Identifizierung und Charakterisierung von μ-Opioidrezeptor-interagierenden Proteinen

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1. Introduction

1.1. Opioid alkaloid and endogenous opioid peptide

Opioids are responsible for a variety of processes including analgesia, sedation, euphoria, respiratory depression and antidiarrhea in organisms.

Preparations of the opium poppy plant papaver somniferum have been used for thousands of years to relieve pain. In 1804, Adam Sertürner isolated the main constituent alkaloid morphine, which was later shown to be almost entirely responsible for the analgesic activity of crude opium. Two other less competent natural opioid alkaloids, codeine and thebaine, were also identified.

Subtle structural changes of opioid alkaloids can lead to dramatic functional changes as to antagonize the original effect, for example, merely converting an N-methyl to an N-allyl substituent transforms the opioid agonist oxymorphone to the opioid antagonist naloxone.

Opioid research was stimulated by the discovery of endogenous opiate peptides. The three main groups of opiate peptides are enkephalins, endorphins and dynorphins (Li and Chung, 1976; Goldstein et al., 1979) (table 1.1). The localization of these peptides to various regions of the spinal cord, brain stem and throughout the brain has been characterized in recent years. Opioid peptides are found in areas known to be involved in pain perception (periaqueductal gray matter, spinal cord, cerebral cortex and thalamus) as well as in other areas of brain including the limbic system (hippocampus) and striatum. The opioid peptides appear to mediate all of the processes induced by opioid alkaloid.

1.2. Opioid receptors

The rigid structural and stereochemical requirements essential for the analgesic actions of morphine and related opioids led to the theory that they produce their effects by interacting with specific receptors (Beckett and Casey, 1954). It is now clear that there are

three classical types of opioid receptor: mu, delta and kappa. Genes encoding these receptors have been cloned (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Minami et al., 1993). All of the cloned opioid receptors possess the same general structure with an extracellular N-terminal region, seven transmembrane domains and an intracellular C-terminal tail structure characteristic for G protein-coupled receptors (Figure 1.2).

Opioid receptors are subdivided on the basis of the selectivity of different agonists for different physiological responses. For example, the mu opioid receptor has high affinity for β-endorphin and morphine, followed by the enkephalins; The delta receptor recognizes the enkephalins better than β-endorphin and morphine; The kappa receptor is more sensitive to dynorphin and ketocyclazocine than morphine. Some opioid alkaloids and endogenous peptides and their selectivity of the receptor subtypes are listed in table 1.1.

Table 1.1. Opioid ligands

Receptor type	Mu opioid receptor	Delta opioid receptor	Kappa opioid receptor
Prototypic agonists	morphine	enkephalins	ketocyclazocine
Selective agonists	DAMGO	DPDPE	enadoline
Endogenous agonists	endomorphins, β-endorphin	enkephalins	dynorphins
Antagonists	naloxone	naltrindole	nor-binaltorphimine

The mu opioid receptor (MOR) mediates the actions of morphine and most clinical analgesic agents, as well as drugs of abuse such as heroin. Numerous pharmacological studies have suggested subtypes of the mu opioid receptor and studies have raised the possibility that some of these may reflect splice variants of the MOR1 gene (Wolozin and Pasternak, 1981; Pasternak, 1993; Reisine and Pasternak, 1996; Pasternak and Standifer, 1995). Two MOR1 variants, MOR1A and MOR1B, were identified shortly after the initial cloning of MOR1 (Bare et al., 1994; Zimprich et al., 1995). Thereafter, additional MOR1 splice variants such as MOR1C, D and E were identified (Pan et al., 1999). The sequence

differences between these splice variants are shown in the following figure 1.1.

