Phosphorylation State of \( \mu \)-Opioid Receptor Determines the Alternative Recycling of Receptor via Rab4 or Rab11 Pathway

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Agonist-induced phosphorylation, internalization, and intracellular trafficking of G protein-coupled receptors are critical in regulating both cellular responsiveness and signal transduction. The current study investigated the role of receptor phosphorylation state in regulation of agonist-induced internalization and intracellular trafficking of \( \mu \)-opioid receptor (MOR). Our results showed that after agonist stimulation, the recycle of a mutant MOR that lacks the C-terminal residues after Asn\(^{362} \) (MOR362T) was greatly decreased, whereas a C-terminal phosphorylation sites-mutated MOR (MOR3A), which is deficient in agonist-induced phosphorylation recycled back to the membrane at a level comparable to that of the wild-type receptor, however, interestingly at a slower rate. Inhibition of functions of either Rab4 or Rab11 by dominant-negative mutants and small interfering RNA both significantly impaired the recycling of the wild-type MOR, whereas the recycling of the phosphorylation-deficient mutant was only inhibited by the dominant-negative mutant and small interfering RNA of Rab11, suggesting that the recycling of nonphosphorylated MOR is exclusively via Rab11-mediated pathway. Furthermore, phosphorylated MOR was observed accumulated in Rab5- and Rab4-, but not Rab11-positive vesicles. Our data indicate that both phosphorylated and nonphosphorylated MOR internalize via Rab5-dependent pathway after agonist stimulation, and the phosphorylated and nonphosphorylated MORs recycle through distinct vesicular trafficking pathways mediated by Rab4 and Rab11, respectively, which may ultimately lead to differential cellular responsiveness or downstream signaling. (Molecular Endocrinology 22: 1881–1892, 2008)

PROTEIN-COUPLED RECEPTORS (GPCRs), as transducers of numerous extracellular signals to interior of cell to generate integrated responses, are stringently regulated by multiple mechanisms acting at different levels of signal propagation. One of the major regulatory mechanisms is internalization and intracellular trafficking of GPCRs (1). After agonist-induced activation, GPCRs internalize into endosomal vesicles through clathrin-coated pits. This event decreases the amount of surface receptors and ultimately leads to attenuation of the receptor-mediated signaling transduction (2). The internalized receptors undergo different intracellular trafficking pathway. Some can be recycled back to the cell surface, contributing to the recovery of cellular responsiveness to agonist. Alternatively, the internalized receptors can be targeted to lysosomes for degradation, resulting in a prolonged attenuation of signal transduction (1, 3). Internalization and intracellular trafficking of GPCRs regulate the responsiveness of cell surface receptors, and in some cases also generate new signal transduction to certain downstream effectors, including MAPK, p53, and nuclear factor-\( \kappa \)B (4, 5), through the formation on endosome membranes of a protein complex including both internalized GPCRs and signal-transducing kinases (such as c-Src) recruited from the cytoplasm (6, 7) or receptor tyrosine kinases (such as epidermal growth factor receptors) cointernalized from the plasma membrane (8).

Many studies have suggested that the carboxyl terminus of GPCRs, which undergoes pronounced conformational changes after agonist stimulation, is the major regulatory domain of the internalization and intracellular trafficking of receptors. Carboxyl terminus of certain GPCRs contains specific sorting domains that are sufficient to dictate receptors to recycling or degradation pathways (9–13). It could also interact with some cytoplasmic sorting proteins to regulate receptor intracellular trafficking (14–22). Studies on the \( \beta2 \)-adrenergic receptor (\( \beta2 \)-AR) have demonstrated that phosphorylation of the carboxyl terminus of the receptor by GRK facilitates its interaction with \( \beta \)-ar-
restins, which promotes the association of activated receptors with clathrin-coated pits to initiate of internalization (23–26). Carboxyl-terminal phosphorylation of receptors and the subsequent β-arrestins recruitment are critical for the internalization of GPCRs. However, increasing evidence indicates that certain GPCRs, such as CXCR4, PTH receptor, substance P receptor, δ- and μ-opioid receptors, internalize independently of carboxyl-terminal phosphorylation (27–31), challenging the critical role of phosphorylation in GPCRs internalization. Our recent work showed that phosphorylation-dependent and -independent internalization of δ-opioid receptor involves different β-arrestin isoforms (32). However, little is known on whether phosphorylation of receptors is necessary for intracellular trafficking of GPCRs.

