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Role of FK506 binding protein 12 in morphine-induced μ -opioid receptor internalization and desensitization

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Abstract

Agonist-activated μ -opioid receptor (OPRM1) undergoes robust receptor phosphorylation by G protein-coupled receptor kinases and subsequent β -arrestin recruitment, triggering receptor internalization and desensitization. Morphine, a widely prescribed opioid, induces receptor phosphorylation inefficiently. Previously we reported that FK506 binding protein 12 (FKBP12) specifically interacts with OPRM1 and such interaction attenuates receptor phosphorylation and facilitates morphine-induced recruitment and activation of protein kinase C. In the current study, we demonstrated that the association of FKBP12 with OPRM1 also affects morphine-induced receptor internalization and G protein-dependent adenylyl cyclase desensitization. Morphine induced faster receptor internalization and adenylyl cyclase desensitization in cells expressing OPRM1 with Pro³⁵³ mutated to Ala (OPRM1P353A), which does not interact with FKBP12, or in the presence of FK506 which dissociates the receptor-FKBP12 interaction. Furthermore, knockdown of cellular FKBP12 level by siRNA accelerated morphine-induced receptor internalization and adenylyl cyclase desensitization. Our study further demonstrated that peptidyl prolyl cis-trans isomerase activity of FKBP12 probably plays a role in inhibition of receptor phosphorylation. In the view that internalized receptor recycles and thus counteracts the development of analgesic tolerance, receptor's association with FKBP12 could also contribute to the development of morphine tolerance through modulation of receptor trafficking.

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Keywords

μ-opioid receptor; FK506 binding protein 12; Morphine; Internalization; Desensitization

1. Introduction

μ-opioid receptor (OPRM1), which belongs to G protein-coupled receptor (GPCR) family, plays important roles in analgesic effects and the development of tolerance and physical dependence induced by opioids administration [1]. Similar to other G-protein-coupled receptors, activation of OPRM1 by opioids leads to receptor coupling to G-proteins and receptor phosphorylation by G protein-coupled receptor kinases (GRKs) and subsequent β-arrestin recruitment. β-arrestins uncouple the receptor from G proteins for internalization (endocytosis) and dampen G protein-dependent signal transduction processes such as adenylyl cyclase inhibition [2].

Morphine, which is widely used in the treatment of pain, has high potential to develop antinociceptive tolerance and physical dependence. Unlike other opioids such as [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO) and etorphine which mainly induce receptor phosphorylation, morphine induces less receptor phosphorylation and internalization, but mainly activates protein kinase C (PKC) and subsequent signaling molecules, which contributes to its analgesic tolerance [3-6]. On the other hand, it has been widely demonstrated that phosphorylation-induced receptor internalization facilitates receptor recycling and subsequent reactivation at cell surface, and thus to reduce the development of opioid tolerance [7-9]. The morphine tolerance could also be attributed to the inability of morphine to induce receptor internalization by the fact that DAMGO can facilitate morphine-induced receptor endocytosis and reduces morphine tolerance development in rats [10].

The function of OPRM1 has been found to be modulated by interaction with various proteins. The interaction of synaptophysin and phospholipase D2 with OPRM1 accelerates receptor internalization [11, 12]; Membrane glycoprotein M6a interacts with OPRM1 and influences receptor endocytosis and recycling [13]; G protein-regulated inducer of neurite outgrowth GRIN1 tethers OPRM1 receptor and G protein in the lipid rafts [14]. In addition to these proteins, previous studies in our lab has demonstrated that FK506 binding protein 12 (FKBP12) specifically interacts with OPRM1 at the Pro³⁵³ residue of OPRM1 [15]. FKBP12, a 12 kDa FK506 binding protein is a peptidyl prolyl cis-trans isomerase (PPIase) [16] belonging to the family of the immunophilins and is highly expressed in the brain [17]. FKBP12 has been found to have versatile functions in central nervous system [18, 19].