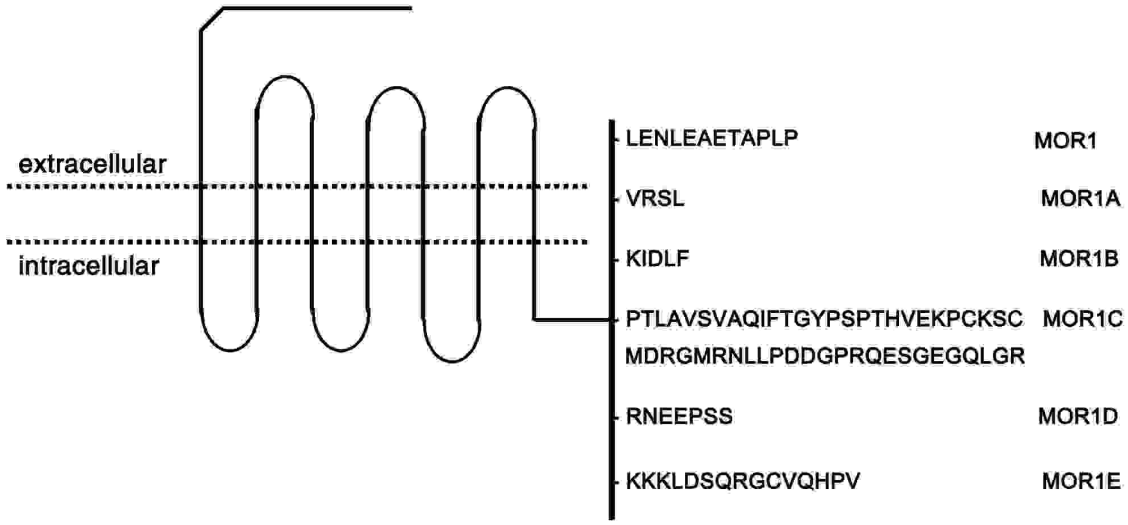


Figure 1.1. Different amino acid sequence of MOR1 and its splice variants (modified from Pan et al., 1999). Amino acid sequence of MOR1 splice variants predicted from the cDNA clones. All represent murine variants, with the exceptions of MOR1A (Bare et al., 1994) and MOR1B (Zimprich et al., 1995), which are from human and rat, respectively.

Although MOR1 and its splice variants are derived from the same gene, there exist markedly different immunohistochemical distributions between the receptor variants indicating region specific processing. For instance, MOR1 immunolabeling was observed in patches in the striatum, whereas MOR1B, C, D, E antisera failed to label this areas (Pan et al., 1999). These regional differences in expression further support the possibility that these variants encode pharmacologically and physiologically relevant receptors.

1.3. Effector mechanisms of opioid receptors

1.3.1. Structure and function of G protein-coupled receptors (GPCRs)

Like other G protein-coupled receptors, the mu opioid receptor has seven domains of 20-25 hydrophobic residues that form α-helices and span the plasma membrane, an extracellular N-terminus, three extracellular loops (e1-3), three intracellular loops (i1-3)

and an intracellular C-terminal tail (Figure 1.2). The binding of ligands to the extracellular domains of these receptors induces a conformational change that allows the cytosolic domains of the receptor to bind to G protein associated with the inner face of the plasma membrane. This interaction activates the G protein, which then dissociates from the receptor and carries the signal to intracellular targets, which may be either enzymes or ion channels.

