Heterologous Activation of Protein Kinase C Stimulates Phosphorylation of \( \delta \)-Opioid Receptor at Serine 344, Resulting in \( \beta \)-Arrestin- and Clathrin-mediated Receptor Internalization*

Bin Xiang‡, Guo-Hua Yu‡, Jun Guo‡, Li Chen‡, Wei Hu‡, Gang Pei‡, and Lan Ma‡¶

From the ‡National Laboratory of Medical Neurobiology, Fudan University Medical Center, Shanghai 200032, and ¶Shanghai Institute of Biochemistry and Cell Biology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, People's Republic of China

The purpose of the current study is to investigate the effect of opioid-independent, heterologous activation of protein kinase C (PKC) on the responsiveness of opioid receptor and the underlying molecular mechanisms. Our result showed that removing the C terminus of \( \delta \) opioid receptor (DOR) containing six Ser/Thr residues abolished both DPDPE- and phorbo12-myristate 13-acetate (PMA)-induced DOR phosphorylation. The phosphorylation levels of DOR mutants T352A, T353A, and T358A/T361A/S363S were comparable to that of the wild-type DOR, whereas S944G substitution blocked PMA-induced receptor phosphorylation, indicating that PKC-mediated phosphorylation occurs at Ser-344. PKC-mediated Ser-344 phosphorylation was also induced by activation of G\(_{\text{q}}\)-coupled \( \alpha_{1A} \)-adrenergic receptor or increase in intracellular Ca\(^{2+}\) concentration. Activation of PKC by PMA, \( \alpha_{1A} \)-adrenergic receptor agonist, and ionomycin resulted in DOR internalization that required phosphorylation of Ser-344. Expression of dominant negative \( \beta \)-arrestin and hypertonic sucrose treatment blocked PMA-induced DOR internalization, suggesting that PKC mediates DOR internalization via a \( \beta \)-arrestin- and clathrin-dependent mechanism. Further study demonstrated that agonist-dependent G protein-coupled receptor kinase (GRK) phosphorylation sites in DOR are not targets of PKC. Agonist-dependent, GRK-mediated receptor phosphorylation and agonist-independent, PKC-mediated DOR phosphorylation were additive, but agonist-induced receptor phosphorylation could inhibit PKC-catalyzed heterologous DOR phosphorylation and subsequent internalization. These data demonstrate that the responsiveness of opioid receptor is regulated by both PKC and GRK through agonist-dependent and agonist-independent mechanisms and PKC-mediated receptor phosphorylation is an important molecular mechanism of heterologous regulation of opioid receptor functions.

Opioid receptors are G protein-coupled receptors (GPCR)\(^1\) and include \( \delta \), \( \kappa \), and \( \mu \) subtypes. Interaction of opioid receptors on the surface of neurons in the central nervous system with endogenous opioid peptides and synthetic alkaloids produces strong analgesic effect, but chronic use of opioid drug results in drug tolerance and dependence. The molecular mechanisms of regulation of the receptor responsiveness and opioid tolerance and dependence are not well understood, although desensitization of opioid receptor has been implicated as one of the major mechanisms.

The responsiveness of opioid receptor reduces upon exposure to opioid agonist, and this agonist-dependent desensitization is defined as homologous desensitization of the opioid receptor. Several mechanisms contribute to desensitization of opioid receptors. It has been demonstrated that, following stimulation of opioid agonist, the opioid receptor becomes phosphorylated rapidly (1–4), the phosphorylated receptor uncouples from G proteins and binds to \( \beta \)-arrestins (5, 6), the receptor is subsequently sequestered in an intracellular compartment (7), and even the expression of the opioid receptor is down-regulated (8). Phosphorylation of opioid receptors is the initial step in opioid receptor desensitization, and phosphorylation of \( \delta \), \( \kappa \), and \( \mu \) subtypes of opioid receptors in response to agonist stimulation has been demonstrated by other laboratories as well as our own (1–4, 9). Experimental results indicate that GPCR kinase (GRK), not PKC and PKA, is the primary protein kinase involved in homologous phosphorylation of opioid receptors stimulated by opioid agonist and plays an important role in agonist-induced homologous desensitization of opioid receptors (1, 6, 9, 10).

