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Receptor heterodimerization leads to a switch in signaling: β -arrestin2-mediated ERK activation by μ - δ opioid receptor heterodimers

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Abstract

Opiates are analgesics of choice in the treatment of chronic pain, but their long-term use leads to the development of physiological tolerance. Thus, understanding the mechanisms modulating the response of their receptor, the μ opioid receptor (μ OR), is of great clinical relevance. Here we show that heterodimerization of μ OR with δ opioid receptors (δ OR) leads to a constitutive recruitment of β -arrestin2 to the receptor complex resulting in changes in the spatio-temporal regulation of ERK1/2 signaling. The involvement of β -arrestin2 is further supported by studies using β -arrestin2 siRNA in cells endogenously expressing the heterodimers. The association of β -arrestin2 with the heterodimer can be altered by treatment with a combination of μ OR agonist (DAMGO) and δ OR antagonist (Tippu), and this leads to a shift in the pattern of ERK1/2 phosphorylation to the pattern observed with μ OR alone. These data indicate that, in the naive state, μ OR- δ OR heterodimers are in a conformation conducive to β -arrestin-mediated signaling. Destabilization of this conformation by cotreatment with μ OR and δ OR ligands leads to a switch to a non- β -arrestin-mediated signaling. Taken together, these results show for the first time that μ OR- δ OR heterodimers, by differentially recruiting β -arrestin, modulate the spatio-temporal dynamics of opioid receptor signaling.

Keywords

G-protein-coupled receptors; morphine; oligomerization; 7TM receptors; enkephalin; narcotic addiction

The opioid receptors μ OR (μ -opioid receptor), δ OR (δ -opioid receptor), and κ OR (κ -opioid receptor) (1) are members of the family A of G-protein-coupled receptors (GPCRs). They are involved in many biological responses, including analgesia, miosis, bradycardia, feeding, and hypothermia (2). Activation of these receptors leads to inhibition of adenylyl cyclase activity, phosphorylation of extracellular signal-regulated kinase 1/2 (ERK), activation of K^+ currents, and inhibition of Ca^{2+} channels (3). Previous studies have shown that morphine functions primarily by activating μ OR (3). Furthermore, studies have suggested that μ OR interacts with δ OR, and this leads to changes in receptor properties (4). For example, mice treated with δ OR antagonists exhibit diminished morphine tolerance and dependence (5, 6). δ OR knockout animals do not develop morphine tolerance (6), and reducing the surface insertion of δ OR abolishes morphine tolerance (7). In addition, chronic morphine treatment up-regulates δ OR (8), which leads to changes in μ OR function (7).

Recent studies have directly examined the interaction between μ OR and δ OR using a variety of techniques such as coimmunoprecipitation and bioluminescence resonance energy transfer, and have shown that dimerization affects ligand binding and receptor signaling (9, 10, 11). These studies have suggested that the μ OR- δ OR heterodimer could represent a functional unit distinct from μ OR or δ OR. This is supported by the fact that μ OR agonist-induced signaling can be enhanced by cotreatment with δ OR-selective ligands, including δ OR-selective antagonists (that do not elicit a signal when administered alone) (9). This raises the exciting possibility that the signaling pathways activated by μ OR- δ OR heterodimers are distinct from those activated by μ OR or δ OR alone. In this study, we show that μ OR- δ OR heterodimers recruit β -arrestin, which leads to changes in the spatio-temporal dynamics of opioid-mediated ERK activation. These findings have major clinical relevance since modulation of ERK phosphorylation has been shown to play an important role in regulating pain and analgesia (12).

MATERIALS AND METHODS

Cell lines, reagents, and plasmids

Chinese hamster ovary (CHO) K1, human embryonic kidney (HEK) -293, Neuro2A, and SKNSH cells from ATCC (Manassas, VA, USA) were maintained in F12 or DMEM + 10% FBS at 37°C in a humidified 5% CO₂ incubator. CHO- δ OR (CHO cell line stably expressing FLAG- δ OR) is described elsewhere (13). Rabbit polyclonal anti-FLAG antibody, Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol (DAMGO), Tyr-D-Ala-Phe-Glu-Val-Val-Gly (Deltorphan II), and G418 were from Sigma (St. Louis, MO, USA). D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) was from Peninsula Inc. (Belmont, CA, USA). Calphostin C and pertussis toxin were from Calbiochem (San Diego, CA, USA). Anti-ERK polyclonal, antiphospho-ERK monoclonal, antiphospho Stat3 (Ser-727, polyclonal, antiphospho-p90rsk polyclonal, antilamin A/C polyclonal, anti-HA monoclonal, and antiphospho- δ OR (Ser-363, polyclonal antibodies were from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-HA polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal β -arrestin2 antibody (A2CT) was a gift from Drs. R. Lefkowitz (Duke University, Durham, NC, USA) and L. Bohn (Ohio State University College of Medicine, Columbus, OH, USA). The anti- β -arrestin polyclonal antibody was from Calbiochem. Monoclonal anti-GAPDH antibody was from Novus Biologicals (Littleton, CO, USA). Horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences (Arlington Heights, IL, USA). Sulfo-NHS-biotin and avidin-coupled agarose were from Pierce (Rockford, IL, USA). Tyr-Tic Ψ (CH₂NH)-Phe-Phe (Tipp Ψ) was a gift from Dr. Peter Schiller (Institut de Recherches Cliniques de Montreal, Canada). The HA- μ OR plasmid was a gift from Dr. Liu Chen (Temple University School of Medicine, Philadelphia, PA, USA).