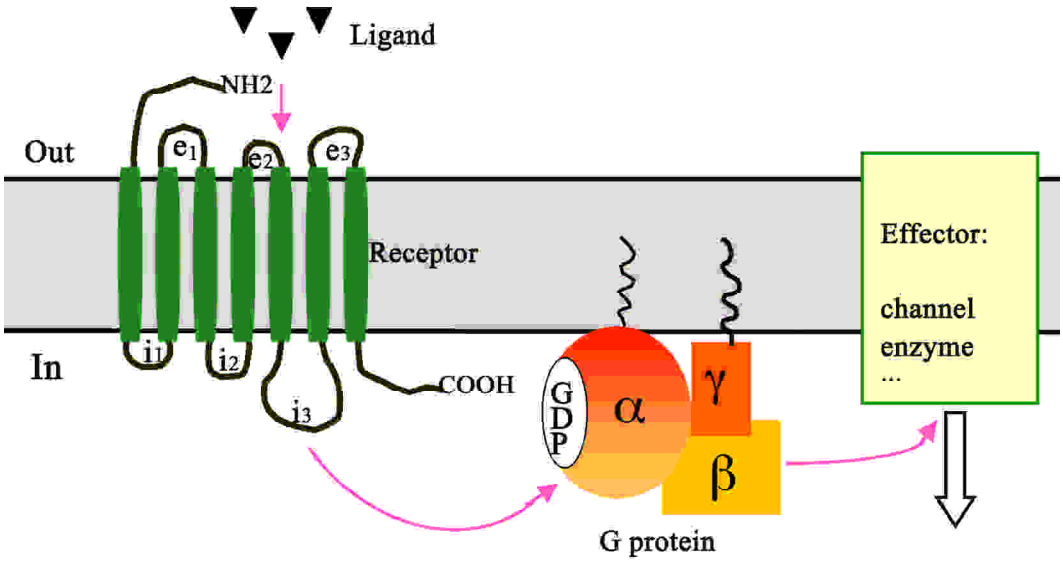


Figure 1.2. Structure of G protein-coupled receptor and G protein. GPCRs have a central common core made of seven transmembrane helices connected by three intracellular (i1, i2, i3) and three extracellular (e1, e2, e3) loops. G protein consists of three subunits, designated α, β and γ. The α subunit binds guanine nucleotide (GDP), which regulates G protein activity. Activated G protein transducts signal by regulating enzymes or ion channels.

1.3.2. Common opioid receptor evoked cellular responses

Activation of any of the three opioid receptor subtypes produces similar cellular actions. The most commonly reported actions are listed as below.

1.3.2.1. Effect on adenylyl cyclase

In general, opioid receptor activation results in inhibition of adenylyl cyclase, an effect which is mediated by the α subunit of the G protein. The G protein associated with the opioid receptor is called Gi/o and inhibits the activity of adenylyl cyclase. Many effects have now been identified as the physiological consequences of the acute inhibition of adenylyl cyclase by opioids. One example is the cAMP-mediated modulation of a voltage-dependent current (Ingram and Williams, 1994; Svoboda and Lupica, 1998).

On the other hand, chronic opioid receptor activation can also lead to a stimulation of AC mediated by the βγ subunit of G proteins (Chakrabarti et al., 1998a). Opioid receptors can also couple to Gs proteins after chronic opioid exposure, leading to an increase in AC activity (Chakrabarti et al., 1998b).

1.3.2.2. Activation of potassium conductance

Opioids have been shown to activate potassium conductances. The most commonly observed is the G protein-activated inwardly rectifying conductance (Alreja and Aghajanian, 1993; Ingram and Williams, 1996; Jiang and North, 1992; Grudt and Williams, 1993). The second messenger pathway is mediated by the βγ subunits of G protein (Aghajanian and Wang, 1986; Jan and Jan, 1997).

1.3.2.3. Inhibition of calcium conductance

There are many examples of the inhibition of calcium currents by activation of all opioid receptor subtypes (Hamra et al., 1999; Gross et al., 1990; Acosta and Lopez, 1999). The inhibition of high threshold calcium currents by opioids is mediated by the βγ subunits of G proteins (Weisskopf and Nicoll, 1995).

1.4. Opioid tolerance and dependence

Although opioids are highly effective for the treatment of pain, they are also known to be intensively addictive. After chronic opioid intake, the drug becomes less effective, so called tolerance. At the same time, a situation develops in which the interruption of taking the drugs results in withdrawal sickness, unmasking a state called dependence (Goldstein, 1994). Both tolerance and dependence result from biochemical changes in the brain.