In addition to agonist-specific receptor desensitization, functions of GPCRs can be regulated by agonist-independent mechanisms, namely, heterologous desensitization. Second messenger-dependent protein kinases such as PKA and PKC mediate receptor phosphorylation, and this has been implicated in heterologous regulation of activities of a number of GPCRs recently (11, 12). Accumulating evidence suggest that the sensitivity of opioid receptor in response to neural signals is

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† To whom correspondence should be addressed: National Laboratory of Medical Neurobiology, Fudan University Medical Center, 138 Yi Xue Yuan Rd., Shanghai 200032, P. R. China. Tel.: 86-21-6404-1900 (ext. 2522); Fax: 86-21-6471-8563; E-mail: lanma@shmu.edu.cn.

\(^1\) The abbreviations used are: GPCR, G protein-coupled receptor; DOR, \( \delta \)-opioid receptor; GRK, G protein-coupled receptor kinase; PKC, protein kinase C; PKA, protein kinase A; PKM, phosphoryl 12-myristate 13-acetate; HEK, human embryonic kidney; DPDPE, \( \alpha \)-[Pen\(_2\),t-Pen\(_3\)]enkephalin; \( \alpha_{1A} \)-AR, \( \alpha_{1A} \)-adrenergic receptor; HA, hemagglutinin; MEM, modified Eagle’s medium; FITC, fluorescein isothiocyanate; WT, wild type; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; BAPTA/AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N’N’-tetraacetic acid tetra(acetoxy-methyl) ester; NMDA, N-methyl-D-aspartic acid.
also regulated by nonopioid pathways. Animal experiments and clinical studies showed that NMDA antagonist potentiates morphine-induced analgesia and prevents opioid tolerance (13). Our previous study demonstrated that activation of NMDA receptor attenuates opioid receptor-mediated cellular signaling, and this is mediated by PKC (14). Osaka and colleagues (15) showed that preadministration of insulin inhibits the antinociceptive effect of (α-Ala2, N-Me-Phe4, Gly5, ol)enkephalin, a specific agonist of μ-opioid receptor in mice, and their results suggest that the effect is mediated by activation of PKC and tyrosine kinase. Increasing Ca2+ concentration in neurons and synaptosomes antagonizes opioid-induced antinociception (16, 17). Suppression of μ, δ, and κ opioid receptor agonist-induced analgesia by PKC activators in animals has also been shown by many laboratories (18–21). It has also been observed that chronic opiate treatment strongly increases PKC activity in specific brain regions, and inhibition of PKC activity attenuates the development of opioid tolerance and dependence (22, 23). These data strongly suggest that opioid signaling is regulated heterologously by agonist-independent pathways in vivo and PKC is likely an important mediator.