Methods

Transfections—Transfections were performed when cells were 80–90% confluent using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, with 2.5 μ g plasmid DNA (unless otherwise indicated).

Coimmunoprecipitation and Western blot—Cells were lysed for 1 h in lysis buffer (1% Nonidet P-40, 10% glycerol, 300 mM NaCl, 1.5 mM MgCl₂, 1 mM CaCl₂, and 50 mM Tris-Cl, pH 7.4) containing protease inhibitor cocktail (Sigma). For immunoprecipitation, 100–200 μ g of protein was incubated with 1 μ g of polyclonal anti-FLAG or monoclonal anti-HA antibody with 10% v/v protein A-agarose (Sigma) overnight at 4°C. The beads were washed twice with lysis buffer and phosphate-buffered saline (PBS) containing 10 mM EDTA. Proteins were eluted in 50 μ l of 2 $\times$ Laemmli buffer containing 1% 2-mercaptoethanol. Proteins were resolved by 10% SDS-PAGE and subjected to Western blot

using monoclonal anti-HA or polyclonal anti- β -arrestin antibodies. Chemiluminescence detection was performed using the SuperSignal West Pico reagent (Pierce, Rockford, IL, USA). Multiple exposures of immunoblots were scanned and densitometric analysis was carried out within linear range by using Image J software. GraphPad PRISM software was used for data analysis.

Phospho-ERK assay—CHO, CHO- δ OR, or HEK-293 cells were transfected with HA- μ OR plasmid. Cells were seeded on 24-well plates 24 h post-transfection. The next day, the cells were starved for at least 6 h in serum-free medium prior to stimulation with 100 nM DAMGO for the indicated times. In some cases cells were preincubated for 30 min with the indicated kinase inhibitor, followed by treatment with DAMGO in the presence of this inhibitor. Cells were solubilized by directly adding $1 \times$ SDS buffer prewarmed to 65°C, followed by sonication with a microtip for 5 s. For each transfection, protein determination (Bradford) was carried out, then 30 μ g protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting with mouse monoclonal antiphospho-p44/42 MAPK (anti-pERK, 1:1000) and rabbit polyclonal anti-p44/42 MAPK (anti-ERK, 1:1000).

Immunofluorescence and confocal microscopy—For ERK localization, CHO and CHO- δ OR cells growing on 6-well plates were transfected with HA- μ OR. The next day cells were plated on 14 mm coverslips. On the following day, cells were starved for at least 6 h and stimulated with 100 nM DAMGO for the times indicated. Cells were stained with the monoclonal pERK antibody according to the manufacturer's protocol. The cells were incubated with DAPI for 5 min, then washed four times with PBS.

β -Arrestin localization—CHO and CHO- δ OR cells growing on 6-well plates were transfected with HA- μ OR and β -arrestin2-EGFP plasmids. Cells were plated on 14 mm coverslips 24 h post-transfection. The next day, cells were fixed in -20°C methanol and immunostained with primary antibodies against HA (1:2000) and Flag (1:150), and against secondary Alexa 594-coupled donkey anti-mouse and Cy5-coupled donkey anti-rabbit antibodies. The same procedure was carried out with Neuro2A and HEK cells transfected with Flag- δ OR, HA- μ OR, and β -arrestin2-EGFP plasmids. Coverslips were mounted with Mowiol and visualized with a Leica TCS SP1 confocal microscope equipped with four external lasers (350, 488, 568, and 633 nm, Leica Microsystem). Images were acquired with an $\times 63/1.32$ PL APO objective lens and analyzed in sequential scanning mode.

Cell fractionation—CHO or CHO- δ OR cells expressing HA- μ OR were grown in 6-well plates, serum starved for 6 h, and stimulated with 100 nM DAMGO for the indicated times. Nuclear and cytoplasmic fractions were prepared as described elsewhere (14). Immunoblotting for pERK and ERK was performed as described above. Immunoblotting of the nuclear protein lamin A/C with the anti-Lamin antibody (1:2000) and of the cytoplasmic protein GAPDH (1:2000) was performed to assess the purity of the fractions.

Membrane biotinylation—SKNSH cells at 70% confluency in 6-well plates were stimulated with 100 nM DAMGO, 20 nM TIPP Ψ , or a mixture of both ligands for 5 min. After stimulation, cells were washed twice with ice-cold PBS/ Ca^{2+} / Mg^{2+} and incubated with sulfo-NHS-Biotin (Pierce) for 1 h at 4°C. Biotin was quenched by incubating the cells with 0.1 M glycine for 30 min at 4°C, then the cells were solubilized in immunoprecipitation lysis buffer for 30 min. The cell lysate was spun down to eliminate insoluble material and the supernatant was incubated with avidin-coupled agarose (Pierce) overnight at 4°C. The beads were washed four times in lysis buffer and eluted in 50 μ l of $2 \times$ SDS sample buffer.

