Agonist-induced Formation of Opioid Receptor-G Protein-coupled Receptor Kinase (GRK)-Gβγ Complex on Membrane Is Required for GRK2 Function in Vivo

Received for publication, March 7, 2003, and in revised form, April 29, 2003
Published, JBC Papers in Press, May 15, 2003, DOI 10.1074/jbc.M302385200

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G protein-coupled receptor kinases (GRKs) catalyze agonist-induced receptor phosphorylation on the membrane and initiate receptor desensitization. Previous in vitro studies have shown that the binding of GRK to membrane-associated Gβγ subunits plays an important role in translocation of GRK2 from the cytoplasm to the plasma membrane. The current study investigated the role of the interaction of GRK2 with the activated δ-opioid receptor (DOR) and Gβγ subunits in the membrane translocation and function of GRK2 using intact human embryonic kidney 293 cells. Our results showed that agonist treatment induced GRK2 binding to DOR, GRK2 translocation to the plasma membrane, and DOR phosphorylation in cells expressing the wild-type DOR but not the mutant DOR lacking the carboxyl terminus, which contains all three GRK2 phosphorylation sites. DORs with the GRK2 phosphorylation sites modified (M3) or with the acidic residues flanking phosphorylation sites mutated (E355Q/D364N) failed to be phosphorylated in response to agonist stimulation. Agonist-induced GRK2 membrane translocation and GRK-receptor association were observed in cells expressing M3 but not E355Q/D364N. Moreover, over-expression of Gβγ subunits promoted GRK2 binding to DOR, whereas over-expression of transducin α or the carboxyl terminus of GRK2 blocked binding. Further study demonstrated that agonist stimulation induced the formation of a complex containing DOR, GRK2, and Gβγ subunits in the cell and that agonist-stimulated formation of this complex is essential for the stable localization of GRK2 on the membrane and for its catalytic activity in vivo.

G protein-coupled receptors (GPCRs)1 constitute a superfamily of plasma membrane receptors. More than 1,000 of 19,000 open reading frames in the genome of Caenorhabditis elegans (1) encode GPCRs, and more than 600 GPCR genes have been identified in the human genome (2). GPCRs transduce a huge number of extracellular signals from hormones, neurotransmitters, chemokines, and other environmental stimuli to the interior of cells and thus play fundamental roles in regulating a variety of cellular functions (3, 4). An important feature of GPCR-mediated signal transduction is that repeated agonist stimulation triggers a negative feedback regulatory mechanism that attenuates GPCR-mediated signal transduction (desensitization). The initial event of receptor desensitization is agonist-stimulated receptor phosphorylation catalyzed by GPCR kinases (GRKs) (5). Seven members of the GRK family have been identified to date, and they have been divided on the basis of structural and functional similarities into rhodopsin kinase (GRK1 and GRK7), the β-adrenergic receptor (βAR) kinase (GRK2 and GRK3), and the GRK4 (GRK4, GRK5, and GRK6) subfamilies (6–8).

The functions of GRKs are highly regulated in the cell. GRKs preferentially catalyze phosphorylation of the activated (agonist-occupied) rather than the inactive or antagonist-occupied GPCR substrates (9). The interactions of GRKs with the activated receptor substrates in turn potently activate GRKs (10). The participation of regulatory mechanisms responsible for the membrane localization and receptor targeting of GRKs is required for agonist-induced GPCR phosphorylation. Studies revealed that the members of the βAR kinase subfamily of GRKs (GRK2 and GRK3) exhibit an agonist-dependent association with cell membranes and that the agonist-induced GRK2 translocation to plasma membrane precedes receptor phosphorylation and desensitization (11). Studies using purified components demonstrated that the Gβγ subunits of the heterotrimeric G proteins (Gβγ) interact with the carboxyl tail of GRK2 and promote the association of GRK2 with lipid vesicles and rod outer segment membranes. Furthermore, this interaction stimulated the phosphorylation of rhodopsin and βAR in vitro (12–15). The association of GRK2 and Gβγ has been demonstrated using purified GRK2 in vitro and recently using co-immunoprecipitation in vivo (14, 16). These studies proposed the following model: following the release of the free Gβγ dimer led by agonist occupancy of a GPCR, GRK2 binds to the membrane-bound Gβγ and subsequently targets GRK2 to the activated GPCR substrate. This model remains to be tested in vivo, and the detailed mechanism of GRK2 translocation to plasma membrane must still be developed.

