0026-895X/95/020173-05$3.00/0

MOLECULAR PHARMACOLOGY, 48:173–177 (1995).

ACCELERATED COMMUNICATION

Agonist-Dependent Phosphorylation of the Mouse δ-Opioid Receptor: Involvement of G Protein-Coupled Receptor Kinases But Not Protein Kinase C

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Received February 21, 1995; Accepted May 16, 1995

SUMMARY

With chronic opiate use, opioid receptor desensitization may be one of the important mechanisms underlying the development of opiate tolerance and addiction. Opioid receptors belong to the G protein-coupled receptor superfamily. In this study, the mouse δ-opioid receptor (δOR) was used in a model system to investigate the role of opioid receptor phosphorylation in receptor desensitization. When expressed in 293 cells and exposed to agonist, the δOR underwent receptor-specific desensitization within 10 min. This agonist-induced desensitization corresponded temporally to a 3-fold increase in receptor phosphorylation. Phorbol ester, but not forskolin, also stimulated phosphorylation of the δOR in 293 cells. Although down-regulation of protein kinase C failed to affect agonist-induced receptor phosphorylation, it abolished phorbol ester-induced receptor phosphorylation. Agonist-induced δOR phosphorylation must therefore involve kinases other than protein kinase C. Whereas overexpression of a dominant negative mutant (K220R) of β-adrenergic receptor kinase-1 (βARK1) in 293 cells significantly reduced agonist-dependent phosphorylation of the δOR, overexpression of βARK1 or G protein-coupled receptor kinase-5 significantly enhanced this phosphorylation. Concordantly, βARK1-K220R overexpression reduced agonist-dependent δOR desensitization, whereas βARK1 overexpression enhanced this desensitization. We conclude that short term desensitization of the δOR involves phosphorylation of the receptor by one or more G protein-coupled receptor kinases.

Opiates exert their various effects on the central and peripheral nervous systems through a family of membrane receptors, which are coupled to the inhibitory G protein G_i. Three major subtypes of opioid receptors, designated as μ, δ, and κ, have been well characterized by pharmacological studies (1–3), and cDNAs for each have been cloned (4–8).

It has long been known that chronic use of opiates produces tolerance and addiction (9), but the molecular mechanisms underlying these phenomena are incompletely defined. With continuous or repetitive stimulation, the opioid receptor/G_i system demonstrates reduced responsiveness (10–13). This process of desensitization is also observed with other G protein-coupled receptors. In the well characterized β_2-adrenergic receptor system, desensitization can transpire over seconds to minutes and involves phosphorylation of the receptor by both PKA and βARK1, a prototypical GRK (14).

Data suggesting the involvement of protein kinases in opioid receptor desensitization have recently emerged. Chen and Yu (8) have shown that activation of PKC can reduce μ-opioid receptor responsiveness in *Xenopus* oocytes. Fukushima *et al.* (15) found that PKC is involved in the functional uncoupling of δORs from G proteins in the striatum of young guinea pigs. Recently, it has also been shown that βARK1 levels in the rat locus coeruleus are increased after chronic morphine administration (16). Furthermore, a dominant negative mutant of βARK1 blocks κ-opioid receptor desensitization in COS-7 cells (17).

This work was supported in part by National Institutes of Health Grants HL16037 (R.J.L.) and HL03008–02 (N.J.F.). N.J.F. is the recipient of a Clinical Investigator Development Award from the National Heart, Lung, and Blood Institute.

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ABBREVIATIONS: PKA, cAMP-dependent protein kinase; βARK, β-adrenergic receptor kinase; δOR, δ-opioid receptor; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; GRK, G protein-coupled receptor kinase; IBMX, 3-isobutyl-1-methylxanthine; LPA, lysophosphatidic acid; PKC, protein kinase C; SDS, sodium dodecyl sulfate; PMA, phorbol-12-myristate-13-acetate.