The internalization and intracellular trafficking of GPCRs also involve the movement of receptors between intracellular membrane vesicles, which is a highly regulated process including a series of the vesicular membrane budding and fusion events. Many studies have demonstrated that Rab GTPases, which belong to the small GTPases superfamily, are key regulators of the trafficking between different vesicles (33). Rab5 mediates transport or fusion of endocytic vesicles with early endosomes (34). Rab4 regulates recycling from early endosomes to the plasma membrane, whereas Rab11 directs transport from the perinuclear recycling vesicles back to the cell surface (35). Rab7 is responsible for the continuous fusion events between late endosomes and lysosomes (36). Although the internalization and intracellular trafficking of several GPCRs have been shown to involve certain Rab GTPases (37–42), the mechanisms sorting the internalized receptors to different vesicular trafficking pathways remain largely unknown.

μ-Opioid receptor (MOR) of the GPCRs superfamily, which is activated by endogenously produced opioid peptides, modulates a number of physiological processes including pain, stress, neurotransmitter release, and immune responses. MOR also serves as the principal physiological target for most clinically important opioid analgesics, such as morphine (43). Similar to other GPCRs, prolonged agonist treatment leads to a rapid attenuation of MOR signal transduction (44). Cellular processes such as the internalization and intracellular trafficking of receptors modulate MOR responsiveness and have been implicated in opioid tolerance. The correlation between MOR phosphorylation and internalization has been extensively studied. Overexpression of GRK2 increases agonist-induced phosphorylation and promotes MOR desensitization and internalization (45). Phosphorylation of Ser363, Thr370, and Ser375 residues in the carboxyl terminus of the receptors differentially regulates the agonist-induced MOR internalization (46). Other reports have also proved that the rate of MOR internalization is controlled by cellular events other than receptor phosphorylation. A truncated mutant at position 354 internalizes constitutively and the internalization of receptors is enhanced in the presence of DAMGO (47). Similarly, a mutant MOR, truncated at the amino acid residue Ser363 to remove the putative phosphorylation sites (Thr370 and Ser375), undergoes internalization in N2A cell line upon etorphine stimulation (30). In the present work, we examined the role of carboxyl-terminal phosphorylation in the vesicular trafficking of MOR after agonist stimulation. Our results suggest that, although the carboxyl-terminal structure is critical, phosphorylation of MOR is only involved in regulating the recycling rate of MOR. Furthermore, after internalized, the phosphorylated MOR recycles to the cell surface through a Rab4-mediated pathway, whereas the nonphosphorylated MOR undergoes recycling via a Rab11-mediated recycling mechanism.

RESULTS

Phosphorylation Is Not Required for Agonist-Induced MOR Internalization

It has been demonstrated in human embryonic kidney (HEK) 293 cells that phosphorylation occurs primarily at Ser363, Thr370, and Ser375 of MOR’s carboxyl terminus (46). To discriminate the role of the specific structure of carboxyl terminus and phosphorylation state of MOR in receptor internalization and intracellular trafficking, we constructed two MOR mutants. One is MOR362T, in which the amino acid residues after Asn362 were deleted. The other is MOR3A, in which the identified phosphorylation sites Ser363, Thr370, and Ser375 were substituted by alanine. As shown in Fig. 1A, the mutants were not phosphorylated upon agonist stimulation, consistent with previous report (46, 47). The internalization of the mutated receptors after agonist stimulation was assessed by flow cytometry. The result (Fig. 1B) showed that the surface wild-type receptors were reduced 38 ± 1.7% after a 30-min exposure to DAMGO, whereas MOR362T, which lacks the amino acid residues including the phosphorylation sites, was merely reduced 12 ± 1.7%. However, the agonist-induced internalization of another phosphorylation-deficient mutant MOR3A was not significantly different from the wild-type receptors (35 ± 1.6%).

Many studies have shown that GPCRs are internalized to early endosomes after agonist stimulation, and Rab5 plays an important role in this process (34). To examine the role of Rab5 in the agonist-induced internalization of MOR, HEK293 cells were transiently transfected to express the wild-type and mutant MORs with the constitutive-active Rab5 (Q79L) or dominant-negative Rab5 (S34N) mutant. The flow cytometry data showed that overexpression of Rab5Q79L promoted the internalization of the wild-type MOR, MOR362T, and MOR3A and overexpression of Rab5S34N nearly abolished the internalization of all these receptors (Fig. 1B). Moreover, in cells overexpressing GFP-Rab5, DAMGO treatment promoted colocalization of the wild-type MOR, MOR362T, and
MOR3A with GFP-Rab5-positive vesicles, suggesting their trafficking to early endosomes (Fig. 2). These data suggest that Rab5 GTPases-mediated vesicular trafficking is required for the internalization of MOR, and it is independent of carboxyl-terminal structure or agonist-induced phosphorylation of receptors.