Our earlier work demonstrated that GRK2 phosphorylates the δ-opioid receptor (DOR) upon opioid challenge and results in desensitization of DOR. This work also identified the GRK2 phosphorylation sites in DOR (17–20). The current study explores the mechanism of GRK2-catalyzed GPCR phosphorylation in a mammalian cellular system. We have demonstrated
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for the first time the formation of a stable G<sub>βγ</sub>-GRK2-DOR complex on the membrane in response to agonist stimulation. Furthermore, we demonstrate the requirement of the agonist-induced formation of the receptor-GRK2-G<sub>βγ</sub> complex for the stable membrane association and function of GRK2 in vivo.

EXPERIMENTAL PROCEDURES

**Materials**—[5-Pen<sup>2</sup>]-[5-Pen<sup>6</sup>]-Enkephalin (DPPDE), [5-Ala<sup>2</sup>]-[5-Leu<sup>6</sup>]-enkephalin (DADDLE), [5-Ala<sup>2</sup>]-N-Me-Phe<sup>3</sup>]-Gly<sup>4</sup>-ol-enkephalin (DAMGO), and naloxone were obtained from Sigma. [35S]Orthophosphate (5000 Ci/mmol) was purchased from Amersham Biosciences and [35S]GTP<sup>γS</sup> (1250 Ci/mmol) from PerkinElmer Life Sciences. Benzamidine and cantharidin were obtained from Calbiochem. Rhodamine phal- lous kidney 293 cells (HEK293) were obtained from the American Type Culture Collection (Manassas, VA). Modified Eagle’s medium (MEM), fetal bovine serum, and phosphate-free Dulbecco’s MEM were purchased from Invitrogen. Protein A-Sepharose was obtained from Amersham Biosciences. Mouse monoclonal antibody against FLAG epitope tag and mouse monoclonal antibody 12CA5, recognizing influenza hemagglutinin (HA) epitope, were supplied by Roche Molecular Biochemicals. Texas Red-conjugated goat anti-mouse IgG was purchased from Jackson Immunoresearch (West Grove, PA). Mouse monoclonal antibody against G<sub>i</sub> was supplied by Calbiochem, and mouse monoclonal antibody against GRK2 was kindly provided by Dr. Martin Oppermann (Goethe University, Frankfurt).

**Plasmid Construction**—Plasmids encoding bovine GRK2, GRK5, and HA-tagged mouse DORs including the wild-type δ-opioid receptor (WT), the carboxy-terminal 31-residue truncated DOR (A31), and the mutant T358A/T361A/S363G (M3) were prepared as described previously (18–20). The HA-tagged mouse DOR mutants E355Q, D364N, and E355Q/D364N were constructed by PCR mutagenesis, and the authenticity of the sequences was confirmed by DNA sequencing. Bovine GRK3 cDNA was provided by Dr. Lin Li (Shanghai Institutes of Life Sciences, Chinese Academy of Sciences) and was subcloned into pcDNA3 (Invitrogen) with a sequence encoding a FLAG tag to generate GRK3 with an amino-terminal tag (FLAG-GRK3). The GRK2-GFP pcDNA construct was a generous gift from Dr. Marc G. Caron (Duke University Medical Center).

**Cell Culture and Transfection**—HEK293 cells cultured in MEM containing 10% fetal bovine serum were seeded in 60- or 100-mm tissue culture dishes at 1–2 × 10<sup>5</sup> cells/dish for 24 h before transfection. In immunofluorescence experiments, cells were seeded in 6-well plates or 35-mm tissue culture dishes. Plasmids (1–5 μg) were transfected into the cells using the calcium phosphate method. The cells were used for the first time the formation of a stable G<sub>βγ</sub>-GRK2-DOR complex on the membrane in response to agonist stimulation.

**Data Analysis**—Data were analyzed using the Student’s t test for comparison of independent means with pooled estimates of common variances.