We therefore designed this study directly to test the hypothesis that opioid receptor desensitization involves phosphorylation of the receptor itself and that GRKs participate in this process. For this purpose, we used a cell culture model system to assess opioid receptor phosphorylation in intact cells.

Materials and Methods

Construction of an epitope-tagged δOR. All recombinant DNA procedures were conducted according to standard protocols (18). The influenza hemagglutinin epitope (YPYDVPDYA) for the monoclonal antibody 12CA5 (19) was added to the amino terminus of mouse δOR cDNA (5) with the polymerase chain reaction. The 5′ primer was 5′-CGCGGGGAATTCACCATGTACCCCTACGACGTCCCCGACTACGCCGAGCTGGTGCCCTCTGCC-3′ (with the start codon being underlined). The 3′ primer was 5′-CGCGGGCTCGAGTCAGGCGGCAGCGCCACC-3′. The 1.2-kilobase polymerase chain reaction product was subcloned into pcDNA I/Amp (Invitrogen) in the *Eco*RI and *Xho*I sites, and the fidelity of cDNA amplification was verified with dideoxy DNA sequencing (Sequenase T7 kit; United States Biochemicals).

Cell culture and receptor expression assays. Human embryonic kidney 293 cells were transiently transfected with the epitope-tagged δOR by the calcium phosphate method (20). Receptor expression 48 hr after transfection was measured by a modification of the radioligand binding assay described by Kieffer *et al.* (5); 10 nM [*N-allyl*-2,3-^{3}H]naloxone (DuPont-NEN) was used, without or with 1 μM naloxone (Sigma) to determine nonspecific binding. Expression levels of the δOR in 293 cells were 1–2 pmol/mg of protein, as measured by saturation binding. There was no detectable [^{3}H]naloxone binding in untransfected 293 cells. Expression of the epitope-tagged δOR was also assessed by determination of immunofluorescence by flow cytometry (21).

cAMP assay and desensitization. δOR-transfected 293 cells were labeled overnight with 2 μCi/ml [2,8-^{3}H]adenine, in minimal essential medium with 3% fetal bovine serum. The cells were prechallenged for the indicated times with control medium or medium containing 5 μM DPDPE (Sigma), at 37°. The cells were washed with warm minimal essential medium containing 500 μM IBMX (Sigma) and were then stimulated with either 10 μM (−)-isoproterenol (Sigma) or 10 μM forskolin (Sigma), alone or with either 5 μM DPDPE or 10 μM LPA (Sigma), for 10 min at 37°. The reaction was terminated with an equal volume of 2× stop solution [0.2 mM cAMP (Sigma) and 9 nCi/ml [^{14}C]cAMP (DuPont-NEN) in 5%, w/v, perchloric acid (Sigma)]. The cAMP accumulated after stimulation was quantitated as described (22). Percentage inhibition of the cAMP formation was calculated as $100 \times [1 - [cAMP_{(Iso+DPDPE)}/cAMP_{Iso}]]$.