Proteins were resolved by 10% SDS-PAGE for immunoblotting using anti- β -arrestin2 antibody.

siRNA transfection—Chemically synthesized, double-stranded siRNAs targeting human β -arrestin2 and human μ OR were purchased from Dharmacon (Lafayette, CO, USA). A nonsilencing RNA duplex was used as a control. SKNSH cells on 6-well plates were transfected with 100 nM of siRNA and HA- μ OR plasmid. The next day cells were split into 24-well plates for pERK assays. HEK cells were similarly transfected with the siRNAs.

Stat3 luciferase assay—CHO or CHO- δ OR cells were transfected with HA- μ OR and Ly6E Stat3-response luciferase reporter construct (15). After 24 h, the cells were split into 6-well plates. The next day cells were starved for 6 h in serum-free media, stimulated for 30 min with 100 nM DAMGO, and lysed in lysis buffer (Promega, Madison, WI, USA). Firefly luciferase activity was measured using a fluorometer as described (15). Renilla luciferase activity was measured as a control.

RESULTS

β -Arrestin2 constitutively associates with the μ OR- δ OR heterodimer

We and others have previously shown that μ OR and δ OR associate to form heterodimers that display new pharmacological properties (9–11, 16). In an effort to explore the signaling mechanisms of μ OR- δ OR heterodimers, we examined the ability of μ OR- δ OR to recruit β -arrestin. Since little is known about β -arrestin recruitment by μ OR- δ OR heterodimers, first we examined the subcellular localization of β -arrestin in HEK cells expressing μ OR- δ OR and compared it to cells individually expressing μ OR or δ OR. Confocal immunofluorescence studies showed that in cells expressing μ OR or δ OR, β -arrestin2 is located exclusively in the cytoplasm and does not colocalize with the receptors, which are found mostly at the plasma membrane. In contrast, in cells coexpressing μ OR and δ OR, β -arrestin2 is primarily located at the plasma membrane, where it colocalizes with the receptors (Fig. 1A). This suggests a constitutive recruitment of β -arrestin2 by the heterodimer. Quantification indicates that in > 94% of cells that coexpress μ OR and δ OR, β -arrestin2 is colocalized with the receptors (R. Rozenfeld and L. A. Devi, unpublished results). To ensure that coexpression of μ OR and δ OR leads to surface localization of β -arrestin2 independent of the cell system used, we carried out studies in two other cell lines. Examination of the neuroblastoma cell line Neuro2A also revealed a plasma membrane localization of β -arrestin2 only in cells coexpressing μ OR and δ OR, and not in cells expressing either one of these receptors alone (Fig. 1B). Similar results were obtained in CHO cells (Fig. 1C). To study the physical association of β -arrestin2 with μ OR- δ OR heterodimers, we carried out immunoprecipitation experiments. We find that β -arrestin co-immunoprecipitates with the receptors only in cells expressing μ OR- δ OR heterodimers but not in cells expressing only μ OR or δ OR (Fig. 1D). The fact that β -arrestin2 associates with μ OR- δ OR in the absence of ligand treatment suggests that μ OR- δ OR heterodimers constitutively recruit β -arrestin2.

Association with δ OR affects the kinetics of μ OR-mediated ERK phosphorylation

β -Arrestins have gained recognition in recent years as modulators of signal transduction of many GPCRs predominantly by activating signaling pathways such as the ERK pathway (17). β -Arrestin2-dependent ERK activation has been shown to be slower in onset and more sustained than the classic (β -arrestin-independent) pathway of ERK activation (18–20). We thus examined heterodimer-mediated ERK phosphorylation and compared it to μ OR-mediated ERK phosphorylation. For this, we examined the time course of DAMGO-induced ERK phosphorylation in HEK cells transiently expressing both μ OR and δ OR, and

compared it with the time course in cells expressing only μ OR. In cells expressing μ OR, DAMGO treatment leads to a rapid and transient increase of phosphoERK (pERK), with a peak signal at ~3–5 min. The level of pERK rapidly declines to < 30% of the maximal response after 10 min (Fig. 2, left panel). In μ OR- δ OR cells, in addition to the first pERK peak, DAMGO treatment leads to a slower increase in pERK, with a sustained second phase at ~15–20 min (Fig. 2, right panel). This suggests that the sustained phase of pERK is mediated by the activation of μ OR- δ OR heterodimers.