Research indicates that phorbol esters, activators of PKC, reduce opioid-induced inhibition on cAMP production (24) and potentiate desensitization of opioid receptor-activated K+ current (2, 25). Furthermore, research from other laboratories and our groups demonstrated that phorbol ester PMA treatment could induce phosphorylation of δ and μ opioid receptors in the absence of agonist stimulation (1, 2, 9, 26). These data indicate that PKC-mediated heterologous phosphorylation of opioid receptor may contribute to desensitization of opioid receptor in neurons. However, there is so far no report on the effect of physiological activation of PKC on opioid receptor phosphorylation and signal transduction, and the mechanism and functional impact of PKC activation on opioid receptor-mediated signaling are not known. In the current study, we identified a PKC-mediated phosphorylation site in the δ-opioid receptor (DOR) and demonstrated that activation of PKC by stimulation of other types of GPCR or increase in intracellular Ca2+ concentration in HEK 293 cells induces heterologous phosphorylation of DOR. Our results further established that DOR phosphorylation at Ser-344 by PKC results in internalization of DOR in HEK 293 cells through a β-arrestin- and clathrin-mediated mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—[α2,β2]-enkephalin (DPDPE), forskolin, ionomycin, 1-methyl-3-isobutylxanthine, PD98059, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). [32P]Orthophosphate (5000 Ci/mmol) was purchased from Amersham Pharmacia Biotech. [1,2-Bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester] (BAPTA/AM), and [N,N,N′-triacetylethylene diamine tetraacetate] tetra(acetoxymethyl) ester (BAPTA/AM) benzamidine-HCl, cantharidin, KN-62, staurosporine, were obtained from Calbiochem (La Jolla, CA). A61603, GF109203X, and WB4101 were supplied by-Tocris (United Kingdom). HEK 293 cells were obtained from the American Type Culture Collection (Rockville, MD). Modified Eagle’s medium (MEM), fetal bovine serum, and phosphate-free Dulbecco’s MEM were purchased from Life Technologies, Inc. (Grand Island, NY). Protein A-Sepharose was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). 12CA5 mouse monoclonal antibody, recognizing influenza hemagglutinin (HA) epitope, was supplied by Roche Molecular Biochemicals. FITC-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Plasmid Construction—Plasmids encoding HA-tagged mouse wild type δ opioid receptor (WT) and 3 T-c-terminal 31 residues truncated DOR (Δ31), and DOR mutants with the C-terminal serine or threonine residue substituted were constructed in pDNA3 as described previously (1, 9). OR mutant, DOR mutant S344G (m3), T352A (m2), and T353A (m3) with HA-tag at the N terminus were constructed by exchanging the NotI/XbaI fragment of DOR with the corresponding fragment in FLAG-tagged mutant DOR (9). The HA-tagged mutant DOR was used in the experiments.

**RESULTS**

As shown in Fig. 1, incubation of PMA with the HEK 293 cells transiently expressing DOR induced DOR phosphorylation. Phosphorylation of DOR in these cells was detected in 3 min and reached a peak level (400% of the basal level) in 5 min of PMA exposure (Fig. 1A). PMA-induced DOR phosphorylation was concentration-dependent. DOR phosphorylation was detectable at 100 nM PMA and approached the plateau at 1 μM PMA (Fig. 1B).

The role of PKC in PMA-stimulated DOR phosphorylation was explored next. Fig. 2 shows that PKC inhibitors staurosporine and GF109203X (28) abolished PMA-induced DOR phosphorylation completely whereas MEK (MAPK/ERK kinase) inhibitor PD98059 had no significant effect under the same conditions. In addition, the PKA activator forskolin did not stimulate DOR phosphorylation (Fig. 2). These data indicate that PMA-stimulated DOR phosphorylation is mediated by PKC, whereas other protein kinases such as PKA and MAPK are not critically involved.

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Our previous research (9) has demonstrated that the C-terminal domain of DOR plays a critical role in PMA-induced DOR phosphorylation, implicating that PKC-mediated DOR phosphorylation occurs at the C-terminal domain of the receptor. To identify the PKC-mediated DOR phosphorylation site, DOR mutants S344G (m1), T352A (m2), and T358A/T361A/S363G (m4/5/6) with one or more potential Ser/Thr phosphorylation sites in the C terminus of DOR substituted and the truncation mutant lacking the 31 residues containing six Ser/Thr potential phosphorylation sites (designated as Δ31), were constructed. Results showed that surface expression, ligand binding, and G protein coupling of the above mutant receptors were comparable to the wild-type DOR (WT) (data not shown). As shown in Fig. 3, removing the C-terminal domain containing all six potential phosphorylation sites blocked PMA-induced DOR phosphorylation completely, whereas eliminating Thr-358, Thr-361, and Ser-363, the three potential phosphorylation residues proximal to the C terminus, by substitution of the three Ser/Thr residues with neutral amino acid had no detectable effect on the phosphorylation level of DOR, indicating that the PKC-mediated DOR phosphorylation site is likely located among Ser-344, Thr-352, and Thr-353 in the receptor cytoplasmic tail.

