Phosphorylation of the δ-Opioid Receptor Regulates Its β-Arrestins Selectivity and Subsequent Receptor Internalization and Adenylyl Cyclase Desensitization*

Yu Qiu1, Horace H. Loh2, and Ping-Yee Law1

From the Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

In the current study, we investigated the role of receptor phosphorylation and β-arrestins in δ-opioid receptor (DOR) signaling and trafficking by using a DOR mutant in which all Ser/Thr residues in the C terminus were mutated to Ala (DTS). We demonstrated that the DOR agonist D-[Pen2, Pen5]enkephalin could induce receptor internalization and adenylyl cyclase (AC) desensitization of DTS, but with comparatively slower kinetics than those observed with wild type DOR. Blockade of the internalization of DTS by the dominant-negative mutant dynamin, dynamin K44E, did not affect AC desensitization. However, depletion of β-arrestins almost totally blocked both internalization and AC desensitization of DTS. A BRET assay suggested that DOR phosphorylation promotes receptor selectivity for β-arrestin 2 over β-arrestin 1. Furthermore, in mouse embryonic fibroblast (MEF) cells lacking either β-arrestin 1 (βarr1−/−) or β-arrestin 2 (βarr2−/−), agonist-induced DTS desensitization and internalization were similar to that observed in wild type MEFs. In contrast, although DOR internalization decreased in both βarr1−/− MEFs and βarr2−/− MEFs, DPDPE-induced DOR desensitization was significantly reduced in βarr2−/− MEFs, but not in βarr1−/− MEFs. Additionally, the BRET assay suggested that depletion of phosphorylation did not influence the stability of the receptor-β-arrestin complex. Consistent with this observation, DTS did not recycle after internalization, which is like wild type DOR. Taken together, these results indicate that receptor phosphorylation confers DOR selectivity for β-arrestin 2 without affecting the stability of the receptor-β-arrestin complex and the fate of the internalized receptor.

The receptor activity upon agonist stimulation of G protein-coupled receptors (GPCRs) is generally regulated by a common pathway that involves receptor phosphorylation by G protein-coupled receptor kinases and subsequent arrestin recruitment. The recruited arrestins promote uncoupling of the GPCRs (desensitization) from G proteins and subsequent internalization (also termed endocytosis or sequestration) via clathrin-coated pits (1–3). Being a member of rhodopsin subfamily of GPCRs, the opioid receptor undergoes receptor phosphorylation, desensitization, and internalization upon agonist activation. Concrete demonstration of opioid receptor phosphorylation has long been reported for all three classes of opioid receptors, µ, δ, and κ receptors (4–6). Furthermore, receptor phosphorylation has been shown to be necessary for the functional regulation of µ-opioid receptor (MOR) (7–9) and δ-opioid receptor (DOR) (10–12). However, a discrepancy between receptor phosphorylation and receptor internalization and desensitization appears to exist (13). Our previous studies have demonstrated that receptor phosphorylation is not absolutely required for MOR desensitization and internalization, but rather plays a role in the resensitization of the internalized receptors (14). The COOH-terminal truncated DOR is shown to undergo phosphorylation-independent agonist-induced desensitization (15, 16). Moreover, a “brake mechanism” theory has been suggested, in which phosphorylation is required for the endocytosis of full-length DOR, but not for the carboxyl tail-truncated receptor (17). However, phosphorylation-independent internalization of full-length DOR was reported recently (18). Thus, the role of phosphorylation in internalization and desensitization of DOR remains to be fully resolved.

β-Arrestin 1 and β-arrestin 2 are ubiquitously expressed and are major regulators for most of the GPCRs. Although agonist-induced receptor phosphorylation has been demonstrated to be of great importance for arrestins binding (19, 20), the ability of opioid receptors to recruit arrestins without being phosphorylated has been illustrated in MOR and DOR where the putative agonist-induced phosphorylation sites are removed or mutated (14, 18). Recently, the different roles of β-arrestin 1 and β-arrestin 2 in the internalization of DOR have been suggested (18). However, how the absence of receptor phosphorylation influences the binding property of the receptor to β-arrestins, and the regulatory profile of β-arrestins on phosphorylation-independent receptor signaling, are not well interpreted.