Phosphorylation of the opioid receptor. 293 cells transfected with the δOR were seeded at 1×10^6 cells/well, in six-well dishes, 36 hr before assay. On the day of assay, cells were washed with phosphate-free Dulbecco's modified Eagle medium and labeled with 100 μCi/ml [^{32}P]orthophosphate (DuPont-NEN) for 60 min. Labeled cells were then stimulated with 5 μM DPDPE, 1 μM PMA (Calbiochem), or 50 μM forskolin (Sigma) plus 500 μM IBMX (Sigma) for 10 min at 37°. After incubation, the cells were placed on ice, washed with ice-cold phosphate-buffered saline, and solubilized at 0° in 0.8 ml of RIPA+ buffer [150 mM NaCl, 50 mM Tris·HCl, pH 8.0 at 25°, 5 mM EDTA, 1%, v/v, Nonidet P-40 (Calbiochem), 0.5%, w/v, sodium deoxycholate, 0.1%, w/v, SDS, with 10 mM NaF, 10 mM disodium pyrophosphate, and 1 μM okadaic acid (Calbiochem) as phosphatase inhibitors and with 10 μg/ml leupeptin, 10 μg/ml benzamidine, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, and 0.2 mM phenylmethylsulfonyl fluoride (all from Sigma) as protease inhibitors]. After centrifugation at 300,000 × *g* for 15 min at 4°, solubilized cell supernatants (0.7 ml) were precleared with Protein A-Sepharose beads (Pharmacia), as described (23), and were then immunoprecipitated for 2 hr at 4° with 13 μg of 12CA5 monoclonal antibody and another aliquot of Protein A beads. The beads were next washed three times with 1 ml of RIPA+ buffer, and immunoprecipitated receptors were desorbed by incubation of the beads at 37° for 30 min in 60 μl of 1× Laemmli sample buffer (23). To load SDS-polyacrylamide (10%) gel lanes with equivalent amounts of δORs, we calculated the number of "receptor equivalents" in each immunoprecipitation tube; the receptor expression (in picomoles/milligram, determined with a nonradioactive aliquot of cells) was multiplied by the amount of protein subjected to immunoprecipitation [in milligrams, determined (DC protein assay kit; Bio-Rad) for the solubilized cells before preclearing]. These values were normalized and used to adjust gel loading volumes. After electrophoresis, gels were dried and autoradiographed with BioMax MR film (Kodak). Receptor phosphorylation was quantitatively analyzed with a PhosphorImager (Molecular Dynamics). Opioid receptor phosphorylation was also examined in 293 cells when the receptor was co-transfected with the plasmid pcDNA I, without or with a cDNA insert encoding bovine βARK1, bovine GRK5, or a dominant negative mutant βARK1-K220R (24, 25).

Western blot analysis. Cell lysates prepared from transfected 293 cells were fractionated on 10% SDS-polyacrylamide gels. The gels were transferred to nitrocellulose membranes and the membranes were blocked with 5% low-fat dried milk dissolved in phosphate-buffered saline containing 0.1% Tween-20. Blots were incubated with diluted primary 12CA5 monoclonal antibody or primary rabbit polyclonal antibodies against βARK isoforms (26) or GRK5 (27) and were then incubated with anti-mouse or anti-rabbit horseradish peroxidase-linked secondary antibodies, following enhanced chemiluminescence Western blotting protocols (Amersham). Immunoreactive proteins were visualized with the enhanced chemiluminescence detection system.

Statistical analysis. Data were analyzed with the *t* test for comparison of independent means, with pooled estimates of common variances. Throughout the text, two-tailed *p* values are given.

Results and Discussion

When expressed in 293 cells, the δOR demonstrated functional coupling to G_i by inhibiting isoproterenol-stimulated cAMP accumulation upon challenge with the agonist DPDPE (Fig. 1). This effect was indistinguishable from that produced by stimulation of the endogenous LPA receptor in 293 cells. When the cells were pretreated with DPDPE for 10 min, a second challenge with DPDPE failed to inhibit cAMP production. This short term desensitization was receptor-specific, or homologous, because the DPDPE pretreatment barely affected LPA-stimulated inhibition of cAMP production.

To determine whether this homologous desensitization of the δOR involves phosphorylation of the receptor, we immunoprecipitated the epitope-tagged receptors from agonist-stimulated cells metabolically labeled with $^{32}P_i$. The opioid receptor was phosphorylated within 3 min of agonist exposure, and the degree of receptor phosphorylation increased another 25% by 10 min, to levels 3.3 ± 0.3-fold greater than that seen in the absence of agonist (Fig. 2). Thus, agonist-promoted phosphorylation of the δOR coincides temporally with homologous desensitization and follows a time course very similar to that of the β_2-adrenergic receptor (28).

