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Carboxyl Terminus of Delta Opioid Receptor Is Required for Agonist-Dependent Receptor Phosphorylation

Jing Zhao,* Gang Pei,† Ya-Lin Huang,* Fu-Min Zhong,† and Lan Ma*,[1]

**National Laboratory of Medical Neurobiology and Department of Neurobiology, Shanghai Medical University, 138 Yi Xue Yuan Road, Shanghai 200032, China; and †Shanghai Institute of Cell Biology and Shanghai Research Center of Life Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China*

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The wild-type delta opioid receptor (DOR) and a carboxyl terminus-truncated mutant DOR lacking the last 31 amino acids (DOR-T) were expressed in neuroblastoma × glioma hybrid NG108-15 cells to investigate the role of the carboxyl terminus of DOR in agonist-dependent receptor phosphorylation. Stimulation of the cells with delta specific agonists significantly induced DOR phosphorylation whereas no phosphorylation of DOR-T was detected under the same conditions. Neither overexpression of G protein-coupled receptor kinases (GRK2 or GRK5) nor activation of protein kinase C promoted agonist-induced phosphorylation of DOR-T, in contrast to their strong stimulatory effect on the agonist-dependent phosphorylation of DOR. Furthermore, DOR-T failed to be internalized after agonist stimulation, probably due to its inability to be phosphorylated. Our results indicate that the carboxyl terminus of DOR is required for agonist-dependent receptor phosphorylation and the phosphorylation site(s) of DOR is likely located at its carboxyl terminus.

Like other members of G protein-coupled receptor (GPCR) superfamily, mu, delta, and kappa opioid receptors undergo rapid desensitization upon exposure to their agonists (1-4). Opioid receptor desensitization, the reduced responsiveness of receptor, has been implied as one of the molecular mechanisms underlying opiate tolerance and dependence (5, 6). From analogy to other GPCRs (7), receptor phosphorylation and internalization may contribute to desensitization of opioid receptors though the detail mechanisms are not fully understood. It has been shown recently that agonist stimulation strongly induces phosphorylation of delta and mu opioid receptors, probably mediated by G protein-coupled receptor kinases (GRKs) and protein kinase C (PKC) (8-10). It has also been reported that agonist occupation causes internalization of delta opioid receptor (DOR) and that the carboxyl terminus of DOR plays an important role in the receptor internalization (11).

Opioid receptors, like other GPCRs, possess an extracellular amino terminus, seven transmembrane domains connected with three extracellular and three intracellular loops, and an intracellular carboxyl terminus (12-14). There are several potential phosphorylation sites of Ser/Thr residues on the first and third intracellular loops and at the carboxyl terminus of DOR (11-13) and some of them could be essential in the agonist-dependent phosphorylation and internalization of DOR. Since recent studies have suggested that the agonist-induced phosphorylation of some other GPCRs occurs at their carboxyl terminal tails (15-19) and that the truncation of DOR carboxyl terminus impairs the receptor internalization (11), we examined the role of the carboxyl terminus of DOR in agonist-dependent receptor phosphorylation in the present study. Our results, using neuroblastoma × glioma hybrid NG108-15 cells as a model system, suggest that the carboxyl terminus of DOR is required for agonist-dependent receptor phosphorylation and the phosphorylation site(s) on DOR is likely located at its carboxyl terminus.

MATERIALS AND METHODS

Cell culture and transfection. NG108-15 cells were cultured in Dulbecco's modified Eagle's medium containing 0.1 mM hypoxanthine, 10 mM aminopterin, 17 mM thymidine, and 10 % calf serum (Sijiqing Institute of Biomaterials). The epitope-tagged wild-type DOR (8) or a carboxyl terminus-truncated mutant of the tagged DOR without last 31 amino acids (DOR-T) (20) was transiently transfected in NG108-15 cells using the calcium phosphate method. In some experiments, bovine G protein coupled receptor kinase GRK2 or GRK5 (8, 21) was co-transfected with each opioid receptor. The cells

[1] To whom correspondence should be addressed. Fax: 21-64718563. E-mail: lanma@shmu.edu.cn.