**Phosphorylation Is Not Required for MOR Recycling after Agonist-Induced Internalization**

Once internalized, GPCRs either return to the cell surface or are delivered to lysosomes for degradation (3). Next, we examined the fate of the wild-type MOR, MOR3A, and MOR362T after internalized. The recycling of cell surface receptors was monitored by flow cytometry as described in Materials and Methods. The recycling time courses (Fig. 3A) showed that cell surface MORs recovered rapidly ($t_{1/2} = 15$ min) after agonist removal and 69 ± 4.0% of the internalized receptors returned to the cell surface within 60 min. Although the phosphorylation-deficient mutant MOR3A exhibited a slower recycling rate ($t_{1/2} = 25$ min), the extent of its recycling at 60 min was comparable to that of MOR (62 ± 4.4%). In contrast, the recycling of MOR362T was severely impaired (13 ± 2.9%, 60 min after agonist removal). The above results suggest that intracellular trafficking pathway of MOR362T might be different from that of MOR and MOR3A. Our immunofluorescence imaging data showed that 60 min after agonist stimulation, MOR362T colocalized extensively with GFP-Rab7, a marker for late endosomes and lysosomes, whereas the wild-type MOR and MOR3A were present primarily in GFP-Rab7-negative vesicles under the same condition (Fig. 3B), suggesting intracellular trafficking of MOR362T may associate with lysosomes.

Above experiments quantified the steady-state levels of surface receptors during and after agonist treatment. To more accurately measure the percentages of internalized receptor trafficking back to the membrane and those of being degraded, we carried out a cleavable-biotin label assay that specifically labels cell surface receptors and monitors their intracellular trafficking after internalization by their inaccessibility to a membrane-impermeable reducing agent as described previously (32, 48). As shown in Fig. 4, biotinylated MOR, MOR3A, and MOR362T internalized after agonist stimulation (Fig. 4A, lanes 2, 6, and 10). After a recovery period after the agonist removal from the culture medium, more than 50% of the internalized MOR and MOR3A recovered rapidly ($t_{1/2} = 15$ min), whereas MOR362T showed a slower recycling rate ($t_{1/2} = 25$ min). The extent of recycling at 60 min was comparable to that of MOR (62 ± 4.4%). In contrast, the recycling of MOR362T was severely impaired (13 ± 2.9%, 60 min after agonist removal). The above results suggest that intracellular trafficking pathway of MOR362T might be different from that of MOR and MOR3A. Our immunofluorescence imaging data showed that 60 min after agonist stimulation, MOR362T colocalized extensively with GFP-Rab7, a marker for late endosomes and lysosomes, whereas the wild-type MOR and MOR3A were present primarily in GFP-Rab7-negative vesicles under the same condition (Fig. 3B), suggesting intracellular trafficking of MOR362T may associate with lysosomes.

**Fig. 1.** Agonist-Induced Internalization of the Wild-Type and Mutant MORs

A, HEK293 cells were transiently transfected with HA-tagged wild-type or mutant receptors. After 48 h, cells were either not treated (−) or treated (+) with 10 μM DAMGO for 30 min. The receptors were immunoprecipitated from cell lysates using anti-HA agarose beads and immunoblotted with a rabbit antiphosphorylated-Ser375 MOR antibody (pMOR, upper panel). Expression of the total receptors was confirmed by immunoblotting with a mouse anti-HA antibody (MOR, lower panel) from the same blot. Shown is a representative autoradiograph from one of three independent experiments. B, HEK293 cells were cotransfected HA-tagged receptors with β-gal (control), flag-tagged Rab5Q79L, or Rab5S34N. Surface receptor fluorescence was determined by flow cytometry. Percentage of internalization refers to the fractional reduction of surface receptor in response to 30-min DAMGO exposure. Data are means ± SE of at least three independent experiments. **+, P < 0.01 vs. the wild-type MOR; ##, P < 0.01 vs. the control groups.**
biotinylated wild-type MOR and MOR3A remained (Fig. 4A, lanes 3 and 7). However, receptors could hardly be detected after the cells were treated with membrane-impermeable biotin cleavage agent (Fig. 4A, lanes 4 and 8), indicating that those internalized receptors largely recycled to the cell surface. The internalization of MOR362T was also examined. In contrast, nearly no biotinylated MOR362T was detected after the recovery period (Fig. 4A, lane 11), indicating that majority of the internalized MOR362T was degraded. Taken together, these experiments reveal that receptor phosphorylation is not essential for MOR recycling. Instead, the distal portion of carboxyl terminus of receptors may contain specific structural element(s) required for the recycling of MOR.