Because agonist-promoted receptor phosphorylation may be effected by both second messenger-dependent kinases and GRKs, we attempted to ascertain the potential contributions of these kinase classes to δOR phosphorylation. For this purpose, we first used PMA to activate PKC and forskolin to activate PKA. Activation of PKC produced opioid receptor phosphorylation 2.7-fold greater then basal levels, whereas

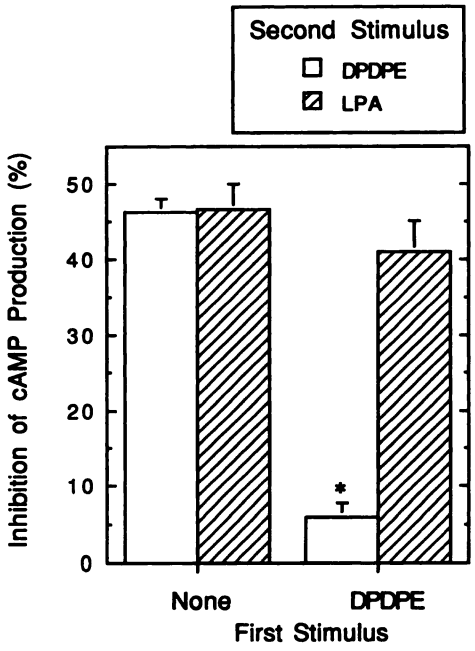

Fig. 1. Homologous desensitization of the δOR in 293 cells. 293 cells expressing the δOR were treated for 10 min with medium containing vehicle (*None*) or 5 μM DPDPE (*DPDPE*), at 37°. Cells were washed and challenged with 10 μM isoproterenol, without or with 5 μM DPDPE or 10 μM LPA, for an additional 10 min. Plotted is the percentage inhibition of isoproterenol-stimulated cAMP formation from three separate experiments (mean ± standard error), calculated as 100 × [$cAMP_{Iso}$ − $cAMP_{(Iso+G_i)}$]/[$cAMP_{Iso}$], where $cAMP_{(Iso+G)}$ is cAMP produced in the presence of the G_i-coupled receptor agonists DPDPE or LPA and $cAMP_{Iso}$ is cAMP production in the absence of any G_i-coupled receptor agonist. In cells challenged with isoproterenol alone, cAMP production was 41 ± 12-fold greater than that measured in unstimulated cells. *, $p < 0.001$, compared with control cells.

forskolin plus IBMX barely affected receptor phosphorylation (Fig. 3). The activation of PKA in 293 cells with 8-chlorophenylthio-cAMP also failed to promote opioid receptor phosphorylation (data not shown). PKA therefore appears an unlikely candidate for agonist-promoted phosphorylation of the δOR.

Because PMA could stimulate phosphorylation of the δOR, we next assessed whether PKC participates in the agonist-promoted phosphorylation of the δOR. Down-regulation of PKC by long term PMA treatment (29) abolished PMA-stimulated δOR phosphorylation but failed to diminish agonist-stimulated δOR phosphorylation (Fig. 3). Thus, although PKC can phosphorylate the δOR, it seems unimportant in the process of agonist-promoted receptor phosphorylation that attends short term δOR desensitization in 293 cells. Congruent with this inference, stimulation of the δOR with DPDPE in 293 cells failed to activate inositol phospholipid hydrolysis (data not shown) and would therefore not be expected to activate PKC.

Because the second messenger-dependent kinases PKA and PKC appear unimportant in the agonist-promoted phosphorylation of the δOR, we examined the possibility that one or more GRKs known to exist in 293 cells (25) might effect this activity. For this purpose, we overexpressed in 293 cells a dominant negative (K220R) mutant of βARK1, which competitively inhibits wild-type βARK1 activity with the β_2-adrenergic receptor (24). Although βARK1-K220R overexpression reduced agonist-stimulated δOR phosphorylation by