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were cultured at 7×10^5 per 60-mm dish overnight and then transfected with 5 - 15 μg of cDNAs to obtain comparable receptor expression for both DOR and DOR-T. Expression of opioid receptors was measured by the radioligand binding assay 48 hr after transfection as described (20) before functional receptor analysis.

Laser confocal immunofluorescence microscopy and flow cytometry. Cells were suspended by gently pipeting and fixed in 1 % polyformaldehyde for 20 minutes. Fixed cells were stained with 12CA5 (a HA-epitope-specific monoclonal antibody from Boehringer Mannheim) and fluorescein-conjugated goat anti-mouse IgG (Biosource International) as described previously (22). Samples for microscopic analysis were mounted in 50 % glycerol-phosphate-buffered saline (PBS). Images were recorded using a Leica TCS NT laser confocal scanning microscope. Samples for internalization experiments were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Basal mean fluorescence intensity was determined with cells not transfected with CCR5 or cells incubated only with the fluorescein-labeled goat anti-mouse antibody.

Immunoprecipitation and Western blot analysis. Immunoprecipitation was carried out essentially as previously described (2, 8, 21, 23). Cells were lysed on ice in buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5 % deoxycholate, 0.1 % SDS and 1 % Nonidet P-40 and immunoprecipitated with 12CA5 and protein A Sepharose. The immunoprecipitated complexes were removed from Sepharose by heating at 65 °C in reducing SDS-PAGE sample buffer for 20 min, and the supernatants were analyzed on 10 % polyacrylamide gels. The gels were either dried and subjected to analysis using a PhosphorImager (Molecular Dynamics) to determine receptor phosphorylation or transferred to nitrocellulose membranes for Western analysis to quantify amount of receptors immunoprecipitated. After blotting, the HA-epitope-tagged receptors were detected with biotinylated 12CA5 and streptavidin-horseradish peroxidase conjugate (Beohringer Mannheim) using the ECL kit (Amersham) according to manufacturer-suggested protocol.

Phosphorylation of opioid receptors. Measurement of opioid receptor phosphorylation was carried out as described previously (2, 8, 21, 23). Briefly, the cells were labeled at 37 °C for 60 min with 100 μCi/ml [^{32}P]orthophosphate (5000 Ci/mmol; DuPont New England Nuclear) in the phosphate-free Dulbecco's modified Eagle's media 48 hr after transient transfection with the epitope-tagged opioid receptors. The labeled cells were then exposed to agonists for 10 min at 37 °C. After stimulation, the tagged receptors were immunoprecipitated with monoclonal antibody 12CA5 and immunoprecipitates were resolved 10 % sodium dodecyl sulfate-polyacrylamide gels. DOR Phosphorylation was visualized and quantitatively analyzed with a PhosphorImager.