**RESULTS**

Our earlier study demonstrated that agonist-induced phosphorylation of DOR occurs on the cytoplasmic carboxy-terminal domain of the receptor and is mediated by GRK2 (18, 19). These studies suggested that the cytoplasmic tail of DOR is also important for interaction with GRK, although interaction between GRK2 and DOR has not been demonstrated directly. As shown in Fig. 1A, stimulation of the HEK293 cells expressing the wild-type DOR with DPDPE, a specific agonist of DOR, induced DOR phosphorylation. Co-expression of GRK2 strongly enhanced agonist-dependent phosphorylation of DOR. In contrast, truncated DOR lacking the carboxy-terminal 31 residues (A31), although possessing unchanged surface expression and G protein coupling (18, 19), failed to be phosphorylated upon DPDPE challenge even in cells over-expressing GRK2. After stimulation with the DOR-specific agonist DPDPE, GRK2 could be co-immunoprecipitated with DOR in cells expressing DOR WT and GRK2 (Fig. 1B). In contrast, no GRK2 was detected in the receptor complex in the absence of agonist challenge. Furthermore, the agonist-dependent association of GRK and DOR was time-dependent and paralleled receptor phosphorylation (data not shown). Binding of GRK2 to the receptor immunoprecipitation complex was detected 1 min following agonist stimulation, and maximal binding was reached in 3–5 min. The binding decreased 5 min after stimulation and was not detectable after 10 min. DADLE, another δ-specific agonist, but not DAMGO, a μ-specific opioid agonist, could stimulate the formation of DOR-GRK2 complex in cells expressing DOR, and agonist-induced formation of DOR-GRK2 complex could be completely blocked by the opioid-specific antagonist naloxone (Fig. 1B). In contrast to observations with the wild-type DOR, no GRK2 was co-immunoprecipitated with the mutant receptor A31. This mutant lacks the cytoplasmic tail and all of the GRK2 phosphorylation sites (Fig. 1B). These data demonstrate directly that agonist stimulation induces GRK2 binding to DOR and that the 31 amino acid residues in the carboxyl-terminal domain of the receptor are critical for its interaction with GRK.
HEK293 cells then were transfected with GRK2-GFP and DOR, and the subcellular distribution of GRK upon agonist stimulation was examined using a laser confocal microscope. As shown in Fig 1C, GRK2 was uniformly distributed in the cytoplasm in the absence of agonist stimulation, whereas it translocated rapidly to plasma membrane and colocalized with DOR upon challenge with DPDPE. The real-time recording of GRK2-GFP confocal fluorescence images in living cells co-expressing GRK2-GFP and the wild-type DOR showed that, in response to agonist challenge, the membrane-associated GRK2-GFP fluorescence increased quickly, whereas the cytoplasmic GRK2-GFP fluorescence decreased. This redistribution was accompanied by apparent changes in membrane shape (data not shown). However, agonist-induced GRK2-GFP translocation to cellular membranes was impaired in the cells co-expressing Δ31, the mutant receptor incapable of binding GRK2 and of being phosphorylated (Fig. 1C). These data demonstrate that the carboxyl-terminal domain of DOR is required not only for agonist-stimulated GRK2-receptor binding but also for agonist-stimulated GRK2 membrane localization.

The agonist-induced membrane translocation of GRK2 precedes GPCR phosphorylation. Earlier studies from Loh and co-workers (23) and our group (19) revealed that GRK-related phosphorylation sites (Thr358 and Ser363) are within the carboxyl-terminal 31 amino acid residues of DOR (19, 23). As shown in Fig. 2A, DOR M3, in which all of the serine and threonine residues in this region are replaced with alanine, fails to support the agonist-induced redistribution of GRK2-GFP (Fig. 2A). These data suggest that agonist-induced GRK2-GFP translocation to cellular membranes requires the presence of a carboxyl-terminal domain of DOR, which is phosphorylated in response to agonist challenge. This conclusion is consistent with the observation that the carboxyl-terminal domain of DOR is required for agonist-stimulated GRK2 membrane localization (23).
Threonine phosphorylation sites in GRK have been replaced by alanines, failed to be phosphorylated following treatment with DPDPE. The E355Q and D364N mutants of DOR bearing single point mutations at the acidic residues adjacent to two GRK phosphorylation sites (Thr$^{358}$ and Ser$^{363}$) showed greatly impaired phosphorylation, and a double mutant (E355Q/D364N) completely blocked receptor phosphorylation (Fig. 2A). Overexpression of GRK2 partially rescued the impaired phosphoryl-

**Fig. 2. Interaction of GRK2 with membrane-bound DOR is required for stable GRK2 membrane localization.** HEK293 cells were transfected with WT, E355Q, D364N, E355Q/D364N, or M3 cDNA alone or co-transfected with or without GRK2 cDNA as indicated and harvested 48 h post transfection. A, transfected cells were metabolically labeled with $^{32}$P$_i$. Left, the cells were incubated at 37 °C for 10 min in the presence or absence of 1 μM DPDPE (DP) as indicated. Right, the cells co-transfected with or without GRK2 cDNA as indicated were incubated in 1 μM DPDPE at 37 °C for 10 min. HA-DORs were then immunoprecipitated with 12CA5, resolved on 8% SDS-polyacrylamide gels, and subjected to phosphorimaging analysis. The level of phosphorylation was quantified and expressed as means ± S.E. of three independent experiments. ***, p < 0.01 as compared with unstimulated control or receptor alone.