To test this further, we examined the effect of varying the levels of μ OR- δ OR on the kinetics of pERK. For this, we used CHO cells stably expressing δ OR (CHO- δ OR) and transfected them with different amounts of HA- μ OR cDNA. As anticipated, transfection with 5, 3.75, and 2.5 μ g of HA- μ OR cDNA yielded decreasing expression levels of μ OR (lanes 1, 3, and 5 in Fig. 3A) without affecting δ OR expression levels (Fig. 3A, IB: anti-FLAG). HA- μ OR could be coimmunoprecipitated with δ OR under the three transfection conditions (lanes 2, 4, and 6); however, the ratio of μ OR associated with δ OR to total μ OR varied with the different expression levels of μ OR, suggesting that μ OR- δ OR heterodimerization is modulated by the expression level of the receptors. Under conditions of low μ OR expression (2.5 μ g of cDNA), heterodimerization with μ OR is favored compared with conditions where μ OR is highly expressed (5 μ g of cDNA); under these conditions, μ OR appears to be mostly in homodimeric form (Fig. 3A). As the ratio of heterodimer to total μ OR increased, we observed a shift in the peak of pERK. For example, at highest heterodimer levels pERK peaks at 15 min, and at lowest heterodimer levels it peaks at 3–5 min (Fig. 3B, C), a time course reminiscent of that seen in μ OR alone (Fig. 3B, upper panel). To ensure that these changes in pERK kinetics were not mediated by δ OR activation, we examined ERK phosphorylation in δ OR-expressing cells on DAMGO treatment. Under these conditions, 100 nM DAMGO treatment did not lead to detectable ERK phosphorylation (data not shown). We also checked that DAMGO treatment did not lead to dissociation of the heterodimer by coimmunoprecipitation experiments. We found that upon stimulation, μ OR is still present in the δ OR immunoprecipitate (Supplemental Fig. 2). Taken together, these results are consistent with the notion that the extent of heterodimerization regulates the dynamics of ERK signaling.

Role of protein kinase C (PKC) in DAMGO-induced ERK phosphorylation

Several studies have reported that μ OR-mediated ERK phosphorylation involves a PKC-dependent mechanism (21–23). We examined the involvement of PKC activity in μ OR- δ OR-mediated ERK phosphorylation using the PKC inhibitor, calphostin C. For this purpose, we examined DAMGO-induced ERK phosphorylation in CHO- δ OR transfected with 2.5 μ g of HA- μ OR cDNA (to favor heterodimer formation) and compared it to cells expressing μ OR alone. In vehicle-treated μ OR cells (Fig. 4A, top panels), DAMGO treatment leads to ERK phosphorylation, with a peak at 3–5 min, whereas in μ OR- δ OR cells this treatment leads to a sustained phosphorylation at 10–20 min. In μ OR-expressing cells, treatment with calphostin C inhibits ERK phosphorylation at early time points. While a component of the response at late time points is calphostin C-resistant, it represents only a minor portion compared with the total pERK response (Fig. 4A, lower panel). In contrast, in μ OR- δ OR-expressing cells, this treatment does not inhibit DAMGO-induced ERK phosphorylation at any time point examined (Fig. 4B, lower panel). On the contrary, pertussis toxin treatment inhibits ERK phosphorylation in both μ OR and μ OR- δ OR cells (Supplemental Fig. 3). We examined the possibility that DAMGO-induced ERK phosphorylation by the heterodimer involves transactivation of δ OR. For this, we used phosphorylation of δ OR C-terminal tail as a readout of its activation. We find that δ OR is not phosphorylated under any condition examined, except on direct activation by the

specific agonist deltorphin II (Supplemental Fig. 4). This suggests that the changes in pERK kinetics seen in μ OR- δ OR cells are not due to transactivation of δ OR.

Together with results showing that the early phase of ERK phosphorylation is PKC dependent but the pERK response at later times is not, these findings support the notion that the signal transduction pathways activated by the μ OR- δ OR heterodimer are distinct from the pathways activated by μ OR.

Depletion of β -arrestin2 affects μ OR- δ OR-mediated ERK phosphorylation

To critically evaluate the role of β -arrestin2 in μ OR- δ OR heterodimer-mediated ERK phosphorylation, we used β -arrestin2 siRNA (to deplete endogenous β -arrestin2) in SKNSH cells that endogenously express μ OR- δ OR heterodimers (9, 11). In these cells, DAMGO treatment leads to a rapid peak of pERK at 5 min, followed by a second, less intense sustained phase of pERK at 10–15 min (Fig. 5). We find that treatment with β -arrestin2 siRNA leads to a substantial decrease in endogenous protein (Fig. 5, inset). Under these conditions, there is a significant decrease in the late sustained phase of ERK phosphorylation (Fig. 5). This treatment also leads to an increase in the early phase of ERK phosphorylation (Fig. 5). As a control, β -arrestin2 siRNA treatment did not affect the kinetics of ERK phosphorylation in HEK cells expressing μ OR alone (not shown). These results showing that depletion of β -arrestin2 affects the μ OR- δ OR heterodimer-mediated signaling suggest that the heterodimer signals through a β -arrestin2-dependent pathway.

Dual occupancy of the heterodimer leads to dissociation of β -arrestin2 from the μ OR- δ OR heterodimer

A provocative set of studies with other GPCRs has shown that a subset of ligands is able to specifically modulate the extent of arrestin association with their receptors (19, 24). We applied this to opioid receptor heterodimers, and examined the effect of a mixture of μ OR and δ OR specific ligands on the association of β -arrestin with the heterodimer. We chose the μ OR agonist DAMGO and the δ OR antagonist TIPP Ψ , since in previous studies this combination was found to enhance G-protein coupling and signaling by the heterodimer (9, 11).

