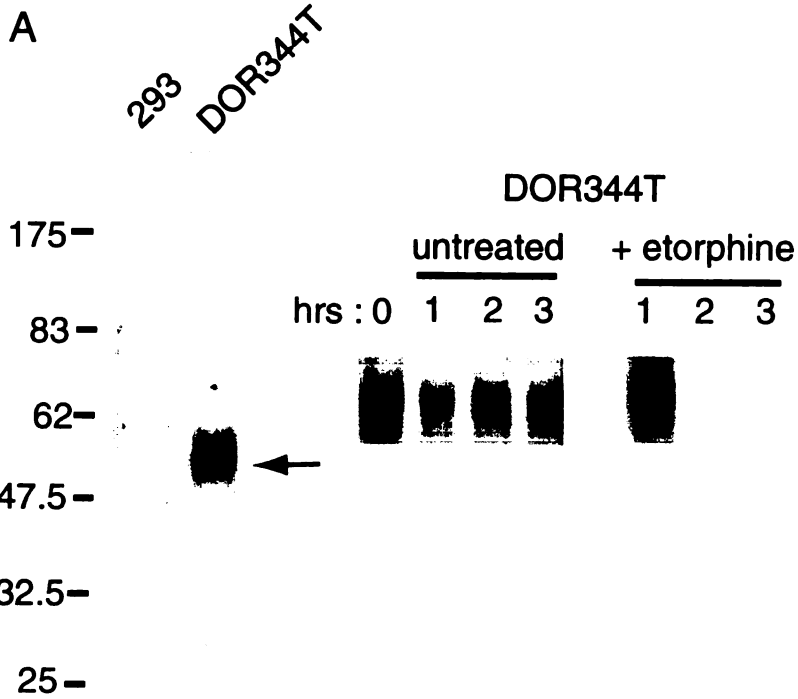nts of cells prepared after incubation of cells in the absence (untreated) or presence of the agonist 10 μM etorphine for the indicated time period. In cells incubated in the absence of agonist, DOR344T was recovered in similar amounts as present in cells lysed immediately after biotinylation (hr:0). In cells incubated in the presence of agonist, a significant reduction in the amount of biotinylated receptor protein was observed. Panels B and C: Biotinylated receptor protein for DOR and DOR344T was quantitated by densitometric scanning of streptavidin overlays from multiple experiments (n≥3 for each time point). Results are plotted as the mean recovery of biotinylated receptor protein (relative to that isolated at t = 0). Error bars represent S.D.. Panel B represents relative amount of biotinylated DOR and DOR344T recovered after incubation of cells for 3 hours in the absence (solid bar) or presence (stippled bar) of the agonist. Panel C displays a time course of the amounts of biotinylated receptor recovered from agonist-treated cells (DOR, filled squares; DOR344T, filled circles).

A

293 DOR344T

DOR344T

175—

83—

hrs : 0 1 2 3   1 2 3

untreated   + etorphine

62—

47.5—

32.5—

25—

B

biotinylated receptor (fraction of control)

1.4
1.2
1.0
0.8
0.6
0.4
0.2

ut   +et   ut   +et

DOR   DOR344T

C

biotinylated receptor (fraction of control)

1.4
1.2
1.0
0.8
0.6
0.4
0.2

DOR344T

DOR

0.5 1 1.5 2 2.5 3

etorphine treatment (hrs)

