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ADP-ribosylation factor 6 regulates mu-opioid receptor trafficking and signaling via activation of phospholipase D2

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ABSTRACT

Endocytosis of the mu-opioid receptor (MOPr) has been shown to play a protective role against the development of tolerance to opioid drugs by facilitating receptor reactivation and recycling. It has been further demonstrated, that the opioid-mediated and ADP-ribosylation factor (ARF)-dependent activation of phospholipase D2 (PLD2) is a prerequisite for MOPr endocytosis. In this study, we investigated which particular ARF protein is involved in opioid-mediated PLD2 activation and what are the mechanisms of ARF function in MOPr trafficking and signaling. By coexpressing the MOPr and dominant negative or constitutively active ARF mutants in human embryonic kidney (HEK) 293 cells and primary cultured cortical neurons as well as by using siRNA technology, we identified the ARF6 protein to be involved in the regulation of MOPr endocytosis. We also found that expression of an effector domain mutant of ARF6, which is incapable of activating PLD, blocked agonist-induced endocytosis suggesting that ARF6 function in MOPr trafficking is PLD2-mediated. Analogously, opioid-mediated activation of PLD2 is blocked in the presence of dominant negative ARF6 mutants. Finally, we also showed that ARF6 protein influences the recycling/reactivation of internalized MOPr and thus modulates agonist-induced MOPr desensitization. Together, these results provide evidence that ARF6 protein regulates MOPr trafficking and signaling via PLD2 activation and hence affects the development of opioid receptor desensitization and tolerance.

1. Introduction

Agonist-induced endocytosis is an important regulatory and signaling event for G protein-coupled receptors (GPCRs) (for review see [1]). For the mu-opioid receptor (MOPr), the investigation of molecular mechanisms regulating this process is of clinical importance because MOPr endocytosis counteracts the development of tolerance to opioid drugs by facilitating the reactivation of desensitized receptors [2].

In search of molecular players involved in MOPr endocytosis, we have recently identified phospholipase D2 (PLD2) as a MOPr interacting protein [3]. This ubiquitously expressed and plasma membrane located enzyme was previously reported to be activated by a great variety of hormones, neurotransmitters, growth factors, cytokines and stimulation of various GPCRs (reviewed in [4]). Activation of PLD2 leads to hydrolysis of phosphatidylcholine (PC), the major phospholipid of biological membranes, to phosphatidic acid (PA) and choline. We have previously shown that MOPr-mediated activation of PLD2 is essential for MOPr endocytosis and recycling [3,5]. We could further demonstrate that this opioid-mediated PLD2 activation is ADP-ribosylation factor (ARF) and not protein kinase C (PKC)-dependent.

The ARF family of small GTPases has six members of which ARF1 and ARF6 are the best described (for review see [6]). These molecules are important components of the molecular machinery that regulates membrane trafficking along endocytic and biosynthetic pathways and are involved in the activation of lipid-modifying enzymes like PLD and phosphatidylinositol-4-phosphate 5-kinase (PIP5K). ARF1 is localized mainly to the Golgi complex and regulates the assembly of different types of 'coat' complexes onto budding vesicles, whereas ARF6 is associated to the plasma membrane and involved in the regulation of plasma membrane/endosome trafficking as well as actin cytoskeleton rearrangements. However, it has been shown recently that ARF1 can be recruited to the plasma membrane upon activation of some GPCRs [7]. In addition, both ARF1 and ARF6 proteins have been reported to interact with different GPCRs and to be involved in the regulation of their trafficking and signaling events [7–10].

ARF1 and ARF6 proteins, although very similar in structure, differ in their effectors and downstream signaling pathways. Thus, the knowledge of whether MOPr trafficking is regulated by ARF1 or ARF6 might provide new insights into MOPr-mediated signaling pathways and may lead to the identification of further regulatory proteins involved in the modulation of MOPr trafficking and signaling. Therefore, in the

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present study we investigated which ARF protein, ARF1 or ARF6, is involved in opioid-mediated PLD2 activation and elucidated the main molecular mechanisms of ARF function in MOPr trafficking and signaling.

To answer these questions, different ARF mutants have been used. Like other small GTPases, ARFs cycle between their inactive, GDP-bound state and active, GTP-bound state. Therefore the function of these proteins is largely investigated by using dominant negative and constitutively active mutants which are thought to be "locked" in GDP- and GTP-bound states, respectively. However, some recent studies have shown that in the case of ARF6 these "classical" mutants, namely dominant negative ARF6/T27N and constitutively active ARF6/Q67L, show some artifacts *in vivo* [11–13]. Namely, it was demonstrated that ARF6/T27N mutant has a high tendency to lose its nucleotide and to denature *in vitro* [11] and thus, is no longer located in the plasma membrane where ARF6 normally resides. To overcome these problems, another dominant negative ARF6 mutant, ARF6/T44N was generated, which has a 30-fold decreased affinity for GTP in comparison to the wild type protein and, importantly, is properly located in the plasma membrane *in vivo* [11]. This mutant has been suggested to be a better choice for investigation of blocking ARF6 function *in vivo* and therefore was used in the present study. On the other hand, regarding active ARF6 mutants, "fast cycling" ARF6/T157N mutant was found to induce phenotypes that have been previously attributed to ARF6 activation without the toxic effects demonstrated after "classical" constitutively active ARF6/Q67L mutant expression [12,13]. This mutant "cycles" i.e. binds and releases GTP more quickly than the wild type protein and therefore has an enhanced activity *in vivo*. Moreover, unlike ARF6/Q67L mutant that is "locked" in its GTP-bound form, "fast cycling" ARF6/T157N retains a full cycle of GTP binding, hydrolysis and release which is necessary for proper function of ARF6 and therefore is suggested to represent better the ARF6 active form *in vivo* [12,13].

