A Novel Noncanonical Signaling Pathway for the μ -Opioid Receptor^S

Lei Zhang, Horace H. Loh, and Ping-Yee Law

Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota

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ABSTRACT

The μ -opioid receptor (OPRM1) signals as a classic G protein–coupled receptor by activating heterotrimeric G_i/G_o proteins resulting in adenylyl cyclase (AC) inhibition. Such AC inhibition is desensitized after prolonged agonist treatment. However, after receptor desensitization, the intracellular cAMP level remains regulated by OPRM1, as demonstrated by the intracellular cAMP level increase or AC superactivation upon removal of an agonist or addition of an antagonist. We now demonstrate that such intracellular cAMP regulation is mediated by a novel noncanonical signaling pathway resulting from OPRM1 being converted to a receptor tyrosine kinase (RTK)-like entity. This noncanonical OPRM1 signaling is initiated by the receptor recruiting and activating Src kinase within the receptor complex, leading to phosphorylation of the OPRM1 Tyr³³⁶ residue. Phospho-Tyr³³⁶ serves as the docking site for growth factor receptor-bound

protein/son of sevenless, leading to the recruitment and activation of the Ras/Raf-1 and subsequent phosphorylation and activation of AC5/6 by Raf-1. Such sequence of events was established by the absence of Ras/Raf1 recruitment and activation by the OPRM1-Y336F mutant, by the presence of Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) or the absence of Src activity, by the presence of specific Raf-1 inhibitor GW5074 (5-iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone) or the absence of Raf-1, or by the dominant negative RasN17 mutant. Src together with Ras activates Raf1 which was established by the inability of the Raf1-Tyr^{340/341} mutant to activate AC. Hence, the phosphorylation of OPRM1 at Tyr³³⁶ by Src serves as the trigger for the conversion of a classic G_i/G_o-coupled receptor into an RTK-like entity, resulting in a noncanonical pathway even after the original G_i/G_o signals are blunted.

Introduction

In G protein–coupled receptor (GPCR) canonical signaling, agonist stimulation leads to a rapid exchange of GDP for GTP on the α subunit of the heterotrimeric G proteins and the subsequent dissociation of α and $\beta\gamma$ subunits. Both α and $\beta\gamma$ subunits are involved in the activation of different effectors, including adenylyl cyclase (AC), phospholipase C, and ion channels (Pierce and Lefkowitz, 2001). However, this linear model does not adequately address the full range of GPCR stimulation. There is evidence to suggest that the overall cellular responses depend on GPCR-mediated stimulation of a large number of complex signaling cascades. Therefore, it is not surprising that cross-regulation occurs in the GPCR signaling pathways. For example, β -arrestins have been demonstrated to not only blunt the GPCR signal but also serve as signal

transducers for the same GPCR. As observed with the signaling of β_2 -adrenergic receptors, β -arrestins mediate agonist-induced receptor desensitization and internalization, and also mediate the recruitment of c-Src to the receptor, which facilitates the activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) cascades (Luttrell et al., 1999a; McDonald et al., 2000; Lefkowitz and Shenoy, 2005). Cross-regulation can also occur by the modulation of either upstream or downstream events of other receptors' signaling pathways on GPCR activation, which results in even more complicated cellular responses. An example for such cross-regulation is the growth-promoting effects of many GPCRs that are mediated through transactivation of the epidermal growth factor receptor (EGFR), which elicits cellular responses mainly through its intrinsic tyrosine kinase activity, as in the case of bradykinin receptors activation in PC12 cells leading to transactivation of EGFR and the activation of the Ras/MAP kinase pathway (Zwick et al., 1997).

In the case of opioid receptors, activation of the receptors results in the inhibition of AC activity, activation of MAPK

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ABBREVIATIONS: AC, adenylyl cyclase; AG-1478, *N*-(3-chlorophenyl)-6,7-dimethoxy-4-quinazolinamine; co-IP, coimmunoprecipitation; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GPCR, G protein–coupled receptor; Grb, growth factor receptor-bound protein; GRK, G protein–coupled receptor kinase; GW5074, 5-iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone; HA, hemagglutinin; HEK293, human embryonic kidney 293 cells; HEKMT, HEK293 cells stably expressing HA-OPRM1; HEKMT-Y336F, HEK293 cells stably expressing HA-OPRM1 with Tyr³³⁶ mutant; Igepal CA-630, octylphenoxypolyethoxyethanol; KO, knockout; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblasts; MEK1, mitogen-activated protein kinase kinase 1; OPRM1, μ -opioid receptor; PD-173074, *N*-[2-[[4-(diethylamino)butyl]amino]-6-(3,5-dimethoxyphenyl)pyrido[2, 3-*d*]pyrimidin-7-yl]-*N*-(1,1-dimethylethyl)-urea; PKA, protein kinase A; PKC, protein kinase C; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; RT-PCR, reverse-transcription polymerase chain reaction; RTK, receptor tyrosine kinase; SH2/3 domain, Src homology 2/3 domain; SOS, son of sevenless; WT, wild-type.

and Kir3 K⁺ channels, inhibition of voltage-dependent Ca²⁺ channels, and modulation of other effectors such as phospholipase C (Law et al., 2000). However, prolonged agonist exposure not only blunts these cellular responses but also results in a compensatory increase in the intracellular cAMP level, which is particularly significant upon the removal of an agonist or the addition of an antagonist such as naloxone (Sharma et al., 1977; Law et al., 1983; Pineyro and Archer-Lahlou, 2007). This compensatory increase in AC activity or AC superactivation phenomenon has been postulated to be the molecular basis of drug tolerance and dependence (Koob and Bloom, 1988). However, the classic linear GPCR signaling pathway cannot account for such change from the initial receptor-mediated AC inhibition to the eventual receptor-mediated AC activation because the G_i/G_o-mediated signaling is blunted after prolonged agonist treatment.

