Biochemical Pharmacology, Vol. 60, pp. 781–792, 2000.

ELSEVIER

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00400-7

Tyrosine Phosphorylation of the δ-Opioid Receptor

EVIDENCE FOR ITS ROLE IN MITOGEN-ACTIVATED PROTEIN KINASE ACTIVATION AND RECEPTOR INTERNALIZATION*

H. Kenneth Kramer,†‡ Matthew L. Andria,† Douglas H. Esposito† and Eric J. Simon†§

DEPARTMENTS OF †PSYCHIATRY AND §PHARMACOLOGY, NEW YORK UNIVERSITY SCHOOL OF MEDICINE, MILLHAUSER LABORATORIES, NEW YORK, NY 10016, U.S.A.

ABSTRACT. The internalization of G-protein-coupled receptors (GPCRs), including the delta opioid receptor (δ-OR), has been shown to involve the phosphorylation of serine and threonine residues. However, recent studies suggest that these residues may not be the only ones phosphorylated in response to prolonged opioid exposure. Tyrosines also appear important for δ-OR signalling, but it remains unclear whether they undergo phosphorylation. We examined whether the δ-OR, stably expressed in Chinese hamster ovary (CHO-K1) cells, was tyrosine-phosphorylated during prolonged agonist treatment. The epitope-tagged δ-OR was purified by immunoprecipitation, and the presence of phosphorylated tyrosines was detected using anti-phosphotyrosine antibodies. Tyrosine residues in the δ-OR were phosphorylated after exposure to the high-affinity agonist [d-Thr^2]-Leu-enkephalin-Thr (DTLET) in a time- and concentration-dependent manner. Tyrosine phosphorylation of the δ-OR appeared to require the actions of a Src-like protein tyrosine kinase, since the Src inhibitor 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)-pyrazolo-[3,4-*d*]-pyrimidine (PP1) attenuated this response. PP1 also attenuated the DTLET-mediated activation of mitogen-activated protein kinase, as well as rapid δ-OR internalization, but not receptor down-regulation. Finally, only opioid agonists that induce receptor internalization via the clathrin-dependent endosomal pathway stimulated significant tyrosine phosphorylation of the δ-OR protein. Evidence is presented that the δ-OR is tyrosine-phosphorylated, and we suggest how this may have an active role in opioid receptor signalling and regulation. BIOCHEM PHARMACOL **60**;6:781–792, 2000.

KEY WORDS. receptor; phosphorylation; tyrosine; MAP kinase; opioid; Src

ORs‖ mediate the rewarding and analgesic properties of opioid drugs such as morphine, fentanyl, and codeine [1]. Pharmacological and cloning studies have demonstrated that these proteins belong to the superfamily of GPCRs, which transmit their signals to effectors in the plasma membrane, cytosol, and nucleus through linkage to heterotrimeric guanine nucleotide binding proteins (reviewed in Ref. 2). The presence of seven transmembrane domains characterizes all GPCRs, including the ORs, and these proteins undergo substantial post-translational modification. The types of post-translational modifications known to occur in ORs include glycosylation, palmitoylation, the formation of disulfide bridges between cysteines, and the phosphorylation of serine and threonine residues. Although it is not known when many of these alterations to the native receptor protein occur, several years of study have shown that μ- and δ-ORs are phosphorylated reversibly in response to repeated or prolonged agonist exposure [3, 4]. Phosphorylation appears to be an important event during the regulation of GPCR function and may be critical for the processes of receptor desensitization, internalization, and down-regulation [5].

ORs appear to be regulated by mechanisms that share considerable similarity with those that act upon the model GPCR, the β_2-AR [6]. Receptor desensitization is a rapid and reversible loss of agonist affinity and receptor function, which is produced by an uncoupling of the receptor from its G-protein. This process is believed to be dependent upon the phosphorylation of serine/threonine residues by GRKs [3, 7] and the binding of cytosolic proteins known as β-arrestins [8]. In concordance with desensitization, the GPCR complex becomes internalized into intracellular endosomes via the clathrin-coated vesicular pathway in a

* Aspects of these studies were presented at the 30th Annual Meeting of the International Narcotics Research Conference in Saratoga Springs, New York (July 10–15, 1999).

‡ Corresponding author: Dr. H. Kenneth Kramer, Department of Psychiatry, New York University School of Medicine, Millhauser Laboratories, Room HN-610, 550 First Avenue, New York, NY 10016. Tel. (212) 263-5638; FAX (212) 263-5591; E-mail: ken.kramer@nyu.edu

‖ *Abbreviations*: OR, opioid receptor; GPCR, G-protein-coupled receptor; β_2-AR, β_2-adrenergic receptor; CHO, Chinese hamster ovary; DTLET, [D-Thr^2]-Leu-enkephalin-Thr; DSLET, [Tyr-D-Ser-Gly-Phe-Leu-Thr]; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)-pyrazolo-[3,4-*d*]-pyrimidine; MAPK, mitogen-activated protein kinase; GRKs, G-protein receptor kinases; ERK, extracellular receptor-regulated kinase; RTK, receptor tyrosine kinase; PTK, protein tyrosine kinase; 5-HT, 5-hydroxytryptamine; PTX, pertussis toxin; DIPR, diprenorphine; DAMGO, [D-Ala^2,N-MePhe^4,Gly-ol]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; and CTOP, [Cys^2,Tyr^3,Orn^5,Pen^7]-amide.

Received 8 December 1999; accepted 1 March 2000.

process dependent upon the GTPase dynamin [9, 10]. Upon sequestration into early endosomes, agonist-bound receptors can be resensitized and recycled back to the plasma membrane after dephosphorylation [11]. Alternatively, internalized receptors may be targeted to different intracellular compartments for degradation, resulting in receptor down-regulation.

Although internalization and desensitization of GPCRs appear to require receptor phosphorylation followed by the binding of β-arrestin [12], a causal relationship between GRK-mediated receptor phosphorylation, internalization, and desensitization is not always well defined. For example, prolonged activation of μ-receptors with [^{3}H]DAMGO, or of δ-ORs with etorphine elicits GRK-dependent phosphorylation and receptor desensitization, and promotes the internalization of these sites into clathrin-coated endosomes [5, 10]. By contrast, prolonged exposure of μ-OR-expressing cells to morphine produces functional desensitization [13] in the absence of any significant GRK-mediated receptor phosphorylation or internalization [14]. In a separate study, when all of the serines and threonines in the third cytoplasmic loop and C-terminus of the μ-OR were mutated to alanines, the prolonged presence of μ-opioid agonists still produced desensitization even though no internalization was observed during a 4-hr incubation period [15]. Furthermore, when each of the serine/threonine residues in the distal portion of the β_2-AR, tentatively identified by *in vitro* phosphorylation as sites for GRK2- and GRK5-mediated phosphorylation [16], were mutated to alanines, these mutant receptors still underwent extensive and rapid agonist-induced phosphorylation, desensitization, and internalization when expressed in HEK-293 cells [17]. Thus, serine/threonine phosphorylation may be required for some processes in certain GPCRs (e.g. internalization of ORs), whereas other processes may be independent of serine/threonine phosphorylation (e.g. μ-OR desensitization). Moreover, some receptors appear to undergo both desensitization and internalization even in the absence of serine/threonine phosphorylation (e.g. β_2-ARs in HEK 293 cells).