EXPERIMENTAL PROCEDURES

Cell Culture and cDNA constructs

Human embryonic kidney cells (HEK293) cells were maintained and passaged in
Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine
serum and 100 U/mL penicillin/streptomycin (University of California, San Francisco
Cell Culture Facility). A stable cell line expressing FLAG-tagged DOR (DOR#5) was
generated as previously described (21). The previously described truncated mutant
DOR (DOR344T) cell line was constructed by engineering a stop codon following
residue 344 in the coding sequence of the FLAG-tagged murine DOR and introducing
the cDNA into HEK293 cells via calcium phosphate precipitation and G418 selection
(25). Relative expression levels were quantitated using flow cytometry,
immunofluorescence microscopy, and radioligand binding using [3H]-diprenorphine
(see below for methods). DOR #5 expressed FLAG-tagged DOR at 0.8 pmol/mg
protein and DOR344T expressed FLAG-tagged truncated receptor at 1-2.5 pmol/mg
protein. Thr 335 and Ser 344 in DOR344T cDNA were mutated to alanines using a
Quik-Change site-directed mutagenesis kit (Stratagene) and multiple clones were
generated by introducing cDNA into HEK293 cells via calcium phosphate precipitation
and G418 selection.

Radioligand Binding

Agonist-induced downregulation of receptors was assayed in intact cells using a
previously described method (34). Briefly, monolayers of cells expressing FLAG-
tagged full-length DOR (DOR#5) and FLAG-tagged truncated mutant DOR

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(DOR344T) were incubated for 3 hours at 37°C in the absence or presence of 10 μM

DADLE (Research Biochemicals). To assure a saturating concentration of peptide

agonist over the incubation period, monolayers incubated with DADLE were

supplemented with fresh peptide every hour during the incubation. At the end of the

incubation, cells were lifted with PBS supplemented with EDTA and washed four

times by centrifugation with 10 ml of warm (37°C) PBS. Then cells were washed once

by centrifugation in 10 ml of Krebs Ringer HEPES buffer (KHRB: 110 mM NaCl, 5

mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 55 mM sucrose, 10 mM

HEPES pH 7.3). Radioligand binding was carried out in 120 μl of KHRB containing

equal amounts of washed cells (50-100 μg protein) with 2 nM [3H] diprenorphine.

Incubations were carried out for 30 minutes at room temperature and terminated by

vacuum filtration through glass fiber filters (Packard Instruments) and repeated washes

with ice-cold Tris-buffered saline, pH 7.4. Bound radioactivity was determined by

scintillation counting (Scintiverse, Fisher) using a Beckman LS 6500 instrument. For

all determinations, bound radioligand represented ≤ 10% of total radioligand present in

the incubation, and nonspecific binding (defined by assays conducted in the presence of

10 μM naloxone) was ≤ 10% of counts isolated on glass fiber filters. All assays were

conducted in triplicate with similar results. Results are expressed as mean picomoles of

radioligand specifically bound per mg of cells assayed (protein concentration was

determined by the method of Bradford et al (35) using reagents from Bio-Rad).

Immunoblotting

Monolayers of cells expressing FLAG-tagged DOR (DOR#5) or truncated mutant DOR (DOR344T) were incubated in the absence or presence of 10 μM etorphine or 10 μM DADLE. For experiments using inhibitors of lysosomal proteolysis, monolayers were preincubated for 1 hour at 37°C with 100 μg/ml leupeptin (Calbiochem), 200 μM chloroquine (Sigma) or 50 mM ammonium chloride (Sigma) before agonist addition, and these reagents were present in the medium during agonist incubation. Equal amounts of cell lysate (prepared by extracting monolayers with 0.1% Triton X100 (Sigma)) were resolved by SDS-PAGE (10% acrylamide), transferred to nitrocellulose membranes and blotted for FLAG-tagged receptor using M1 monoclonal antibody (Sigma) followed by detection using HRP-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) and ECl (Amersham). Immunoblots were quantitated by densitometric scanning of films exposed in the linear range.