Recycling of MOR Is Mediated by Both Rab4 and Rab11, Whereas Recycling of Nonphosphorylated Receptor Is Exclusively via Rab11

The functional recovery of desensitized MOR involves recycling of internalized receptors to the cell surface (49, 50). The processing of internalized receptors from early endosomes has been well characterized and is known to involve Rab4-mediated fast recycling pathway and Rab11-mediated slow recycling pathway (33). Considering the slower recycling kinetics of MOR3A (Fig. 3A), we then explored whether nonphosphorylated MOR recycles via distinct vesicular trafficking pathway from the phosphorylated MOR. Our result showed that overexpression of dominant-negative Rab4 (Rab4S24N) or Rab11 (Rab11S25N) had no effect on the agonist-stimulated internalization of MOR.
and MOR3A (Fig. 5, A and B). Interestingly, overexpression of Rab11S25N significantly impaired the recycling of both MOR and MOR3A, whereas overexpression of Rab4S24N only impaired the recycling of MOR, not MOR3A (Fig. 5, C and D). Although the expression of the wild-type or mutant receptors on the surface of nonstimulated cells did not change (data not shown), overexpressing GFP-Rab11S25N (Fig. 6), the surface distribution of both MOR and MOR3A after agonist removal was reduced and most of receptors retained in the cytoplasm of cell. However, in cells overexpressing GFP-Rab4S24N, most phosphorylation-deficient mutant MOR3As were distributed on the cell surface, whereas the surface distribution of the wild-type MOR on the cell was remarkably reduced under the same condition.

To further clarify the relationship between MOR phosphorylation state and the recycling pathway, we determined the effect of Rab4 and Rab11 siRNAs on the recycling of the wild-type MOR and MOR3A. Introducing the siRNA of either Rab4 or Rab11 into HEK293 markedly reduced corresponding endogenous Rab GTPases expression (Fig. 7A). Similar to the results obtained from cells transiently expressing dominant-negative mutants, reduction of the protein level of endogenous either Rab4 or Rab11 by siRNA had no significant effect on the internalization of both receptors (Fig. 7B). Consistent with the result obtained with dominant-negative Rabs, Rab11 siRNA significantly impaired the recycling of both MOR and MOR3A, whereas Rab4 siRNA only inhibited the recycling of the wild-type MOR, not MOR3A (Fig. 7C).

Together, the above results indicate that the recycling of MOR involves both Rab4 and Rab11 GT-Pases, and imply that phosphorylated and nonphosphorylated MORs return to the cell surface probably through different vesicular trafficking pathways: phos-
phorylated MOR recycles through a Rab4-mediated pathway, whereas the nonphosphorylated MOR recycles via a Rab11-mediated mechanism.

Dephosphorylation in Rab5- and Rab4-Positive Vesicles Are Required for Phosphorylated MOR Recycling to the Cell Surface

Agonist-induced phosphorylation of GPCRs is thought to be quickly reversed upon removal of the agonist by receptor internalization and dephosphorylation catalyzed by phosphatases (51–54). Studies on the β-adrenergic receptor have shown that okadaic acid, a potent inhibitor of protein phosphatase 1 (PP1)/2A (PP2A), inhibited β-adrenergic receptor dephosphorylation and thereby its recycling, supporting that dephosphorylation of receptor plays critical role in receptor recycling (51). Because the recycling kinetics of β-adrenergic receptor and MOR are very similar, we explored whether receptor dephosphorylation also play a role in recycling of the MOR. As shown in Fig. 8A, the level of phosphorylated MOR decreased after agonist removal, indicating a process of dephosphorylation. Treating cell with low-dose okadaic acid (100 nm), which had no effect on surface expression and agonist-induced internalization of receptors (data not shown), were efficacious to block dephosphorylation of MOR, inducing an increase in the basal phosphorylation level of MOR and the phosphorylation level of receptors remained for at least 60 min after agonist removal. Furthermore, okadaic acid treatment significantly decreased the recycling of MOR but had no detectable effect on the recycling of MOR3A (Fig. 8B). Our findings suggest that the regulation of dephosphorylation of MOR is primarily mediated by PP1/PP2A, and receptor dephosphorylation may play an important role for the phosphorylated MOR to efficiently recycle to the membrane, although the effect of okadaic acid on other dephosphorylation processes could not be excluded.