Besides serine and threonine residues, tyrosine residues on proteins, including those on some GPCRs, can be phosphorylated by RTKs or PTKs [18]. However, the effect of such a modification on GPCR physiology is not well known. Opioid agonists induce the tyrosine phosphorylation of numerous protein substrates within OR-expressing cell lines, suggesting that PTKs or RTKs are activated after OR stimulation [19, 20]. The aim of the present study was to determine whether the δ-OR, itself, becomes phosphorylated at tyrosine residues in response to prolonged agonist exposure. Among the 11 tyrosines found in the primary sequence of the δ-OR, two are located in regions known to be important for G-protein coupling and agonist-dependent regulation. One (Y308) is located in the distal portion of the seventh transmembrane segment, and a second (Y318) is found in the proximal portion of the carboxyl tail. It has been shown that deletion of the entire carboxyl tail in CHO-δ-OR cells completely abolishes both internalization and down-regulation [21, 22]. Thus, the phosphorylation of C-terminal tyrosines may have a substantial effect on δ-OR signalling and/or regulation.

We used CHO cells transfected with the cDNA of the FLAG-epitope-tagged murine δ-OR to test our hypothesis. Identical CHO-δ-OR cells have been used by other investigators to examine OR signalling and the effects of chronic agonist exposure on internalization and down-regulation [21]. We first measured whether the δ-OR was tyrosine-phosphorylated in an agonist-dependent manner. Then, in addition to assaying for the effects of tyrosine phosphorylation on receptor internalization, we chose to measure the activation of the MAPK cascade as a reporter assay to characterize the role of PTKs in δ-OR signalling. Several G-protein-coupled receptors, including ORs [23, 24], β_2-ARs [25], 5-HT_{1A} [26], and thrombin [27] receptors, rapidly activate the MAPK cascade. Moreover, it has become clear that GPCRs share common biochemical components with RTKs that activate the MAPK cascade, including the need for tyrosine phosphorylation of adapter proteins.

These experiments demonstrate that opioid agonists induce tyrosine phosphorylation of the δ-OR, and we describe how this event contributes to OR physiology. Tyrosine phosphorylation occurred with a time course that closely paralleled opioid-mediated MAPK activation and receptor internalization. We show that inhibitors of Src-like PTK also attenuate OR internalization. Lastly, we demonstrate that only those opioid agonists that induce receptor internalization into clathrin-coated endosomes produce significant tyrosine phosphorylation of the δ-OR protein.

MATERIALS AND METHODS

Drugs

All chemicals used for these studies were of reagent grade and were obtained from the sources listed. PP1, PD 98059 [2-(2-amino-3-methoxyphenyl)oxanaphthalen-4-one], and wortmannin were purchased from Calbiochem. The opioid agonists DAMGO, DTLET, DPDPE, etorphine, and morphine were from the National Institute of Drug Abuse. OR antagonists were from the following sources: naloxone (National Institute of Drug Abuse), CTOP (Bachem Bioscience), and naltrindol (RBI). All other compounds were obtained from the sources mentioned in the individual method descriptions.

Cell Culture

CHO-K1 cells were stably transfected with the cDNA encoding the epitope-tagged (FLAG) murine δ-OR (CHO-δ-OR) and were a gift from Dr. Lakshmi A. Devi (New York University School of Medicine). CHO-δ-OR cells, along with untransfected cells, were plated at an initial density of 5.0×10^5 cells in 75-cm^2 flasks. CHO-δ OR cells were grown in Ham's F-12 medium (Gibco-BRL), and

C6-μ-OR cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL). The culture medium was supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Gemini Bio-Products), and 100 μg/mL of gentamycin sulfate (Gemini Bio-Products), and the cells were grown at 37° in 7% CO_2/humidified air.

Immunoprecipitation of the Epitope-Tagged δ-OR and Immunoblotting for Phosphorylated MAPK and Phosphorylated Tyrosines

CHO-δ-OR cells were seeded and grown in 6-well plates until they reached 80–90% confluency. Sixteen hours before the addition of drugs, the culture medium was removed and replaced by fresh F-12 serum-free medium. The use of serum-free medium has been shown to reduce the basal level of MAPK activation by inhibiting its phosphorylation [28]. All drugs were added at least 30 min before the addition of opioid agonists. After the appropriate incubation period, cell monolayers were rinsed twice with ice-cold PBS, and the cells were solubilized by the addition of 200 μL of solubilization buffer (10 mM Tris–HCl, 1% SDS, and 1 μM $NaVO_3$, pH 7.4) for 15 min. Extracts were boiled for 5 min and sonicated briefly. Soluble proteins were isolated by centrifugation at 30,000 *g* for 10 min at 4°. Cell lysates were analyzed for protein concentration using the Bio-Rad detergent-compatible method. A mouse monoclonal antibody raised against the FLAG-epitope [Flag-M_1 antibody (1:1000 dilution), Sigma Chemical Co.] was used to immunoprecipitate the tagged δ-OR from the cell lysates or for immunoblots to examine the expression of the δ-OR from transfected cells. Immunoprecipitation of the FLAG-tagged δ-OR was performed using magnetic Dynabeads (Dynal Bioscience) coated with anti-mouse IgG and used according to the manufacturer's instructions. The proteins were prepared for SDS–PAGE by boiling them in a sample buffer of 4% SDS, 37.5 mM Tris–HCl, 20% glycerol, and 0.02% bromophenol blue, pH 6.8, containing 0.2 M dithiothreitol. Proteins (5–10 μg/lane) were separated by SDS–PAGE (12.5% acrylamide running gel, 4% acrylamide stacking gel) and electroblotted onto nitrocellulose (0.45 μm) at 250 mV for 90 min. Nitrocellulose membranes were rinsed three times with PBS and blocked overnight in a solution of 50 mM Tris–HCl, 100 mM NaCl, 3% nonfat dry milk, 1 mM $CaCl_2$, and 0.05% Tween-20, pH 7.5 (TBS–Tween buffer). Membranes were exposed for 12 hr at 4° to mouse monoclonal antibodies raised against phosphorylated MAPK (1:5000 dilution in blocking solution, p-ERK, Santa Cruz Biotechnology) or phosphorylated tyrosines (1:2000, PY99, Santa Cruz Biotechnology). After three washes in blocking buffer, the membranes were incubated for 60 min in the presence of a goat anti-mouse secondary antibody linked to horseradish peroxidase (1:2,000–10,000) at room temperature. The nitrocellulose was washed four times in TBS–Tween buffer to remove all nonspecifically bound antibodies before the visualization of immunopositive bands. Immunoreactive proteins were visualized using a horseradish peroxidase-sensitive enhanced chemiluminescent western blotting kit (Pierce).