Analysis of amino acid sequences flanking the three Ser/Thr residues predicated Ser-344 and Thr-352 as putative PKC phosphorylation sites (29). To further examine the role of Ser-344, Thr-352, and Thr-353 in PMA-induced DOR phosphorylation, phosphorylation levels of DOR mutants m1, m2, and m3 stimulated by PMA were determined. As shown in Fig. 3, following stimulation with PMA, the extents of phosphorylation of m2 and m3 mutants were comparable to that of the wild type DOR (WT), but in contrast, phosphorylation of m1 DOR was not detectable under the same conditions. These data indicate that Ser-344 in the cytoplasmic tail of DOR is the phosphorylation site in PKC-mediated DOR phosphorylation. We have demonstrated that PKA and MAPK are not involved in PKC-mediated DOR phosphorylation. Furthermore, the Ser-344 flanking sequence does not resemble phosphorylation consensus sequences for PKA, MAPK, or calcium/calmodulin-dependent protein kinase II, but Ser-344 is located in a typical PKC phosphorylation motif. Therefore, Ser-344 is very likely the in vivo phosphorylation site of PKC in PMA-stimulated DOR phosphorylation.

PKC is one of the most important signal molecules in cells and its activity is regulated through a number of different pathways. Phosphorylation status of opioid receptors could be therefore regulated, via PKC, by activation of one or more signal molecules or signal transduction cascades other than opioid pathway. To explore the potential physiological significance of PKC-stimulated opioid receptor phosphorylation, we examined the effect of activation of Gα protein-coupled receptor, α1A-adrenergic receptor (α1A-AR), on the phosphorylation of DOR. As shown in Fig. 4A, stimulation of HEK 293 cells transiently coexpressing DOR and α1A-AR with A61603, a selective agonist of α1A-AR, resulted in strong phosphorylation of DOR at a level comparable to that of stimulated by PMA. A61603-stimulated DOR phosphorylation was abolished by preincubation with either the selective α1A-AR antagonist WB4101 or the PKC inhibitors staurosporine and GF109203X (Fig. 4A). The mutant DOR lacking PKC phosphorylation site Ser-344 failed to become phosphorylated following stimulus with A61603 under the same conditions (Fig. 4A). These data indicate that heterologous activation of PKC through activation of α1A-AR, a receptor coupled to Gα protein, results in DOR phosphorylation at PKC site Ser-344. Furthermore, ionomycin, a Ca2+ ionophore, elevated Ca2+ concentration in HEK 293 cells (data not shown) and stimulated DOR phosphorylation at Ser-344, and the effect of ionomycin could be blocked by inhibitors of PKC but not by an inhibitor of calcium/calmodulin-dependent protein kinase II, KN-62 (Fig. 4B). In addition to ionomycin, A23187, another Ca2+ ionophore, and ATP, a natural ligand of purinoceptor, increased intracellular Ca2+ concentration and stimulated DOR phosphorylation in HEK 293 cells (data not shown). BAPTA/AM, a Ca2+ chelator, abolished PKC-mediated DOR phosphorylation stimulated by PMA, A61603, and ionomycin (Fig. 4C).