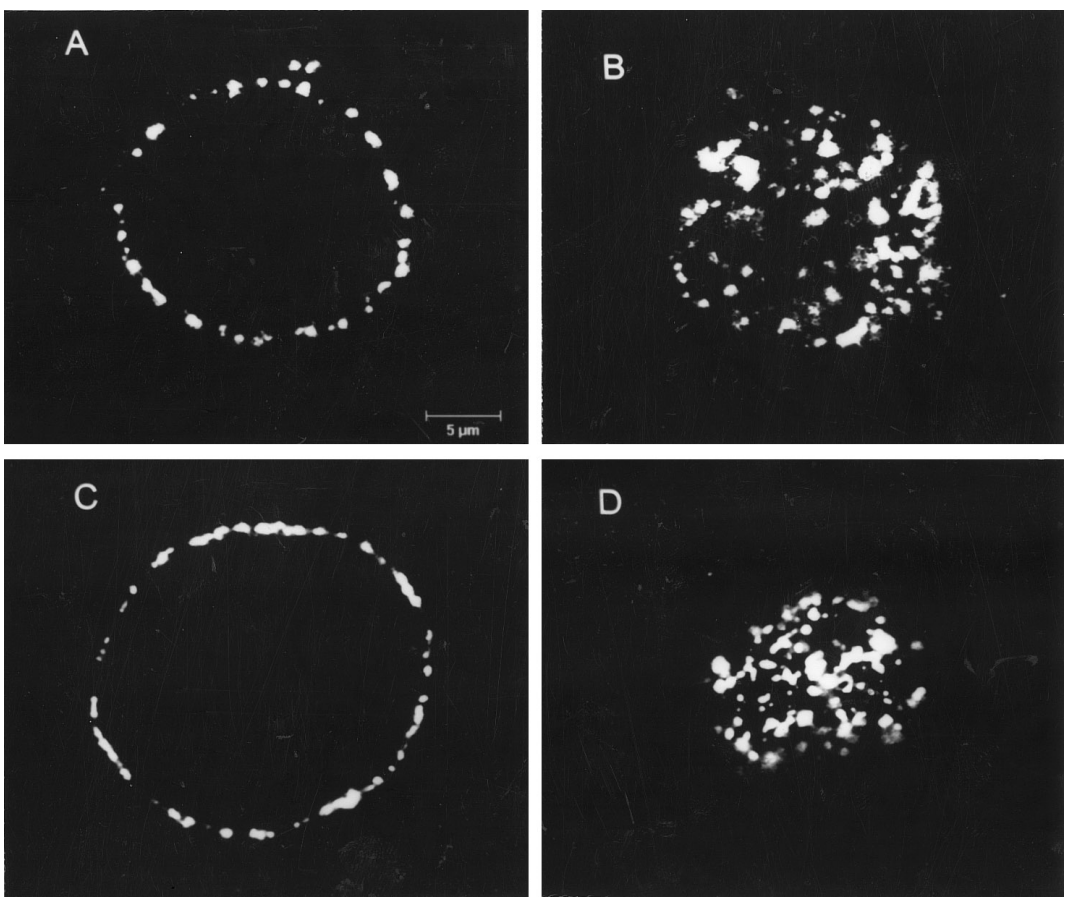

FIG. 1. Analysis of surface expression of DOR and DOR-T by confocal microscopy. NG108-15 cells transiently expressing the epitope-tagged wild-type DOR (panels A and B) or C-terminal truncated DOR-T (panels C and D) were analyzed 48 h after transfection. Immunofluorescence staining of the receptors were performed using antibody 12CA5 against the epitope tag and goat anti-mouse Ig-fluorescein conjugate. Cells were imaged by laser confocal fluorescence scanning microscopy. A cross-section through the center of the cells were shown in panels A and C. A cross-section through the surface of the cells were shown in panel B and D.

Statistical analysis. Data were analyzed with the student's *t*-test for comparison of independent means, with pooled estimates of common variances. Throughout the text, two-tailed *p* values are given.

RESULTS

Expression of epitope-tagged DOR and DOR-T was examined 48 hr following transiently transfecting NG108-15 cells. The surface expression of DOR or DOR-T was visualized under laser confocal scanning microscope after staining with 12CA5 and fluorescein-conjugated secondary antibody. Scanning images of cross-sections through the center and the surface of the cells showed that both DOR and DOR-T were expressed on the cell surface in a similar distribution pattern (Fig. 1). Typically approximately 20 % of the transiently transfected cells were expressing the HA-tagged opioid receptors while the remainder was not fluorescently positive and not visible in the background. This was consistent with our previous results when the epitope-tagged DOR and DOR-T were transiently expressed in human embryonic kidney 293 cells (20).

Surface expression of DOR and DOR-T in NG108-15 cells was also quantitatively determined by radioligand binding assay with [^{3}H] diprenorphrine, a widely-used opioid receptor ligand (20). The level of expression of DOR-T was comparable to that of DOR as measured in ligand binding assays (data not shown), consisting well with the data obtained from the fluorescence staining. To ensure quantitative analysis of receptor functions, expression levels of both DOR and DOR-T were stabilized at approximately 2 pmol/mg protein with fluctuation less than 10 % after carefully controlling cell culture and transfection conditions.