OR Internalization and Down-Regulation Studies

Rapid receptor internalization was measured 0–24 hr after opioid agonist exposure, using a method that simultaneously measures the disappearance of cell surface receptors relative to the total number of receptors [29]. Opioid receptor binding was performed following the method of Slowiejko and Fisher [29] with minor modifications. Briefly, CHO-δ-OR cells were harvested by incubation in a solution comprised of PBS (pH 7.4)/5 mM EDTA/0.025% trypsin and placed into 50-mL conical tubes on ice. Cells were centrifuged at 300 *g* for 1–2 min at room temperature and then washed in working buffer (buffer A), which contained 142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM d-glucose at pH 7.4. The pellet was resuspended once in buffer A, divided into two aliquots (A and B; A = agonist-treated; B = buffer control), and placed in a 37° water bath for 5 min. The cells then were incubated in the presence of opioids or other drugs for 0–60 min at 37°. Drug exposure was halted by the addition of 6 vol. of ice-cold buffer A. The cells were washed extensively to remove residual agonist, centrifuged at 3000 rpm at 4° for 5 min in a Sorvall centrifuge using an SS-34 rotor, resuspended a final time in assay buffer, and placed on ice. These whole cell suspensions were subjected to [^{3}H]DIPR (0.1 to 1.5 nM) binding to measure total receptor number (surface plus internalized receptors) and to [^{3}H]DSLET (21.6 Ci/mmol, Multiple Peptide Systems; 0.5 to 5.0 nM) binding to measure surface δ-receptors. [^{3}H]Agonist binding was performed at 4° for 16–18 hr to reach equilibrium, to limit any nonspecific penetration of the hydrophilic ligand into subcellular organelles, and to prevent the recycling of internalized receptors back to the cell surface [30]. For [^{3}H]DIPR, equilibrium binding was done at 25° for 2 hr to allow for complete penetration of the ligand into intracellular endosomes. Nonspecific binding was determined in the presence of 10 μM naloxone. The tissue was harvested onto Titertek filtermats (coated with 0.1% polyethylimine to reduce nonspecific binding) using a Brandel cell harvester, and the filters were placed in scintillation vials containing 3.0 mL Liquiscint (National Diagnostics). Samples were counted for 5.0 min in a Beckman liquid scintillation counter (model LS 6500; ^{3}H-counting efficiency: 60%). Data are expressed as picomoles of [^{3}H]radioligand bound per milligram of protein.

Agonist-Stimulated [^{35}S]GTPγS Binding to Cell Membrane Homogenates

Agonist-stimulated [^{35}S]GTPγS binding to cell membrane homogenates was performed essentially following the method of Selley *et al.* [31]. Crude membranes of each cell type were prepared by homogenizing the tissue in 20 vol. of ice-cold 50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH

7.4. The resulting homogenate was centrifuged at 30,000 *g* at 4° for 15 min. The pellet was resuspended in buffer and centrifuged again at 30,000 *g* at 4° for 15 min. The resulting pellet was resuspended in assay buffer containing 50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4, and stored at −80° until assayed. Samples of homogenates containing approximately 10 μg protein were incubated at 30° for 1 hr in assay buffer in the absence or presence of DAMGO (μ) or DTLET (δ) (0.01 to 30 μM), GDP (20 μM), and 0.05 nM [^{35}S]GTPγS in a 1-mL total volume. Basal binding was assessed in the absence of agonist and in the presence of GDP, and nonspecific binding was assessed in the presence of a 10 μM concentration of unlabelled GTPγS. The reaction was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with 3 mL of ice-cold 50 mM Tris buffer. Samples were counted for 5.0 min in a Beckman liquid scintillation counter (model LS 6500; ^{3}H-counting efficiency: 60%). Data are expressed as picomoles of [^{35}S]radioligand bound per milligram of protein.

Data Analysis and Statistical Methods

Radioligand binding curves (to determine K_d and B_{max}) were generated, and all regression analyses were performed using the LIGAND curve-fitting program [32]. Graphs were produced using Sigmaplot for Windows (version 4.0). The IC/EC_{50} values were determined using the equation of Cheng and Prusoff [33]. Images of immunoreactive bands were captured on x-ray film and analyzed using the MCID morphometric system (Imaging Research Inc.). All immunochemical assays were performed at least three times, although results from a single experiment sometimes are shown for clarity. One-way and two-way ANOVA and the post-hoc Tukey test were used for multiple comparisons at a minimum significance level of $P \leq 0.05$. Student's *t*-test was substituted for the ANOVA for simple two-sample tests at the same significance level. Statistical data are expressed as means ± SEM of the indicated number of observations.

RESULTS

Receptor Binding and Function in CHO-δ-OR Cells

CHO-δ-OR cells demonstrated saturable and high-affinity binding for both the opioid antagonist [^{3}H]DIPR (K_d = 0.7 ± 0.1 nM and B_{max} = 1.1 ± 0.2 pmol/mg protein) and the agonist [^{3}H]DSLET (K_d = 2.3 ± 0.5 nM and B_{max} = 0.6 ± 0.3 pmol/mg protein). These receptors were found to be coupled to functional G-proteins when assayed for DTLET-stimulated [^{35}S]GTPγS binding to prepared membranes [EC_{50} = 71 ± 0.3 nM and maximal stimulation (E_{max}) = 210% binding above basal levels]. The μ-selective opioid agonist DAMGO was a very low-affinity agonist for stimulating [^{35}S]GTPγS binding (EC_{50} = 5.1 ± 0.2 μM) in CHO-δ-OR cells, whereas morphine acted as a partial agonist, producing 47% of the maximal DTLET-mediated stimulation of [^{35}S]GTPγS binding (EC_{50} = 437 ± 37 nM).

Activation of MAPK by δ-OR Agonists

The activation of $p44^{MAPK}$ (ERK1) and $p42^{MAPK}$ (ERK2) is characterized by the appearance of the phosphorylated forms of these proteins on immunoblots. The δ-agonist DTLET increased the expression of both phospho-ERK1 and ERK2 in a time- and concentration-dependent manner. DTLET-induced MAPK activation reached a maximum at 10 nM (Fig. 1A) and showed a $T_{1/2}$ of 2 min (Fig. 1B). MAPK stimulation by DTLET (100 nM) was prevented by preincubation with PTX (100 ng/mL, overnight) and the selective δ-OR antagonist naltrindol (10 μM), but not the μ-selective antagonist CTOP (10 μM) (data not shown). This demonstrated that this response occurs via stimulation of a δ-opioid receptor that is coupled to a $G_{i/o}$ protein. DTLET (100 nM)-mediated MAPK activity was sensitive to inhibition by the Src-type PTK inhibitor PP1, the MEK-1 inhibitor PD 98059, and the phosphatidylinositol-3 kinase (PI3-K) blocker wortmannin (Fig. 1C). PD 98059 also appeared to reduce, significantly, the basal expression of phosphorylated ERKs, whereas the others had very little effect on basal MAPK levels. These results suggest that DTLET activates the MAPK cascade through a mechanism that is dependent upon a Src-type PTK, which is similar to what has been postulated for other GPCRs [34].

Tyrosine Phosphorylation of the δ-OR

We examined whether the δ-OR protein becomes tyrosine-phosphorylated as part of the overall mechanism that results in the activation of MAPK. CHO-δ-OR cells were exposed to an escalating range of DTLET concentrations known to induce time-dependent activation of the MAPK cascade. Cell lysates were immunoprecipitated with an antibody against the FLAG-epitope to purify the tagged δ-OR from the other cellular proteins, and the resulting immunopurified protein was subjected to SDS–PAGE and immunoblotting. When the immunoblots were probed with an anti-phosphotyrosine antibody, a tyrosine-phosphorylated protein of ~66 kDa appeared from cells exposed to DTLET (Fig. 2A). This phosphotyrosine band was of the same molecular mass as the protein identified in immunoblots of cell lysates probed only with the anti-FLAG-M_1 antibody (data not shown). Panels A and B of Fig. 2 show that tyrosine phosphorylation of the δ-OR occurred in a time- and concentration-dependent manner with an EC_{50} = 16 ± 2.3 nM and an apparent $T_{1/2}$ of ~2 min. Interestingly, the increase in tyrosine phosphorylation was long-lasting, remaining significantly above control for 120 min after DTLET exposure began. The phosphorylation of tyrosines by DTLET (1 μM) in the δ-OR was inhibited by PTX (100 ng/mL) and naltrindol (10 μM) (Table 1). Moreover, no other proteins in our CHO cells carried the FLAG-epitope.