Biochemical Analysis of Receptor Degradation Using Noncleavable Biotin

Proteolysis of surface-biotinylated receptors was estimated using a previously described protocol (36). Briefly, stably transfected cells expressing FLAG-tagged DOR or truncated mutant DOR (DOR344T) were surface biotinylated by incubation at 4°C with 30 mg/mL sulfo-NHS-biotin (Pierce), rinsed with Tris-buffered saline to quench the biotinylation reaction, warmed to 37°C and incubated with or without 10 μM etorphine, and then chilled on ice to stop further membrane trafficking. Cells were extracted and receptor immunoprecipitates were prepared, and biotinylated receptor protein recovered in immunoprecipitates was detected by streptavidin overlay. Receptor proteolysis was

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indicated by a loss of biotinylated protein recovered in immunoprecipitates, and was

quantitated by densitometric scanning of streptavidin overlays exposed in the linear

range. Samples representing equal numbers of cells were loaded in each lane.

FOOTNOTES

Abbreviations used: GPCR – G protein-coupled receptor; DOR – δ-opioid receptor;

HEK, human embryonic kidney; DADLE – [D-ala2, D-leu5] enkephalin, PBS-

phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PAGE,

polyacrylamide gel electrophoresis

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Overview

Extensive study of the regulatory processes of GPCR signalling has been conducted using pharmacological techniques. These studies have provided important insights; however, they are limited in their ability to identify the specific mechanisms and proteins critical for thoroughly understanding how these processes work and how to manipulate these processes for therapeutic benefit.

By adapting rigorous biochemical and cell biological techniques used to analyze other plasma membrane receptors, this thesis contributes significantly to our understanding of GPCR sorting after endocytosis. I have identified a novel fate for *nonproteolytically* activated GPCRs, rapid degradation following endocytosis. Secondly, I have shown that distinct receptors can differ significantly in their trafficking after endocytosis by the same mechanism. Therefore, these studies have identified a new level of *selectivity* which differentiates GPCR trafficking and can consequently have an important influence on receptor signaling. In other words, it appears that a receptor's fate is determined not only at the plasma membrane by its decision to internalize, but also in the endosome by its choice between the recycling and degradative pathways.

The data presented in the second part of Chapter 2 supports the following model for GPCR sorting to lysosomes. After endocytosis via clathrin-coated pits, DOR is targeted to lysosomes by being pulled out of the recycling endosomes and packaged into it's own distinct set of transferrin-negative endosomes. Interestingly, once a receptor is segregated into these vesicles, it appears to be destined to degrade even without the cascade of signaling events activated by agonist stimulation. Finally, data in

Chapter 3 suggests that despite the critical role of phosphorylation in endocytosis and recycling, this entire chain of events can occur efficiently without receptor phosphorylation.

In addition to providing significant insights into the specificity and mechanisms of postendocytic sorting, the series of experiments utilized to analyze the trafficking of the B2AR and the DOR can serve as an example of the types of techniques useful for addressing these questions. Chapter 2 begins by using the classic pharmacologic (radioligand) and biochemical (immunoblotting) techniques to ascertain how GPCRs may differ in behavior following agonist treatment. It then takes a closer look at the cell biological mechanisms underlying this difference using several techniques that have only been recently developed and refined – immunofluoresence to visualize differences in postendocytic trafficking, biotinylation to measure stability of receptors previously on the surface, flow cytometry to measure receptor recycling, a modified biotinylation technique to measure receptor retention, and finally, an immunofluorescence pulse-chase assay to determine colocalization with transferrin. The application of these techniques to the study of other GPCRs is expected to be enormously useful for more fully elucidating their membrane trafficking.

Future Directions

An important area of further study is to delineate the fundamental membrane mechanisms by which DORs are sorted to lysosomes. The biochemical and immunohistochemical techniques used in this study have been invaluable for elucidating some basic principles. My studies demonstrate that the DOR is rapidly

sorted out of the early endocytic/recycling pathway, presumably via a selective membrane fission event. Thus, DOR sorting to lysosomes may differ from that of ligands like LDL, which are proposed to fail to recycle and retained in a maturing endocytic compartment (1). Further elucidation of mechanisms will benefit greatly from analysis by live cell imaging techniques, which are currently being developed in this laboratory. In fact, preliminary studies of live cells co-expressing B2AR and DOR visualize rapid movement of internalized DOR-containing vesicles, while B2AR-containing vesicles are relatively static. These data are consistent with the hypothesis that DOR vesicles are rapidly segregated away from the recycling endosomes. Future studies can use live cell imaging to determine the precise kinetics of the sorting operation and to identify specific compartments participating in this process.