Fig. 7. The Effect of Rab4 and Rab11 siRNAs on the Recycling of MOR and MOR3A

HEK293 cells expressing MOR or MOR3A were transfected with control, Rab4 or Rab11 siRNA. Forty-eight hours after transfection, cells were treated with 10 μM DAMGO for 30 min and then washed and incubated in media without agonist for 60 min. A. The whole cell lysates were subjected to Western blotting. The reduction of Rab4 or Rab11 expression induced by siRNA targeting was probed with mouse anti-Rab4 or -Rab11 antibodies (actin as loading control). The internalization (B) and the recycling (C) of MOR and MOR3A were measured and analyzed by fluorescence flow cytometry assay. Data are means ± SE of at least three independent experiments. *, P < 0.05; **, P < 0.01 vs. the control groups.

Fig. 8. Effect of Okadaic Acid on the Recycling of MOR and MOR3A

HEK293 cells expressing HA-tagged MOR were pretreated with dimethylsulfoxide (Control) or 100 nm okadaic acid for 45 min. Treatment was then continued for an additional 30 min with 10 μM DAMGO, and for another 60 min in the agonist-free medium. A. The receptors were immunoprecipitated using anti-HA agarose beads and immunoblotted with rabbit antiphosphorylated-Ser375 MOR antibody (pMOR, upper panel). Expression of the total wild-type and mutant receptors was confirmed by immunoblotting with an mouse anti-HA antibody (MOR, lower panel) from the same blot. Shown is a representative autoradiograph from one of three independent experiments. B. Surface receptor fluorescence was determined by flow cytometry, the recycling of receptors was analyzed. Data are means ± SE of at least three independent experiments. **, P < 0.01 vs. the control groups.
We also characterized the vesicular trafficking pathway where dephosphorylation of MOR took place. Immunofluorescence images (Fig. 9) of cells treated with phosphatase inhibitor showed that the clustered MOR structures colocalized with Rab5-, Rab4-, and Rab11-positive vesicles after agonist stimulation, whereas the phosphorylated MOR clusters were only found in Rab5- and Rab4-positive vesicles but not Rab11-positive vesicles. These observations suggest that dephosphorylation of the internalized MORs mediated by PP1/PP2A may occur in both Rab5 and Rab4 vesicles or in vesicles in transition between the Rab5- and Rab4-positive early endosomal vesicles, which is critical for the recycling of phosphorylated MOR.

Fig. 9. Colocalization of the Phosphorylated MOR with Different Rab GTPases
HEK293 cells expressing HA-tagged MOR were pretreated with dimethylsulfoxide or 100 nM okadaic acid for 45 min. Treatment was then continued for an additional 30 min with medium containing 10 μM DAMGO, a mouse anti-HA antibody, and a rabbit antiphosphorylated-Ser375 MOR antibody at 37 C. After fixed and permeabilized, the cells were probed by Cy3-conjugated antimouse and Cy5-conjugated antirabbit antibodies. Cells were visualized with confocal microscope by triple excitation and corresponding emission filter sets. The colocalization of receptors (MOR, red; pMOR, blue) with GFP-RabGTPases (green) was indicated by arrowheads. Representative confocal images are shown from approximately 10 cells visualized per condition in each experiment and over three independent experiments. The scale bars represent 10 μm.

DISCUSSION
The heterogeneous nature in the active conformations of a given GPCR suggests the existence of different pharmacological properties and downstream signal pathways of these active states, creating an entirely new field of investigations. Differences between the signaling properties of multiple agonists through a single GPCR have been extensively studied. It has been reported that both CCL19 and CCL21, the endogenous agonist for CCR7, induce G protein activation and calcium mobilization with equal potency; however, only activation by CCL19, not CCL21, promotes robust desensitization of endogenous CCR7 through the induction of receptor phosphorylation and β-arrestin recruitment (55). Our preliminary work has also found that TIPP, which has been shown to have the same agonist activity as DPDPE and SCN 80 (56, 57), failed to promote phosphorylation and internalization of δ-opioid receptor (unpublished data). MOR has also been shown to be differentially regulated depending on agonist occupancy. For example, both morphine and etorphine promote receptor desensitization and analgesic tolerance, whereas morphine seems to be much less effective than etorphine in promoting receptor phosphorylation. β-arrestin recruitment, and MOR internalization (45, 48, 58), and each of these limitations can be overcome by overexpression of GRK2. Salvinorin A, a potent κ-opioid receptor agonist, is capable to activate MOR but induces very little receptor phosphorylation and no β-arrestin recruitment, or receptor internalization even in the presence of overexpressed GRK2 (59). Altogether, these findings strongly indicate that phosphorylation can place the receptor in different conformations for activation of downstream signaling and regulation of GPCRs. The present study has provided a detailed analysis on the role of phosphorylation state of receptors in agonist-induced MOR internalization and intracellular trafficking, thus providing insight into whether different active state of receptors may undergo diverse vesicular trafficking pathways and ultimately lead to distinct functional regulation of MOR.