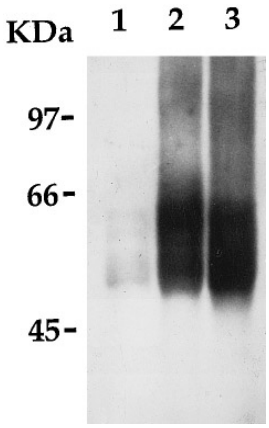

FIG. 2. Analysis of expression of DOR and DOR-T by immunoprecipitation. Untransfected NG108-15 cells (lane 1) and NG108-15 cells transiently expressing epitope-tagged DOR (lane 2) and DOR-T (lane 3) were lysed and immunoprecipitated with 12CA5. Proteins bound to protein A Sepharose were analyzed on 10 % SDS-polyacrylamide gels. The epitope-tagged proteins were detected with biotinylated 12CA5 and streptavidin-peroxidase conjugate after blotting. Biotinylated proteins (Bio-Rad) were used as molecular weight markers.

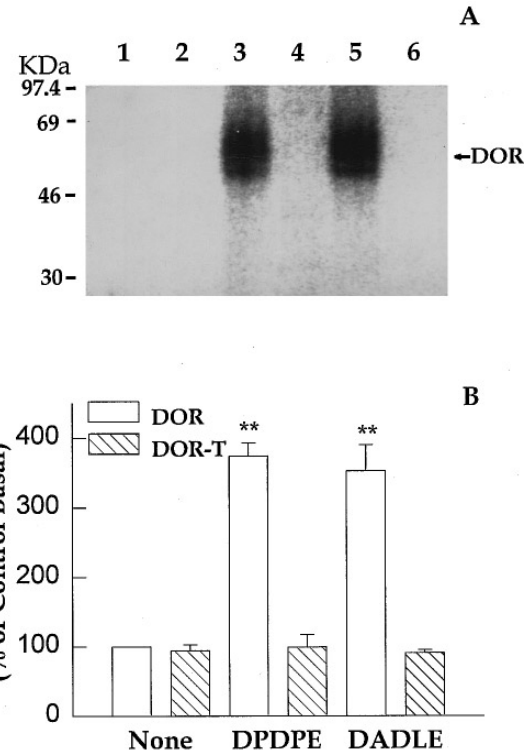

FIG. 3. Agonist-induced phosphorylation of DOR and DOR-T. (**A**) NG108-15 cells expressing the epitope-tagged DOR (lanes 1, 3, 5) and DOR-T (lanes 2, 4, 6) were labeled with [^{32}Pi] and stimulated without (lanes 1 and 2) or with 1 μM DPDPE (lanes 3 and 4), 1 μM DADLE (lanes 5 and 6) for 10 min. The epitope-tagged DOR and DOR-T were then immunoprecipitated with 12CA5, resolved on 10 % SDS-polyacrylamide gels, and subjected to phosphorimaging. ^{14}C-methylated proteins (Amersham) were used as molecular weight markers. (**B**) Receptor phosphorylation was quantitatively analyzed with a PhosphorImager and represented as a percentage of the basal level of receptor phosphorylation seen in control cells. Data shown are means ± SD of two separate experiments. **, $p < 0.01$

Exogenous expression of the wild-type and the C-terminus-truncated DORs were also examined by immunoprecipitation with the HA epitope antibody (Fig. 2). As shown in Fig. 2, lane 1, no exogenously expressed epitope-tagged opioid receptor could be detected in untransfected NG108-15 cells (Fig. 2, lane 1), whereas in sample derived from cells expressing the epitope-tagged wild-type DOR, a major protein band of ~ 50 kDa was specifically detected by 12CA5 (Fig. 2, lane 2). Sample derived from cells expressing the epitope-tagged DOR-T revealed a protein band running slightly faster than DOR (Fig. 2, lane 3), corresponding to the migration pattern expected for the truncated mutant.