Tolerance and dependence occur predominantly at the cellular level. Critical of opioid tolerance and dependence is receptor desensitization, endocytosis and down regulation. Receptor desensitization is mediated by uncoupling of activated receptors from G proteins, and effectively terminates the signaling. Receptor endocytosis depletes the plasma membrane of high-affinity receptors and contributes to both desensitization and resensitization of signaling. Receptor down-regulation is a loss of receptors from a cell that results from long-term (hours to days) continuous exposure of cells to agonists.

1.4.1. Receptor phosphorylation

The early events of signalling by opioid receptor are usually rapidly attenuated by receptor desensitization (Lohse, 1993; Freedman and Lefkowitz, 1996). An important component of desensitization, which occurs within seconds to minutes of receptor activation, is uncoupling of the activated receptor from its G-proteins by receptor phosphorylation.

Agonist-induced phosphorylation of the opioid receptor is mediated by G protein-coupled receptor kinases (GRKs) (Kovoor et al., 1997) and second messenger-regulated protein kinases, such as Ca^{2+}/calmodulin-dependent kinase II (Koch et al., 1997; Mestek et al., 1995) and mitogen activated protein (MAP) kinase (Polakiewicz et al., 1998; Schmidt et al., 2000), but not by protein kinase A (Chen and Yu, 1994) or protein kinase C (Zhang et al., 1996).

1.4.1.1. GRKs-mediated phosphorylation

Phosphorylation of an activated receptor by a GRK terminates signaling by initiating the binding of ß-arrestin and consequently by uncoupling of the receptor from heterotrimeric G proteins. Specific GRK2 phosphorylation sites involved in the agonist-induced receptor desensitization have been identified. A cluster of serine/threonine residues (T354/S355/S356/T357) in the C terminus of the mu opioid receptor has been shown to play an important role in GRKs mediated receptor desensitization (Wang, 2000). Another important phosphorylation site is T394 (Wolf et al., 1999; Deng et al., 2000; Pak et al., 1997).

1.4.1.2. CaM kinase II-mediated phosphorylation

Direct evidence of the involvement of CaM kinase II in the desensitization of the rat mu opioid receptor was provided by the identification of serine-266 in the intracellular loop as the critical phosphorylation site for the CaM kinase II-mediated receptor desensitization (Koch et al., 1997; Koch et al., 2000). It was further demonstrated that the CaM kinase II is colocalized with the mu opioid receptor and contributes to the development of tolerance to opioid analgesics (Brüggemann et al., 2000).

1.4.1.3. MAP kinase-mediated phosphorylation

Evidence has been provided for an involvement of the MAP kinase pathway in the homologous desensitization of the mu opioid receptor. Specific inhibitors of the MAP kinase diminish the agonist-induced desensitization and phosphorylation of the mu opioid receptor in a dose-dependent manner (Polakiewicz et al., 1998; Schmidt et al., 2000). The mu opioid receptor signaling via the MAP kinase cascade is also desensitized upon prolonged agonist exposure in cultured cells. The MAP kinase cascade also appears to undergo neuroadaptation during chronic opioid exposure in vivo. By monitoring the activation state of the MAP kinase using phosphospecific antibodies, neuronal MAP kinase

activity in the rat brain was potently repressed after repeated morphine administration (Schulz and Höllt., 1998).