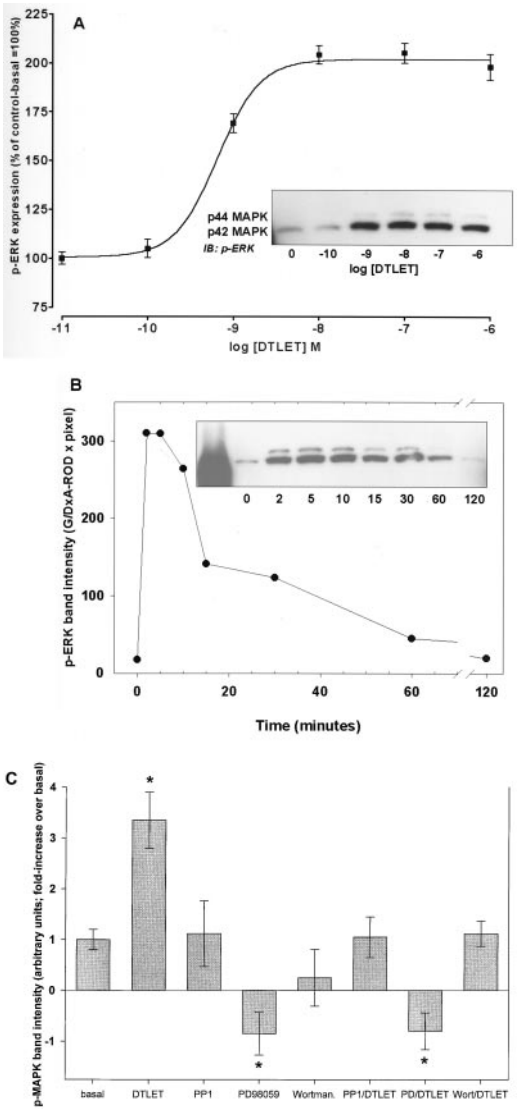

FIG. 1. MAPK activation by the δ-OR agonist DTLET in CHO-δ-OR cells. Confluent cultures were exposed to serum-free medium 12 hr before the addition of opioid agonist or medium (controls) for the times indicated. In some experiments an OR antagonist, a MAPK inhibitor, or a PTK inhibitor was added 30 min before DTLET. Cells were washed extensively after agonist treatment and prepared for MAPK extraction as explained in Materials and Methods. Five micrograms of total protein was separated via SDS–PAGE, followed by immunoblotting using a monoclonal antibody raised against phosphorylated MAPK. (A) Concentration dependency of DTLET (0.1 to 1000 nM)-induced MAPK activation, as measured by the appearance of p-ERK immunopositive bands. Cells were exposed to DTLET for 5 min before drug washout and cell solubilization. (B) Time dependency of DTLET (100 nM)-mediated MAPK activation. For panels A and B, immunopositive bands were detected using enhanced chemiluminescence and quantified using the MCID system. Band intensities are expressed graphically as: grain density x band area –[region of detection (ROD)] × pixel. Immunopositive bands corresponding to control and opioid-treated cultures appear in the inset. (C) Effect of PP1 (50 μM), wortmannin (10 μM), and PD 98059 (100 μM) on the appearance of phospho-MAPK by DTLET. All data points for panels A, B, and C represent the means ± SEM of three experiments. Key: (*) $P < 0.05$ compared with basal conditions as determined by ANOVA and the Tukey post-hoc test.

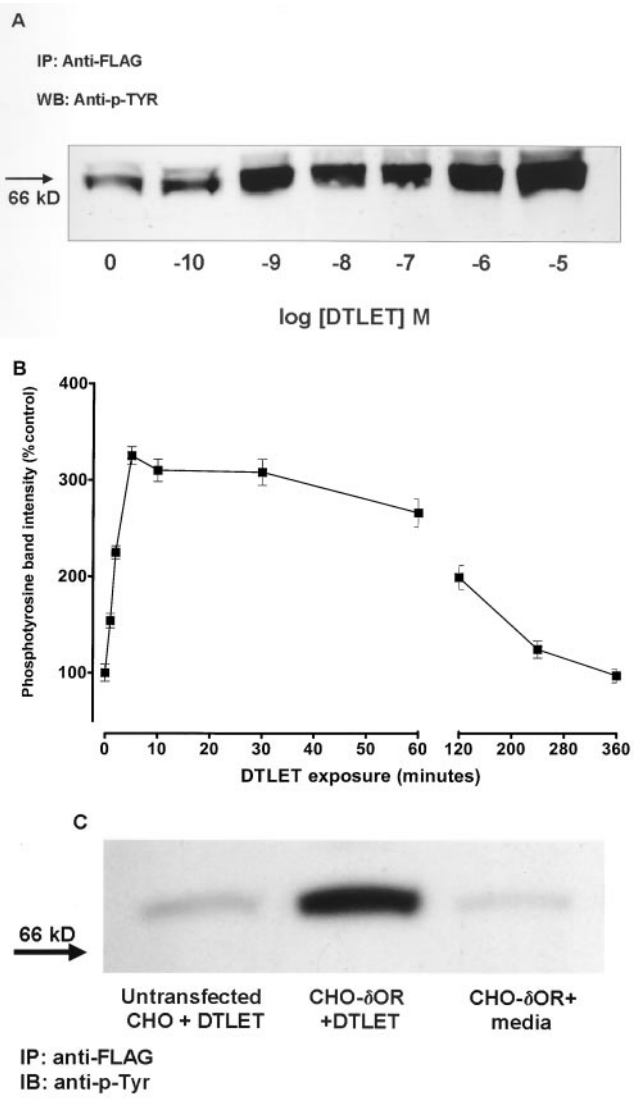

FIG. 2. Tyrosine phosphorylation of the δ-OR. Confluent cultures were exposed to serum-free medium for 12 hr before the addition of opioid agonist or medium (controls) for the times indicated. Cells were washed extensively after agonist treatment, and the FLAG-tagged δ-OR was immunoprecipitated as explained in Materials and Methods. The immunopurified protein (5 μg) was subjected to SDS–PAGE, followed by immunoblotting using mouse monoclonal antibodies raised against phosphorylated tyrosine. In addition, whole cell lysates were prepared to identify the presence of the FLAG epitope (DYKDDDDK). (A) Concentration dependency of DTLET (0.1 to 10,000 nM for 10 min)-induced tyrosine phosphorylation of the δ-OR. (B) Time dependency of DTLET (1 μM)-induced tyrosine phosphorylation of the δ-OR. Immunoblot A is representative of three similar experiments, and the data points in B represent the means ± SEM of three experiments. (C) Tyrosine phosphorylation of the δ-OR after DTLET (1 μM for 10 min) treatment requires the δ-OR to be present in CHO cells. CHO-δ-OR or untransfected CHO-K1 cells were exposed to DTLET (1 μM for 10 min) or medium before the cells were prepared for immunoprecipitation of the δ-OR. The proteins were then probed for the presence of phosphorylated tyrosine residues as indicated in Materials and Methods. Immunopositive bands were detected using enhanced chemiluminescence (ECL) techniques.