Agonist-induced phosphorylation of the DOR was investigated in intact NG108-15 cells. Cells transiently

expressing the epitope-tagged DOR or DOR-T was metabolically labeled with [^{32}Pi] and the extent of DOR phosphorylation following agonist treatment was observed after immunoprecipitation of the epitope-tagged exogenous opioid receptor with antibody 12CA5 (Fig. 3). The result of phosphorimaging showed that DOR was present in unstimulated NG108-15 cells primarily in an unphosphorylated form (Fig. 3, lane 1). Treatment of the cells transiently expressing the wild-type DOR with selective delta agonists, [D-Pen^2, D-Pen^5]-enkephalin (DPDPE) or [D-Ala2, D-Leu5]-enkephalin (DADLE), resulted in a significant increase (approximately 400 % over the unstimulated cells) in [^{32}Pi] incorporation into the receptor (Fig. 3, lanes 3 and 5), indicative of agonist-dependent phosphorylation of DOR in a neuronal environment.

Similar to the wild-type receptor, DOR-T transiently expressed in NG108-15 cells was also present predominantly in an unphosphorylated form prior to agonist stimulation (Fig. 3, lane 2). However, in strong contrast to the wild-type receptor, phosphorylation of DOR-T was not observed after treatment of cells with the either delta agonist, DPDPE or DADLE (Fig. 3, lanes 4 and 6). We have shown in this study that DOR-T was efficiently expressed on the cell surface and was able to be immunoprecipated with 12CA5 (Fig. 1 and 2). Therefore, these data clearly indicate that DOR-T lacks agonist-induced phosphorylation and the carboxyl terminal 31 amino acid residues are required for the agonist-induced DOR phosphorylation.

Our previous research suggests that GRKs may mediate agonist-dependent phosphorylation of DOR as overexpression of GRK2 and GRK5 enhances agonist-induced phosphorylation of DOR (8). In the present study in NG108-15 cells, co-expression of DOR with either GRK2 (Fig. 4, lane 3) or GRK5 (Fig. 4, lane 5) also enhanced DPDPE-induced DOR phosphorylation. Whereas, overexpression of GRK2 or GRK5 failed to stimulate phosphorylation of the carboxyl terminus-truncated receptor after the same agonist treatment (Fig 4, lanes 4 and 6). This observation is consistent with our previous results (8) and indicate further that the 31 residues at the carboxyl terminus are also required for the GRK-mediated receptor phosphorylation.

Our previous studies have demonstrated that interaction of delta opioid with DOR activates PKC (24), that PKC activator phorbol 12-myristate 13-acetate (PMA) enhances DOR phosphorylation (8) and that PKC is required for the desensitization of DOR in NG108-15 cells (2). DOR has several putative phosphorylation sites for PKC including two on the carboxyl terminal tail (12, 13). Therefore, PMA-stimulated phosphorylation of DOR and DOR-T was examined in NG108-15 cells in this study. As shown in Fig. 5, stimulation with PMA resulted in phosphorylation of the