Although the exact mechanism for such signal changes is yet to be elucidated, activation of specific protein kinases and subsequent phosphorylation of AC isoforms (Avidor-Reiss et al., 1996, 1997) and other signaling molecules, such as G protein-coupled receptor kinases (GRK) 2/3 (Chakrabarti et al., 2001) have been suggested to be the key for the observed AC activation. Among all the protein kinases studied, protein kinase C (PKC), MAP kinases and Raf-1 have been implicated in the AC superactivation (Li and Chang, 1996; Varga et al., 2002; Schallmach et al., 2006). Alternative mechanisms, such as agonist-induced receptor internalization and the increase in the constitutive activities of the receptor or the switching from G_i/G_o-coupled to G_s-coupled, also have been suggested to play a role in AC superactivation (Szucs et al., 2004; Walwyn et al., 2007). Regardless of the mechanism, the exact molecular events that lead to the switching of opioid receptor from an inhibitory response to a stimulatory response remain elusive.

In one of our previous studies, we demonstrated that AC superactivation required the recruitment and activation of Src and subsequent Src-mediated phosphorylation of the μ -opioid receptor (OPRM1) at the Tyr³³⁶ residue (Zhang et al., 2009). In our current study, we further detail the cellular events after Tyr³³⁶ phosphorylation that lead to AC superactivation by demonstrating that phosphorylation of OPRM1 at Tyr³³⁶ serves as the docking site for growth factor receptor-bound protein (Grb)/son of sevenless (SOS), leading to the recruitment and activation of Ras/Raf-1 and the subsequent phosphorylation and activation of AC5/6 by Raf-1, resulting in the ultimate AC superactivation. Hence, the phosphorylation of OPRM1 at Tyr³³⁶ by Src kinase serves as the switch for the conversion of a classic G_i/G_o-coupled receptor into an receptor tyrosine kinase-like (RTK-like) entity, resulting in a noncanonical signal pathway even after the canonical G_i/G_o signaling has been attenuated.

Materials and Methods

Cell Culture. Human embryonic kidney 293 (HEK293) cells stably expressing hemagglutinin (HA)-OPRM1 (HA-OPRM1HEK293; HEKMT) and HEK293 cells stably expressing HA-OPRM1 with tyrosine mutants (HEKMT-Y336F) were cultured at 37°C in advanced minimum Eagle's medium (Invitrogen/Life Technologies, Carlsbad, CA) supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 2 mM GlutaMAX (GIBCO/Life Technologies, Grand Island, NY), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.1 mg/ml G418 (geneticin; GIBCO) in a 5% CO₂ incubator. Mouse embryonic fibroblasts (MEF)-Raf-1 wild type and cells isolated from Raf-1 knockout mice were

cultured at 37°C in advanced Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal bovine serum, 2 mM GlutaMAX, 100 units/ml penicillin and 100 μ g/ml streptomycin in a 10% CO₂ incubator. HEKMT or MEF-Raf1 wild-type or MEF cells isolated from Raf-1 knockout mice were transiently transfected with various cDNA plasmids using Nucleofector transfection kits designed for HEK293 cells or for MEF cells (Lonza, Basel, Switzerland).

cAMP Assay. Cells were seeded in 96-well plates the day before and morphine was added to the medium 4 hours before the assays. The culture medium was removed and cells were washed with medium at 37°C once and then 100 μ l treatment buffer (0.5 mM isobutylmethylxanthine and 10 μ M forskolin in Krebs-Ringer HEPES buffer) with different concentration of naloxone was added to each well. The cells were incubated for 15 minutes at 37°C and then the assay was terminated by placing the plate in an 85°C water bath for 6 minutes to lyse the cells and release the intracellular cAMP. The cAMP level was measured by using AlphaScreen cAMP Detection Kit (PerkinElmer Life and Analytical Sciences, Waltham, MA) and Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Brea, CA). The results were analyzed and summarized with Prism 4.0 software (GraphPad Software, San Diego, CA).

Immunoprecipitation and Western Blotting. Cells were treated with or without morphine and/or naloxone, then the lipid raft fraction was prepared following the method described elsewhere (Zhang et al., 2009). The prepared lipid raft was incubated with mouse monoclonal anti-HA antibody (1:200; Covance, Princeton, NJ) in 1 ml of buffer A (100 mM NaCl, 10 mM Tris, pH 7.4) in the presence of 0.1% digitonin (Sigma-Aldrich, St. Louis, MO) at 4°C overnight. Proteins in the samples were resolved by 10% SDS-polyacrylamide gel electrophoresis and were electrophoretically transferred to polyvinylidene difluoride membranes (0.45 μ m; Amersham Biosciences/GE Healthcare, Little Chalfont, UK). The presence of pRaf-1, pSrc, AC5/6, and OPRM1 in the SDS-PAGE-resolved immunoprecipitates was determined with anti-pRaf1-Tyr340/341 (1:500; Millipore, Billerica, MA), anti-pSrc-Tyr416 (1:500; Millipore), anti-AC5/6 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-HA (1:1000; Covance) antibody respectively. The band intensities were quantified and analyzed with the ImageQuant software (Amersham Biosciences/GE Healthcare).

Ras Activation Assay. The Ras activity was determined by using a Ras activation assay kit (Millipore) as described by the manufacturer's protocol. Briefly, the cells were lysed by adding 0.5 ml of MLB buffer (Mg lysis/wash buffer: 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630 (octylphenoxypolyethoxyethanol), 10 mM MgCl₂, 1 mM EDTA, and 10% glycerol). The solution was cleared, and the protein concentration was determined by bicinchoninic acid assay. The cell lysis solution from each treatment with same amount of protein was mixed with 5 μ g of Raf-1 Ras-binding domain agarose and incubated at 4°C for 45 minutes with slow rotation. The agarose beads were pelleted down by brief centrifugation and washed 3 times with 0.5 ml of MLB (Mg lysis/wash buffer) each. The agarose beads were resuspended in 40 μ l 2 $\times$ Western sample loading buffer and were boiled for 5 minutes. The samples were loaded on a 12% SDS-PAGE gel, and Western blot was performed as described; the blot was probed with mouse anti-Ras antibody (1:1000; Millipore). A portion of the cell lysis solution from each sample with an equal amount of protein was loaded to another gel, and the result was used to determine the amount of input Ras.