First, we examined whether treatment with these two ligands would lead to a dissociation of β -arrestin2 from the heterodimer. Immunoprecipitation experiments showed that in untreated cells or in cells treated with individual ligands, β -arrestin2 was associated with the heterodimer (Fig. 6A). In contrast, in cells treated with a combination of ligands, β -arrestin2 was not associated with the heterodimer, as evidenced by the lack of β -arrestin2 in the δ OR immunoprecipitate (Fig. 6A). Next, using cell surface biotinylation, we examined whether this combination of ligands would lead to the dissociation of β -arrestin2 from endogenous receptors in SKNSH cells. In untreated cells or in cells treated with DAMGO or TIPP Ψ , β -arrestin2 was present in the biotinylated fraction (Fig. 6B), suggesting that it associates with membrane proteins (*i.e.*, opioid receptors). Confocal microscopy experiments also show that in SKNSH cells, a large fraction of transfected β -arrestin2-EGFP is localized at the plasma membrane (Supplemental Fig. 1). In contrast, in cells treated with a combination of ligands, β -arrestin2 was not detected in the biotinylated fraction. These results are consistent with the notion that, in the unstimulated state, heterodimerization stabilizes μ OR- δ OR in a conformation conducive to association with β -arrestin2. Dual occupancy of the heterodimer (by simultaneous treatment with the δ OR antagonist and DAMGO) leads to destabilization of this conformation, resulting in a dissociation of β -arrestin2.

Next we examined the effect of β -arrestin2 dissociation (induced by the combination of ligands) on ERK phosphorylation. We find that cotreatment with DAMGO and TIPP Ψ leads

to a significant decrease in the late phase of ERK phosphorylation and to an increase in the early phase of ERK phosphorylation (Fig. 6C). This pattern is similar to that observed with β -arrestin2 siRNA treatment (Fig. 5). Taken together, these results suggest that dual occupancy of μ OR- δ OR (by cotreatment with μ OR and δ OR ligands) leads to changes in the conformation of the heterodimer that results in the dissociation of β -arrestin2, enabling rapid ERK phosphorylation.

Heterodimerization affects δ OR signaling in a reciprocal fashion

In a parallel series of studies, we examined the effects of heterodimerization on δ OR signaling. In CHO cells expressing δ OR alone, treatment with deltorphin II leads to a rapid and transient increase in ERK phosphorylation (Fig. 7A). In contrast, in μ OR- δ OR-expressing SKNSH cells, this treatment leads to a slow and sustained late pERK response in addition to the rapid first peak (Fig. 7B, top panel). We examined whether heterodimerization with μ OR could account for this pattern of ERK phosphorylation. For that we used siRNA to μ OR to down-regulate the μ OR expression level. SiRNA to μ OR decreased μ OR expression by 70–80% (Supplemental Fig. 5A) and abolished μ OR-mediated ERK signaling (Supplemental Fig. 5B). Under these conditions, there is a significant decrease in the late sustained phase of deltorphin II-induced ERK phosphorylation (Fig. 7B). These results indicate that heterodimerization with μ OR is necessary for the second phase of δ OR-mediated signaling. We tested whether this regulation of δ OR signaling by heterodimerization involved a β -arrestin2-dependent mechanism. SiRNA-mediated β -arrestin2 down-regulation leads to a similar shift in deltorphin II-induced pERK kinetics. Then we examined whether occupying μ OR and δ OR binding sites affects the time course of ERK phosphorylation. Cotreatment with deltorphin II and a μ OR antagonist (10 nM CTOP) also leads to a shift in pERK kinetics to a pattern similar to that observed in cells expressing δ OR alone.

Taken together, these results indicate that heterodimerization affects the signaling properties of δ OR, and this regulation involves a β -arrestin2-mediated pathway, suggesting a common β -arrestin2-mediated modulation of μ OR and δ OR signaling by heterodimerization.

DAMGO-induced ERK phosphorylation is mostly restricted to the cytoplasm

We examined whether, in addition to changes in the kinetics of ERK phosphorylation, heterodimerization leads to alterations in the subcellular localization of pERK. In μ OR-expressing cells, DAMGO-induced pERK is largely localized to a nuclear compartment, as revealed by confocal microscopy (Fig. 8A, left panel). In contrast, in μ OR- δ OR-expressing cells, the majority of pERK is confined to the cytoplasm (Fig. 8A, right panel). Nuclear localization was not seen even on prolonged (20 min) DAMGO exposure (not shown). To confirm the differential localization of pERK, we carried out subcellular fractionation studies. The purity of the cytoplasmic and nuclear fractions was verified by probing fractions with antilamin A/C antibody (nuclear marker) and anti-GAPDH antibody (cytoplasmic marker) (Fig. 7B). In μ OR-expressing cells, μ OR agonist treatment results in localization of the majority (>70%) of pERK to the nuclear fraction within 5 min. In contrast, in μ OR- δ OR cells the same treatment results in localization of < 10% of pERK to the nuclear fraction even after 15 min stimulation; the majority of pERK (>80%) is restricted to the cytoplasm (Fig. 8B). Thus, while opioid treatment in μ OR-expressing cells results in mobilization of pERK to the nucleus, the same treatment in μ OR- δ OR heterodimer-expressing cells results in retention of pERK in the cytoplasm.