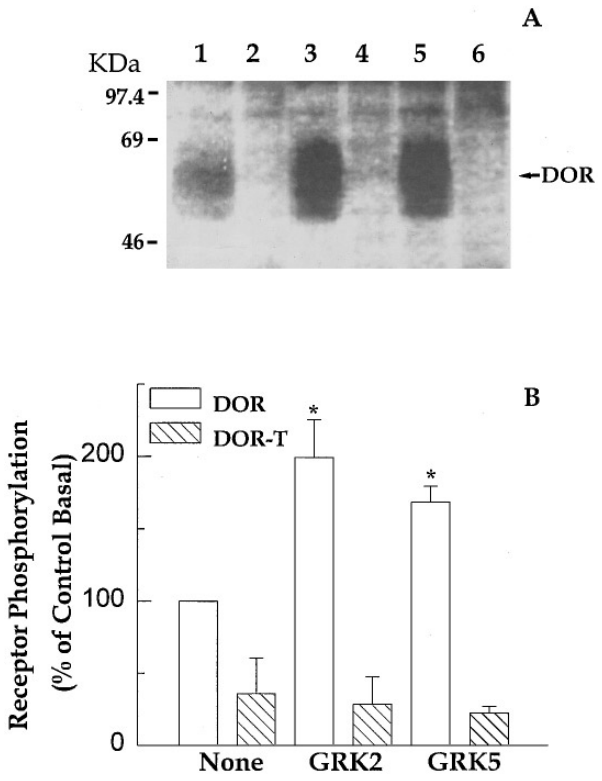

FIG. 4. Effects of overexpression of GRKs on agonist-induced receptor phosphorylation. (**A**) NG108-15 cells co-transfected with GRK2 (lanes 3 and 4) or GRK5 (lanes 5 and 6) and the epitope-tagged DOR (lanes 1, 3, 5) or DOR-T (lanes 2, 4, 6) were metabolically labeled with [^{32}Pi] and stimulated with 1 μM DPDPE for 10 min. DOR and DOR-T were then immunoprecipitated with 12CA5, resolved on 10 % SDS-polyacrylamide gels, and subjected to phosphorimaging. (**B**) Receptor phosphorylation was quantitatively analyzed with a PhosphorImager and represented as a percentage of the basal level of receptor phosphorylation seen in control cells. Data shown are means ± SD of two separate experiments. *, $p < 0.05$

wild-type DOR (lane 5) as compared to the unstimulated control (lane 1), consistent with our previous result (8). But under the same conditions, PMA failed to stimulate phosphorylation of DOR-T (lane 6), suggesting that the major target of PKC, as those of GRKs, may reside in the carboxyl terminus of the DOR.

Receptor internalization was determined using flow cytometry in NG108-15 cells transiently expressing the epitope-tagged DOR or DOR-T. As shown in Fig. 6, treatment of the cells expressing DOR with 1 μM DPDPE for 30 min caused approximately 50 % reduction in cell surface fluorescence, indicating a considerable loss of the wild-type DOR from the cell surface (Fig. 6). However, the surface immunofluorescence for the cells expressing DOR-T did not change significantly after the same treatment (Fig. 6), suggesting that the carboxyl terminal 31 residues are involved in the agonist-induced DOR internalization.

DISCUSSION

Opioid receptors are mainly distributed in the central nerve system and they mediate the actions of opiate drugs and exogenous opioid neuropeptides in producing euphoria, modulating pain perception, and altering other important functions of the brain (25, 26). Recent *in vitro* studies, using non-neuronal cell lines, have demonstrated that agonist stimulation strongly induces phosphorylation of opioid receptors, likely mediated by GRKs and PKC (4, 8-10). In the present study, we attempted to apply NG108-15 cells, which endogously expresses DOR and has been widely used as a model cellular system in the opiate research, in the investigation of opioid receptor phosphorylation. Our results clearly showed that in neuronal cells DOR underwent agonist-induced phosphorylation, which was enhanced by overexpression of GRKs and paralleled to agonist-stimulated internalization of DOR. These data support for an important role of receptor phosphorylation in agonist-induced desensitization of opioid receptors observed in neuronal cells.