Raf-1 Activity Assay. Raf-1 kinase activity was determined with the assay kit supplied by Millipore. Briefly, after various treatments, cells were lysed in lysis buffer containing 1% Triton X-100, 10 mM Tris, pH 7.5, 120 mM NaCl, 25 mM KCl, 100 μ M Na₃VO₄, and 1 $\times$ complete protease inhibitor (Roche Applied Science, Indianapolis, IN). The cell lysate was cleared by centrifuging at 16,000g for 5 minutes, and the supernatant was incubated with 2 μ g anti-Raf-1 polyclonal antibody (Santa Cruz Biotechnology) overnight at 4°C, followed by another 3 hours of incubation after the addition of 20 μ l of protein G agarose beads. Afterward, the agarose beads were washed

3 times with buffer A. Then, the agarose beads pellets were resuspended in 20 μ l assay dilution buffer [20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2, 25 mM β -glycerol-phosphate, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol], 10 μ l Mg/ATP cocktail (75 mM MgCl₂, 500 μ M ATP), and 1.6 μ l (0.4 μ g) inactive mitogen-activated protein kinase kinase 1 (MEK1). The mixture was incubated for 30 minutes at 30°C with shaking. The reaction was terminated by adding 10 μ l 6 $\times$ Western sample loading buffer and boiled for 5 minutes. The samples were loaded on a 10% SDS-PAGE gel, and the blot was probed with anti-pMEK1 (1:1000; Millipore) or anti-Raf-1 (1:500; Santa Cruz Biotechnology) antibody. The kinetics of Raf-1 kinase activation were determined by the use of [γ -³²P]ATP.

The immunoprecipitation was performed in a similar manner except the agarose beads pellets were suspended in 50- μ l kinase buffer (50 mM Tris-HCl, pH 7.5; 10 mM MgCl₂, 10 mM MnCl₂, 25 μ M ATP, 1 mM dithiothreitol, and 100 μ M Na₃VO₄) containing 5 μ Ci of [γ -³²P]ATP (New England Nuclear/PerkinElmer Life and Analytical Sciences, Waltham, MA). The mixture was incubated at 30°C, and 10- μ l aliquots were removed at the time points of 0, 5, 10, and 20 minutes. The reaction was terminated by addition of 10 μ l of 40% trichloroacetic acid. The sample was then spotted onto a P81 phosphocellulose paper square (Millipore). The paper was washed extensively with 1% phosphoric acid 3 times and with acetone 1 time. The radioactivity retained on the P81 paper was quantified by liquid scintillation counting.

Reverse-Transcription Polymerase Chain Reaction. The total RNA of cultured cells was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. The RNA samples were then treated with DNase I (Ambion/Life Technologies, Austin, TX). The reverse-transcription polymerase chain reaction (RT-PCR) reaction was performed by using One Step RT-PCR Kit (Qiagen, Valencia, CA) following the manufacturer's recommendations, and 1.0 μ g of DNase I-treated total RNA was added to each reaction. The RT-PCR conditions were as follows: 30 minutes at 50°C followed by 15 minutes at 95°C and then 35 cycles of 1 minute at 94°C, 1 minute at 60°C (54°C for β -actin), and 1 minute at 72°C followed by 1 cycle of 10 minutes at 72°C. The PCR primers for Raf-1 were forward: AAGAAAGCACGCTTAGATTGG, reverse:

CAAGAGCTGTCTGATATT; for OPRM1, forward: CATCAAAGCACTGATC ACGATTCC, reverse: TAGGGCAATGGAGCAGTTTCTGC; and for β -actin, forward: AGACCTCTATGCCAACACAGT, reverse: ATCTCGGTGGTTAGGTGTGTC.

Results

Our previous study suggested that during the chronic morphine treatment OPRM1 recruited and activated Src, which in turn phosphorylated the receptor at Tyr³³⁶. This is the pivotal event that eventually leads to AC superactivation (Zhang et al., 2009). However, the function of this phosphorylated Tyr³³⁶ in OPRM1 signaling leading to AC superactivation has not been addressed. Classically, phosphorylated tyrosine residue often serves as a docking site for proteins with Src homology 2 domain (SH2) leading to the recruitment of the downstream signaling proteins to the receptor's proximity (Karoer et al., 1998; Fan et al., 2001; Tobin, 2008). Among them, the Grb/SOS complex has been shown to bind to the phosphorylated Tyr of RTK and transduce the signal to the downstream Ras/MAP pathway (Satoh et al., 1990). If the Src-mediated recruitment of Grb/SOS to the phosphorylated OPRM1 Tyr³³⁶ is the key for AC superactivation, then the disruption of such a complex formation should affect AC superactivation. When HEK293 cells stably expressing OPRM1 (HEKMT cells) were treated with the SOS1 Src homology 3 domain (SH3) domain-binding peptide, which specifically blocks the interaction of SOS1 and Grb2, the chronic morphine-induced AC superactivation was significantly blunted ($155.0\% \pm 15.6\%$ compared with $309.1\% \pm 19.6\%$ in presence of 10^{-4} M naloxone; Fig. 1A). Because Grb2/SOS1 usually binds and activates Ras, we examined whether Ras is involved in the AC superactivation process. Transient expression of the dominant negative RasN17 mutant reduced morphine-induced AC superactivation to $223.6\% \pm 20.7\%$ as compared with the control