μ OR- δ OR heterodimerization differentially affects the phosphorylation of cytoplasmic and nuclear pERK substrates

We next examined the functional consequences of differential localization of pERK in μ OR vs. μ OR- δ OR cells by examining the phosphorylation of cytoplasmic (p90rsk) and nuclear (serine 727 of Stat3) pERK substrates (pERK-mediated Stat3 activation by μ OR has been reported; ref. 25). We find that DAMGO treatment leads to a rapid and transient phosphorylation of p90rsk in μ OR cells and a slow and sustained phosphorylation of p90rsk in μ OR- δ OR cells (Fig. 9A). This pattern is similar to that of ERK phosphorylation and agrees with the cytoplasmic localization of pERK in μ OR- δ OR cells. In contrast, Stat3 phosphorylation at its nuclear phosphorylation site, Ser-727 (26), is seen only in μ OR-expressing cells and not in μ OR- δ OR-expressing cells (Fig. 9A), even after 30 min stimulation (not shown). This agrees with the nuclear localization of pERK in μ OR cells. Next we examined whether the differential phosphorylation of Stat3 affects its transcriptional activity. Stat3-mediated transcriptional activity was measured using a Stat3 luciferase reporter gene. We find that DAMGO treatment leads to a significant increase in Stat3 activity only in μ OR- but not in μ OR- δ OR-expressing cells (Fig. 9B). These results support the idea that μ OR- δ OR heterodimerization restricts pERK to the cytoplasm, thereby preventing its ability to activate nuclear substrates such as the transcription factor Stat3. Thus, the subcellular localization of pERK, which is critical to the outcome of downstream signaling pathways, is modulated by μ OR- δ OR heterodimerization.

DISCUSSION

In the present study, we show that heterodimerization with δ OR alters μ OR-mediated signaling, resulting in spatio-temporal changes in the dynamics of ERK phosphorylation. We find that the slow and sustained ERK phosphorylation (seen as a second phase) is mediated by the μ OR- δ OR heterodimer. In cells that express high levels of heterodimers (CHO cells, Fig. 3C), there is a robust second phase, and as the levels of the heterodimer are reduced, the relative robustness of the second phase decreases (Fig. 3A–C). Under these conditions there is a reciprocal increase in the levels of the μ OR homodimer, and this corresponds to an increase in the first peak of ERK phosphorylation. Thus, the level of the first peak of ERK phosphorylation correlates with the level of homodimers, and the level of the second phase correlates with the level of heterodimers. This is further supported by our data with various cell systems expressing different levels of the heterodimer (Figs. 2, 3, and 6). The μ OR- δ OR heterodimer-induced second phase of ERK response is mediated by β -arrestin2, and thus the heterodimer constitutes a unique signaling unit. Depletion of β -arrestin2 from cells expressing μ OR- δ OR leads to a pattern of ERK phosphorylation similar to that observed in cells expressing μ OR alone. This is consistent with other studies that have shown altered G-protein coupling and channel activation by μ OR- δ OR heterodimers (16, 27).

β -Arrestin-mediated ERK phosphorylation has been described for several GPCRs, albeit the majority of these studies have been in cells expressing only homodimers (for a review, see ref. 28). The best-studied example is that of the AT1 receptor, for which two distinct pathways of ERK activation have been uncovered: a PKC-dependent pathway that leads to transient ERK phosphorylation and targets pERK to the nucleus, and a β -arrestin-dependent pathway that leads to sustained ERK phosphorylation and targets pERK to the cytosol and endosomes (18). This spatial and temporal segregation of ERK activated by PKC and β -arrestin pathways has been shown to lead to the activation of distinct downstream signaling cascades (29, 30). This raises the possibility that conditions that selectively stimulate or inhibit one of these pathways could have significant physiological relevance. Indeed, the activation of distinct signaling pathways by specific ligands has been described recently for β 2-adrenergic (24) and parathyroid hormone (20) receptors. In both studies, specific ligands

were shown to stabilize their cognate receptor in conformations conducive to either G-protein-dependent or β -arrestin-dependent ERK phosphorylation. Here we show that heterodimerization can also stabilize receptors in a conformation conducive to activating a specific signaling pathway. This allows the same ligand (DAMGO) to activate distinct pathways of ERK phosphorylation (*i.e.*, a PKC-dependent or a β -arrestin-dependent pathway), based on the absence or presence of the dimerizing partner (in this case, δ OR). Note that β -arrestin-mediated ERK activation is sensitive to pertussis toxin, suggesting that this also requires G-protein activation. It is conceivable that the homo- and heterodimeric receptors are organized as higher order complexes that include G-proteins, scaffolding molecules (such as β -arrestin), and other signaling molecules. Within the complex, receptor activation could initiate a sequence of events involving G-protein activation, a component of which could induce changes in the conformation of β -arrestin needed to enhance ERK phosphorylation. Further studies are needed to validate such a model. Taken together, these results show for the first time that μ OR- δ OR heterodimers, by recruiting β -arrestin, modulate the dynamics of receptor signaling and its downstream effects. These findings have potential clinical relevance for modulating morphine effects.