In our previous study, the interaction of FKBP12 with OPRM1 is demonstrated to reduce receptor phosphorylation and facilitate the recruitment and activation of PKCε [15]. However, whether the interaction of FKBP12 with OPRM1 affects receptor internalization and desensitization is unknown. In the current study, we demonstrated that the association of FKBP12 with OPRM1 also affects morphine-induced receptor internalization and desensitization.

2. Materials and Methods

2.1 Materials

Human recombinant glutathion-S-transferase (GST)-FKBP12 and its double mutant GST-FKBP12 (D37L, F99Y) were generous gifts from Dr. Yves Engelborghs, (Laboratory of Biomolecular Dynamics, K. U. Leuven, Belgium) [20]. Enzyme bovine G protein-coupled receptor kinase 2 (GRK2) was kindly provided by Dr. Jeffrey L. Benovic (Thomas Jefferson University, Philadelphia, PA) [21]. Morphine was supplied by the National Institute on Drug Abuse. FK506 was purchased from A.G. Scientific, Inc. (San Diego, CA, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Cell culture

Human embryonic kidney 293 (HEK293) cells were maintained in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The transfection of HEK293 cells with pCDNA3 plasmids containing the hemagglutinin (HA) epitope tagged rat OPRM1 (HA-OPRM1) and its mutant at Pro³⁵³ to Ala (HA-OPRM1P353A) and the establishment of stably transfected cells was carried out as described previously [15]. The expression levels of wild type OPRM1 and mutant OPRM1P353A were 1.5 pmol/mg protein and 1.1 pmol/mg protein respectively. Stably transfected cells were maintained in the same medium supplemented with 200 µg/ml G418.

2.3 Knockdown of FKBP12

HEK293 cells stably expressing OPRM1 were transfected with the reported short interfering RNA (siRNA) corresponding to the target sequence GCTTGAAGATGGAAAGAAA of FKBP12 gene [22] (GenePharma, Shanghai, China) or a scrambled sequence as control at the final concentration of 50 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and were analyzed 48 h later as indicated. The effect of siRNA on protein expression was determined by Western blot.

2.4 *In vitro* peptidyl prolyl isomerization and subsequent receptor phosphorylation assay

The method was modified from assay for peptidyl prolyl isomerization [23] and *in vitro* assay of G protein-coupled receptor kinase (GRK) activity [24]. Briefly, 1 µg glutathion-S-transferase (GST) fused OPRM1 (GST-OPRM1CT) or GST-OPRM1CTP353A was incubated with 0.4 µg GST-FKBP12 or its double mutant GST-FKBP12 (D37L, F99Y) in 35 mM Hepes pH 7.8 in a total volume of 5 µl at 10°C for 10 min. Then the reaction was added 25 µl GRK assay buffer (25 mM Hepes, pH 7.5, 2.5 mM EDTA, and 7.5 mM MgCl₂) supplemented with 0.5 µg GRK2 and 1 mM ATP. After incubation at 30°C for 5 min, the reaction was terminated by adding SDS-PAGE sample buffer and boiled for 3 min. When FK506 was used, it was added 5 min at the concentration of 10 µM before the addition of GST-FKBP12. The samples were resolved by SDS-PAGE and the phosphorylated OPRM1 C-tail was detected by anti-phosphoSer³⁷⁵ of OPRM1 antibody (OPRM1phosphoSer³⁷⁵) (Cell Signaling, Danvers, MA, USA). The intensity of individual bands was determined with the analysis software ImageQuant (GE Healthcare, Piscataway, NJ, USA).

2.5 Determination of receptor internalization by FACS analysis

Receptor internalization was quantified by FACS analysis as previously described [7]. Briefly, after incubation with 1 μ M morphine for 0.5, 1, 2, 3, 4 h, cells were chilled on ice to terminate receptor trafficking, and cell surface receptors were visualized by incubating the cells with anti-HA antibody (Convance, Princeton, NJ, USA; 1:1000), followed by incubation with the Alexa 488-conjugated anti-mouse IgG antibody (Invitrogen, 1:1000). Surface receptor staining intensity of the antibody-labeled cells was analyzed using fluorescence flow cytometry (FACScan, BD Biosciences, San Jose, CA, USA).