2. Materials and methods

2.1. Plasmids

The rat mu-opioid receptor was tagged at the NH_2-terminus with the T7 epitope tag sequence MASMTGGQQMGK using polymerase chain reaction and then subcloned into the pcDNA3 expression vector (Invitrogen, Karlsruhe, Germany), generating pcDNA3:T7-MOPr plasmid. Plasmids containing HA-tagged ARF6 wild type protein and mutants (pCMV5-HA-ARF6/wt, pCMV5-HA-ARF6/T44N, pCMV5-HA-ARF6/N48I and pCMV5-HA-ARF6/T157N) and HA-tagged ARF1 wild type and ARF1/T31N dominant negative mutant were kind gifts from Dr. Jacek Jaworski (IIMCB, Poland) and Dr. Rory Mitchell (CIP, UK), respectively. pEAK10:HA-MOPr plasmid and pcDNA3.1:PLD2 plasmid used for generation of HEK293 stable cell line coexpressing MOPr and PLD2 were generated as previously described [3,5].

2.2. Generation of cell lines coexpressing mu-opioid receptor, ARF mutants and/or PLD2

All transfections were done using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. For HEK293 stable cell line expressing T7-MOPr, the cells were transfected with pcDNA3:T7-MOPr plasmid containing G418 resistance gene. Stable transfectants were selected in the presence of 1 mg/ml G418 (PAA Laboratories GmbH, Pasching, Germany). Receptor expression was monitored using Western blot analysis and confocal microscopy. Generation of stable cell line coexpressing HA-MOPr and PLD2 that was used for PLD2 assay was done as previously described [3,5]. The expression of MOPr and ARF mutants after transient transfection of T7-MOPr-expressing cells with HA-ARF mutants was monitored by confocal microscopy, revealing that about 50% of the cells coexpressed both proteins.

2.3. RNA interference

The sequence of double-stranded siRNA targeting human ARF6 gene was 5′-AAGGUCUCAUCUUCGUAGUGG-3′ (ARF6 siRNA). Non-specific control sequence was 5′-AGGUAGUGUAAUCGCCUUGTT-3′. Both sequences were manufactured by Eurofins MWG Operon, Ebersberg, Germany and the efficiency of ARF6 siRNA in endogenous protein knock down was tested by Western blot analysis. HEK293 cells transfected with indicated concentrations of appropriate siRNAs were lysed 48 h after transfection and lysate was used directly in Western blot analysis with mouse monoclonal anti-ARF6 antibody (1:100, Santa Cruz Biotechnology, Inc.). Quantitative analysis of MOPr endocytosis and recycling was done 48 h after transfection with siRNAs in 6-well plates with Lipofectamine 2000 according to the manufacturer's instructions. 24 h after transfection the cells were seeded in 48-well plates and one day later tested in ELISA assay.

2.4. Radioligand binding assays

Binding studies were performed on membranes prepared from stably transfected cells. The dissociation constant (K_D) and number of binding sites (B_{max}) for [^{3}H]DAMGO [(D-Ala^2,NMe-Phe^4,Gly-ol^5)-enkephalin] (Bachem, Heidelberg, Germany) were calculated by Scatchard analysis using at least six concentrations of [^{3}H]DAMGO in a range from 0.3 to 9 nm as previously described [5]. Non-specific binding was determined as radioactivity bound in the presence of 1 μM unlabeled DAMGO.

2.5. Quantitative ELISA assay

24 h after transfection with ARF mutants or adequate siRNAs, T7-MOPr-expressing HEK293 cells were seeded in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% FCS and grown onto poly-L-lysine-treated 48-well plates overnight. In experiments with N-myr-ARF6, DMEM medium was also supplemented with 25 μM N-myr-ARF6 peptide (Calbiochem, Darmstadt, Germany). To estimate endocytosis, cells were specifically surface labeled with T7-antibody (1:1000; Novagen, Darmstadt, Germany) in UltraMEM (Cambrex Bio Science Verviers S.p.r.l., Verviers, Belgium) for 1.5 h at 4 °C and then ELISA assay was done as previously described [5,14]. During the assay, the cells were kept on 4 °C for 30 min (4 °C control), treated with indicated agonists in UltraMEM for 30 min at 37 °C or not treated (37 °C control). Constitutive endocytosis in the absence of agonist was calculated as percentage loss of surface receptors in 37 °C control to 4 °C control. Agonist-induced receptor endocytosis was calculated by subtraction of constitutive endocytosis from total endocytosis that was detected in agonist-treated samples in comparison to 4 °C control (100%), or total endocytosis was shown as indicated. To measure recycling, after 30 min of treatment with the μ-agonist DAMGO [(D-Ala^2,NMe-Phe^4,Gly-ol^5)-enkephalin] (Bachem, Heidelberg, Germany) the cells were washed with warm media to remove the agonist and then incubated for further 30 min at 37 °C in the presence of 1 μM receptor antagonist naloxone (Pfizer/Gödecke, Freiburg, Germany) in order to block residual DAMGO-stimulated endocytosis of MOPr. After fixation of cells, surface receptors were detected as described in this section. In these experiments, 37 °C control was taken as 100% for calculation of receptor endocytosis in agonist-treated samples. The recycling of internalized receptor was estimated as the percentage of recovered surface receptors from endocytosed receptors.

2.6. Immunocytochemistry

24 h after transfection, HEK293 cells coexpressing T7-MOPr and indicated HA-ARF mutants were seeded on poly-L-lysine-coated coverslips and grown overnight. After surface labeling of receptors with T7-antibody for 1.5 h at 4 °C, the cells were stimulated or not with indicated