Fig. 1. Grb/SOS/Ras is involved in chronic morphine-induced AC superactivation. (A) HEKMT cells were treated with 1 μ M morphine for 4 hours (■ HEKMT) or were added to 10 μ M Grb2 and SOS1 interaction inhibitor, SOS1 SH3 peptide, for 3 hours during the 4 hour-morphine treatment (● + SH3 peptide). The cAMP assays were performed as described in *Materials and Methods*. ** $P < 0.01$; *** $P < 0.001$ ($n = 6$) when compared with the 4-hour morphine treatment (■), unpaired t test. (B) HEKMT cells were either transiently transfected with 0.5 μ g mock cDNA (■ HEKMT) or dominant negative RasN17 cDNA (◇ + RasN17). After 48 hours, the cells were treated with 1 μ M morphine for 4 hours, then cAMP assays were performed. The Ras inhibitor farnesylthiosalicylic acid (FTS; 50 μ M) was added for 3 hours during the morphine treatment (● + FTS). *** $P < 0.001$ ($n = 6$) compared with the 4-hour morphine treatment (■), one-way analysis of variance. Forskolin-induced cAMP in cells without drug treatment is used as 100% control.

of $336.3\% \pm 25.6\%$ in presence of 10^{-4} M naloxone (Fig. 1B). A similar result was obtained by pretreating the cells with the Ras inhibitor farnesylthiosalicylic acid, which decreased AC superactivation to $194.4.1\% \pm 21.7\%$ (Fig. 1B).

Measurements of Ras activation revealed there was a time-dependent increase in Ras activity during chronic morphine treatment (Supplemental Fig. 1). Addition of the antagonist naloxone after chronic morphine (4 hours) further potentiated Ras activation ($193.7 \pm 15.8\%$ compared with $161\% \pm 14.9\%$ for 4-hour morphine without naloxone; Fig. 2A). Furthermore, pretreatment of cells with the Src kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) or Ras inhibitor farnesylthiosalicylic acid totally abolished chronic morphine-induced Ras activation ($106.0\% \pm 22.5\%$ and $83.7\% \pm 25.1\%$, respectively, compared with morphine and naloxone treatment only; Fig. 2A). The ability to blunt the morphine-induced Ras activation by these inhibitors correlates with the ability of these inhibitors to block morphine-induced AC superactivation (Fig. 1B) (Zhang et al., 2009).

More importantly, Ras is observed to be within the OPRM1 signaling complex during chronic morphine treatment, as revealed by coimmunoprecipitation (co-IP) studies ($166.0\% \pm 22.1\%$ compared with control, Fig. 2B). Coincidentally, addition

of naloxone further increased the amount of Ras co-IP with OPRM1 by $271.0\% \pm 34.4\%$ as compared with control (Fig. 2B). This Ras and OPRM1 interaction can be prevented by transient expression of the dominant negative RasN17 mutant ($39.3\% \pm 14.7\%$ compared with the morphine and naloxone treatment only; Fig. 2C). As discussed earlier, phosphorylated Try³³⁶ could act as a docking site for downstream signal molecules to facilitate AC superactivation. Hence, it is not unexpected that when morphine-induced, Src-mediated Tyr-phosphorylation was not observed (Zhang et al., 2009) in HEK293 cells expressing the OPRM1 Y336F mutant (HEKMT-Y336F), Ras activation could not be detected (Fig. 2D) and co-IP of Ras with OPRM1 was not observed either (L. Zhang, unpublished observations).

Because Ras activation is often the consequence of RTK activation and transactivation of RTK by opioid receptors has been reported (Belcheva et al., 2002), whether OPRM1-mediated Ras activation and subsequent AC superactivation is the consequence of RTK transactivation was examined. HEKMT cells were incubated with the EGFR inhibitor lavendustin C at different concentrations as indicated (Fig. 3A). Even at the highest concentration of lavendustin C tested (100 nM), attenuation of AC superactivation was not observed

Fig. 2. Ras is activated and colocalized with OPRM1 during chronic morphine treatment. (A) The effect of naloxone or Src and Ras inhibitors on Ras activation. HEKMT cells were treated with 1 μ M morphine for 0 minutes, 10 minutes, or 4 hours, as indicated, followed by the addition of 0 or 10 μ M of naloxone (Nal) for 15 minutes. Src inhibitor (PP2; 2 μ M) or Ras inhibitor [farnesylthiosalicylic acid (FTS), 50 μ M] was added for 1 or 3 hours during the 4-hour morphine treatment. The assays were performed as described in *Materials and Methods*. $^{*}P < 0.01$ compared with control (the ratio of the density of active Ras to the density of input Ras from untreated cells was used as 100% control), unpaired *t* test. $^{*}P < 0.05$ compared with 4-hour morphine treatment, unpaired *t* test. $^{**}P < 0.01$ compared with 4-hour morphine and naloxone treatment, unpaired *t* test. (B) Co-IP of Ras with OPRM1. HEKMT cells were treated with or without 1 μ M morphine for 4 hours with or without 10 μ M naloxone for 15 minutes, as indicated. The co-IP was performed as described in *Materials and Methods*. The ratio of the density of Ras to the density of OPRM1 from untreated cells was used as 100% control. $^{*}P < 0.01$; $^{***}P < 0.001$ compared with control, unpaired *t* test. The no-IP Ab control was performed the same as earlier with the absence of IP Ab (anti-HA). (C) RasN17 inhibited Ras and OPRM1 interaction. HEKMT cells were either transiently transfected with 0.5 μ g mock cDNA (Ctrl) or dominant negative RasN17 cDNA (RasN17). After 48 hours, the cells were treated with morphine (1 μ M, 4 hours) and naloxone (10 μ M, 15 minutes), then the co-IP was performed as described in *Materials and Methods*. The ratio of the density of Ras to the density of OPRM1 from mock cDNA transfected cells was used as 100% control. $^{*}P < 0.01$ compared with control, unpaired *t* test. (D) Ras was not activated in cells expressing Y336F mutant OPRM1. Cells were treated with or without 1 μ M morphine for 4 hours followed by with or without 10 μ M naloxone for 15 minutes, and assays were performed as described in *Materials and Methods*. Each experiment was repeated 3 times. Ctrl, control; Mor, morphine.

Fig. 3. AC superactivation is EGFR or fibroblast growth factor receptor (FGFR) independent. HEKMT cells were treated with 1 μ M morphine for 4 hours (■) or pretreated with EGFR inhibitor lavendustin C (HDBA) (A) or AG-1478 (B) at a concentration of 10 nM (▼), 50 nM (◆), or 100 nM (●); or pretreated with FGF inhibitor PD-173074 (C) at a concentration of 20 nM (▼), 50 nM (◆), or 100 nM (●) for 2 hours during the 4-hour morphine treatment before cAMP assays were performed ($n = 6$). Forskolin-induced cAMP in cells without drug treatment is used as 100% control.