2.6 Determination of receptor desensitization by measurement of intracellular cAMP Levels

Receptor desensitization by measuring intracellular cAMP Levels was determined as previously described [7]. Briefly, cells were exposed to 1 μ M morphine for 0.5, 1, 2, 4, 6 h. The medium was then removed and replaced with 100 μ l of treatment buffer, with or without agonist. The treatment buffer consisted of 0.5 mM isobutylmethylxanthine and 10 μ M forskolin in Krebs-Ringer-HEPES buffer (KRHB; 110 mM NaCl, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, pH 7.4). The cells were incubated at 37°C for 15 min. The reaction was terminated by heating the cells at 90°C for 6 min. The cAMP level in the supernatant was measured by the AlphaScreen™ cAMP detection kit (PerkinElmer Life Science, Waltham, MA, USA), as described previously [25].

2.7 Statistical Analysis

The data are presented as mean $\pm$ S.E.M. of at least three independent experiments. Unpaired Student's *t* test (two-tailed) was performed for statistical comparisons.

3. Results

3.1 PPIase activity of FKBP12 probably contributes to the regulation of phosphorylation of OPRM1

We previously showed that phosphorylation of OPRM1 was attenuated by receptor's association with FKBP12. Molecular dynamics simulations further demonstrated the steric hindrance incurred by the interaction affected the receptor phosphorylation. FKBP12 possesses PPIase activity, whether such activity contributes to the modulation of OPRM1 phosphorylation by causing conformational change of receptor carboxyl tail needs elucidation. Thus *in vitro* peptidyl prolyl isomerization and subsequent receptor phosphorylation assay were performed. Incubation of GST-OPRM1CT with equimolar amount of GST-FKBP12, a fusion protein having same enzymatic activity as FKBP12 [20], significantly reduced the phosphorylation of OPRM1 carboxyl tail detected at Ser³⁷⁵ by 45 $\pm$ 9.9%, whereas incubation of GST-OPRM1CTP353A which was shown to bind FKBP12 poorly with GST-FKBP12 did not influence the phosphorylation of OPRM1 carboxyl tail (Figure 1A and 1B). Moreover, co-administration of 10 μ M FK506 which binds FKBP12 and inhibits the receptor-FKBP12 association abolished the effect of FKBP12 (Figure 1A and 1B). These results further confirm that the phosphorylation of OPRM1 suppressed by FKBP12 was from the direct interaction of FKBP12 and OPRM1. Then double mutant of

FKBP12 (D37L, F99Y), which has been shown to significantly reduce the PPIase activity of FKBP12 [26] was employed. As shown in Figure 1A and 1B, incubation of GST-OPRM1CT with double mutant of FKBP12 (D37L, F99Y) did not significantly reduce the OPRM1 carboxyl tail phosphorylation. These data suggest that the PPIase activity of FKBP12 probably contributes to its suppression of agonist-induced receptor phosphorylation.

3.2 Receptor-FKBP12 association affects morphine-induced receptor internalization

The robust receptor phosphorylation facilitates β -arrestins binding to the receptors and thus induces receptor internalization. Therefore, we investigated whether the changes in the morphine-induced receptor phosphorylation influenced by the OPRM1-FKBP12 interaction can influence the morphine-induced receptor internalization. As shown in Figure 2A, morphine induced minimal internalization of OPRM1. Only $6.3 \pm 1.6\%$ receptor internalized after 4 h of morphine pretreatment. Whereas FK506 pre-incubation which can dissociate FKBP12 from the receptor increased receptor internalization to $30 \pm 5.1\%$ after 4 h of morphine treatment. Similarly, morphine also induced faster internalization of the OPRM1P353A mutant, a receptor mutant that we have shown not to interact with FKBP12 [15]. $42 \pm 2.0\%$ of receptor was internalized after 4 h of morphine treatment. Moreover, knockdown of FKBP12 with siRNA which results in an increase in receptor phosphorylation [15] also accelerated receptor internalization to $17 \pm 0.9\%$ after 4 h of morphine treatment, compared to $3.7 \pm 1.4\%$ of receptors internalized in cells transfected with control siRNA (Figure 2B). These data suggest that morphine-induced receptor internalization could be promoted by dissociation of OPRM1-FKBP12 interaction.