BRET, bioluminescence resonance energy transfer; AC, adenylyl cyclase; FACS, fluorescence-activated cell sorter; HA, hemagglutinin; GFP, green fluorescent protein variant at 405 nm excitation.

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1 To whom correspondence should be addressed: 6-120 Jackson Hall, 321 Church St. S.E., Minneapolis, MN 55455-0217. Tel.: 612-626-6539; Fax: 612-625-8408; E-mail: qiuoxi014@umn.edu.

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Moreover, receptor phosphorylation has been shown to play a role in the intracellular fate of the internalized receptors. Overexpression of G protein-coupled receptor kinase 2 can increase the down-regulation of the β2-adrenergic receptor (21). Phosphatase inhibitors can prevent effective receptor recycling of the cannabinoid CB1 receptor and the β2-adrenergic receptor (22, 23). Oakley and colleagues (24, 25) put forward a classification of GPCRs according to their binding status to β-arrestins. Class A GPCRs bind β-arrestins transiently and tend to recycle rapidly, whereas class B receptors form stable complexes with β-arrestins and tend to recycle slowly. Stable complexes between receptor and β-arrestin are shown to be determined by the phosphorylation of specific clusters of Ser and Thr residues in the carboxyl tail (26), in which depletion of phosphorylation reverses the internalized receptor from lysosome targeting to rapid recycling. In addition, a study using a “preactive” mutant of β-arrestins indicated that an unphosphorylated receptor forms a less stable complex with β-arrestin than a phosphoreceptor, and is more likely to recycle (21). DOR, exhibiting a high affinity for β-arrestins as compared with MOR (27), activates β-arrestins more efficiently (28) and traffics to lysosome after endocytosis (29). Hence, if observations with other GPCRs apply to the DOR function, then eliminating phosphorylation sites would destabilize the interaction between the receptor and β-arrestin, thus changing the eventual fate of the internalized receptor.

Therefore, in our current study, we investigated the role of receptor phosphorylation and β-arrestins in DOR adenyl cyclase (AC) signaling and trafficking. Our study demonstrated that full-length DOR could undergo phosphorylation-independent internalization and AC desensitization. In addition, the stability of the receptor-β-arrestin complex and the fate of the internalized receptor were not affected by the phosphorylation status. However, receptor phosphorylation affected DOR preference to β-arrestins, underlying the differential roles of β-arrestin 1 and β-arrestin 2 in the internalization and desensitization of opioid receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Plasmid encoding dynamin K44E (in pcDNA3 zeo(+)) was a gift from Dr. Mark von Zastrow (University of California, San Francisco, CA). β-Arrestin 1 and β-arrestin 2 tagged with GFP2 at the COOH terminus (in pEGFP-N1) were kindly provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA). GFP2 fusion protein expression vector (GFP2-C3) and DeepBlueC coelenterazine were from PerkinElmer Life Science. D-[Pen2,Pen5]Enkephalin (DPDPE) was supplied by the National Institute on Drug Abuse. Mouse monoclonal anti-he-magglutinin protein (HA) antibody (HA.11) was from Conva-nce (Richmond, CA). Anti-β-arrestin1/2 antibody (SGODDA, 2004) was kindly provided by Dr. Martin Oppermann (University of Göttingen, Göttingen, Germany). Anti-GFP antibody was from Santa Cruz (Santa Cruz, CA). AlexaTM-488 goat anti-mouse IgG was purchased from Molecular Probes (Eugene, OR). Other chemicals were purchased from Sigma.

**Plasmids and Adenovirus Construction**—The construction of human influenza virus HA epitope-tagged mouse wild type δ-opioid receptor (DOR) and HA-tagged mutant DOR with all 7 Ser and Thr residues in the carboxyl tail mutated to Ala (DTS) in pcDNA3 has been described previously (30).