(IC₅₀, 12 nM). These results were confirmed by using various concentrations of another EGFR inhibitor, AG-1478 (*N*-(3-chlorophenyl)-6,7-dimethoxy-4-quinazolinamine; IC₅₀, 3 nM; Fig. 3B). It was also reported that opioid receptors could transactivate fibroblast growth factor receptor (Belcheva et al., 2002); however, pretreatment of the cells with various concentrations of the fibroblast growth factor receptor inhibitor PD-173074 (*N*-[2-[[4-(diethylamino)butyl]amino]-6-(3,5-dimethoxyphenyl)pyrido[2, 3-d]pyrimidin-7-yl]-*N*-(1,1-dimethylethyl)-urea; IC₅₀, 21.5 nM) did not result in any inhibition of chronic morphine-induced AC superactivation (Fig. 3C). Based on these observations, it is unlikely that the Ras activation observed in the presence of naloxone after chronic morphine treatment is the consequence of RTK transactivation.

Upon activation, the active Ras binds to Raf kinases with high affinity and causes Raf kinases to translocate to the cell membrane where Raf activation takes place. The involvement of Raf-1 in chronic opioid administration had been implicated based on the fact that pretreatment with the Raf-1 specific inhibitor GW5074 (5-iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone) significantly blunted AC superactivation (Varga et al., 2002). By performing co-IP studies, we could demonstrate that activated Raf-1 (pRaf-1), activated c-Src (pSrc),

and AC5/6 formed a signal complex with OPRM1 in a chronic morphine-dependent manner ($264.6\% \pm 43.0\%$, $215.3\% \pm 22.8\%$ and $155.6\% \pm 21.6\%$, respectively, compared with the control; Fig. 4, A and B). Pretreatment with the Raf-1 inhibitor GW5074 totally blocked the co-IP of pRaf-1 with OPRM1 but did not alter morphine-induced Src activation ($124.7\% \pm 39.1\%$ and $187.2\% \pm 22.0\%$ respectively). However, pretreatment of Src kinase inhibitor PP2 not only eliminated pSrc from the complex but also prevented the appearance of pRaf-1 in the complex ($92.5\% \pm 23.0\%$ and $113.0\% \pm 36.7\%$, respectively), suggesting that the presence of pRaf-1 within the OPRM1 complex requires Src kinase activation. Both PP2 and GW5074 pretreatment also reduced the presence of AC5/6 in the OPRM1 complex in the presence of morphine and naloxone from $155.6\% \pm 21.6\%$ to $82.0\% \pm 13.2\%$ and $73.3\% \pm 6.5\%$, respectively (Fig. 4, A and B).

The measurements of Raf-1 kinase activity revealed that Raf-1 activity was significantly enhanced within the OPRM1 signaling complex as determined by the phosphorylation of the Raf-1 substrate MEK1 (pMEK1) after chronic morphine treatment ($168.0\% \pm 26.7\%$) and after chronic morphine followed by naloxone treatment ($191.0 \pm 36.0\%$) when compared with the control (Fig. 4, C and D). A similar result was obtained from a Raf-1 kinase kinetics assay using [γ -³²P]ATP to label the substrate (Fig. 4E). All these results demonstrate that the phosphorylation of OPRM1 Tyr³³⁶ is the key for recruiting Ras to the receptor complex, leading to the translocation and activation of Raf-1 to cell membranes via the binding of protein kinase to Ras. If this is the scenario, then it should follow that without Tyr³³⁶ being phosphorylated, Raf-1 translocation and activation should not occur. Indeed, in cells expressing the OPRM1 Y336F mutant, the phosphorylation of Raf-1's substrate MEK1 was not observed after chronic morphine treatment (Fig. 5, A and B).

To further confirm the involvement of Raf-1 in chronic morphine-induced AC superactivation, MEF cells from both wild-type (WT) and Raf-1 knockout (KO) mice were used to transiently express OPRM1 and/or Raf-1. Figure 6A shows the RT-PCR results of Raf-1 levels that were similar in various transfection combinations as indicated. The cAMP assays using these transfected cells suggested that only when cells expressed both OPRM1 and Raf-1, AC superactivation was significantly potentiated (Raf-1WT + OPRM1, $194.8\% \pm 15.6\%$ and Raf-1 KO + OPRM1 + Raf-1, $278.5\% \pm 19.7\%$). Other combinations with the absence of either OPRM1 or Raf-1 did not exhibit AC superactivation (Fig. 6, B and C). These MEF cells studies support the Raf-1 inhibitor GW5074 studies and clearly indicate that Raf-1 activation is critical for the observed AC superactivation.

At present, the exact mechanism for Raf-1 kinase activation remains elusive. However, the Src kinase phosphorylation at 340 and/or 341 tyrosine residues is one of the keys for Raf-1 activation (Marais et al., 1995). Because activated Raf-1 and activated Src can be observed within the OPRM1 signaling complex in co-IP experiments (Fig. 4A), it is likely that Src within the receptor complex could phosphorylate and thus activate Raf-1, leading to AC superactivation. To test this hypothesis in AC superactivation, Tyr³⁴⁰ or Tyr³⁴¹ was mutated to Ala singly or in combination to generate the Raf1^{Y340A} or Raf1^{Y341A} single mutant or Raf1^{Y340/341AA} double mutant. To mimic the phosphorylated state at these Tyr residues, they were mutated to Asp to generate the constitutively active