1.4.2. Receptor endocytosis

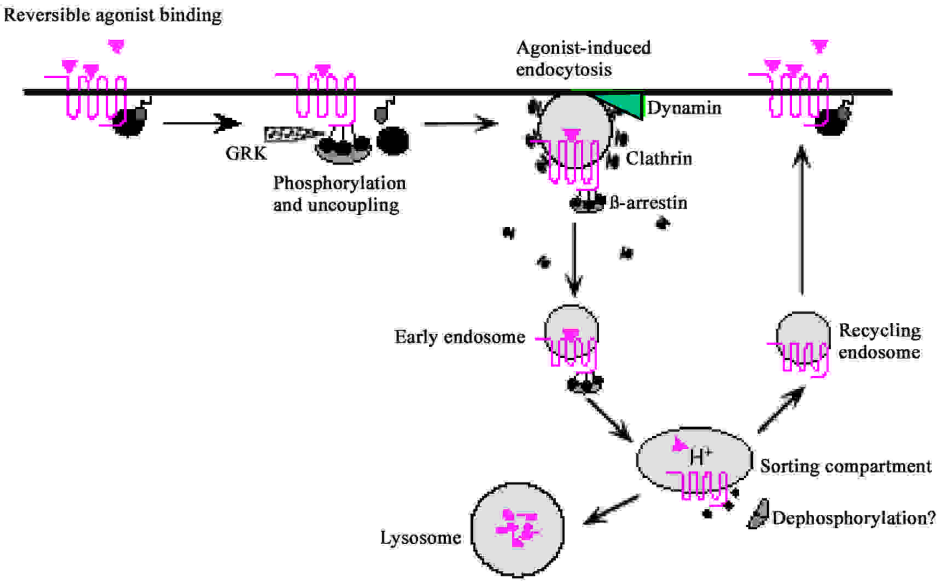


Figure 1.3. Typical pathways of endocytosis of opioid receptor (modified from Böhm et al., 1997a).

Like other GPCRs, agonist-induced endocysis (internalization) of opioid receptor causes a rapid spatial uncoupling of the endocytosed receptor from the cell surface, providing an additional mechanism for terminating signaling (Koenig and Edwardson, 1997). Reversible agonist binding to the receptor is followed by receptor phosphorylation. The binding of ß-arrestins to the phosphorylated receptor leads to receptor uncoupling from G-proteins, which causes receptor desensitization. The ligand-receptor complex is internalized via clathrin-coated pits. Once the plasma membrane is invaginated, dynamin-GTP wraps around and constricts the necks upon GTP hydrolysis, thus vesiculation occurs (Hinshaw, 2000). These vesicles soon shed their clathrin coat and become early endosomes. Ligand and receptor are separated in an acidified perinuclear compartment.

Dissociation of ß-arrestins also occurs. Cytosolic phosphatases may dephosphorylate the receptor. The ligand is degraded, the receptor is either recycled to the plasma membrane or degraded in the lysosome. Internalization, processing and recycling is necessary for resensitization of receptors (Böhm et al., 1997a). The process of opioid receptor endocytosis is shown in Figure 1.3.

1.4.2.1. Domains involved in receptor endocytosis

Clathrin-mediated endocytosis requires the interaction of specific receptor domains with components of the endocytic machinery (Trowbridge et al., 1993). Endocytic domains have been characterized for several GPCRs by receptor mutation experiments (Böhm et al., 1997b; Thomas et al., 1995; Huang et al., 1995) but a common endocytic motif has not been identified (Rodriguez et al., 1992; Parker et al., 1995).

1.4.2.2. Different endocytotic profile of the splice variants of the mu opioid receptor

Our lab has previously shown that MOR1 and the C terminal splice variant MOR1B differ in their DAMGO-mediated internalization and resensitization rates. The rapid internalizing variant MOR1B revealed a faster resensitization and consequently a slower desensitization as compared with rMOR1 (Koch et al., 1998). It was found that DAMGO treatment resulted in a rapid internalization and similar desensitization and resensitization rates of receptor splice variants MOR1C, MOR1D and MOR1E, whereas after morphine treatment only the splice variants MOR1D and MOR1E were internalized and showed a faster resensitization, a slower desensitization as compared with MOR1 and MOR1C (Koch et al., 2001). All of theses indicate that the carboxyl terminus of the mu opioid receptor can influence the agonist selectivity and rate of endocytosis.