TABLE 1. Tyrosine phosphorylation of the δ-OR and its inhibition

	Tyrosine phosphorylation of the δ-OR (% of basal)	
	No DTLET	With DTLET (1 μM)
Control	100	323 ± 22*
PTX	107 ± 12	110 ± 21
Naltrindol	101 ± 16	105 ± 7.1
PP1	96 ± 11	99 ± 11
Wortmannin	108 ± 2.3	372 ± 62*

Confluent cultures were exposed to serum-free medium 12 hr before the addition of opioid agonist or medium (controls) for 10 min. Some cultures were pretreated with PTX (100 ng/mL for 18 hr), naltrindol (10 μM for 30 min), wortmannin (10 μM for 30 min), or PP1 (10 μM for 30 min) before the addition of DTLET. Cells were washed extensively after agonist treatment and immunoprecipitated for the FLAG-tagged δ-OR as explained in Materials and Methods. The immunopurified protein (5 μg) was submitted to SDS-PAGE, followed by immunoblotting using mouse monoclonal antibodies against phosphorylated tyrosine as indicated in Materials and Methods. Immunopositive bands were detected using enhanced chemiluminescence (ECL) techniques and quantified using the MCID system. The values (means ± SEM of four separate experiments) represent the increase in tyrosine phosphorylation of the δ-OR compared with controls (% of basal).

* $P \leq 0.05$ compared with the no DTLET value (by ANOVA).

No phosphotyrosine bands were visible when untransfected CHO cells were exposed to DTLET, nor was an increase in tyrosine phosphorylation of the immunoprecipitated δ-OR apparent when CHO-δ-OR cells were exposed to growth medium alone (Fig. 2C). All this strongly indicates that the tyrosine-phosphorylated protein is a δ-OR. DTLET-mediated tyrosine phosphorylation of the δ-OR was also prevented by the Src inhibitor PP1 (10 μM), but not by the PI3-K antagonist wortmannin (Table 1). In summary, the stimulation of tyrosine phosphorylation of the δ-OR is agonist-dependent and appears to be catalyzed by a Src-type protein tyrosine kinase, which is possibly the one involved in DTLET-dependent MAPK activation. Furthermore, the insensitivity to wortmannin indicates that the phosphorylation of the δ-OR is upstream of the opioid activation of ERKs.

Effect of PTK Inhibitors on δ-OR Internalization and Down-Regulation

The rapid internalization of GPCRs is believed to be important for the resensitization of ORs after they have been desensitized by prolonged agonist exposure [6]. β-Arrestin and dynamin appear to be essential protein components during clathrin-dependent endocytosis of GPCRs, and recent evidence suggests that dynamin is tyrosine-phosphorylated by Src-like protein tyrosine kinases [35]. In addition, β-arrestin appears to form a multi-protein complex with the agonist-occupied GPCR and Src before receptor internalization begins [36]. These data suggest that receptor internalization may have some dependency on the activity of PTKs, and possibly, tyrosine phosphorylation of the δ-OR. Therefore, we wished to examine whether the MAPK kinase (MEK) inhibitor PD 98059 or the protein tyrosine kinase inhibitor PP1 had any effect on agonist-stimulated δ-OR internalization. DTLET (5 μM for 30 min) decreased the number of surface δ-OR labeled by the membrane-impermeable peptide agonist [^{3}H]DSLET without altering the total number of receptors labeled by the membrane-permeable antagonist [^{3}H]diprenorphine (Fig. 3A). We have determined previously the kinetics of δ-OR internalization by DTLET in CHO-δ-OR cells to have an EC_{50} = 111 nM and a $T_{1/2}$ of 12.5 min (data not shown). δ-OR internalization was inhibited totally by pretreatment with 0.4 M sucrose, which suggests that this occurs via a clathrin-dependent endocytotic pathway (data not shown). Both the Src-kinase inhibitor PP1 (50 μM) (Fig. 3A) and the MEK inhibitor PD 98059 (100 μM) markedly attenuated DTLET-mediated δ-OR internalization (Fig. 3B). In contrast, neither inhibitor prevented receptor down-regulation that was produced by a 24-hr exposure to DTLET (5 μM) (Fig. 4).

Tyrosine Phosphorylation of the δ-OR by Agonists That Induce Receptor Internalization

A disparity exists between agonists that activate ORs and those that promote their internalization. For instance, DADLE, etorphine, and morphine all decrease forskolin-stimulated adenylate cyclase accumulation [9, 37] and increase MAPK activity [38] in δ-OR-expressing cell lines. However, morphine does not induce OR internalization [10, 39]. Therefore, we examined the ability of DTLET, etorphine, DPDPE, and morphine to promote tyrosine phosphorylation of the δ-OR, and then correlated this with the capacity of each drug to induce receptor internalization. During a 30-min incubation with each drug, only DPDPE (1 μM), etorphine (1 μM), and DTLET (1 μM) produced significant receptor internalization, whereas we confirmed the earlier finding by others [9] that the number of surface δ-ORs remained unchanged in cells exposed to morphine (10 μM) (Fig. 5A). The rank order of these drugs for inducing receptor internalization was DTLET = etorphine > DPDPE [tmt] morphine. The potency of these compounds for producing receptor internalization appeared to correlate closely with the capacity of each compound to produce tyrosine phosphorylation of the δ-OR protein (r^2 = 0.95 by first order regression analysis) (Fig. 5, A and B). Interestingly, when cells were exposed to 5 μM DTLET for 30 min in the presence of 0.4 M sucrose, there was no decrease in the tyrosine phosphorylation (Fig. 6A) of the δ-OR, although receptor internalization was totally blocked (Fig. 6B). Based on this finding and our kinetic data, it appears that the phosphorylation of tyrosines in the δ-OR precedes the physical internalization of these sites into clathrin-coated endosomes (Figs. 2B and 3A).

DISCUSSION

Similarly to the phosphorylation of serine and threonine residues, the phosphorylation of tyrosines can alter the function of cytoskeletal and signal-transduction proteins