A second important area of further study is to identify the intricate protein machinery which determines and regulates GPCR trafficking in the recycling and degradative pathways. This thesis establishes that these proteins operate at the endosome level, rather than at the plasma membrane. One approach to identifying these proteins would be to determine the specific sequences that direct a receptor to lysosomes and to use these sequences as probes for interacting proteins via genetic (yeast two-hybrid) or biochemical (affinity chromatography) techniques.

Although consensus sequences have been identified for receptor internalization, relatively little is known about what receptor sequences determine a receptor's fate after endocytosis. Early work by Fred Maxfield demonstrated that transferrin receptors recycle at the same rate as endosome lipids (2), suggesting that receptor recycling occurs by default and that sorting signals must exist to direct receptors to lysosomes.

Site-directed mutagenesis studies of other proteins that degrade rapidly (i.e. P-selectin, lysosomal membrane proteins, EGFR) have identified specific sequences in the cytoplasmic tail that appear to determine receptor targeting to lysosomes (3-7) but have not been able to identify any clear consensus sequences or motifs. Interestingly, my demonstration that DORs are targeted to lysosomes via a specific sorting event, taken together with those of Cao et al. (8), suggest that endocytic sorting of GPCRs may involve an interplay between distinct signals controlling sorting into the recycling and degradative pathways.

The truncated mutant opioid receptor described in this thesis not only establishes that GPCR lysosomal targeting is phosphorylation-independent, but also provides some clues as to what receptor sequences determine targeting to lysosomes. The ability of the truncated mutant DOR to undergo degradation indicates that either a redundant lysosomal targeting signal exists in other regions of the DOR or that the signal sequence resides in the proximal region of the tail. Interestingly enough, a receptor chimera containing the body of another closely homologous opioid receptor subtype which recycles, the mu-opioid receptor, and the proximal stub of the DOR cytoplasmic tail does indeed degrade rapidly after agonist treatment, suggesting that this region contains a transplantable degradation signal. Our results with the truncated opioid receptor predict that this degradation signal should be phosphorylation-independent. However, this remains to be determined.

Based on the putative role of the DOR proximal stub in lysosomal targeting, preliminary studies were conducted to identify a lysosomal sorting protein. A yeast two-hybrid screen previously conducted in the laboratory had identified a pool of DOR-

interacting proteins. One particular protein was found to interact specifically with the proximal stub of the DOR tail and this interaction did not appear to require receptor phosphorylation. However, when this protein was overexpressed with the DOR in a stable cell line, no significant impairment of lysosomal targeting was observed. A important goal of current and future studies in the laboratory is to further verify the sequence of the putative DOR lysosomal targeting sequence, to continue to investigate whether the candidate protein influences lysosomal targeting, and to continue the search for the other proteins mediating rapid lysosomal sorting of GPCRs.

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APPENDIX A

Downregulation of G protein-coupled Receptors

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Summary

Ligand-induced activation regulates the number of many G protein-coupled receptors (GPCRs) present in cells and tissues. In this review we summarize previous studies indicating that multiple, highly conserved mechanisms contribute to agonist-induced downregulation of GPCRs in both neural and non-neural cell types. Then we review recent progress in understanding proteolytic mechanisms of GPCR downregulation, with an emphasis on membrane trafficking mechanisms that mediate receptor targeting to lysosomes.

Introduction

The majority of known neurotransmitters and neuromodulators mediate their physiological effects by binding to heptahelical GPCRs. In addition, these receptors are targets of many therapeutic and abused drugs. GPCRs are so-named because they mediate signal transduction by regulating guanine nucleotide exchange on a conserved family of heterotrimeric GTP-binding proteins, although additional protein interactions may also function in signaling by these receptors [1]. GPCRs are extensively regulated by multiple processes, which have important effects on signal transduction.