Fig. 4. Raf-1 is activated during chronic morphine treatment. (A) Raf-1 is activated within the OPRM1 signal complex in a chronic morphine- and Src kinase-dependent manner. The cells were treated with or without morphine (Mor; 1 μ M, 4 hours) and naloxone (Nal, 10 μ M, 15 minutes), as indicated. Src inhibitor (PP2; 2 μ M) or Raf-1 inhibitor GW5074 (GW; 10 μ M) was added for 1 or 2 hours during the 4-hour morphine treatment, respectively. Co-IP was performed as described in *Materials and Methods*. (B) Bar graph summary of Western blots as shown in A. The ratio of the density of pRaf1, pSrc, or AC5/6 to the density of OPRM1 from untreated cells was used as 100% control. * P < 0.05; ** P < 0.01; *** P < 0.001 compared with control; # P < 0.05; ## P < 0.01 compared with morphine and naloxone treatment, unpaired t test. The bars represent averages from three separate Western blots. (C) Raf-1 activation requires chronic morphine treatment. The cells were treated with or without morphine (1 μ M, 4 hours) and naloxone (10 μ M, 15 minutes), as indicated. Raf-1 activity assays were performed as described in *Materials and Methods*. The blot was probed with anti-pMEK1 and anti-Raf-1. (D) Bar graph summary of Western blots as shown in C. The ratio of the density of pMEK1 to the density of Raf-1 from untreated cells was used as 100% control. ** P < 0.01 compared with control, unpaired t test. The bars represent averages from three separate Western blots. (E) Kinetics of Raf-1 kinase activation. The cells were treated with or without morphine (1 μ M, 4 hours) and naloxone (10 μ M, 15 minutes), as indicated. The assays were performed following the procedure in *Materials and Methods*. * P < 0.05; ** P < 0.01; *** P < 0.001 compared with control; # P < 0.05 for morphine and naloxone treatment (▼ + Mor + Nal) compared with morphine alone treatment (▲ + Mor), unpaired t test. Each experiment was repeated 3 times.

Raf1^{Y340/341DD} mutant. These mutants or WT Raf-1 were transiently expressed in MEF-Raf1 KO cells, and their expression levels were similar as determined by RT-PCR (Fig. 7A). To test their effect on chronic morphine induced AC superactivation, WT or mutant Raf-1 was cotransfected with OPRM1 to the MEF-Raf1 KO cells. Both single mutant Raf-1^{Y340A} and Raf-1^{Y341A} and double mutant Raf1^{Y340/341AA} significantly blunted the AC superactivation to $155.2 \pm 14.0\%$, $177.5 \pm 16.0\%$, and $131.4 \pm 12.1\%$, respectively, as compared with the cells expressing WT Raf-1, where AC superactivation was at $286.3 \pm 21.9\%$ above the control level. In contrast, expression of the constitutively active mutant Raf-1^{Y340/341DD} significantly potentiated AC superactivation to $436.9 \pm 29.7\%$ above the control level (Fig. 7, B and C). These studies suggest that the phosphorylation of Raf-1 at these Tyr residues is another key for the observed AC superactivation.

Discussion

In past decades, the classic view of GPCRs as simple heterotrimeric G protein activators has gradually evolved into

a concept of intracellular signaling pathways with complex modulations. Emerging evidence suggests that the efficiency, specificity, and fidelity of GPCR signaling can be achieved by a more confined and organized system, which may involve cross-talk, fine-tuning, and specific regulation at multiple levels, even within the same GPCR signal pathway. For example, β -arrestins have been shown to recruit different downstream signal molecules to control the outcome of the signal transduction in addition to their roles as signal terminators (Cao et al., 2000; Lefkowitz and Shenoy, 2005). Crosstalk can also happen between different GPCRs' pathways, leading to distinct signal pathways that are different from the originals. For example, activation of phospholipase C-coupled P2Y₂ receptors specifically inhibited β_2 -adrenergic receptor-mediated cAMP production via G_i proteins in pGT- β cells (Suh et al., 2001). The cross-regulations may also happen between GPCRs and other signal pathways; among them, the transactivation between GPCR and RTK has been intensively studied (Daub et al., 1996). It has been reported that stimulation of various kinds of GPCRs, including the receptors for bradykinin, angiotensin, thrombin, lysophosphatidic acid, bombesin, endothelin,

Fig. 5. Raf-1 activation is not observed in cells expressing Tyr mutant OPRM1. (A) Raf-1 activation. HEKMT or HEKMT-Y336F cells were treated with or without 1 μ M morphine (Mor) for 4 hours followed by 0 or 10 μ M naloxone (Nal) for 15 minutes, as indicated. Raf-1 activity assays were performed as described in *Materials and Methods*. The blots were probed with anti-pMEK1, anti-Raf-1, and anti-G β . (B) Bar graph summary of Western blots as shown in A. The ratio of the density of pMEK1 to the density of Raf-1 from untreated cells was used as 100% control. * P < 0.05; ** P < 0.01 compared with control, unpaired t test. The bars represent averages from 3 separate Western blots. Ctrl, control.

and muscarinic acetylcholine, can transactivate the EGFR system (Prenzel et al., 1999; Kalmes et al., 2000; Gschwind et al., 2001; Pierce et al., 2001).

For opioid receptors, agonist-induced acute inhibition of AC activity is a classic GPCR signaling event (Zhang et al., 2006). However, after prolonged treatment, the inhibition effect is blunted; it gradually converts to a compensatory increase in intracellular cAMP level, and is particularly significant upon the removal of the agonist or the addition of an antagonist (Sharma et al., 1977; Law et al., 1982; Pineyro and Archer-Lahlou, 2007). This observed change from receptor-mediated AC inhibition to receptor-mediated AC activation cannot be explained by the classic GPCR signal paradigm and may reflect intracellular signal switching. Our previous studies suggested that Src kinase activation and subsequent Src-mediated phosphorylation of OPRM1 at Tyr³³⁶ were the keys for such signal switching. Similar GPCR phosphorylation-induced signal switching had been intensively studied in β -arrestin-mediated signaling. Lefkowitz and coworkers were the first to report that β -arrestins bound to the GRK-phosphorylated receptor were not only a signal termination event, but also the β -arrestins could serve as adaptors to recruit Src to the receptor complex, leading to Src-dependent, receptor-stimulated activation of downstream effectors such as the MAP kinases (Luttrell et al., 1999b). Because β -arrestins can recruit a broad spectrum of signaling molecules, the GRK/ β -arrestin system has been demonstrated