To investigate the functional consequence of PKC-mediated DOR phosphorylation, DOR internalization in response to PKC activation induced by PMA, ionomycin, and A61603 was measured using flow cytometry. As shown in Fig. 5, a 30-min PMA treatment resulted in a significant reduction in cell surface fluorescence in the cells expressing the wild type DOR, indicating a considerable loss of the DOR from the cell surface.
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Fig. 4. PKC-mediated phosphorylation of DOR was Ca2+-dependent. The transfected HEK 293 cells were labeled with 32P, and treated as indicated. DORs were immunoprecipitated, and receptor phosphorylation was analyzed. The figures are representative of three independent experiments performed. A, the cells coexpressing α7-AR and WT or m1 were incubated with or without 2 μM WB4101 (WB) for 30 min, 0.2 μM staurosporine for 20 min, or 2 μM GF19203X for 20 min prior to stimulation with 1 μM A61603 (A6), 1 μM PMA, or PBS for 10 min. B, the cells expressing WT or m1 were incubated with or without 0.2 μM staurosporine for 20 min, 2 μM GF19203X for 20 min, 10 μM KN-62 (KN) for 20 min prior to stimulation of 1 μM ionomycin (Iono), 1 μM PMA, or PBS for 10 min. C, the cells expressing DOR or coexpressing DOR and α2δ-AR (for A61603 treatment) were incubated with or without 50 μM BAPTA/AM (BAP) for 20 min prior to stimulation of 1 μM PMA, 1 μM ionomycin, 1 μM A61603, or PBS for 10 min.

(40–50%), which was similar to the extent of DOR internalization induced by the DOR agonist DPDPE (Fig. 6). In contrast, the same PMA treatment caused no significant change in the surface immunofluorescence in the cells expressing DOR mutant m1 lacking PKC site Ser-344 in the receptor C-tail (Fig. 5). However, m1 mutant DOR internalized rapidly in response to DOR-selective agonist DPDPE (data not shown). Similarly, activation of PKC by stimulation of α1A-AR or mobilization of Ca2+-induced internalization of the wild type DOR but had no significant effect on the surface density of DOR mutant m1 (Fig. 5C). Similar to agonist pretreatment, incubation of PMA with the HEK 293 cells transiently expressing DOR strongly attenuated DPDPE-induced inhibition on cAMP accumulation; however, substituting Ser-344 to alanine did not block the PMA-induced desensitization of DOR-mediated inhibition of cAMP accumulation (Fig. 5D). These results are consistent with the previous studies that PKC is capable of phosphorylating Ge2 (13, 30) and adenylyl cyclase V (31), which couple with opioid receptors (32) and suggest that PKC may regulate opioid signal transduction at levels of G protein and/or cyclase in addition to phosphorylation of opioid receptor. Our results indicate that induction of DOR internalization is one of the functional consequences of agonist-independent phosphorylation of DOR following activation of PKC via a heterologous signaling pathway (stimulation of another GPCR or mobilizing Ca2+), whereas PKC-mediated reaction targeting molecules downstream from the receptor may also play important roles in desensitization of opioid-induced cellular responses.

The mechanisms of PKC-mediated DOR internalization were examined next. As shown in Fig. 6A, flow cytometric analysis of surface receptor indicates that PMA-induced DOR internalization is reversible: The surface DOR fluorescence gradually recovered 1 h after PMA was removed. Studies showed that opioid-induced receptor internalization involves β-arrestins and clathrin-coated pits (33, 34). Hence, the effects of coexpression of the wild-type β-arrestin 1 or β1V53D, a dominant negative mutant of β-arrestin 1, on PKC-mediated DOR internalization were assessed. Overexpression of β-arrestin 1 enhanced PMA-stimulated DOR internalization and overexpression of β1V53D blocked this effect (Fig. 6B). It has been shown that β-arrestins function in agonist-induced GPCR internalization as an adapter of clathrin in formation of clathrin-coated pits (35). To examine the role of clathrin in PKC-stimulated DOR internalization, HEK 293 cells transiently expressing DOR were incubated with PMA following 0.4 M sucrose pretreatment, which blocks formation of clathrin-coated pits (36). Analysis of cell surface DOR fluorescence indicates that exposure of the cells to hypertonic sucrose completely blocked PMA-induced DOR internalization (Fig. 6C). These data clearly indicate that, like agonist-dependent homologous DOR internalization, the PKC-mediated heterologous internalization of DOR requires β-arrestin and occurs via clathrin-coated pits.