B, cells expressing DOR as indicated were pretreated with or without 1 μM DPDPE. [35S]GTP$^y$S binding to the membranes was determined. Data are means ± S.E. of at least three independent experiments. ***, p < 0.01 as compared with corresponding controls without agonist pretreatment. C, transfected cells were incubated in the presence or absence of 1 μM DPDPE at 37 °C for 3 min, and the cell lysate was prepared. Immunoprecipitation of the receptor complex was carried out using 12CA5. Western analysis was done using antibody against GRK2 (top panel), and the same blot was reprobed with 12CA5 against HA-DOR after stripping (middle panel). Direct Western analysis of the cell lysates using GRK2 antibody for GRK2 expression is shown (bottom panel). D, the amounts of GRK2 in the DOR immunoprecipitation complex were quantified and are expressed as means ± S.E. of three independent experiments. ***, p < 0.01 as compared with WT.
ation capability of E355Q and D364N, but it did not have any significant effect on E355Q/D364N (Fig. 2B). Ligand (data not shown) and [³⁵S]GTPγS (Fig. 2B) binding experiments showed that these mutated receptors were able to bind agonists, be activated, and couple to G protein normally. Thus, the impaired phosphorylation of M3, E355Q, D364N, and E355Q/D364N is not likely to be due to a deficiency in receptor activation and signaling. Furthermore, the E355Q/D364N double mutant as well as mutation of all the GRK phosphorylation sites on DOR resulted in remarkable inhibition of agonist-induced receptor desensitization (Fig. 2B). The above results indicate that the acidic residues near receptor phosphorylation sites play a critical role in GRK-mediated receptor phosphorylation.

To explore the contributions of the GRK phosphorylation sites and the adjacent acidic residues on agonist-stimulated GRK2 membrane localization, GRK2-DOR interaction, and GRK-catalyzed DOR phosphorylation, the wild-type and mutant DORs were co-expressed with GRK2 in HEK293 cells. As shown in Fig. 1C, DPDPE treatment stimulated membrane redistribution of GRK2-GFP in the cells expressing M3, which lacks all of the GRK phosphorylation sites. Furthermore, the level of GRK2 membrane translocation was comparable with that in the cells expressing wild-type DOR. Co-immunoprecipitation experiments revealed that in response to opioid agonist stimulation, M3 interacted with GRK2 as efficiently as the wild-type receptor (Fig. 2, C and D), although it could not be phosphorylated (Fig. 2A). On the other hand, DPDPE-stimulated GRK2 membrane association and colocalization with the opioid receptor was entirely blocked in cells expressing the E355Q/D364N double mutant (Fig. 1C). Both the E355Q and D364N mutant showed a 50–60% reduction in agonist-induced GRK2 binding in co-immunoprecipitation experiments, whereas the E355Q/D364N double mutant showed a total loss of activity in this assay (Fig. 2, C and D). The above results indicate that those GRK serine and threonine phosphorylation sites on DOR are not critically involved in the interaction with GRK2 and in the membrane association of GRK2 induced by agonist. In contrast, the glutamic and aspartic acid residues near these phosphorylation sites are critical. The data also hint that the GRK2-DOR interaction is required not only for DOR phosphorylation but also for stable GRK2 membrane localization.

Agonist-stimulated DOR phosphorylation is regulated by both the βAR kinase (GRK2 and GRK3) and GRK4 (GRK4, GRK5, and GRK6) subfamilies of GRKs. The function of Glu³⁵⁵ and Asp³⁶⁴ of DOR in the interaction with other members of the GRK family was examined in HEK293 cells co-expressing DORs and either GRK3 or GRK5. The co-immunoprecipitation experiments revealed that similar to GRK2, GRK3 and GRK5 co-immunoprecipitated with the wild-type DOR in response to opioid agonist stimulation. Both of these kinases showed reduced binding to E355Q and D364N and a total loss of binding to E355Q/D364N (Fig. 3). Furthermore, E355Q/D364N was not phosphorylated by GRK5 upon DPDPE stimulation (data not shown). The above results provide the initial evidence demonstrating the interaction of DOR with GRK3 and GRK5, in addition to GRK2. These data also indicate that the acidic residues Glu³⁵⁵ and Asp³⁶⁴ adjacent to phosphorylation sites on DOR are critically involved in the interaction with GRKs in both the βAR kinase and GRK4 families.