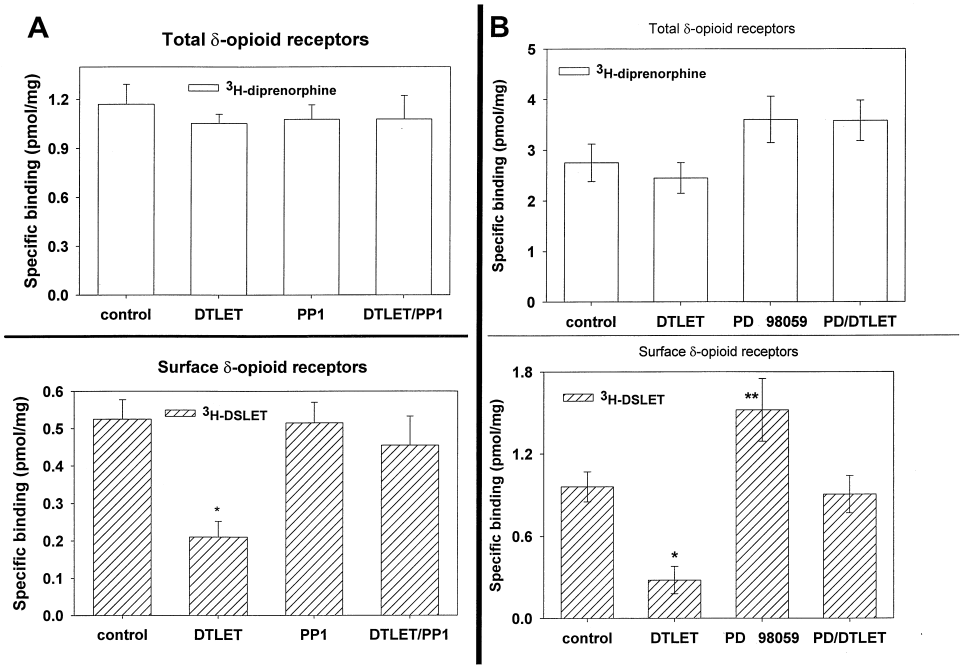

FIG. 3. Effect of PP1 (A) or PD 98059 (B) on DTLET-mediated internalization of δ-ORs in CHO-δ-OR cells. CHO-δ-OR cells were pretreated with serum-free medium, 50 μM PP1 (30 min), or 100 μM PD 98059 (30 min). Then cells were exposed to 1 μM DTLET at 37° for 30 min and assayed for surface ORs by hydrophilic radioligand binding as explained in Materials and Methods. Cells exposed to serum-free growth medium were the controls. Nonspecific binding was determined in the presence of 10 μM naloxone and represented ≤ 10% of the total binding for each ligand. A decrease in cell surface receptors, in the absence of a measurable change in total receptors, represents receptor internalization. Data are expressed as picomoles of radioligand bound per milligram of protein. Each bar represents the mean ± SEM of four experiments. Key: (*) $P < 0.05$ compared with control by ANOVA and the Tukey post-hoc test; and (**) $P < 0.01$ compared with control by ANOVA and the Tukey post-hoc test.

dramatically. The most notable example of this is the effect of tyrosine phosphorylation on growth factor receptors that contain endogenous tyrosine kinase activity [40]. A substantial body of evidence indicates that some G-protein-coupled receptors activate cytosolic tyrosine kinases, which results in the tyrosine phosphorylation of several known substrates [19, 20]. The hypothesis that ORs become tyrosine-phosphorylated is derived from a body of evidence showing that (a) opioids activate PTKs and RTKs, and (b) opioids stimulate the MAPK pathway, which has been shown to require the tyrosine phosphorylation of membrane-bound proteins [23, 40].

We investigated whether tyrosines were phosphorylated in the δ-OR after stimulation by a high-affinity agonist, since no evidence for tyrosine phosphorylation of ORs has appeared in the literature. In the present report, we provide the first evidence for the agonist-induced tyrosine phosphorylation of the δ-OR. We demonstrated that DTLET induces a concentration- and time-dependent increase in the appearance of phosphorylated tyrosines in the FLAG-tagged δ-OR when it is specifically immunoprecipitated from CHO-δ-OR cells using a FLAG-M_1 antibody. The following evidence supports our claim that the protein, which undergoes tyrosine phosphorylation after DTLET, is the δ-OR. First, the appearance of a phosphotyrosine band in the immunopurified protein at ~66 kDa was where the FLAG-tagged δ-OR migrates in immunoblots of whole cell

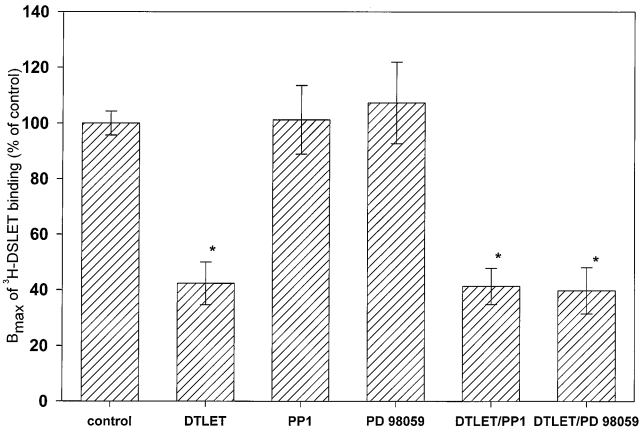

FIG. 4. Lack of effect of inhibitors of PTK or the MAPK cascade on δ-OR down-regulation by a 24-hr exposure to DTLET. Confluent CHO-δ-OR cultures were serum-starved for 12 hr prior to the addition of medium (control) or DTLET (5 μM) in the presence or absence of PD 98059 (100 μM) or PP1 (50 μM). PD 98059 or PP1 was added 30 min before DTLET. Control-treated cells expressed δ-ORs at a density of 1.23 ± 0.2 pmol/mg protein as assessed by [^{3}H]DSLET binding. After 24 hr of drug exposure, cells were washed and prepared for whole cell [^{3}H]DSLET binding at room temperature as described in Materials and Methods. Nonspecific binding was determined in the presence of 10 μM naloxone. The bars represent the B_{max} of [^{3}H]DSLET binding (percent of medium-treated control) expressed as the means ± SEM of three independent experiments. Key: (*) $P < 0.05$ from control.