For the construction of receptor-luciferase fusion proteins, a multisite mutation of HA-tagged mouse DOR and mutant DTS pcDNA3 plasmids was performed to insert an XbaI site after the coding sequence and to remove the stop codon using the QuikChange® multisite-directed mutagenesis kit (Stratagene, La Jolla, CA). Renilla luciferase coding sequence was amplified from pRL-null vector (Promega, Madison, WI), using sense and antisense primers harboring unique cloning sites (XbaI and Apal). The PCR fragments were then inserted after DOR and DTS coding sequences to yield constructs that were named HA-DOR-Rluc and HA-DTS-Rluc. Construction of plasmids for GFP2-β-arrestin fusion proteins were carried out by digesting β-arrestin 1 and β-arrestin 2 coding sequences from their expression plasmids (in pEGFP-N1) with HindIII and Apal, and then inserting them in-frame into GFP2-C3 vector to generate GFP2-β-arrestin 1 and GFP2-β-arrestin 2.

Recombinant HA-tagged mouse DOR and DTS adenovirus was constructed using AdEasy™ adenoviral vector system (Stratagene, La Jolla, CA). DOR and DTS in pcDNA3 were digested with Asp718 and XbaI and cloned into the pHShuttle vector. Homologous recombination of the pShuttle vector with AdEasy-1 vector in the BJ5183 cells was carried out as recommended by the manufacturer. Plasmids from the identified and isolated recombinants were digested with PacI before transfecting into HEK293 for virus production. Successful production of the virus was determined by opioid receptor binding of the cell pellets. Titer of the amplified virus was determined using the Adeno-X™ rapid titer kit (BD Biosciences).

**Transfection of HEK293 Cells**—Human embryonic kidney (HEK) 293 cells were maintained in modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The establishment of pools of stably transfected HEK293 cells expressing DOR or DTS has been described previously (30). Transient transfections were performed using FuGENE 6 reagent (Roche Applied Science), and experiments were performed 48 h after transfection.

**Infection of MEF Cells**—Mouse embryonic fibroblast (MEF) cell lines with or without β-arrestins were generously provided by Dr. Robert Lefkowitz (Duke University, Durham, NC). Five MEF cell lines were established (31): MEFs lacking β-arrestin 1 and its wild-type littermate controls WT1–7 MEFs; MEFs lacking β-arrestin 2 and its wild-type littermate controls WT-1 MEFs; MEFs lacking both β-arrestin 1 and 2; and WT-1 MEFs also served as its wild type controls. All MEFs were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For overexpression of DOR and DTS, MEF cells were infected with recombinant DOR or DTS adenovirus at a multiplicity of infection sufficient for expression of the receptor around 200–400 fmol/mg of protein.

**Determination of Receptor Internalization and Recycling by FACS Analysis**—Receptor internalization was quantified by FACS analysis as previously described (14). Briefly, after incubation with 1 μM DPDPE for the indicated time intervals, cells were chilled on ice to terminate receptor trafficking, and cell
surface receptors were visualized by incubating the cells with anti-HA antibody (1:1000), followed by incubation with the Alexa 488-conjugated anti-mouse IgG antibody (1:1000). Surface receptor staining intensity of the antibody-labeled cells was analyzed using fluorescence flow cytometry (FACScan, BD Biosciences). For recycling experiments, the cells were treated with 10 μg/ml cycloheximide for 2 h. Then, DPDPE was added at 1 μM, and the cells were further treated for 30 min for DOR or 2 h for DTS. The cells were washed repeatedly to remove DPDPE, followed by incubation in agonist-free medium containing 10 μg/ml cycloheximide at 37 °C for the indicated time intervals before staining the cell surface receptors.

**Determination of Receptor Desensitization by Measurement of Intracellular cAMP Levels**—Receptor desensitization was determined as previously described (14). Briefly, cells were exposed to 1 μM DPDPE for the indicated time intervals. The medium was then removed and replaced with 100 μl of treatment buffer, with or without agonist. The treatment buffer consisted of 0.5 mM isobutylmethylxanthine and 10 μM forskolin in Krebs-Ringer-HEPES buffer (KRHB; 110 mM NaCl, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, pH 7.4). The cells were incubated at 37 °C for 15 min. The reaction was terminated by heating the cells at 90 °C for 6 min. The cAMP level in the supernatant was measured by the AlphaScreen™ cAMP detection kit (PerkinElmer Life Science), as described previously (32).