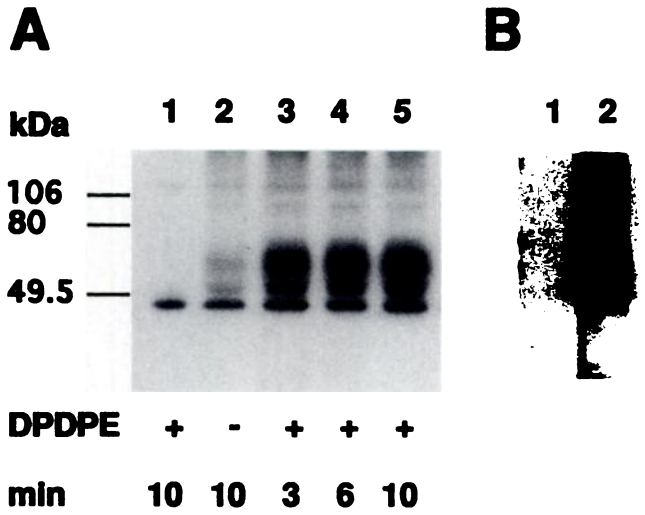

Fig. 2. Agonist-promoted phosphorylation of the δOR. A, 293 cells, transfected (*lanes 2–5*) or not (*lane 1*) with the epitope-tagged δOR, were labeled with $^{32}P_i$ for 60 min and stimulated without or with 5 μM DPDPE for the indicated times. δORs were then immunoprecipitated with the monoclonal antibody 12CA5, resolved on SDS-polyacrylamide gels, and subjected to autoradiography as described in Materials and Methods. Pictured is the result of a single experiment representative of three performed. B, Cell lysates, prepared from 293 cells untransfected (*lane 1*) or transfected (*lane 2*) with the 12CA5 epitope-tagged δOR, were size-fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted with 12CA5 as described in Materials and Methods. Shown is an autoradiogram representative of three experiments.

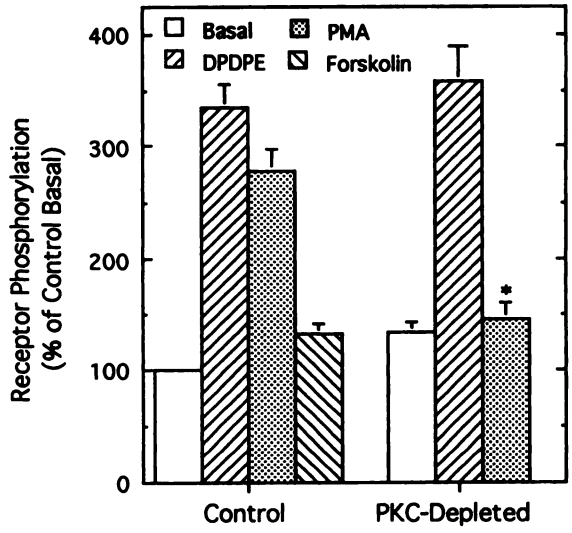

Fig. 3. PKC effects on phosphorylation of the δOR in 293 cells. δOR-transfected 293 cells were pretreated without (*Control*) or with (*PKC-Depleted*) 1 μM PMA for 23 hr before labeling with $^{32}P_i$ for 60 min. The cells were then stimulated for 10 min with either 5 μM DPDPE, 1 μM PMA, 50 μM forskolin plus 500 μM IBMX, or vehicle (*Basal*). δORs were then immunoprecipitated with 12CA5 and resolved by SDS-polyacrylamide gel electrophoresis, as described in Materials and Methods. Receptor phosphorylation was quantitatively analyzed with a PhosphorImager and expressed as a percentage of the basal level of receptor phosphorylation seen in control cells. Data shown are means ± standard errors of at least three separate experiments. *, $p < 0.003$, compared with the control cell value.

about 50%, it did not affect the PMA-stimulated receptor phosphorylation (Fig. 4A). This ability of βARK1-K220R specifically to inhibit agonist-stimulated δOR phosphorylation indicates that, in intact 293 cells, one or more GRKs are