It has been demonstrated that Rab5 mediates internalization of several GPCRs, including dopamine D2 receptor (D2DR), β2AR, neurokinin 1 receptor (NK1R), and angiotensin II type 1A (AT1AR) (37, 38, 60, 61). In this study, we observed that the internalized wild-type or phosphorylation-deficient MORs prominently colocalized with Rab5-positive vesicles and that overexpression of constitutively active or dominant-negative Rab5 mutants respectively promoted or impeded the internalization of all these receptors (Figs. 1 and 2), suggesting that phosphorylation is not essential for the internalization of MOR through the Rab5-mediated vesicular trafficking pathway. Our data indicate, however, that phosphorylation state is important for MOR trafficking through different Rab vesicles after internalization. We observed that most of internalized MOR
and MOR3A recycled to the cell surface and exhibited no detectable localization in GFP-Rab7-positive vesicles, whereas MOR362T largely colocalized with GFP-Rab7 and underwent degradation (Figs. 3 and 4), indicating that subsequent steps mediating recycling of the internalized MOR require carboxyl-terminal structure of receptors. Consistent with our results, it has been reported that a sequence in the cytoplasmic tail of mouse μ-opioid receptor is both necessary and sufficient for the rapid recycling (62).

Internalized receptors may return to the cell surface by at least two distinct recycling pathways, either rapidly from sorting endosomes or slowly from recycling endosomes. Rab4 is partially colocalized with Rab5 and governs the rapid cell-surface recycling of proteins from early endosomes, whereas Rab11 controls the slow recycling route of proteins and nutrients from perinuclear recycling endosomes back to the plasma membrane (35). It has been demonstrated that Rab11 mediates the recycling of V2 vasopressin receptor (V2R), somatostatin-3 receptor (SST3R), M4 muscarinic acetylcholine receptor (m4 mAChR), and CXCR2, whereas Rab4 mediates the recycling of corticotropin-releasing factor 1a receptor (CRF1 R) (39, 40, 63–65). Recent studies established that both Rab4 and Rab11 regulate the recycling of AT1aR, β2AR and NK1R (38, 41, 42, 66, 67), indicating the involvement of more than one Rab GTPases in regulating the recycling of a certain GPCR. Our data of the colocalization of MOR with both Rab4- or Rab11-positive vesicles (Fig. 9) indicate that MOR uses both of Rab4- and Rab11-mediated recycling pathways. Using dominant-negative mutants or siRNA of Rab4 and Rab11, we showed that the recycling of nonphosphorylated MOR is exclusively via Rab11-mediated mechanism (Figs. 5–7). In addition, okadaic acid treatment resulted in accumulation of the phosphorylated MOR in Rab5- and Rab4-positive vesicles and significantly attenuated the recycling of the wild-type MOR (Figs. 8 and 9). It suggests that dephosphorylation of the internalized MOR mediated by PP1/PP2A occurs in Rab5- or Rab4-positive early endosomal vesicles and this process is critical for the recycling of phosphorylated MOR.

Based on the present results and the data available in the literature, a proposed model depicting the internalization and intracellular trafficking of MOR and possibly other GPCRs is shown in Fig. 10. A specific motif dictating internalization and recycling of MOR is located in the carboxyl terminus containing the phosphorylation sites and disrupting the integrity of such motif may result in inability to enter Rab4/Rab11-associated sorting pathways and ultimately missorting to Rab7-mediated degradation pathway (Figs. 3 and 4). Additionally, the internalized MOR could recycle via two distinct pathways mediated by different profiles of Rab GTPases, dependent on their phosphorylation state. Phosphorylated MOR recycles through the Rab4-mediated rapid pathway, whereas nonphosphorylated MOR recycles through Rab11-dependent slow recycling pathway, leading to the differential resensitization and responsiveness of surface receptors.