As shown in Fig. 7A, PKC inhibitors staurosporine and GF19203X did not block DPDPE-stimulated DOR phosphorylation, indicating that PKC is not required for agonist-dependent DOR phosphorylation (also called homologous phosphorylation). Our previous studies indicated that agonist-dependent DOR phosphorylation occurs in the C-terminal region of DOR and is mediated by GRKs (1, 9). Removal of the C-terminal 31 residues of DOR containing six potential Ser/Thr phosphorylation sites (mutant Δ31) abolished both DPDPE- and PMA-induced DOR phosphorylation (Figs. 3 and 7B), whereas substitution of PKC phosphorylation site Ser-344 (mutant m1) blocked PKC-mediated receptor phosphorylation but had no significant effect on agonist-stimulated DOR phosphorylation (Figs. 3, 7B, and 8A). Substituting the last three Ser/Thr residues (mutant m4/5/6) abolished DPDPE-induced receptor phosphorylation completely but left PMA-stimulated DOR phosphorylation intact (Figs. 3, 7B, and 8A). These data demonstrate that agonist-dependent GRK-mediated homologous phosphorylation and agonist-independent PKC-mediated heterologous phosphorylation both occur at DOR cytoplasmic tail at comparable levels but at clearly different sites and suggest that responsiveness and phosphorylation of opioid receptor are regulated by both PKC and GRK through agonist-dependent (homologous) and agonist-independent (heterologous) mechanisms.

Although both the PKC-mediated heterologous and GRK-mediated homologous DOR phosphorylation could occur in the absence of the other at comparable levels (~4-fold over the basal level), costimulation with PMA and DPDPE caused no considerable increase in DOR phosphorylation, as compared with the level of receptor phosphorylation stimulated with either PMA or DPDPE alone (Fig. 8A). The phosphorylation level of the wild type DOR (~4.3-fold over basal) was not significantly different from that of DOR mutant m1 or m4/5/6 following costimulation of PMA and DPDPE (Fig. 8A). This is unlikely due to a limitation in the labeling or detection system, because, under similar conditions, the level of DOR phosphorylation increased to ~9-fold of the basal level following GRK coexpression (data not shown). These data argue that DOR phosphorylation is regulated by both PKC- and GRK-mediated...
mechanisms, but it seems that only one type of phosphorylation could occur if the receptor is exposed to activated PKC and GRK at the same time. This could be a result of the inhibitory effect brought by receptor phosphorylation at one site.

In an effort to estimate the impact of the initial phosphorylation event on the subsequent GRK- or PKC-mediated receptor phosphorylation, DOR mutants T358D, T361D, and S363D, mimicking GRK-phosphorylated receptors, and S344D, to imitate the PKC-induced phosphorylation state, were constructed. As shown in Fig. 8B, T358D, T361D, and S363D phosphorylated poorly following PMA stimulation as compared with wild type DOR, whereas agonist-stimulated S344D phosphorylation was at a level similar to that of the wild type DOR (Fig. 8C). This result suggests that the negative charges brought by agonist-stimulated phosphorylation may strongly inhibit PKC-mediated heterologous phosphorylation of DOR at Ser-344, and this is consistent with the results shown in Fig. 8A. To verify this, receptor phosphorylation and internalization levels following sequential PMA and DPDPE treatments were determined. The results show that incubation of PMA followed by DPDPE treatment increased DOR phosphorylation to ~2.5-fold of phosphorylation stimulated by DPDPE or PMA alone (~10-fold of the basal level), whereas receptor phosphorylation in response to incubation with DPDPE followed by PMA stimulation was not significantly different from that stimulated by DPDPE or PMA alone (Fig. 8D). These data are in agreement with our results obtained with phosphorylation state receptor mimics. Furthermore, the effects of sequential treatment of PMA and DPDPE on DOR internalization were similar to those observed in DOR phosphorylation (Fig. 8E). These results suggest further that, although phosphorylation induced by either homologous agonist stimulation or heterologous PKC activation alone is sufficient to bring about changes in responsiveness of the receptors, opioid signaling is still regulatable by GRK following PKC-mediated DOR phosphorylation.