**BRET Assay**—HEK293 cells were co-transfected with constructs coding receptor-Rluc and GFP2 or GFP2-β-arrestin proteins. 48 h after transfection, cells were harvested and washed once with phosphate-buffered saline. Then, the cells were suspended in Dulbecco’s phosphate-buffered saline (phosphate-buffered saline + 0.1% glucose + 0.01% CaCl2 + 0.01% MgCl2) and distributed into 96-well microplates (White Optiplate, PerkinElmer Life Science) at a density of 100,000 cells per well. The cells were warmed to 37 °C. DeepBlueC coelenterazine was added at a final concentration of 5 μM, and 1 or 2 readings were collected. Vehicle or 1 μM DPDPE was then added and readings were taken for 10 min. The reading was performed using the Fusion-α system (Packard Bioscience) that allows the sequential reading of the signals detected in the 370–450- and 500–530-nm windows. The BRET signal was determined by the ratio of the light emitted by the GFP2 or GFP2-β-arrestin (500–530 nm) over the light emitted by the receptor-Rluc (370–450 nm). The agonist-induced BRET ratio increase was calculated by subtracting the BRET ratio observed for vehicle-treated cells containing both Rluc and GFP2 or GFP2 fusion proteins from the ratio observed for the same cells treated with the agonist. Expression levels of receptors were determined by radioligand binding assay with [3H]diprenorphine. GFP2 and GFP2-β-arrestins transfected were determined by Western blot.

**Statistical Analysis**—Data are presented as mean ± S.E. Either unpaired Student’s t test (two-tailed) or one-way analysis of variance was performed for statistical comparisons. When analysis of variance was used and when this analysis indicated significance (p < 0.05), Dunnett’s multiple comparison test was used to determine which conditions were significantly different from the controls.

**RESULTS**

**Internalization and AC Desensitization of DTS**—Phosphorylation of DOR at Thr358 and Ser363 residues within the carboxyl tail upon agonist activation has been reported (30, 33), where Ser363 is the primary phosphorylation site. Our previous study with a mutation of Ser363 of DOR suggested that DOR could undergo phosphorylation-independent internalization and AC desensitization (12). However, a brake mechanism theory has been put forward that suggests that phosphorylation is required for the endocytosis of full-length DOR (17). To exclude the possibility of undetectable phosphorylation of other Ser and Thr residues at the carboxyl tail, a mutant of DOR with all Ser and Thr residues within the carboxyl tail mutated to Ala (designated as DTS), which fails to show any detectable phosphorylation under agonist treatment (30), was used to further investigate the role of receptor phosphorylation in full-length DOR desensitization and internalization.

Receptor internalization evaluated by FACS analyses showed that 1 μM DPDPE treatment could cause internalization of
phosphorylation-deficient mutant receptor DTS, although the rate was slower than that of DOR (Fig. 1A). The half-life ($t_{1/2}$) of DTS internalization was $3.4 \pm 1.3$ h, whereas DOR internalized with $t_{1/2} = 0.40 \pm 0.55$ h. The levels of DOR and DTS being internalized after 6 h of agonist exposure were $87 \pm 2.2$ and $73 \pm 0.68\%$, respectively. When the desensitization kinetics of DOR and DTS were compared, the ability of DPDPE to inhibit forskolin-induced cAMP production was measured in cells pretreated with agonist, but with slower kinetics.

Effect of Dominant-negative Dynamin on Internalization and AC Desensitization of DTS—Internalization and desensitization are two independent but related processes. The observed slower desensitization of DTS may be due to slower internalization. Thus, we used a dominant-negative dynamin, dynamin K44E, a mutant shown to inhibit the internalization of receptor, to block the internalization of DOR and DTS. As shown in Fig. 2A, dynamin K44E significantly inhibited the internalization of both DOR and DTS. However, dynamin K44E did not significantly alter the ability of either DOR or DTS to desensitize after agonist activation (Fig. 2B), suggesting that internalization contributes little to desensitization of either wild type DOR or mutant DTS.