The mechanisms underlying the differential trafficking between the phosphorylated and nonphosphorylated receptors remain to be understood. Oakley et al. (68, 69) demonstrated that the stability of the receptor/β-arrestin complex regulates the rate of receptor recycling and resensitization. Receptors that dissociate from β-arrestin at or near the plasma membrane are rapidly dephosphorylated and recycled, whereas receptors that remain associated with β-arrestin are slowly dephosphorylated and recycled (69). However, our preliminary studies did not find a significant difference between phosphorylated and nonphosphorylated MORs in their dissociation from β-arrestins. Recent work demonstrated that phosphorylation-dependent and -independent DOR internalization involves different β-arrestin isoforms (32). Our result has
established that the recycling of phosphorylated and nonphosphorylated MORs is mediated by different profiles of Rab GTPases. These data raise the possibility that distinct β-arrestin isoforms may mediate the phosphorylated and nonphosphorylated MORs associating with different sorting proteins such as Rab GTPase itself or its downstream effectors and hence targeted to diverse trafficking or signaling transduction pathways.

In conclusion, our results suggest that there may exist remarkably complex mechanism in agonist-induced internalization and intracellular trafficking of MOR, perhaps most importantly, highlight a previous unknown role of receptor phosphorylation state in regulating the recycling of internalized MOR through different Rab pathways. Whether these regulatory mechanisms are conserved among other members of the GPCR superfamily remains to be verified. Nevertheless, it may be exploitable for the development of pharmaceuticals that will selectively interact with one conformation of receptor and thus exert impact specifically on the targeted signaling pathway and receptor function.

MATERIALS AND METHODS

Plasmid Construction

The human influenza virus hemagglutinin (HA) epitope-tagged rat MOR was kindly provided by Dr. H. Loh (University of Minnesota School of Medicine, Minneapolis, MN), and subcloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). A truncated mutant MOR362T and a point mutant MOR3A were constructed by PCR mutagenesis as previously described (46, 47). GFP-Rab5, GFP-Rab11 and GFP-Rab7 were kindly provided by Dr. D. Pei (Tsinghua University, Beijing, China) and subcloned into pcDNA3. The Rab4 construct were made by PCR from human universal cDNA library, 5′-Oligonucleotide primers introduced an amino-terminal BamHI restriction site, and 3′-Oligonucleotide primers introduced a carboxyl-terminal Xhol restriction site. The BamHI, Xhol digested PCR product was fused at the C terminus to GFP or flag motif to allow subcloned into pcDNA3. All mutant Rab GTPases plasmids were constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequence integrity of the DNA constructs was confirmed by DNA sequencing.

Cell Culture and Transfection

HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in MEM containing 10% fetal bovine serum (Invitrogen Life Technologies, Gaithersburg, MD). Cell monolayer was transiently transfected using the calcium phosphate-DNA coprecipitation method with the cDNAs described in the figure legends. Cells were used for experiments 42–48 h after transfection.

Stealth/siRNA duplex oligonucleotides of negative control, Rab4, Rab11 were purchased from Invitrogen (Carlsbad, CA) for siRNA-mediated interference. HEK293 cells were seeded at a density of 2.5 × 10^4/100-mm dish and were transiently transfected by a calcium phosphate method with the plasmids encoding the receptor cDNAs described in the figure legends. After transfection (around 20 h), the cells were reseeded into six-well dishes for internalization and recycling studies. Four microliters of Lipofectamine RNAiMAX (Invitrogen) and 5 μl of 20 nM siRNA were added separately to 100 μl of Opti-MEM, and incubated for 5 min. Then both solutions were mixed and incubated for 20 min. The transfection mixture was added to each well of culture dishes containing 0.8 ml of fresh MEM and 10% fetal bovine serum without antibiotics. All experiments were performed 48 h later.

Quantitative Analysis of Receptor Internalization and Recycling by Fluorescence Flow Cytometry Assay

Monolayer of transiently transfected cells expressing the indicated plasmids was incubated in the absent (untreated condition) or presence of 10 μM DAMGO (Sigma, St Louis, MO) for 30 min at 37 C. Cells were chilled on ice to stop receptor trafficking (agonist-treated condition) or subsequently incubated with MEM for different time periods indicated in the figure legends (washout condition). Surface receptors were labeled with mouse anti-HA antibody (Covance, Berkeley, CA) for 1 h at 4 C. After sufficient washing with ice-cold Dulbecco’s PBS (D-PBS), cells were incubated with fluorescein isothiocyanate-conjugated antimouse antibody (Jackson ImmunoResearch, West Grove, PA) for another 1 h at 4 C. Then the cells were collected and fixed, and the fluorescence intensity of surface receptor was measured using a FACS Calibur instrument (Becton Dickinson, Mountain View, CA). The percentage of receptor internalization and recycling were calculated from mean surface receptor fluorescence values (F) as follows: % internalization = (F_{untreated} - F_{agonist-treated})/F_{untreated} × 100, % recycling = (F_{washout} - F_{agonist-treated})/F_{untreated} × 100. Basal cell fluorescence intensity was determined with cells stained with the second antibody alone.