**DISCUSSION**

Opioid receptor desensitization plays an important role in opioid drug-induced analgesia, tolerance, and dependence. Chronic opiate treatment strongly increases GRK levels and PKC activity in specific brain regions, and inhibition of PKC activity attenuates the development of morphine tolerance (37–39). β-Arrestin 2 knocking-out mice with impaired opioid receptor desensitization exhibit enhanced morphine analgesia (40). Phosphorylation of opioid receptors in response to agonist or phorbol ester stimulation has been observed by a number of laboratories, including our own (1–4, 9). Studies revealed that overexpression of GRK2 enhances agonist-dependent receptor desensitization and causes desensitization and overexpression of a dominant-negative mutant of GRK2 or inhibition of GRK activity blocks desensitization of opioid receptors (1, 6, 7, 10). PKC inhibitors attenuate homologous desensitization of some of opioid-mediated responses (41) but fail to block agonist-stimulated opioid receptor phosphorylation (1, 2, 9, 26). Accumulating evidence indicates that GRK is the primary mediator in agonist-induced opioid receptor phosphorylation and desensitization and that GRK-catalyzed opioid receptor phosphorylation is an initial step and important mechanism for opioid agonist-dependent, homologous regulation of the receptor func-
tion (1, 6, 7, 9, 10, 42). However, the mechanism of regulation of responsiveness of opioid receptor by PKC is not clear. In this study, we have demonstrated, for the first time, that activation of PKC through physiological means, stimulating another class of neurotransmitter receptor, or increasing the intracellular Ca\(^{2+}\) concentration, induces phosphorylation of opioid receptor in an agonist-independent manner. Our data showed that the PKC-mediated heterologous opioid receptor phosphorylation occurs at a site distinctly different from that of GRK catalyzed phosphorylation (4) on the receptor cytoplasmic tail and that phosphorylation of DOR by PKC results in \(\beta\)-arrestin- and clathrin-mediated internalization of the receptor. Our study indicates that PKC-mediated opioid receptor phosphorylation is the molecular basis of PKC-mediated receptor desensitization, thus uncovering a molecular mechanism for agonist-independent, heterologous regulation of opioid receptor-mediated signal transduction.