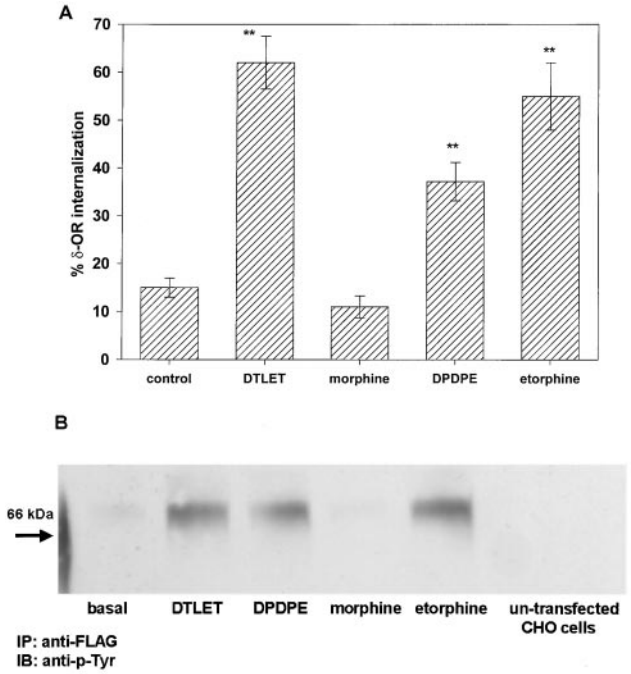

FIG. 5. Comparison of the ability of morphine, DTLET, DPDPE, and etorphine to induce receptor internalization and tyrosine phosphorylation of the δ-OR. (A) Confluent cultures of CHO-δ-OR cells were serum-starved for 12 hr before exposure to medium (control), morphine (10 μM), DPDPE (1 μM), DTLET (1 μM), or etorphine (1 μM) to induce receptor internalization. Cells were exposed to one of the opioid agonists at 37° for 30 min and assayed for surface ORs by [^{3}H]DSLET (5 nM) binding as explained in Materials and Methods. Cells exposed to serum-free growth medium were used as controls and expressed δ-ORs at a level of 1.7 ± 0.5 pmol/mg. Nonspecific binding was determined in the presence of 10 μM naloxone and represented ≤ 10% of the total binding for each ligand. Receptor internalization was measured as a decrease in the number of [^{3}H]DSLET-labeled sites on the cell surface. These values were converted into percentage of receptor sequestration over control. Each bar represents the mean ± SEM of four experiments. Key: (**) $P < 0.05$ compared with control. (B) Confluent cultures of CHO-δ-OR cells were serum-starved for 12 hr before exposure to medium (basal), morphine (10 μM), DPDPE (1 μM), DTLET (1 μM), or etorphine (1 μM) for 5 min. Cells were washed extensively after agonist treatment, and the FLAG-tagged δ-OR was immunoprecipitated as explained in Materials and Methods. The immunopurified protein (5 μg) was subjected to SDS–PAGE, followed by immunoblotting using mouse monoclonal antibodies raised against phosphotyrosine. The tyrosine-phosphorylated protein migrated to 66 kDa, which is very close to the calculated molecular mass of the FLAG-tagged δ-OR. The lane at the far left shows that exposure to DTLET produced no phosphotyrosine bands in untransfected CHO-K1 cells.

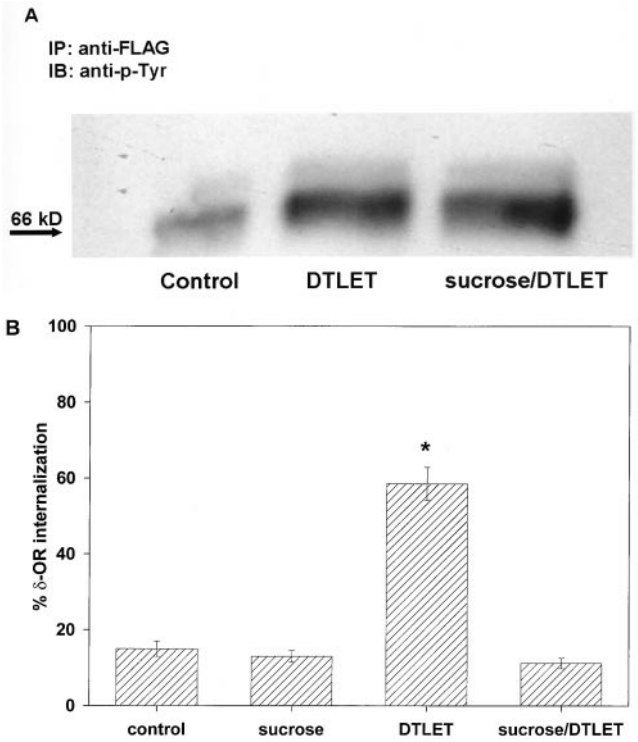

FIG. 6. Effect of sucrose (0.4 M) on δ-OR internalization and agonist-dependent tyrosine phosphorylation. (A) CHO-δ-OR cells were pretreated (4 hr) with serum-free medium or 0.4 M sucrose, and then exposed to either serum-free medium (control) or DTLET (5 μM) for 30 min. Cells were washed extensively and subjected to immunoprecipitation of the δ-OR or MAPK extraction as explained in Materials and Methods. Protein (5 μg) was subjected to SDS–PAGE, followed by immunoblotting using a monoclonal antibody raised against phosphorylated tyrosine residues. Immunopositive bands were detected using enhanced chemiluminescence, and were quantified using the MCID system. (B) CHO-δ-OR cells were pretreated with serum-free medium or 0.4 M sucrose (4 hr). Cells were exposed to opioid agonists at 37° for 30 min and assayed for surface ORs by [^{3}H]DSLET (5 nM) binding as explained in Materials and Methods. Cells exposed to serum-free growth medium were used as controls and expressed δ-ORs at a level of 1.1 ± 0.2 pmol/mg. Nonspecific binding was determined in the presence of 10 μM naloxone and represented ≤ 10% of the total binding for each ligand. Receptor internalization was measured as a decrease in the number of agonist-labeled sites on the cell surface, and these values were converted into percentage of control. Each bar represents the mean ± SEM of four experiments. Key: (*) $P < 0.05$ compared with control.

lysates when the anti-FLAG-M_1 antibody is used as the probe. Second, no phosphotyrosine bands were visible when untransfected CHO cells were exposed to DTLET, nor was an increase in tyrosine phosphorylation of the immunoprecipitated δ-OR apparent when CHO-δ-OR cells were exposed to growth medium alone (Fig. 2C). Third, no other proteins in our CHO cells carried the FLAG-epitope. We conclude that the phosphotyrosine band we have identified is the FLAG-tagged δ-OR protein.

In addition, both PTX and naltrindol blocked this effect, which suggests that the δ-OR is tyrosine-phosphorylated upon activation of this receptor by DTLET.

DTLET-mediated tyrosine phosphorylation of the δ-OR was prevented by PP1, which is an inhibitor of Src-like protein tyrosine kinases. Thus, our results suggest that the δ-OR protein is tyrosine-phosphorylated by the same family of protein kinases shown to phosphorylate Shc [20], which is an important adapter protein involved in MAPK activation. The tyrosine phosphorylation of an integral, plasma membrane-bound protein is believed to be a requirement for MAPK activation by GPCRs [41]. Briefly, upon activa-

tion of G_i- and G_s-coupled receptors, the βγ subunits of the G protein are liberated and act as the main signal transducer to stimulate MAPK activity. These free $G_{\beta/\gamma}$ subunits stimulate activity within the c-Src family of non-receptor PTKs [42, 43]. Several members of the Src group, including Src, Fyn, Lck, Yes, and Lyn, have emerged as candidate enzymes involved in this process [44]. Increased Src activity promotes the tyrosine phosphorylation of plasma-membrane-associated proteins, which may include, but are not limited to, focal adhesion kinase, other RTKs, or as shown here, δ-ORs, possibly creating SH2/SH3 binding domains conducive to the binding of Shc docking proteins [40]. After Shc binds to the tyrosine-phosphorylated substrate, it acts as a protein scaffold for the recruitment of the $p21^{Ras}$ guanine exchange factors Grb2 and mSos to the cytoplasmic face of the plasma membrane [40]. Once bound to the plasma membrane, mSos facilitates GDP/GTP exchange on Ras and the translocation/activation of Raf-1 kinase (MAPK kinase kinase). Upon Raf activation, a series of sequential protein phosphorylation reactions occurs, which culminates in the phosphorylation and activation of specific MAPK subtypes by MAPK kinase (MEK-1/2).

The identity of the membrane-bound protein that is tyrosine-phosphorylated during δ-OR-mediated MAPK activation was unknown in earlier studies. Two possibilities have been advanced to explain, partially, the identity of the tyrosine-phosphorylated protein that binds Shc during δ-OR-stimulated MAPK activity. The first is that the agonist-bound GPCR leads to the activation of Src, and this induces the phosphorylation (transactivation) of tyrosines on focal adhesion kinases or other RTKs (for a review, see Ref. 45). Among the tyrosine kinases activated after opioid receptor stimulation are focal adhesion kinases [46] and the epidermal growth factor receptor. For example, activation of the G_i-coupled lysophosphatidic acid receptor has been reported to elicit the transactivation/tyrosine phosphorylation of the epidermal growth factor receptor by a PTX and Src-dependent process [47]. This mechanism of GPCR stimulation and RTK transactivation is also believed to occur during MAPK activation produced by the G_s-coupled β_2-AR [48].