The Role of β-Arrestins in the Internalization and AC Desensitization of DTS—β-Arrestins are key elements in terminating receptor signaling, and act as scaffolding proteins in coupling GPCRs to clathrin-coated vesicles (34, 35). To further characterize the observed internalization and desensitization of DTS, the role of β-arrestins in phosphorylation-independent internalization and desensitization was examined. MEF cells lacking both β-arrestin 1 and β-arrestin 2 (βarr1−/−/βarr2−/− MEF) were employed. As shown in Fig. 3A, 1 μM DPDPE-induced internalization of wild type DOR was blocked in βarr1−/−/βarr2−/− MEF, with only $20 \pm 3.5\%$ of the receptor internalized after 6 h of incubation. In contrast, $80 \pm 1.5\%$ of DOR was internalized after 6 h of agonist treatment in corresponding wild type MEFs (WT-1). These data confirm that the internalization of DOR is mainly β-arrestin-dependent. Similar to wild type DOR, most of DTS remained at the cell surface of WT-1 MEFs upon agonist treatment, with only $11 \pm 4.4\%$ of the receptor internalized, whereas the internalization was not blocked in WT-1 MEFs ($64 \pm 8.2\%$, Fig. 3A).

A similar approach was used to examine the AC desensitization profile of wild type DOR and DTS in βarr1−/−/βarr2−/− MEFs. As depicted in Fig. 3B, 6 h of treatment with 1 μM DPDPE on DOR-expressing βarr1−/−/βarr2−/− MEFs only desensitized the receptor by $18 \pm 4.0\%$, whereas agonist-induced desensitization of DOR in WT-1 MEFs reached $75 \pm 5.1\%$ after 6 h of incubation. For the phosphorylation-deficient mutant DTS, similar characteristics of agonist-induced desensitization were observed. Fig. 3B also shows that lacking both β-arrestins strongly inhibited the desensitization of DTS, with only $14 \pm 2.7\%$ receptor desensitization observed after treating βarr1−/−/βarr2−/− MEFs with 1 μM DPDPE for 6 h. Whereas DTS in WT-1 MEFs showed $58 \pm 2.6\%$ loss of DPDPE ability to inhibit forskolin-stimulated cAMP production after 6 h of agonist exposure.

Taken together, these findings indicate that phosphorylation-independent internalization and desensitization of DOR, like wild type DOR, are β-arrestin-dependent processes. However, the small amount of DOR internalized in MEFs lacking both β-arrestins suggests DOR may undergo internalization through other pathway(s).
The Interaction of β-arrestins with DTS—We next examined the ability of β-arrestins to interact with DTS upon agonist treatment, using BRET assay as described under “Experimental Procedures.” The expression levels of luciferase-tagged receptors were controlled to be within 10% difference (Fig. 4B). The GFP and GFP-β-arrestins were blotted with anti-GFP antibody (Fig. 4B) and quantified to be at about the same level (data not shown). DPDPPE induced rapid and sustained interaction of wild type DOR with β-arrestins 1 and 2 (Fig. 4A). More interestingly, DOR showed a stronger association with β-arrestin 2 than with β-arrestin 1; the difference was significant after 4 min of treatment with DPDPPE (p < 0.05). The plateau value of DPDPPE-induced BRET ratio increase for DOR and β-arrestin 2 was 0.026 ± 0.001, whereas for DOR and β-arrestin 1, it was 0.014 ± 0.003 (p < 0.05). DPDPPE also induced a prolonged interaction of unphosphorylated DTS with β-arrestins 1 and 2. The interaction profiles of DTS with both β-arrestins were similar to that of DOR with β-arrestin 1 (Fig. 4A, p > 0.05). The plateau values of DPDPPE-induced BRET ratio increases with β-arrestins 1 and 2 were 0.016 ± 0.004 and 0.018 ± 0.003, respectively (p > 0.05). Thus, these data suggest that phosphorylation mainly increases the affinity of DOR to β-arrestin 2.