Heterodimerization of μ OR and δ OR has been implicated in the regulation of morphine-induced analgesia (11). We previously showed that modulating μ OR- δ OR heterodimer activity by occupying the binding site of one protomer (δ OR) changes the agonist-induced response of the partner (μ OR) (9, 11). For example, coadministration of a δ OR ligand with morphine results in an enhancement of morphine-induced analgesia. Animals lacking β -arrestin2 also show enhanced morphine-induced analgesia (31). Here we show that β -arrestin knockdown or dual occupancy of the heterodimer (that leads to dissociation of the heterodimer- β -arrestin complex) leads to a pattern of ERK phosphorylation similar to that of μ OR. In preliminary studies we find that cotreatment with a δ OR antagonist leads to changes in morphine-mediated signaling similar to those observed with DAMGO (R. Rozenfeld and L. A. Devi, unpublished results). This presents an opportunity to selectively target heterodimers with a combination of morphine and a δ OR antagonist. This would lead to a switch in signaling from a μ OR- δ OR (β -arrestin-dependent) to a μ OR (G-protein-dependent)-mediated pathway resulting in an increase in the analgesic response of μ OR, and thus enhanced morphine effects.

Interactions between μ OR and δ OR have also been implicated in the development of morphine tolerance (32). Previous studies have reported the modulation of morphine tolerance by δ OR using animals lacking δ OR (6) or with impaired δ OR expression (7), or by cotreatment with δ OR ligands (5). This, taken with the finding that animals lacking β -arrestin2 fail to develop morphine tolerance (33), supports a role for μ OR- δ OR-mediated recruitment of β -arrestin2 in regulating opiate tolerance.

Taken together, these findings showing that heterodimerization of μ OR and δ OR modulates μ OR agonist-induced ERK activation and its downstream signaling pathways suggest a provocative role for heterodimerization in opiate analgesia and tolerance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Localization of β -arrestin2-EGFP in μ OR, δ OR, and μ OR- δ OR-expressing HEK, Neuro2A, and CHO cells. A) HEK-293 cells, B) Neuro2A cells, or C) CHO cells transfected with β -arrestin2-EGFP and HA- μ OR, FLAG- δ OR, or HA- μ OR+FLAG- δ OR were stained with primary antibodies against the HA and FLAG tags and imaged by confocal microscopy. D) Lysates from CHO cells transfected with HA- μ OR (μ OR) or cells stably expressing FLAG- δ OR (δ OR) transfected with HA- μ OR (μ OR- δ OR) were immunoprecipitated using polyclonal anti-FLAG or monoclonal anti-HA antibodies. β -Arrestins in the immunoprecipitate were detected by immunoblotting using a polyclonal anti- β -arrestin antibody.

Figure 2.

Kinetics of DAMGO-induced ERK activation in μ OR and μ OR- δ OR-expressing HEK cells. HEK-293 cells transfected with μ OR or both μ OR and δ OR were treated with 100 nM DAMGO for the indicated periods. Cell extracts were subjected to Western blot using pERK or ERK antibodies as described in Materials and Methods. The amount of ERK phosphorylation in each lane was quantified by densitometry and normalized by expressing the data as a ratio of pERK over total ERK. Results are expressed as a percentage of the maximum response at 5 min. Data represent mean $\pm$ SE ($n=3$). ** $P < 0.01$ indicates statistically significant difference compared with μ OR.

Figure 3.

Kinetics of DAMGO-induced ERK activation in μ OR and μ OR- δ OR-expressing cells. **A)** Cell lysates from CHO-FLAG- δ OR transfected with increasing amounts of HA- μ OR cDNA were subjected to immunoprecipitation using polyclonal anti-FLAG antibody, and associated HA- μ OR was detected by immunoblotting using a monoclonal anti-HA antibody. Relative levels of HA- μ OR in FLAG- δ OR immunoprecipitates after different transfection conditions were quantified as described in Materials and Methods and shown as a ratio of the level in immunoprecipitate to total levels. The mature form of μ OR (~90 kDa) was detected in the δ OR immunoprecipitate. Level of δ OR under each transfection condition is shown by immunoblotting with anti-FLAG antibody. **B)** Kinetics of DAMGO-induced ERK phosphorylation under each transfection condition. Lysates from cells expressing increasing amounts of HA- μ OR were examined for ERK phosphorylation as described in Materials and Methods. **C)** Quantification of results from at least 3 independent experiments are expressed as mean $\pm$ SE ($n=3$). The maximum response in μ OR cells at 5 min is taken as 100%.

Figure 4.

Effect of the PKC inhibitor calphostin C on the kinetics of DAMGO-induced ERK phosphorylation. μ OR was expressed in A) CHO (μ OR) or B) CHO- δ OR (μ OR- δ OR) cells. Cells were pretreated with vehicle (top panel) or 1 μ M calphostin C for 30 min and stimulated with 100 nM DAMGO for the indicated periods. Cell extracts were subjected to Western blot using pERK or ERK antibodies and the level of pERK was quantified as described in Materials and Methods. Peak value at 5 min was taken as 100% for μ OR cells and a value at 15 min for μ OR- δ OR cells. Data represent mean $\pm$ SE ($n=3$).