Processes of GPCR regulation have been distinguished traditionally by differences in kinetics. This is illustrated by classic studies of the beta-2 adrenergic receptor (B2AR), reviewed extensively elsewhere [2-5]. Rapid attenuation or *desensitization* of B2AR signaling occurs within seconds to minutes after agonist-induced activation. This process is not associated with any detectable change in the number of receptors present in cells and is rapidly reversible following removal of

agonist. A highly conserved mechanism of rapid desensitization is mediated by activation-induced phosphorylation of the receptor protein followed by receptor interaction with cytoplasmic accessory proteins called beta-arrestins, which interfere with receptor-G protein coupling and promote rapid endocytosis of receptors [6]. Upon prolonged or repeated activation of receptors (typically over a period of several hours), a process called receptor *downregulation* causes a more gradual attenuation of cellular signal transduction. Downregulation is associated with a reduced number of receptors detected in cells or tissues and is reversed relatively slowly following removal of agonist. Downregulation of GPCRs has been observed in various neural cell types [7,8] and is thought to be clinically relevant to the actions of important neuropsychiatric drugs [9].

In this review, we briefly summarize previous studies leading to our present understanding of GPCR downregulation, and we highlight recent progress in elucidating mechanisms by which mammalian GPCRs are targeted to lysosomes.

What is downregulation?

Downregulation of GPCRs is traditionally defined as a reduced number of receptor sites measured using radioligand binding techniques in a total membrane fraction prepared from cells or tissues. In principle, this process could reflect conformational changes of the receptor protein that prevent detectable ligand binding or a decrease in the actual amount of receptor protein present in the cells or tissue. There is evidence that, in some cases, downregulation of receptors may be mediated by receptor conformational changes without detectable loss of receptor protein [10].

However, in many cases downregulation is thought to be associated with net loss of receptor protein, as suggested initially by studies demonstrating that recovery from downregulation is dependent on new protein synthesis [11].

Extensive studies of various GPCRs suggest that changes in the rates of both receptor biosynthesis and degradation control the number of receptors present in cultured cells. For example, B2AR mRNA levels are modulated by both transcriptional regulation of the receptor gene [12] and modulation of mRNA stability [13]. Studies using pharmacological assays of receptor turnover strongly suggest that GPCRs undergo ligand-regulated degradation, and receptor proteolysis is believed to be the predominant mechanism of downregulation for several GPCRs [14].

Downregulation of GPCRs is observed in many cell types

Many studies addressing specific mechanisms of downregulation have been carried out in non-neural cell types. However, there is evidence that similar mechanisms mediate GPCR downregulation in many cell types, including neuroblastoma cells and cultured neurons. Furthermore, certain features of GPCR downregulation are conserved in diverse organisms.

For example, GPCR-mediated signal transduction mediates the actions of mating pheromones in the budding yeast Saccharomyces cerevisiae. The *STE2* gene encodes a GPCR activated by secreted α-factor. Prolonged incubation of cells with α-factor causes attenuated responsiveness that is associated with proteolysis of Ste2p. This process is mediated by ligand-induced endocytosis followed by targeting of Ste2p to the vacuole, a proteolytic organelle analogous to the mammalian lysosome. Elegant

studies, reviewed in detail elsewhere [15,16], have identified post-translational

modifications of the receptor protein that control endocytosis and vacuolar targeting of

Ste2p.