The Differential Role of β-Arrestin 1 and β-Arrestin 2 on Receptor Internalization and AC Desensitization of DOR and DTS—Because the BRET assay demonstrated that distinct differences between the interaction profiles of DOR and DTS with β-arrestins are mainly between the phosphorylated receptor and β-arrestin 2, whether the effects of β-arrestin 1 and β-arrestin 2 on phosphorylation-dependent and -independent receptor internalization and desensitization have different patterns needs to be addressed. Treating MEFs lacking β-arrestin 1 (βarr1<sup>−/−</sup>) and MEFs lacking β-arrestin 2 (βarr2<sup>−/−</sup>) with 1 μM DPDPPE resulted in DOR internalization to a similar extent, with 44 ± 3.8 and 60 ± 2.1% of the receptor internalized after 6 h of treatment, respectively (Fig. 3A). These data were significantly different from those observed in their corresponding wild type MEFs (βarr1<sup>+/+</sup> versus WT1–7 with 72 ± 0.6%; βarr2<sup>+/+</sup> versus WT-1 with 83 ± 1.5%). Moreover, the internalization rates observed in βarr1<sup>−/−</sup> MEFs and βarr2<sup>−/−</sup> MEFs were still significantly faster than those observed in βarr1<sup>−/−</sup>/βarr2<sup>−/−</sup> MEFs (Fig. 3A). Taken together, these results indicate that β-arrestin 1 and β-arrestin 2 mediate the phosphorylation-dependent internalization of DOR almost equivalently and their effects are complementary. As for the phosphorylation-deficient mutant DTS, no appreciable difference was observed in agonist-induced receptor internalization in βarr1<sup>−/−</sup> MEFs or βarr2<sup>−/−</sup> MEFs compared with their corresponding wild type cells (Fig. 3A). 60–70% internalization of DTS was observed in all 4 MEF cell lines after 6 h of DPDPPE treatment. This observation demonstrates that either of the two β-arrestins can trigger phosphorylation-independent internalization of DOR sufficiently.

We next investigated the ability of β-arrestin 1 and β-arrestin 2 to mediate agonist-induced AC desensitization of DOR and DTS. As shown in Fig. 3B, DPDPPE-stimulated desensitization of DOR in βarr1<sup>−/−</sup> MEFs was not significantly impaired compared with wild type MEFs, with desensitization rates of up to 80–90% observed. However, the rate of DOR desensitization in βarr2<sup>−/−</sup> MEFs was reduced significantly, with only 43 ± 3.7% of the receptor desensitized, compared with its control WT-1 MEFs (75 ± 5.1% desensitized) after 6 h of DPDPPE treatment (Fig. 3B). Furthermore, this data were significantly different from that obtained in βarr1<sup>−/−</sup>/βarr2<sup>−/−</sup> MEFs. Thus, β-arrestin 2 is primarily responsible for regulation of phosphorylation-dependent desensitization of DOR, whereas β-arrestin 1 is a secondary factor and can complement the effect of
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FIGURE 4. Differential recruiting of β-arrestins by phosphorylated and unphosphorylated DOR upon agonist activation. HEK293 cells transiently co-transfected with DOR-RluC or DTS-RluC and GFP-β-arrestin 1 or GFP-β-arrestin 2 or GFP vector were incubated in the presence of DeepBlueC coelenterazine at a final concentration of 5 μM. Then, vehicle or 1 μM DPDPE was added, and the BRET measurements were immediately collected for 10 min. The interaction between receptors and β-arrestins was represented by the agonist-induced increase in the BRET ratio obtained by subtracting the BRET ratio observed for vehicle-treated cells from the ratio observed for the cells treated with the agonist. Data are mean ± S.E. of at least three independent experiments performed in triplicate (A). The expression level of RluC and GFP fusion proteins was detected by radioligand binding assay with [3H]diprenorphine and Western blotting using anti-GFP antibody. An anti-β-arrestin1/2 antibody (SGODDA, 2004) was used to compare GFP-β-arrestin 1 or GFP-β-arrestin 2 on phosphorylation-independent desensitization of DOR or DTS desensitized quickly in δar1−/− MEFs and in δar2−/− MEFs, reaching the maximal levels of 60 ± 9.3 and 53 ± 5.5%, respectively, after 6 h of agonist exposure. These observations were comparable with those observed in their corresponding wild type MEFs, demonstrating that lacking one of the two β-arrestins is not sufficient to affect agonist-induced AC desensitization of DTS.