3.3 Receptor-FKBP12 association influences morphine-induced adenylyl cyclase desensitization

Adenylyl cyclase desensitization is reported to be triggered by receptor phosphorylation and β -arrestins binding, and can be influenced by receptor internalization and recycling [7, 27, 28]. We next examined whether the observed differences in receptor internalization would affect the rate of agonist-induced desensitization. As expected, morphine induced adenylyl cyclase desensitization inefficiently. After 6 h of pretreatment, the maximal inhibition level was reduced by $14 \pm 3.6\%$ (Figure 3A). FK506 pre-incubation resulted in the reduction of morphine's maximal inhibition of adenylyl cyclase activity by $30 \pm 1.3\%$ after 6 h of morphine pretreatment. Similarly, in cells expressing OPRM1P353A, pretreatment of morphine increased adenylyl cyclase desensitization by showing $32 \pm 3.7\%$ loss in morphine maximal inhibition activity after 4 h's agonist exposure (Figure 3A). Moreover, knockdown of FKBP12 with siRNA accelerated adenylyl cyclase desensitization to $22 \pm 2.3\%$ after 6 h of morphine pretreatment, whereas transfection of control siRNA caused $9.6 \pm 1.7\%$ reduction of morphine's inhibition of adenylyl cyclase (Figure 3B). These data indicate that the receptor association with FKBP12 contributes to the inability of morphine to induce adenylyl cyclase desensitization.

4. Discussion

As a member of rhodopsin subfamily of GPCRs, upon activation by etorphine and DAMGO, OPRM1 undergoes robust receptor phosphorylation by GRKs and subsequent β -arrestin recruitment. However, morphine induces receptor phosphorylation and β -arrestin recruitment inefficiently, but activates PKC and subsequent signaling pathways efficiently [3, 29, 30]. In the present study, we demonstrated that the inability of morphine to induce receptor internalization and adenylyl cyclase desensitization could be attributed to the association of the receptor with FKBP12. Although receptor internalization can lead to decreased receptor signaling and therefore is involved in the development of opioid tolerance, numerous studies have demonstrated that desensitized and internalized mu-opioid receptors can recycle back to the cell surface in a reactivated state and thereby to counteract opioid tolerance [31]. Thus, OPRM1-FKBP12 interaction could also play a role in the development of morphine tolerance by inhibiting receptor internalization and adenylyl cyclase desensitization through dampening receptor phosphorylation.

FKBP12 possesses PPIase activity which could change the carboxyl tail conformation. Our present study further demonstrated that the PPIase activity of FKBP12 probably participates in the attenuation of receptor phosphorylation. FKBP12 (D37L, F99Y), the activity-deficient mutant of FKBP12, blocked its inhibitory effect on OPRM1 C-tail phosphorylation. Such mutation of FKBP12 does not affect its secondary structure [26]. This result does not preclude the effect of steric hindrance incurred by direct C-tail-FKBP12 interaction on C-tail phosphorylation, because OPRM1 C-tail mutant which does not interact with FKBP12 or dissociation of C-tail-FKBP12 complex by FK506 also eliminated the inhibitory effect of FKBP12, though FK506 also suppress PPIase activity of FKBP12. Our previous study demonstrated that the recruited calcineurin to the receptor complex by FKBP12 modulates the receptor phosphorylation. Thus, the inhibition of receptor phosphorylation through receptor's association with FKBP12 could be due to multiple steps.