Figure 5.

Effect of β -arrestin2 siRNA on DAMGO-induced ERK activation in SKNSH cells. SKNSH cells were transfected with control or β -arrestin2 siRNAs. Three days later, cells were stimulated with 100 nM DAMGO for the indicated periods. Cell extracts were examined for pERK and total ERK levels as described in Materials and Methods. Peak value at 5 min in control siRNA cells was taken as 100%. Data represent mean $\pm$ SE ($n=3$). * $P < 0.05$; *** $P < 0.001$, statistically significant differences compared with control siRNA. Inset shows a representative Western blot of β -arrestin2 in cells transfected with control or specific β -arrestin2 siRNA. Blots were reprobed with anti-actin antibody to ensure equal transfer and loading.

Figure 6.

Effect of treatment with a combination of μ OR and δ OR ligands on ERK activation in SKNSH cells; ligand-induced changes in the μ OR- δ OR- β -arrestin2 complex. **A**) HA- μ OR (μ OR) or HA- μ OR+FLAG- δ OR (μ OR- δ OR) -expressing CHO cells were treated with 100 nM DAMGO, 20 nM Tipp_ψ, or both for 5 min, then immunoprecipitated with anti-FLAG antibody to isolate FLAG- δ OR associated proteins as described in Materials and Methods. β -Arrestin2 in the immunoprecipitate was detected by Western blot using anti- β -arrestin2 antibody as described. **B**) SKNSH cells were treated with the indicated ligands for 5 min, then cell surface proteins were biotinylated and isolated by incubation with agarose-coupled avidin. β -Arrestin2 in the biotinylated fraction was detected by Western blot using anti- β -arrestin2 antibody as described in Materials and Methods. **C**) SKNSH cells were stimulated with 100 nM DAMGO without or with 20 nM of Tipp_ψ for the indicated periods. Cell extracts were examined for pERK and total ERK levels using Western blot and pERK was quantified as described in Materials and Methods. Peak value at 5 min in DAMGO-treated cells is taken as 100%. Data represent mean $\pm$ SE ($n=3$). ** $P < 0.01$; *** $P < 0.001$, statistically significant differences compared with DAMGO treatment.

Figure 7.

Effect of μ OR- δ OR dimerization on δ OR-mediated ERK phosphorylation. *A*) δ OR-expressing CHO cells were treated with 100 nM Deltorphan II for the indicated periods. Cell extracts were subjected to Western blot using pERK or ERK antibodies as described in Materials and Methods. The amount of ERK phosphorylation in each lane was quantified by densitometry and normalized by expressing the data as a ratio of pERK over total ERK. Results are expressed as a percentage of the maximum response at 5 min. Data represent mean $\pm$ SE ($n=4$). *B*) SKNSH cells transfected or not with μ OR siRNA or β -arrestin2 siRNA were stimulated with 100 nM Deltorphan II without or with 10 nM of CTOP for the indicated periods. Cell extracts were examined for pERK and total ERK levels using Western blot and pERK was quantified as described in Materials and Methods. Peak value at 5 min in Deltorphan II-treated control cells is taken as 100%. Data represent mean $\pm$ SE ($n=2-5$).

Figure 8.

Effect of μ OR- δ OR heterodimerization on the subcellular distribution of pERK. *A*) μ OR expressed in CHO (μ OR) or in CHO- δ OR (μ OR- δ OR) cells was stimulated with 100 nM DAMGO for the periods indicated and localization of pERK was examined by immunostaining and confocal microscopy as described in Materials and Methods. pERK (red) was detected with an anti-pERK antibody and the nuclei were stained with DAPI (blue). *B*) μ OR expressed in CHO (μ OR) or CHO- δ OR (μ OR- δ OR) cells was stimulated with 100 nM DAMGO for the periods indicated. Cytoplasmic and nuclear extracts were prepared as described in Materials and Methods and analyzed for pERK, ERK, lamin A/C, and GAPDH levels by Western blot. Representative data from 3 to 5 independent experiments are shown.

Figure 9.

Effect of heterodimerization on the phosphorylation of cytoplasmic and nuclear pERK substrates. *A*) μ OR expressed in CHO (μ OR) or CHO- δ OR (μ OR- δ OR) cells was stimulated with 100 nM DAMGO for the periods indicated. Cell lysates were analyzed for phospho-Stat3 (Ser-727), phospho-p90rsk, and pERK by Western blot as described in Materials and Methods. Total ERK is shown as loading control. *B*) To examine the Stat3-mediated luciferase activity, cells were cotransfected with the Stat3 reporter and Renilla luciferase plasmids. Cells were treated without (–) or with (+) 100 nM DAMGO for 30 min and lysed for luciferase assay as described in Materials and Methods. Luciferase activity in μ OR cells in the absence of DAMGO was taken as control. Data represent mean $\pm$ SE ($n=3$). * $P < 0.05$, statistically significant difference from untreated cells.