**DISCUSSION**

Several reports address phosphorylation-independent internalization and desensitization of opioid receptors (14–16, 18). However, the requirement of receptor phosphorylation in the internalization and desensitization processes has been controversial (17, 37). The present study, with a phosphorylation-deficient mutant of DOR in which all 7 Ser and Thr residues in the carboxyl terminus were mutated to Ala, demonstrated that full-length DOR can undergo phosphorylation-independent receptor desensitization and internalization. These results are contrary to the brake mechanism theory, which states that phosphorylation is required for the endocytosis of full-length DOR (17). The reason for the discrepancy is unknown. However, our results are consistent with another study that demonstrates phosphorylation-independent internalization of DOR (18). Our current study and our previous study with MOR (14) used the measurement of adenylyl cyclase activity as the functional end point. Both studies demonstrated phosphorylation-independent desensitization during agonist treatment. However, the requirement of receptor phosphorylation in rapid desensitization of MOR and DOR has been suggested in the Xenopus oocyte expression system (38–40), where the activity of G-protein-coupled K+ channels was monitored. This discrepancy may be due to the different effectors involved in the studies. In the other studies, G-protein-coupled K+ channels are Gβγ-mediated, whereas in our study, adenylyl cyclase is Gα-mediated. This discrepancy provides evidence that mechanisms for receptor desensitization may be different in different signaling pathways.

Our study further demonstrated that blockade of internalization by dynamin K44E did not significantly alter the extent of AC desensitization for either DOR or DTS. This result suggests that the slower desensitization rate of DTS is not due to the unknown. However, our results are consistent with another study that demonstrates phosphorylation-independent internalization of DOR (18). Our current study and our previous study with MOR (14) used the measurement of adenylyl cyclase activity as the functional end point. Both studies demonstrated phosphorylation-independent desensitization during agonist treatment. However, the requirement of receptor phosphorylation in rapid desensitization of MOR and DOR has been suggested in the Xenopus oocyte expression system (38–40), where the activity of G-protein-coupled K+ channels was monitored. This discrepancy may be due to the different effectors involved in the studies. In the other studies, G-protein-coupled K+ channels are Gβγ-mediated, whereas in our study, adenylyl cyclase is Gα-mediated. This discrepancy provides evidence that mechanisms for receptor desensitization may be different in different signaling pathways.

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Our study of the interaction between β-arrestins and receptors was performed by BRET assay in HEK293 cells. Although there are endogenous β-arrestins in HEK293 cells, the expression levels of GFP2-β-arrestin 1 and GFP2-β-arrestin 2 were much higher than the endogenous β-arrestins (Fig. 4B). Thus, endogenous β-arrestins are unlikely to compete with GFP2-β-arrestins efficiently. Our results demonstrated that, without phosphorylation, agonist-activated DOR interacted with β-arrestin 1 and β-arrestin 2 similarly, whereas phosphorylation promoted the receptor selectivity for β-arrestin 2 over β-arrestin 1. It has been reported that β-arrestins are able to bind to the third intracellular loop and carboxyl terminus of DOR (27). This study also showed that mutation of Thr/Ser residues to alanine in the carboxyl tail of DOR almost totally blocked the interaction between β-arrestins and the carboxyl tail. Therefore, we hypothesize that the interaction between DTS and β-arrestins in our study are mainly introduced by the third intracellular loop. The same affinity of DTS to β-arrestin 1 and β-arrestin 2 suggests that the third intracellular loop does not contribute to the preferentiality shown in wild type DOR. Based on the finding that the carboxyl tail of DOR does not show any preference for β-arrestin in an in vitro assay (27), and the fact that DOR is mainly phosphorylated in its carboxyl tail (30, 33), it is reasonable to hypothesize that phosphogroups in the carboxyl terminus are attributed to DOR preferential binding to β-arrestin 2. We therefore propose that phosphorylation of activated DOR leads the receptor to preferentially bind to β-arrestin 2. Although it has been suggested that receptor preferentiality for arrestin is determined by the domains and surface charges of arrestins (47, 48), our data provide another view that phospho-Ser and -Thr residues of the receptor may also modulate the preference.