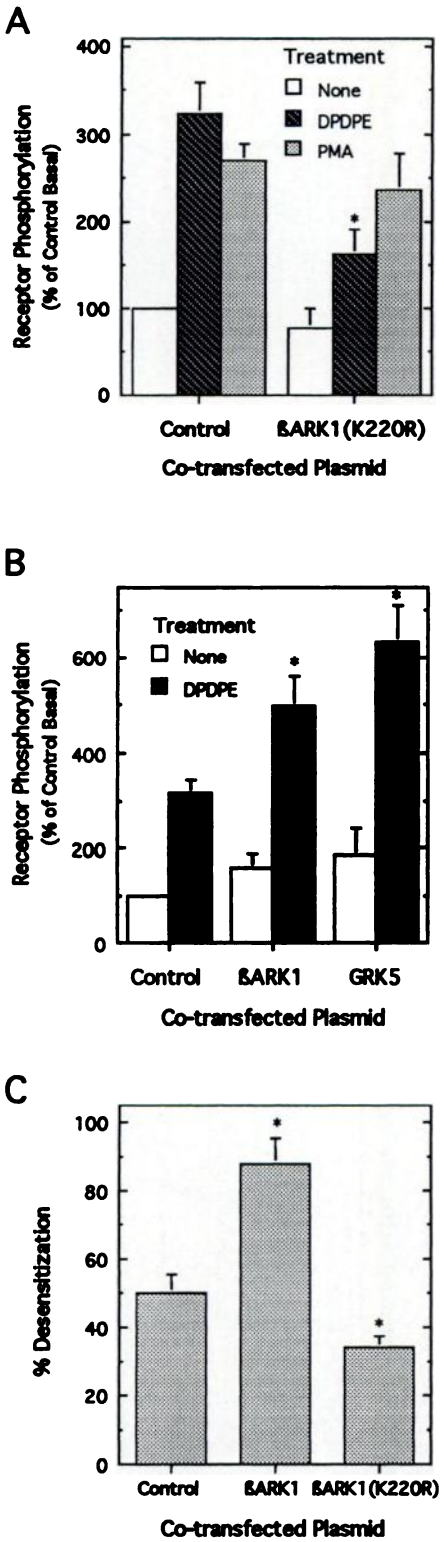

Fig. 4. Agonist-induced δOR phosphorylation and desensitization: reciprocal effects of a GRK inhibitor and overexpressed GRKs. 293 cells were co-transfected with the δOR and the expression vector pcDNA I without (*Control*) or with cDNA inserts for βARK1-K220R (A), βARK1 or GRK5 (B), or βARK1 or βARK1-K220R (C). A and B, Receptor phosphorylation was stimulated with 5 μM DPDPE or 1 μM PMA for 10 min and quantitated by PhosphorImager analysis after immunoprecipitation and gel electrophoresis, as described in Materials and Methods. The degree of receptor phosphorylation is expressed as a percentage of that seen in unstimulated control cells. Plotted are the means ± standard deviations from four separate experiments performed in triplicate. *, $p < 0.002$, compared with the corresponding control cell values. C, Cells were exposed for 4 hr to medium with ("prestimulated") or without ("naive") 5 μM DPDPE. After being washed, prestimulated and naive cells were exposed for 10 min to medium containing 10 μM forskolin and 500 μM IBMX, in the presence or absence of 5 μM DPDPE, and cAMP formation was assessed as described in Materials and Methods. Inhibition of forskolin-stimulated cAMP formation was calculated in the same manner as described for Fig. 1 for the inhibition of isoproterenol-stimulated cAMP formation. Percentage desensitization was calculated as a function of the DPDPE-dependent inhibition of forskolin-stimulated cAMP formation observed in naive and prestimulated cells, i.e., 100 × (naive − prestimulated)/naive. Plotted are the values (mean ± standard error) for percentage desensitization from four (βARK1) or six (βARK1-K220R) separate experiments performed in triplicate. For the transfected cell types, the values for percentage inhibition of forskolin-stimulated cAMP formation in naive cells were 50 ± 14% (control cells), 39 ± 5% (βARK1-transfected cells), and 41 ± 8% (βARK1-K220R-transfected cells). *, $p \leq 0.03$, compared with cells transfected with just the δOR and control plasmids.