Quantitative Analysis of Recycling and Degradation of Internalized MOR with Surface Biotin-Labeling Assay

Cells transfected with HA-tagged wild-type or mutant receptors were biotinylated at 4 C with 400 μg/ml Sulfo-NHS-Ss-biotin (Pierce, Rockford, IL) in D-PBS for 30 min. After rinsed with Tris-buffered saline (150 mM NaCl; 50 mM Tris, pH 7.4) and warmed to 37 C for 1 h, cell were incubated in the absent or presence of 10 μM DAMGO for 30 min to trigger receptor internalization. Surface biotin was cleaved by stripping buffer (50 mM glutathione, 100 mM NaCl, 60 mM NaOH, and 1% FBS) at 4 C for 20 min. Cells were then chilled on ice (untreated condition, agonist-treated condition) or rinsed with ice-cold PBS and gradually recovered in MEM at 37 C for 120 min without agonist (recovery condition). A second strip was used to determine whether there was any biotinylated receptor recycling to the cell surface (second strip condition). After being quenched with 50 mM iodoacetamide resolved in D-PBS at 4 C for 20 min, cells were extracted in lysis buffer (10 mM Tris, pH 7.4; 150 mM NaCl; 25 mM KCl; 0.5% Triton X-100). Receptors were immunoprecipitated with HA-conjugated Sepharose (Sigma), and resolved by SDS-PAGE. Protected biotinylated receptors were visualized with horseradish peroxidase-conjugated streptavidin (Sigma) and quantified by densitometer analysis. The percentage of receptor recycling or degradation was calculated from densitometer values (D) as follows: % recycling = (D_{recovery} - D_{and strip})/(D_{agonist-treated} - D_{untreated}) × 100, % degradation = (D_{recovery} - D_{recovery})/(D_{agonist-treated} - D_{untreated}) × 100. The densitometer values were normalized to those obtained from total receptors which was detected with mouse anti-HA antibody.

Western Blotting

Whole cell lysates or proteins eluted from the HA-conjugated Sepharose were resolved on 10% or 12% SDS-PAGE. Re-
solved proteins were transferred to polyvinylidene difluoride membrane (Amersham Biosciences, Arlington, IL). The membranes were incubated with a rabbit antiphosphorylated-Ser378 MOR antibody (Cell Signaling Technology, Beverly, MA), mouse anti-HA antibody, mouse M1 anti-flag antibody (Sigma), mouse anti-Rab4 or Rab11 antibody (BD Transduction Laboratories, San Diego, CA) respectively as indicated in figure legends, followed by incubation with specific secondary antibody and detection using an enhanced chemiluminescence detection system (Sigma). Immunoblots were quantified by Image-Pro-Plus 5.1 software (Media Cybernetic, Silver Spring, MD).

Confocal Immunofluorescence Microscopy

Confocal microscopy was performed on a Zeiss (Carl Zeiss, Jena, Germany) LSM-510 laser scanning microscope using a Zeiss 63 × 1.3 numerical aperture oil immersion lens. HEK293 cells coexpressing HA-tagged receptors with GFP-tagged Rab proteins were grown on poly-L-lysine-coated cover slips in 12-well clusters and incubated with mouse anti-HA antibody or rabbit antiphosphorylated-Ser378 MOR antibody at 37°C for 30 min. After treatment with reagents as indicated in figure legends, cells were fixed with 4% paraformaldehyde for 10 min, and then permeabilized with 0.2% Triton (vol/vol) for 15 min at room temperature. After incubation with Cy3-conjugated antimouse or Cy5-conjugated antirabbit antibody (Jackson ImmunoResearch) for 1 h at 4°C, the cover slips were mounted and visualized with the confocal microscope using dual excitation (488, 543 nm) or triple excitation (488, 543, 633 nm), and corresponding emission filters. Specificity of labeling and absence of signal crossover were established by examination of single-labeled samples.

Statistical Analysis

Data are expressed as the means ± se for values obtained for the number of independent experiments indicated in the figure legends. A Student's t test (two tailed) or one-way ANOVA was used and indicated significance (P < 0.05), Dunnett's multiple comparison test was used to determine which conditions were significantly different from control.

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