Proteolysis of GPCRs in mammalian cells

Studies using subcellular fractionation, biochemical inhibition of lysosomal

proteolysis, and immunocytochemical localization of receptors strongly suggest that

downregulation of several mammalian GPCRs is associated with translocation of

receptors to lysosomes, including in neuronal cell types [17]. However, despite the

similarity of mammalian lysosomes to the yeast vacuole, it is not clear to what extent

specific mechanisms of GPCR downregulation are conserved in diverse organisms

[15,16]. Moreover, there is emerging evidence that distinct, non-lysosomal

mechanisms mediate proteolysis of mammalian GPCRs in some cases. Previous

studies of the V2 vasopressin receptor demonstrated ligand-induced endoproteolytic

cleavage by a plasma membrane-associated metalloprotease [18]. More recent studies

of B2AR downregulation suggest the operation of an alternate proteolytic mechanism

in some cell types but not others [19]. In principle, one might expect such non-

lysosomal degradation of GPCRs to be mediated by proteasomes. However, the

alternate mechanism of B2AR proteolysis is insensitive to inhibitors of both lysosomal

and proteasome-mediated proteolysis [19].

Endocytic trafficking of GPCRs to lysosomes

Recent studies have begun to address specific mechanisms by which mammalian GPCRs are targeted to lysosomes. In general, delivery of membrane proteins from the plasma membrane to lysosomes is a multi-step process mediated by endocytosis followed by transport of internalized proteins via specific endocytic pathway(s) [20]. While many GPCRs undergo ligand-induced endocytosis, internalization of GPCRs often occurs with more rapid kinetics than receptor downregulation. Furthermore, endocytosis of GPCRs can mediate multiple, distinct physiological functions in addition to proteolytic downregulation. Trafficking of receptors through a rapid recycling pathway promotes functional resensitization of signal transduction [2-4]. There is also evidence that endocytosis of GPCRs may promote signal transduction to the nucleus [21,22]. Recent evidence suggests a specific role of GPCR endocytosis in activating the MAP kinase cascade [23,24], which may involve a Src-associated endocytic signaling complex [25]. However, several studies question the precise role of endocytosis of the receptor in this signaling pathway [26-30] and suggest possible alternate functions of dynamin or dynamin-dependent endocytosis in MAP kinase activation [28,31]. Moreover, recent studies of the V2 vasopressin receptor suggest that endocytosis can target GPCRs to an intracellular membrane compartment in which receptors are stably retained without detectable recycling or proteolysis, thereby leading to a prolonged state of functional desensitization that is not associated with concomitant downregulation of receptors [32•, 33•].

These considerations raise the question of whether distinct endocytic mechanisms sort GPCRs between different membrane pathways. Supporting this idea,

previous studies identified mutations of the B2AR that differentially affect agonist-induced sequestration and downregulation (e.g., [34]). Furthermore, naturally occurring subtypes of alpha-2 adrenergic receptor downregulate with similar rates [14] despite significant differences in rapid endocytosis [35]. Perhaps most compelling, elegant studies indicate that divergent residues located in the carboxyl-terminal cytoplasmic domain specify differences in membrane trafficking of thrombin and substance P receptors between lysosomal and recycling pathways, respectively [36••]. Based on mathematical models [37], these observations are consistent either with completely separate pathways mediating rapid endocytosis and proteolytic degradation of GPCRs, or with the operation of partially overlapping pathways controlled by distinct rate-limiting steps. Thus it is important to define precisely membrane mechanisms and pathways mediating endocytic trafficking of specific mammalian GPCRs.

Multiple mechanisms of GPCR endocytosis

Many GPCRs undergo ligand-induced endocytosis via clathrin-coated pits, which is promoted by a highly conserved mechanism mediated by beta-arrestins [6]. However, distinct GPCRs differ significantly in their ability to undergo endocytosis by coated pits, and there is strong evidence for the existence of receptor-specific and cell type-specific differences in precise mechanisms of GPCR endocytosis. For example, while the B2AR is endocytosed by clathrin-coated pits in several cell types [38•], morphological studies suggest that this receptor can endocytose in other cells by membrane invaginations that resemble caveolae [39,40]. CCK receptors have been

observed in both clathrin-coated pits and caveolae in the same cells [41]. Endocytosis of several GPCRs is not detectably inhibited by a dominant-negative mutant form of dynamin, which blocks endocytosis of both clathrin-coated pits and caveolae [42-44], suggesting that additional mechanism(s) of GPCR endocytosis may function in some cases [45-47]. Furthermore, co-expressed D1 and D2 dopamine receptors are observed in separate vesicles immediately after internalization, suggesting that distinct pathways of GPCR endocytosis can operate in parallel in neuroblastoma cells. However, such heterogeneity in the early endocytic pathway may not control the specificity of GPCR targeting to lysosomes, as internalized D1 and D2 receptors exhibit similar rates of proteolytic degradation [47].