Binding of GRK2 to membrane-associated free Gβγ subunits is required for the activation and membrane localization of GRK2 (12). Thus the roles of GRK2-Gβγ interaction in agonist-dependent GRK2 membrane translocation and in the interaction with the receptor further were investigated. GRK2 was co-expressed in HEK293 cells with the wild-type or mutant DORs and Gβγ2 subunits, the carboxyl-terminal domain of GRK2 (GRK2ct), or transducin α. As shown in Fig. 4, after
The current study has demonstrated that upon agonist stimulation, GRK2 forms a complex with Gβγ subunits and agonist-occupied opioid receptor on the membrane and that Gβγ subunits play a critical role in the interaction of GRK with activated opioid receptor in vivo. Our results from immunoprecipitation and laser confocal fluorescent microscopy experiments indicate that agonist-stimulated formation of DOR-GRK2-Gβγ complex is essential for the stable localization of GRK2 on the membrane and its catalytic activity toward GPCR substrate in vivo.

On the basis of these observations, a refined model for GRK2-catalyzed GPCR phosphorylation is proposed in Fig. 5. In this model, receptor activation induces GRK2 interaction with the free Gβγ subunits associated with the membrane. This interaction is followed immediately by the binding of the GRK2 in the GRK2-Gβγ binary complex to the activated receptor to form a stable receptor-GRK2-Gβγ complex on the membrane. This process targets GRK2 to its substrate and results in the subsequent phosphorylation of the activated receptor. A low level of agonist-stimulated GRK2 membrane translocation was observed in cells over-expressing E355Q/D364N and Gβγ (data not shown), suggesting the presence of GRK2-Gβγ binary complexes on the membrane in the presence of excess free Gβγ. The agonist-induced GRK2 interaction with Gβγ may not be as stable as the receptor-GRK2-Gβγ complex and may be converted into the receptor-GRK2-Gβγ complex as soon as it is formed on the plasma membrane. The agonist-stimulated receptor-GRK-Gβγ complex may contain other molecules such as scaffold protein β-arrestins, which have a high affinity for GPCR phosphorylated by GRKs and have been demonstrated to be associated with the activated receptors (23). The presence of additional scaffold proteins may increase the stability of the receptor-GRK-Gβγ complex on the membrane. Furthermore, of the three functional domains of GRK2, the amino-terminal domain is proposed to mediate receptor interaction; the central domain to exert catalytic function; and the carboxyl-terminal domain, containing a pleckstrin homology domain essential for interactions with the Gβγ subunits and phosphatidylinositol 4,5-bisphosphate, to be involved in agonist-stimulated GRK
Membrane translocation (24). Our observation of a receptor-GRK-Gβγ complex in response to receptor activation is consistent with these structure-function studies on GRKs and suggests that the complex is formed through interaction of the amino-terminal domain of GRK2 with the activated receptor and of the carboxyl-terminal domain of GRK2 with the Gβγ subunits on the membrane.

Previous work has shown that small synthetic peptides are extremely poor substrates of GRKs and that GPCR-derived peptides specifically inhibit phosphorylation of the receptor as opposed to the peptide substrate (25). These studies suggested that GRKs interact with activated GPCRs at sites distinct from their phosphorylation sites (25). Our current data show that the 31 residues in the carboxyl terminus of DOR contain sites for both GRK2 phosphorylation and interaction and that the DORs with phosphorylation sites mutated interact with GRK2 and form a stable complex with GRK2 and the Gβγ subunits on the membrane effectively. These data demonstrate in vivo that the GRK2 phospho-receptor residues in DOR contain sites not necessary for receptor-GRK2 interaction, although these residues are in very close proximity to residues essential for GRK2 interaction.

GRK phosphorylation sites are identified in only a few GPCR substrates, and no clear consensus substrate sequence has been found among them (25). Studies with synthetic peptide substrates and purified GRKs suggest that kinases in the GRK2 family preferentially phosphorylate peptides containing acidic residues flanking the target serines or threonines (26). Studies with the M2 muscarinic receptor and α1 adrenergic receptor have demonstrated that acidic amino acid residues are important in the agonist-dependent phosphorylation and desensitization of these receptors (27–29). In this study, we have found that substitution of glutamate and aspartate residues adjacent to GRK phosphorylation sites in DOR blocks agonist-stimulated DOR phosphorylation and attenuated receptor desensitization and have further revealed that these two acidic residues are essential for GRK2, GRK3, and GRK5 binding. Our results have demonstrated, in a receptor context, that the negatively charged acidic residues flanking phosphorylation sites are critically involved in the interaction of the receptor with GRKs of both the βAR kinase and GRK4 families in vivo and thus are required for GRK-mediated receptor phosphorylation and desensitization.

Acknowledgments—We thank Dr. P. Wang for technical help, Dr. M. G. Caron for providing GRK2-GFP plasmid, Dr. M. Oppermann for GRK antibody, and Y.-J. Lu for preparation of illustrations. We also thank Dr. L. L. Spremulli for critical reading and editorial work.
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