We have demonstrated in the present study that PMA-stimulated DOR phosphorylation is mediated by PKC. The enzymatic pathway involved in PKC-mediated heterologous DOR phosphorylation could involve either direct phosphorylation of the receptor by PKC or activation of other type of kinase by PKC. The PKC-mediated phosphorylation site identified is Ser-344, which is located in a typical PKC (S/T)X(K/R) consensus sequence (29). Therefore, direct phosphorylation of DOR by PKC is very likely, although we can not exclude the possibility of phosphorylation of the receptor by another PKC-activated kinase. PKC-catalyzed phosphorylation has been shown to regulate activity of certain GRKs (43, 44). But our results obtained from overexpression of GRK in HEK 293 cells show that Ser-344 is not a site targeted by GRK (data not shown), and the current study indicates that PKC-mediated DOR phosphorylation occurs in the absence of agonist stimulation and at a site distinct from agonist-stimulated phosphorylation sites. These data argue that PKC-mediated DOR phosphorylation is unlikely a result of GRK activation. Our results show that DOR phosphorylation by PKC was stimulated by activation of \(\alpha_{1A}\)-AR coupled to PKC\(\alpha\), \(\delta\), and \(\epsilon\) but not \(\zeta\) (45) or ionomycin, an ionophore facilitating Ca\(^{2+}\) uptake by cells, blocked by BAPTA/AM, a Ca\(^{2+}\) chelator, or GF109203X, an inhibitor of Ca\(^{2+}\)-dependent PKC\(\alpha\), \(\beta I\), \(\beta I I\), and \(\gamma\) (28). These results suggest that the PKC mediating heterologous phosphorylation and...
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Many neurotransmitters communicate with the cell interior via PKC. However, little is known about substrates and functional impacts of PKC activated by these heterologous pathways. We demonstrated that, at least one mechanism, receptor internalization, is accountable for PKC-mediated heterologous desensitization of opioid receptor. We have shown here that activation of PKC promotes receptor phosphorylation and internalization, and the effects of PKC on receptor phosphorylation and internalization are linked. Mutation of the PKC site in DOR abolished PKC-stimulated receptor internalization, indicating that phosphorylation of Ser-344 in DOR by PKC is required for sequestration of the receptor. Receptor phosphorylation and internalization may occur in sequential steps. Experiments utilizing the wild type and a dominant negative mutant β-arrestin and hypertonic sucrose demonstrated clearly that PKC-mediated phosphorylation of Ser-344 leads DOR internalization through clathrin-coated pits in a β-arrestin-independent manner. The cytoplasmic tail of DOR contains multiple Ser/Thr residues (six in the last 31 amino acids), and there are two consensus PKC phosphorylation sites (Ser-344 and Ser-352). Removal of all Ser/Thr residues in the C-tail (truncated mutant Δ31) abolished both agonist- and PKC-induced receptor desensitization, indicating that the agonist-dependent, GRK phosphorylation site and agonist-independent PKC site are both located in the C terminus of DOR. Phosphorylation of the receptor C-tail is involved in both the homologous and heterologous desensitization of DOR. We have demonstrated that agonist-independent PKC-mediated phosphorylation of DOR occurs at Ser-344, whereas Thr-358, Thr-361, and Ser-363 contribute to agonist-induced receptor phosphorylation. Our results showed that the agonist-induced homologous receptor phosphorylation/internalization and PKC-mediated heterologous receptor phosphorylation/internalization are additive to each other. These data indicate that agonist-stimulated phosphorylation and PKC-catalyzed phosphorylation occur at distinctly different sites at the DOR C-tail and suggest that DOR phosphorylation via the two different mechanisms plays a complementary role in down-regulation of opioid receptor functions.

Studies have demonstrated the cross-regulation between receptors coupled to different signal transduction pathways. Recent studies indicated that, through PKC, a number of receptors cross-talk to other receptors on the membrane of the cell (11, 13). We have shown in the current study that, under physiological conditions, stimulation of α1A-AR and purinoceptor could heterologously activate PKC and result in receptor phosphorylation and desensitization. This study reveals a molecular basis for the observed agonist-independent regulation of opioid receptor desensitization. These observations may have important implications for our understanding of both opioid pharmacology and pathophysiological changes associated with drug tolerance and addiction. The regulation of opioid receptor function in the central nervous system via heterologous activation of PKC is likely to be of substantial physiological importance. Our previous research showed that activation of the NMDA receptor attenuates opioid receptor-
mediated signaling (14), and clinical study showed that coadministration of ketamine, an antagonist of NMDA receptor, potentiated morphine's analgesic effect (13). Insulin and phorbol ester have been also shown to attenuate the analgesic effect induced by morphine (15, 20, 21) Ohsawa et al. (17) demonstrated that the analgesic effect of morphine is attenuated in diabetic mice and involvement of PKC has been implicated. In this study, we have shown that the agonist-independent, heterologous activation of PKC induces desensitization of opioid receptors and this is a molecular mechanism of regulation of opioid signal transduction. Taken together, these data suggest that coadministration of opioid analgesics with other medicine may reduce the effect of opioid drugs. The basal PKC activity and basal Ca2+ level in cytoplasm could affect the analgesic effect of opioid drugs.

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