1.4.3. Adenylate cyclase superactivation after chronic opioid treatment

Opioid tolerance after chronic treatment is not only due to a rapid decrease in receptor activity following phosphorylation and G protein uncoupling, but also due to a compensatory mechanism counteracting receptor function. Studies revealed that opioids acutely inhibit the activity of adenylyl cyclase (AC) in NG108-15 cells, but in the continued presence of morphine, there was an upregulation of the activity of AC (Sharma et al., 1975). This phenomenon, by which chronic activation of inhibitory $G_{i/o}$-coupled receptors lead to an increase in cAMP signaling in the cells, has been termed AC superactivation (Nevo et al., 1998).

However, to date, the mechanism by which AC activity is up-regulated by chronic exposure to inhibitory agonists remains largely unclear. It is known that opioid receptors are predominantly coupled to AC via G_{α} subunits of G proteins. Some recent reports show that there is also an enhanced opioid receptor signaling via $G_{\beta\gamma}$ subunits during the development of opioid tolerance (Chakrabarti et al., 1998a, 2001). Chronic morphine exposure also induces a shift in the relative preponderance of opioid receptor G_{α} inhibition to $G_{\beta\gamma}$ stimulation of AC activity.

1.5. Receptor associated proteins

To date, the regulation of opioid receptor trafficking and signaling is far from clear, although some participants like beta arrestins, GRKs and MAP kinase are known. A receptor-associated protein may play at least four distinct roles. 1) It may regulate receptor signaling through controlling receptor localization and/or trafficking. For example, it is known that the recycling of the mu opioid receptor (MOR) to the plasma membrane after endocytosis promotes rapid resensitization of signal transduction, whereas targeting of the delta opioid receptor (DOR) to lysosomes causes proteolytic down-regulation. Recently, a G protein-coupled receptor-associated sorting protein (GASP) that binds preferentially to the cytoplasmic tail of the DOR compared with MOR has been identified. Disruption of

the DOR-GASP interaction inhibited receptor trafficking to lysosomes and promoted recycling. The GASP family of proteins may modulate lysosomal sorting and functional down-regulation of a variety of G protein-coupled receptors (Whistler et al., 2002). For the mu opioid receptor, filamin A is known to be involved in receptor trafficking (Onoprishvili et al., 2003). 2) A receptor-associated protein may directly mediate receptor signaling, as in the case of G proteins, or interupt agonist-mediated G protein activation as periplakin (Feng et al., 2003). 3) It may act as an allosteric modulator of receptor conformation, altering receptor pharmacology and/or other aspects of receptor function. 4) It may act as a scaffold, physically linking the receptor to various effectors. These four roles are by no means mutually exclusive. Each receptor-associated protein may fill one, two, three, or all four of these roles.

1.6. Aim of the project

Based on the significance of receptor-associated proteins in disclosing the molecular mechanism of opioid receptor functioning, we searched for proteins interacting directly with rat mu opioid receptor, rMOR1 and rMOR1B. Our plan was to first identify the putative interaction by use of a yeast two hybrid screen and subsequently to confirm the physiological interaction by coimmunoprecipitation and bioluminence resonance energy transfer (BRET). The full length of rat mu opioid receptor (rMOR1), the C-termini of the mu opioid receptors, rMOR1 and rMOR1B, and the C-terminus plus the third intracellular loop of rMOR1 were used as bait proteins separately in two hybrid screen. Once the potential interacting proteins were found, functional studies were carried out.