Molecular sorting of GPCRs after endocytosis by a common mechanism

Recent studies suggest that GPCRs can be sorted to distinct destinations after endocytosis by the same membrane mechanism. Elegant studies demonstrating that a dominant-negative mutant form of dynamin inhibits both agonist-induced sequestration and downregulation of the B2AR in HEK293 cells suggest that endocytosis of receptors by clathrin-coated pits is an obligate first step common to membrane pathways leading to recycling endosomes and lysosomes [48••]. Recent studies of endocytic sorting of co-expressed adrenergic and opioid receptors support this hypothesis and suggest that receptor sorting occurs at an early stage in the endocytic pathway and can be observed within several minutes after endocytosis of both receptors by clathrin-coated pits (Tsao P, von Zastrow M, unpublished data).

The possibility that GPCRs can be targeted to different membrane pathways after endocytosis raises important questions of how the sorting of internalized receptors is mediated and regulated. It is likely that lysosomal trafficking of GPCRs involves the formation of multivesicular endocytic intermediates similar to those involved in targeting the EGF receptor tyrosine kinase to lysosomes [49]. Antibody-labeled B2ARs have been observed in lumenal membranes of multivesicular endosomes [40], and analogous endocytic carrier vesicles may function in Ste2p trafficking to the yeast vacuole [50]. However, as genetic studies in yeast indicate that Fab1p lipid kinase activity is absolutely required for translocation of carboxypeptidase S but not Ste2p to intralumenal membranes, it is possible that specialized machinery contributes to endocytic sorting of GPCRs [50].

A recent study of B2AR trafficking in mammalian (HEK293) cells has proposed an endocytic sorting mechanism that requires a PDZ domain-mediated interaction of the receptor with NHERF/EBP50-family proteins as well as additional protein interaction(s) with the cortical actin cytoskeleton [51•]. Interestingly, interaction of the B2AR with NHERF/EBP50-family proteins (including NHERF2/E3KARP) has been shown previously to mediate receptor signaling in the plasma membrane via regulation of the NHE3 Na^+/H^+ exchanger [52•,53]. The functional implications of this striking similarity between protein machinery mediating of B2AR signaling and endocytic sorting are not yet understood. However, signaling via NHE3 is not required for B2AR sorting, as NHERF/EBP50-dependent sorting of the B2AR is observed in cells that do not express detectable amounts of NHE3.