A second scenario, which formed the hypothetical basis of the present work, was that the activated δ-OR could be tyrosine-phosphorylated by Src, which would create an acceptable SH2 binding site for Shc proteins. Opioid agonists have been reported to increase the tyrosine phosphorylation of cellular proteins by activating Src-like PTKs or by transactivating RTKs, and both processes appear to be in place in some cell lines [19, 20]. Since PTKs are bound to the inner portion of the plasma membrane at their N-terminal residues, this allows them access to opioid receptors [41]. During the preparation of this manuscript, it was reported by Pak *et al.* [49] that the μ-OR is tyrosine-phosphorylated in response to DAMGO via a G-protein-independent pathway. In the present report, we show that the δ-OR is tyrosine-phosphorylated, and our studies using the Src-kinase inhibitor PP1 suggest that a Src-like PTK is involved. Further studies, including the *in vitro* phosphorylation of the δ-OR by purified Src, are needed to confirm the exact identity of this tyrosine kinase. Interestingly, Luttrell *et al.* [36] have shown that Src binds with high affinity to the agonist-bound β_2-AR, suggesting that GPCRs are Src substrates. DTLET-mediated activation of MAPKs, ERK-1 and ERK-2, was also found by us to be dependent upon the activities of PTX-sensitive G-proteins, PI3-K, and MEK-1/2. These results are consistent with other reports with respect to concentration- and time-dependencies for δ-opioid-mediated MAPK activation and the need for receptor–G-protein coupling [23, 50]. Our results show that OR stimulation of ERKs and the tyrosine phosphorylation of the δ-OR are blocked by the same Src inhibitor (PP1), suggesting that the multi-protein scaffold may be built autologously upon the δ-OR. However, δ-OR-mediated MAPK activation, working through the transactivation of RTKs, cannot be ruled out at this time.

Opioid receptors, like many GPCRs, have conserved tyrosine residues within regions known to be critical for G-protein coupling (third intracellular loop) and agonist-dependent internalization (C-terminus) [51–53]. While it is not known conclusively what the phosphorylation state of these tyrosines is under basal conditions, the point mutation Y308F in the δ-OR significantly alters the signalling property of this receptor [37]. Receptors that carry this mutation show enhanced basal and agonist-stimulated [^{35}S]GTPγS binding. Moreover, many receptors that are internalized via the clathrin-endosomal pathway express single tyrosines in a highly conserved sequence (NPXXY), and this region appears to be important for agonist-mediated receptor internalization [54–56]. In the δ-OR, this tyrosine (Y318) is present in the proximal portion of the C-terminus, which is a critical region for agonist-mediated receptor internalization [22]. Mutation of the homologous tyrosine (Y326A) in the β_2-AR significantly reduces isoproterenol-induced receptor phosphorylation by GRKs, as well as the internalization of this receptor into clathrin-coated endosomes [56, 57]. It is unknown whether Y326 is phosphorylated in the β_2-AR, or if the presence of the bulky side chain is important for establishing the proper tertiary structure of the protein. We have found that mutation of the equivalent C-terminal tyrosine (Y318F) in the δ-OR significantly attenuates agonist-mediated tyrosine phosphorylation of this receptor, as well as MAPK activation and receptor internalization. A preliminary report of these studies has appeared [58].

The time course of DTLET-mediated tyrosine phosphorylation of the δ-OR (Fig. 2B) indicates that tyrosine(s) becomes phosphorylated before the receptor is internalized into early endosomes. Surprisingly, the Y326A mutation did not alter β_2-AR desensitization or down-regulation, which is consistent with our finding that PP1 did not modulate δ-OR down-regulation. We plan to examine whether Src inhibitors affect functional δ-OR desensitization in future experiments.

It has been proposed by Ferguson *et al.* [56] that the

presence of Y326 in the β_2-AR, which is equivalent to Y318 in the δ-OR, is critical for GRK binding to the agonist-bound β_2-AR. Thus, the loss of this tyrosine may impair GRK binding and receptor phosphorylation significantly, which would decrease the affinity of β-arrestin for the receptor and perturb receptor internalization [6]. Moreover, if tyrosine phosphorylation of the C-terminus acts as a signal for the binding of GRKs to agonist-bound opioid receptors and the subsequent phosphorylation of serine and threonine residues, this may account for the limited ability of morphine to promote GRK-mediated phosphorylation and internalization of the δ-OR [9, 14]. In our studies, morphine was a poor facilitator of tyrosine phosphorylation of the δ-OR (Fig. 5B). Therefore, the weak ability of morphine to induce tyrosine phosphorylation of C-terminal residues may prevent GRKs from phosphorylating serine and threonine residues, resulting in impaired receptor internalization.

In recent years, the phosphorylation of serines and threonines has become widely accepted as being important for agonist-dependent GPCR internalization [6]. In this report, we show that tyrosine phosphorylation of ORs may also contribute to agonist-mediated receptor signalling and regulation. Thus, sites other than the traditional serine and threonine residues may share a substantial regulatory role in opioid receptor physiology. For example, multiple tyrosine residues are phosphorylated in the β_2-AR after *in vitro* exposure to insulin [18]. In addition, pretreatment of mice with insulin reduces DAMGO-mediated antinociception [59]. However, it remains to be determined whether insulin promotes tyrosine phosphorylation of ORs *in vivo*. Nevertheless, it appears that homologous and heterologous mechanisms are present to induce tyrosine phosphorylation of GPCRs when expressed *in vivo* or *in vitro*.

Several GPCRs, including β_2-adrenergic, angiotensin-1, and neurotensin receptors, and ion channel receptors such as the *N*-methyl-d-aspartate receptor, have been shown to undergo agonist-induced tyrosine phosphorylation [60, 61]. Moreover, the mutation of tyrosine residues within the amino acid sequence of the δ-OR significantly alters receptor signalling [37]. Therefore, the phosphorylation of tyrosines in the δ-OR may be important for one or more signal transduction or regulatory pathways controlled by this receptor. Thus, δ-ORs can both stimulate tyrosine kinase activity and act as a substrate for phosphorylation by these enzymes.

In summary, our results demonstrate that the δ-OR is phosphorylated at tyrosine residues in response to its stimulation by an agonist. δ-OR phosphorylation at tyrosines requires that the receptor be coupled to a PTX-sensitive G-protein, which suggests that the activation of PTKs may be another second messenger system modulated by the opioid system. The phosphorylation of one or more tyrosine residues appears to be important for opioid receptor signalling (MAPK activation) and agonist-dependent receptor regulation (internalization), as both responses are inhibited by PP1. Although the exact identity of the responsible PTK(s) is not known conclusively, our initial data suggest that it belongs to the Src family. We currently are investigating which of the tyrosines in the third cytoplasmic loop (Y308) and C-terminus (Y318) of δ-OR are phosphorylated by purified Src in an *in vitro* setting. The knowledge that opioids can activate tyrosine kinases will be important in determining the function of the opioid system in cell growth, differentiation, and the interaction between ORs and growth factor receptor systems.

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