FKBP12 ligand, FK506, has been shown to inhibit the development of morphine tolerance and the effect is speculated to be related to its inhibitory effects on constitutive nitric oxide production [32]. Furthermore, administration of FK506 inhibits the development of morphine tolerance and dependence [32]. However, in the light of our finding that FK506 accelerated receptor internalization and desensitization, FK506 may also exert its effects by dissociating the interaction between FKBP12 and OPRM1 and promoting receptor internalization and thereby resulting in subsequent recycling.

In summary, we demonstrated that the association of FKBP12 with OPRM1 affects morphine-induced receptor internalization and G protein-dependent adenylyl cyclase desensitization. Moreover, PPIase activity of FKBP12 probably plays a role in inhibition of receptor phosphorylation. FKBP12 is up-regulated in the brains of morphine tolerance rats [33]. The modulation of receptor internalization and desensitization, together with PKC activation by FKBP12 could underlie the pathological role of up-regulated FKBP12 in the tolerant brains and the beneficial effect of FK506.

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Abbreviations

GPCRs	G protein-coupled receptors
OPRM1	μ -opioid receptor
GRK	G protein-coupled receptor kinase
DAMGO	[D-Ala ² , N-Me-Phe ⁴ , Gly ⁵ -ol]-enkephalin
PKC	protein kinase C
FKBP12	FK506 binding protein 12
PPIase	peptidyl prolyl isomerase
OPRM1P353A	the mutation of Pro ³⁵³ to Ala of OPRM1
GST	glutathion-S-transferase.

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Highlights

OPRM1-FKBP12 interaction affects morphine-induced receptor internalization.

OPRM1-FKBP12 interaction affects morphine-induced adenylyl cyclase desensitization.

PP1ase activity of FKBP12 may contribute to inhibition of receptor phosphorylation.

Figure 1.

FKBP12 affected OPRM1 C-tail phosphorylation *in vitro*. 1 μ g GST-OPRM1CT or GST-OPRM1CTP353A was incubated with equal molar 0.4 μ g GST-FKBP12 or its double mutant GST-FKBP12 (D37L, F99Y) in 35 mM HEPES pH 7.8 at 10°C for 10 min to allow the peptidyl prolyl isomerization. Then phosphorylation assay was performed by adding GRK2 and ATP as described in “Materials and Methods” (A) and relative density was quantified (B). *, $P < 0.05$ versus GST-OPRM1CT.

Figure 2.

FKBP12 modulated morphine-induced receptor internalization. A, HEK-OPRM1 cells were pretreated with 10 μ M FK506 for 30 min, then the cells and HEK-OPRM1P353A cells were treated with 1 μ M morphine for the indicated intervals. Cell surface receptors were detected by labeling the receptor with anti-HA antibody and fluorescence-labeled secondary antibody. *, $P < 0.05$, **, $P < 0.01$ versus matched internalization rate of OPRM1. B, HEK-OPRM1 cells were transfected with FKBP12 siRNA or control siRNA, 48 h later, cells were treated with 1 μ M morphine for the indicated intervals. The embedded western blot showed the efficiency of knockdown. *, $P < 0.05$, **, $P < 0.01$ versus matched internalization rate of cells transfected with control siRNA.

Figure 3.

FKBP12 modulated morphine-induced receptor desensitization. A, HEK-OPRM1 cells were pretreated with 10 μ M FK506 for 30 min, then the cells and HEK-OPRM1P353A cells were treated with 1 μ M morphine for the indicated intervals. The ability of morphine to inhibit forskolin-stimulated cAMP accumulation was measured. *, $P < 0.05$ versus matched desensitization rate of OPRM1. B, HEK-OPRM1 cells were transfected with FKBP12 siRNA or control siRNA, 48 h later, cells were treated with 1 μ M morphine for the indicated intervals. *, $P < 0.05$, **, $P < 0.01$ versus matched desensitization rate of cells transfected with control siRNA.