Concluding remarks

Significant advances have been made in recent studies of mechanisms that mediate downregulation of GPCRs. We have focused in particular on proteolytic downregulation of GPCRs by membrane trafficking to lysosomes, which appears to occur in both neural and non-neural cell types. Much is known about early stages of GPCR endocytosis, and recent studies have provided new insight into later stages of membrane trafficking leading to lysosomes. Studies of these important membrane sorting mechanisms are at an extremely exciting, albeit early, stage of development. It is anticipated that further studies will provide fundamental insight into the diversity of regulatory mechanisms within the large superfamily of GPCRs, and will more fully examine the intimate relationships that appear to exist between specific mechanisms of GPCR signaling and membrane trafficking. Moreover, as recent studies suggest that highly specialized biochemical mechanisms regulate the membrane trafficking of distinct GPCRs, elucidating these mechanisms may identify targets for the development of novel classes of highly specific drugs useful for therapy of pathological states associated with dysregulation of particular GPCRs.
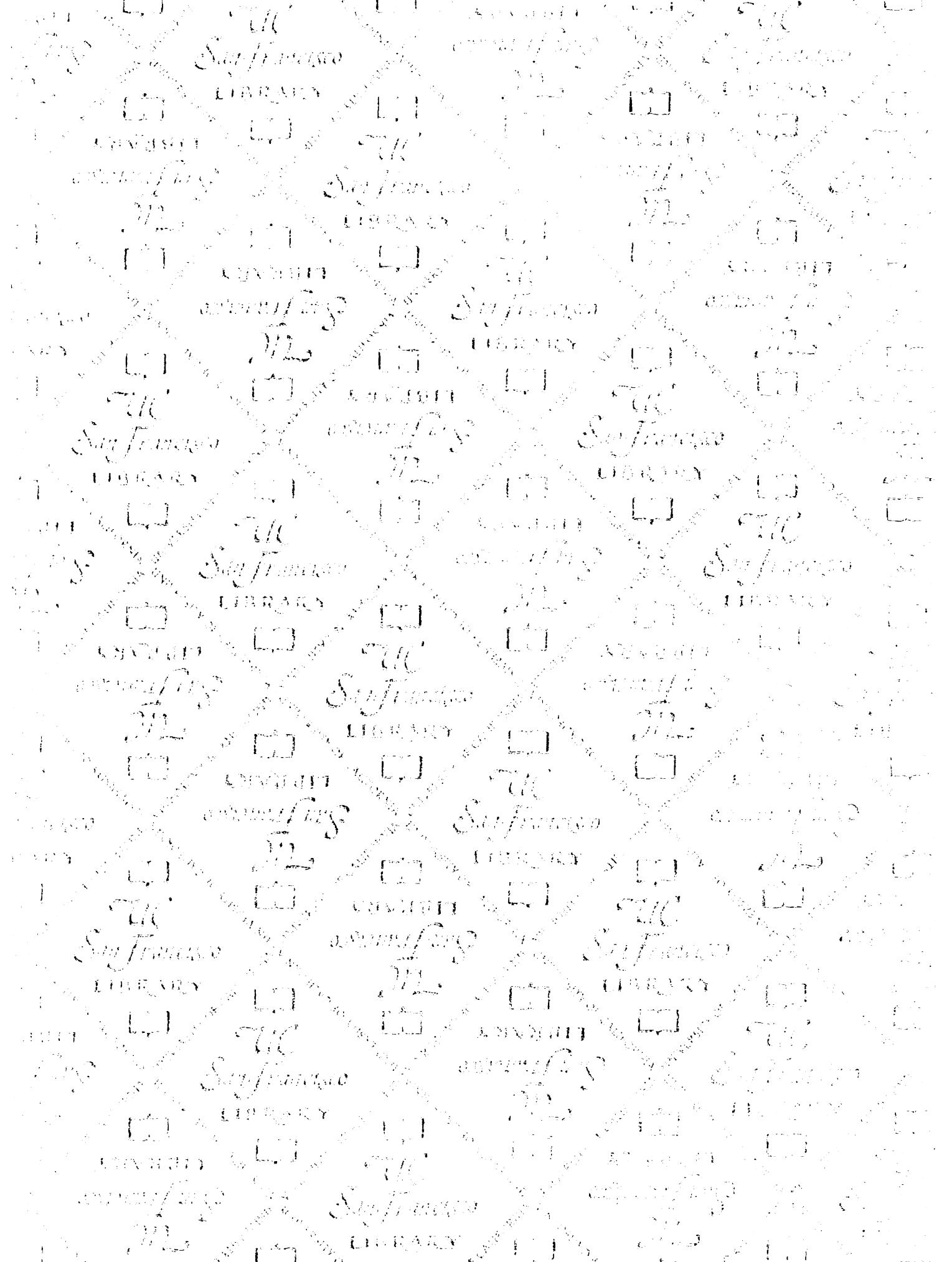
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