Further study with MEFs lacking β-arrestin 1 or β-arrestin 2 indicated that agonist-induced phosphorylation-independent internalization and AC desensitization could be sufficiently initiated by either of the two β-arrestins, supporting the view that unphosphorylated DOR can bind the two β-arrestins with no preference. In addition, based on the finding that the unphosphorylated receptor cannot induce conformational changes of β-arrestin 2 (49), our result is consistent with a previous report that β-arrestin possesses intrinsic signaling effects independent of conformational changes (50). However, the preference of phosphorylated DOR for β-arrestins cannot fully explain the observation that β-arrestin 1 and β-arrestin 2 play differential roles in phosphorylation-dependent internalization and desensitization of DOR, where β-arrestin 1 and β-arrestin 2 were almost equally efficacious in the internalization, but β-arrestin 2 was favored in the desensitization process. This result suggests that the ability of β-arrestins as signaling modulators is affected by the phosphorylation status of the interacting receptor. This influence may be due to the conformational changes of β-arrestins induced by the phosphorylated receptor. The conformational change of β-arrestin 2 has been explored (49, 51) and is demonstrated to depend on phosphorylation of the receptor (49). Accordingly, we propose that β-arrestin 1 and β-arrestin 2, in their basal conformational status, have the same ability to uncouple activated receptors from G proteins and to couple activated receptors to clathrin. Receptor phosphorylation confers the differential signaling properties to β-arrestin 1 and β-arrestin 2, which may be due to the conformational changes conducted by the activated phosphorylated receptor.
In addition, we note that the internalization rates of wild type DOR in two control MEFs (WT-1 and WT1–7) were significantly different (Fig. 3A), suggesting that factors other than β-arrestins could influence receptor internalization and desensitization.

Receptor internalization can enhance desensitization by removing functional receptors from the cell surface, or can reduce desensitization by promoting resensitization of the receptor via receptor recycling. The observation that blockade of internalization by dynamin K44E has little effect on desensitization of DOR and DTS after up to 6 h of agonist treatment indicates that both wild type DOR and phosphorylation-deficient mutant DTS do not recycle, and may undergo similar intracellular trafficking after internalization. It has been proposed that the properties of the receptor-arrestin complex play a significant role in determining the ultimate fate of the internalized receptors (42). Receptor phosphorylation enhances the stability of the receptor-arrestin complex (52). Mutation of clusters of Ser and Thr residues in the carboxyl tails of the neurotensin-1 receptor, the oxytocin receptor, and the angiotensin II type 1A receptor allows these receptors to recycle back to the cell surface after internalization, instead of remaining intracellular (26). Therefore, it is reasonable to expect that elimination of agonist-induced phosphorylation, with all Ser and Thr residues in the carboxyl tail mutated, would affect the stability of the receptor-β-arrestin complex and the intracellular trafficking of the receptor. However, the BRET assay suggests that the phosphorylation-deficient mutant of DOR, like wild type DOR, can form a stable complex with β-arrestin 2. Moreover, the recycling experiment showed no obvious recycling of the phosphorylation-deficient mutant of DOR, suggesting that the intracellular trafficking of unphosphorylated DOR remains similar to its phosphorylated wild type. Thus, it appears that other signal determinant(s) play(s) a larger role in the intracellular trafficking of DOR. These results suggest that receptor phosphorylation does not affect the stability of the receptor-arrestin complex and the fate of the internalized DOR.

In conclusion, the present study confirms that β-arrestins play a critical role in adenylyl cyclase signaling and trafficking of the phosphorylation-deficient mutant of DOR. More importantly, our study uncovers the role of receptor phosphorylation in the recruitment of β-arrestins, and clarifies the contribution of β-arrestins to the phosphorylation-dependent and independent internalization and desensitization of DOR. Additionally, the current study suggests that the stability of the DOR receptor complex with β-arrestins, unlike some other receptors, is not influenced by phosphorylation. Therefore, this study provides new insight into the role receptor phosphorylation plays in the signaling and trafficking of DOR and in the regulation of the function of β-arrestins.

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