Fig. 6. Raf-1 is required for chronic morphine-induced AC superactivation. (A) RT-PCR analysis of Raf-1 wild type (Raf-1 WT) or Raf-1 knockout (Raf-1 KO) MEF cells transiently expressing OPRM1 and/or Raf-1. The cells were transfected with cDNA, as indicated. After 48 hours, the cells were lysed, and the total RNA was purified. RT-PCR was performed as described in *Materials and Methods*. β -Actin was used as the control. (B) Both OPRM1 and Raf-1 are required for AC superactivation. MEF-Raf-1 WT cells were transfected with OPRM1 (Δ Raf-1 WT + OPRM1) or Raf-1 ($\diamond$ Raf-1 WT + Raf-1) cDNA. Raf-1 KO cells were transfected with OPRM1 ($\square$ Raf-1 KO + OPRM1), Raf-1 ($\bullet$ Raf-1 KO + Raf-1), or both ($\blacktriangledown$ Raf-1 KO + OPRM1 + Raf-1) cDNA. After 48 hours, the cells were treated with morphine (1 μ M, 4 hours), and cAMP assays were performed in the presence of various concentrations of naloxone. Forskolin-induced cAMP in cells without drug treatment is used as 100% control. (C) Bar graph representation of the results from B when 10^{-4} M naloxone was added in the assay. ** P < 0.01; *** P < 0.001 compared with control, unpaired t test (n = 6).

to function not solely in the receptor desensitization process but also in signaling pathways that participate in biased agonism (Lefkowitz and Shenoy, 2005).

In our current study, identifying various molecular events after chronic morphine administration has revealed a novel signal pathway for the opioid receptor. Distinct from the canonical G_i/G_o-mediated receptor signaling, the phosphorylation of OPRM1 Tyr³³⁶ serves as the docking site for Grb/SOS (Supplemental Fig. 2), leading to the recruitment and activation of Ras/Raf-1 and the subsequent phosphorylation and activation of AC5/6 by Raf-1. A number of GPCRs have been

Fig. 7. Tyr 340 and 341 on Raf-1 are critical for chronic morphine-induced AC superactivation. (A) RT-PCR analysis of MEF-Raf-1 KO cells transiently expressing OPRM1 and WT or Tyr mutant Raf-1. Cells were transfected with cDNA, as indicated. After 48 hours, cells were lysed, and total RNA was isolated. RT-PCR was performed as described in *Materials and Methods*. β -Actin was used as control. (B) AC activity in the presence of naloxone after 4 hours of morphine treatment. MEF-Raf-1 KO cells were transfected with OPRM1 and WT Raf-1 (■ OPRM1 + Raf-1), or Raf-1 Y340A mutant (● OPRM1 + Y340A), or Raf-1 Y341A mutant (□ OPRM1 + Y341A), or double Tyr mutant Y340/341AA (▼ OPRM1+Y340/341AA), or constitutively active mutant Y340/341DD (◇ OPRM1+Y340/341DD) cDNA. After 48 hours, the cells were treated with 1 μ M morphine for 4 hours, then cAMP assays were performed in the presence of the indicated naloxone concentrations. Forskolin-induced cAMP in cells without drug treatment is used as 100% control. (C) Bar graph representation of the results from B in the presence of 10^{-4} M naloxone. ** $P < 0.01$ compared with the cells expressing OPRM1 and WT Raf-1, unpaired t test ($n = 6$).

shown to activate or inhibit the MAPK cascade by either a Ras-dependent or Ras-independent pathway requiring GPCR transactivation of RTKs such as EGFR (Daub et al., 1996). For example, expression of polypeptides that sequester free $G\beta\gamma$ subunits blocks Ras-dependent MAPK/ERK activation via the lysophosphatidic acid receptor (Herrlich et al., 1998), α_2A -adrenergic receptor (van Biesen et al., 1995), M_2 -muscarinic receptor (Koch et al., 1994), and D_2 -dopamine receptor (Faure et al., 1994). Our data suggest that Ras activation occurs during chronic morphine treatment and is even more pronounced upon the addition of the antagonist naloxone, which mimics the drug withdrawal state (Fig. 2A). This Ras activation does not require the transactivation of RTKs (Fig. 3); instead, it occurs within the

OPRM1 signal complex in a Src-dependent manner (Fig. 2A). Furthermore, in cells expressing the Tyr³³⁶ mutant receptor, Ras activation was not observed, nor could Ras be detected in the OPRM1 signal complex (Fig. 2D; L. Zhang, unpublished observations). Together with the observation that disruption of SOS1 and Grb2 interaction significantly blunted AC activation (Fig. 1), we conclude that phospho-Tyr³³⁶ is a docking site for the recruitment of Grb2/SOS1/Ras to OPRM1, thus forming a signal complex in which Ras is activated.