responsible for this process. The incomplete nature of the inhibition seen with βARK1-K220R may derive from the action of a non-GRK mechanism or from incomplete efficacy of βARK-K220R, as observed with purified proteins in the presence of G protein βγ subunits (24).

If GRKs naturally expressed in 293 cells are responsible for agonist-stimulated δOR phosphorylation, then overexpression of these kinases would be expected to augment this phosphorylation. We therefore overexpressed βARK1 and GRK5 along with the δOR and found that overexpression of each kinase increased agonist-induced δOR phosphorylation by at least 50% (Fig. 4B). Overexpression of the GRKs in these experiments was estimated, by immunoblotting, to be >20-fold greater than endogenous expression levels in 293 cells (data not shown).

To correlate changes in agonist-induced δOR phosphorylation with changes in receptor desensitization, we evaluated the effects of βARK1 and βARK1-K220R overexpression on δOR-mediated inhibition of forskolin-stimulated cellular cAMP accumulation (Fig. 4C). Whereas exposure to agonist for 4 hr diminished the maximal response of control cells by 50%, this treatment of cells overexpressing βARK1 or βARK1-K220R diminished the maximal δOR response to agonist by as much as 88% or as little as 34%, respectively. Thus, overexpression of βARK1 increased not only agonist-induced δOR phosphorylation but also δOR desensitization. Conversely, inhibition of cellular GRK activity by βARK1-K220R decreased both agonist-induced δOR phosphorylation and desensitization.

This study provides the first direct evidence that agonist-induced desensitization of any opioid receptor coincides with phosphorylation of the receptor itself. Additionally, we have established a significant role for GRKs in agonist-dependent phosphorylation of the δOR. The time course of this receptor phosphorylation, furthermore, is consistent with that of the homologous desensitization observed in opiate-treated neurons from locus coeruleus (11, 12).

PKA activators failed to elicit δOR phosphorylation in this study. This observation is not surprising, because the activation of opioid receptors inhibits, rather than stimulates, the formation of the PKA activator cAMP. This result also agrees with earlier reports that PKC, but not PKA, is involved in the functional uncoupling of the δOR from G_i in striatal membranes (15) and that PKA fails to desensitize the μ-opioid receptor in *Xenopus* oocytes (8).

Our data contrast the ability of PKC to phosphorylate the δOR in 293 cells with the failure of cellular PKC depletion to alter agonist-induced receptor phosphorylation. This apparent contrast mirrors findings with the thrombin receptor expressed in Rat 1 cells (30). In the 293 cell model system, βARK1 or perhaps GRK5, but not PKC, appears to be responsible for the agonist-induced δOR phosphorylation that accompanies desensitization. However, although δOR-mediated activation of G_i fails to stimulate phosphatidylinositol-specific phospholipase C activity in 293 cells, the ability of G_i-coupled receptors to stimulate phospholipase C-β isoforms, and subsequently PKC, is well documented in other cell systems (31). Our results do not preclude, therefore, the possibility that PKC may be important for agonist-induced δOR phosphorylation and desensitization in other cell types.

In the rat model of opiate tolerance, up-regulation of βARK1 expression in the locus coeruleus has been demonstrated (16). Coupled with these *in vivo* observations, our results substantially strengthen the hypothesis that βARK1 is a regulator of the δOR, and they suggest that therapies modulating GRK action may be important in circumventing opiate tolerance.

Acknowledgments

We thank Dr. Martin Oppermann for helpful suggestions and discussions, and Grace Irons and Sabrina T. Exum for expert technical assistance with cell culture.

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