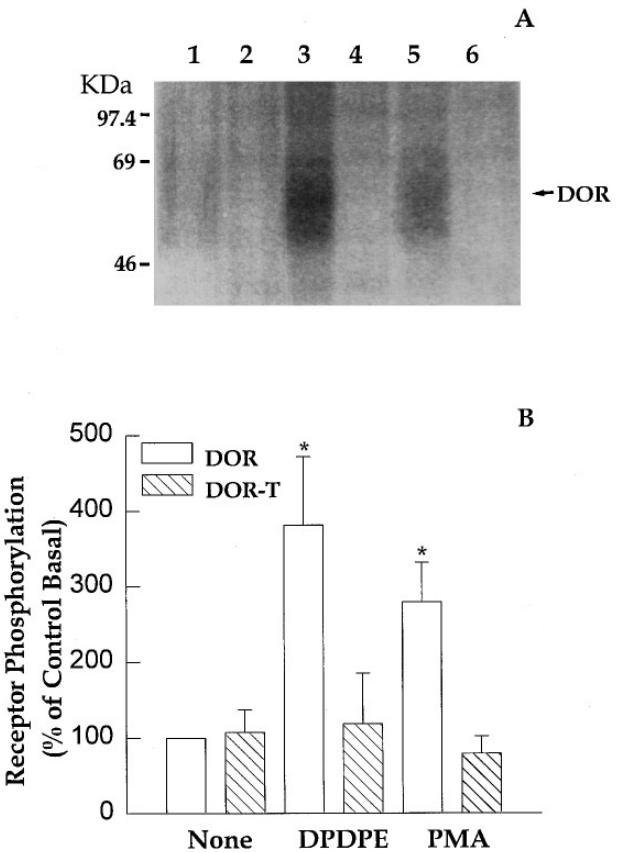

FIG. 5. Effect of PKC on agonist-induced receptor phosphorylation. (**A**) NG108-15 cells transfected with the epitope-tagged DOR (lanes 1, 3, 5) and DOR-T (lanes 2, 4, 6) were metabolically labeled with [^{32}Pi] and stimulated without (lanes 1 and 2) or with 1 μM DPDPE (lanes 3 and 4) or 0.5 μM PMA (lanes 5 and 6) for 10 min. DOR and DOR-T were then immunoprecipitated with 12CA5, resolved on 10 % SDS-polyacrylamide gels, and subjected to phosphorimaging. (**B**) Receptor phosphorylation was quantitatively analyzed with a Phosphorimager and representated as a percentage of the basal level of receptor phosphorylation seen in control cells. Data shown are means ± SD of two separate experiments. *, $p < 0.05$.

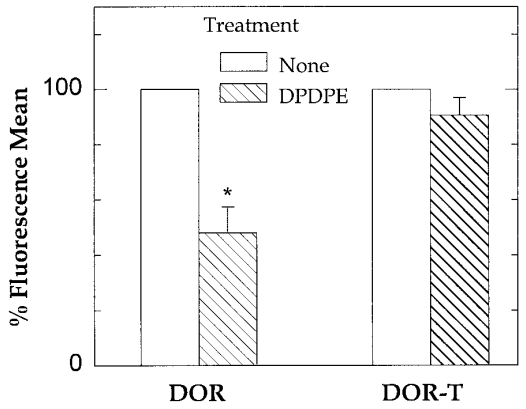

FIG. 6. Internalization of DOR and DOR-T. NG108-15 cells transiently expressing DOR or DOR-T were treated with none or 1 μM DPDPE at 37 °C for 30 min and surface receptors were analyzed by flow cytometry using 12CA5. Internaliaztion of the receptors is indicated by a reduction in mean surface fluorescence in the cells measured. Results were presented as means ± SD. The autofluorescence of the cells is subtracted from the mean fluorescence and the value obtained without DPDPE treatment is taken as 100 %. *, $P<0.05$

Opioid receptors belong to GPCR superfamily. For a growing numbers of GPCRs, the agonist-induced phosphorylation sites for GRKs or the second messenger-dependent kinases such as cAMP-dependent protein kinase or PKC isoforms have been located at the carboxyl terminus (15-19). However, no experimental data are available on the locations of the phosphorylation sites of opioid receptors, which could be either on the intracellular loops or/and in the carboxyl terminal region of opioid receptors according to their amino acid sequences (12-14). It has been reported recently that the truncation of the carboxyl terminus of DOR significantly reduces the agonist-induced receptor internalization, which is dependent on receptor phosphorylation and binding of β-arrestin in the case of β_2 adrenergic receptor (7). The present study, therefore, was undertaken to test the role of the carboxyl terminus of DOR in the agonist-dependent receptor phosphorylation. Our data, taken together, have shown that though DOR-T was expressed efficiently at the cell surface and was able to be immunoprecipated, it did not undergo agonist-induced phosphorylation, even provided with overexpressed GRKs and activated PKC. It indicates that the carboxyl terminal 31 amino acid residues are required for the agonist-induced DOR phosphorylation and further implies that the phosphorylation site(s) for

GRKs or/and PKC is likely located within the carboxyl terminus of DOR.

Multiple mechanisms including receptor phosphorylation, internalization, as well as down-regulation of receptor expression may contribute to desensitization of GPCRs. Receptor phosphorylation and internalization, both induced by agonist stimulation, are distinct but closely related events, as in the well established system of β_2 adrenergic receptor (7). The results from this study that agonist stimulation failed to induced either phosphorylation or internalization of DOR-T in the same neuronal cellular system provided more evidence for such correlation.

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