In the classic view, activated Ras will bind to Raf-1 and recruit protein kinase to the cell membrane, where Raf-1 is activated (Baccarini, 2005). Based on the observation that the Raf-1 inhibitor GW5074 attenuates AC activation, Raf-1 has been implicated in opioid receptor-mediated chronic morphine-induced AC superactivation (Varga et al., 2002). Our current study is the first to suggest that Raf-1 is recruited to the OPRM1 signal complex and activated during chronic morphine/naloxone treatment, and that this Raf-1 activation is Src kinase dependent (Fig. 4, A and B). Based on our previous studies, which indicated that OPRM1 could recruit and activate Src during chronic morphine treatment, together with the fact that Raf-1 activation is not observed in cells expressing Tyr³³⁶ mutant OPRM1 (Fig. 5), we conclude that phospho-Tyr³³⁶ recruits Raf-1 to the OPRM1 signal complex via activated Ras, where Raf-1 is activated by Src. By using MEF-Raf-1 WT and KO cells, we demonstrated Raf-1 activation as a prerequisite for AC superactivation; this activation is mediated by OPRM1 directly, not by other receptor signal pathways (Fig. 6). The currently accepted model for Raf-1 activation involves recruitment to the plasma membrane by activated Ras via the Ras-binding domain in Raf-1, dephosphorylation of the inhibitory protein kinase A (PKA)/protein kinase B site Ser²⁵⁹ at Raf-1 by phosphatase PP2A, and eventual phosphorylation at Tyr³⁴⁰ and/or Tyr³⁴¹ probably by Src kinase (Marais et al., 1995; Kolch, 2002; Baccarini, 2005). Our data have shown that Src is required for Raf-1 activation (Fig. 4, A and B); mutation at Tyr³⁴⁰ or Tyr³⁴¹ significantly attenuates AC activation, and double mutation at these sites almost totally abolishes the AC activation, suggesting that these two sites are equally important for Raf-1 activation. The observation that the constitutively active mutant Tyr^{340/341DD} significantly enhanced AC activation further substantiates this conclusion (Fig. 7, B and C). One may expect that the constitutively active Raf-1-Tyr^{340/341DD} mutant itself is enough to cause AC superactivation, but without the chronic morphine/naloxone treatment, Src activation and subsequent phosphorylation at Tyr³³⁶ of OPRM1 were not observed. Furthermore, without recruitment of Ras to and activation of Ras by OPRM1, Raf-1 was not recruited to the OPRM1 signal complex. Hence, constitutively active Raf-1 itself cannot lead to AC superactivation. In addition to phosphorylation and activation by Src, other protein kinases such as PKC (Kolch et al., 1993), Akt (Zimmermann and Moelling, 1999), PAK3 (King et al., 1998), and PKA (Dhillon et al., 2002) have been suggested to be involved in Raf-1 regulation either positively or negatively. Whether these protein kinases are involved in Raf-1 activation during chronic morphine administration remains to be examined.

In the RTK/Ras/MAPK cascade, activated Raf-1 will directly phosphorylate MEK1/2, leading to the activation of MAPK/ERK, which in turn can activate AC. However, during chronic morphine treatment, MAPK activation is not observed

Fig. 8. Schematic summary of OPRM1 differential signal pathways upon acute and chronic morphine administration. During acute morphine treatment, OPRM1 interacts with G_{i2} (Zhang et al., 2006) and causes inhibition of adenylyl cyclase activity and a decrease of the intracellular cAMP level. Under prolonged treatment, Src kinase is recruited by the OPRM1 signal complex, where it is activated. The activated Src phosphorylates OPRM1 at Tyr³³⁶. The phosphorylated Tyr³³⁶ serves as a docking site to recruit Grb/SOS/Ras and Raf-1, which converts the OPRM1 from a classic G_i/G_o -coupled receptor into an RTK-like entity. The activated Raf-1 eventually phosphorylates and activates the AC isozymes, most likely AC5/6, resulting in AC superactivation. By doing so, OPRM1 can lead to an alternative signaling pathway that is initiated by the same agonist but is different depending on the duration of agonist exposure.

(L. Zhang, unpublished observation). Instead, several studies have suggested that the Raf-1 can phosphorylate several AC isoenzymes directly, such as AC2, AC5, and AC6 (Ding et al., 2004). Avidor-Reiss et al. (1997) reported that AC activation can be reproduced in COS-7 cells with cotransfection of AC5 and OPRM1. It also has been shown that Raf-1-mediated phosphorylation of AC6 leads to the sensitization of this isoenzyme to stimulators (Tan et al., 2001) such as forskolin (i.e., AC activation). Furthermore, mutagenesis studies from the same investigators have suggested that Ser⁷⁵⁰ on AC6 is phosphorylated by Raf-1 and that mutation of Ser⁷⁵⁰ to Ala significantly impaired AC6 activity (Ding et al., 2004). Our current data clearly indicate that AC5/6 was recruited to the OPRM1 signal complex during chronic morphine treatment in a Src- and Raf-1-dependent manner (Fig. 4, A and B). Because other protein kinases such as PKA and PKC and also some regulatory proteins such as RGS2 (Roy et al., 2006), SNAPIN (SNAP-25/Snare complex) (Chou et al., 2004), and PAM (protein associated with myc) (Gao and Patel, 2005) have been suggested to be capable of modulating the AC5/6 activity, chronic morphine-induced AC activation could involve multiple protein kinases in addition to Src and Raf-1 and the regulatory proteins as discussed earlier.

In conclusion, our current study provides a new model for OPRM1-mediated chronic morphine-induced AC superactivation. The phosphorylation at Tyr³³⁶ by Src kinase serves as a docking site to recruit Grb/SOS/Ras and Raf-1. This chronic agonist-induced, site-specific phosphorylation results in a distinct signaling outcome. As a result, OPRM1 is converted from a classic G_i/G_o -coupled receptor into an RTK-like entity, resulting in a noncanonical signaling pathway even when canonical G_i/G_o signaling has been attenuated (summarized in Fig. 8). It is noteworthy that a similar model has been suggested for RTK signaling in which RTKs exert some of their effects by engagement of GPCR signaling molecules, including heterotrimeric G proteins and β -arrestins, suggesting that RTKs can also act as GPCR-like receptors (Luttrell et al., 1990; Liang and Garrison, 1991; Dalle et al., 2001; Povsic et al., 2003; Cao et al., 2009). Because chronic agonist-induced AC superactivation has been implicated in drug addiction processes, if the Src-mediated Tyr³³⁶ phosphorylation can be prevented while still maintaining the overall cellular Src activity, then a specific treatment paradigm for opioid drug addiction might be attainable.

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Authorship Contributions

Participated in research design: Zhang, Law.

Conducted experiments: Zhang.

Performed data analysis: Zhang, Loh, Law.

Wrote or contributed to the writing of the manuscript: Zhang, Law.

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Address correspondence to: Dr. Lei Zhang, Department of Pharmacology, University of Minnesota Medical School, 6-120 Jackson Hall, 321 Church St. SE, Minneapolis, MN 55455. E-mail: zhang247@umn.edu