2. Materials

2.1 Lab instruments and materials

UV-visible Spectrophotometer (Pharmacia Biotech, Germany)

PTC-0200 DNA Engine (MJ Research, Inc. USA) for PCR

Gene Pulser II and Pulse Controller Plus (Bio-Rad) for Eletroporation

dNA sequencer, model 4000 (Li-cor, Germany)

Electrophoresis power supply (Bio-Rad)

Gel electrophoresis system (Bio-Rad)

Semi-dry Transfer Cell (Bio-Rad) for electroblotting

Leica TCS-NT laser-scanning confocal microscope (Leica Microsystems, Germany)

Fusion™ Universal Microplate Analyzer (BioSignal Packard Biosciences)

96-well white Optiplate (BioSignal Packard Biosciences)

Flask, plate and dish for cell culture (Greiner bio-one GmbH, Germany)

Incubator and super clear bench for cell culture (Kendro, Germany)

PCR purification Kit, Gel extraction Kit, Plasmid Midi Kit (QIAGEN, Germany)

2.2 Chemicals

X-gal (Sigma)

Herring testis Carrier DNA (Clontech)

Sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech)

Fetal calf serum (FCS) (Bachem, Heidelberg, Germany)

DAMGO (Sigma)

[^{3}H] DAMGO (NEN, Köln, Germany)

Morphine (Synopharm, Barsbüttel, Germany)

Naloxone (Tocris)

Forskolin (Biotrend, Köln, Germany)

PMA (Sigma)

Ammonium persulfate (Sigma)

30% acrylamide mix (Carl Roth GmbH & Co)

TEMED (Serva)

Lipofectamine (Life technologies, Invitrogen)

DSP (Pierce)

HEPES (Serva)

Enhanced chemiluminescence detection system (Amersham Biosciences).

DPX (Fluka, NeuUlm, Germany)

Deep Blue C (coelenterazine) (BioSignal Packard Biosciences)

Protein A-agarose beads (Amersham Biosciences, Sweden)

Effectene (QIAGEN, Germany)

G418 (Gibco)

Puromycin, Ampicillin, Penicillin and Streptomycin (Sigma)

2.3 Bacterial, yeast and eukaryotic cell line

E. coli XL1 (Promega)

Yeast AH109, Y187 (Clontech)

Human embryonic kidney HEK 293 cell (ATCC CRL 1573).

2.4 Plasmids

pRc/CMV - rMOR1 from Dr. Lei Yu (Indianapolis, IN)

pcDNA3.1- rMOR1B from Alexander Zimprich (Zimprich et al., 1995)

pcDNA3.1- PLD2 from Dr. S. Ryu (Pohang, South Korea)

pGBKT7, pGADT7, pGBKT7-53, pGBKT7-Lam, pGADT7-T (Clontech)

pCMV-Myc (Clontech)

pcDNA3.1 (Invitrogen)

pCMV-SPORT6-M6a (RZPD clone ID: IRAKp961F0453Q2, Germany)

pRluc-N_3 and $pGFP^2$-C_3 vectors (BioSignal Packard Biosciences)

rat brain cDNA library (in E.coli BNN132, Clontech)

2.5 Mediums

Dulbecco's modified Eagle's medium (Gibco)

YPDA (Gibco)

LB media and LB-Agar media (Gibco)

–Leu/–Trp Dropout Supplement (Clontech)

–Ade/–His/–Leu/–Trp Dropout Supplement (Clontech)

–Trp Dropout Supplement (Clontech)

2.6 Enzymes

All endonuclease from New England Biolab

Taq DNA polymerase, T4 ligase, PCR kit from Promega

RNase A from Sigma

2.7 Antibodies

Rabbit anti-human PLD antibody (provided by Dr. S. Ryu, Pohang, South Korea)

Mouse anti-Myc monoclonal antibody (Clontech)

Cyanine 3.18-conjugated anti-mouse antibodies, cyanine 3.18 (Cy3)-conjugated anti-rabbit antibodies and cyanine 5.18-conjugated anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA)

2.8 Buffers and Solvents

Z-buffer:

$Na_2HPO_4.7H_2O$, 16.1g/l; $NaH_2PO_4.H_2O$, 5.5g/l; KCL, 0.75g/l; $Mg_2SO_4.7H2O$, 0.246g/l; pH 7.0.

X-gal stock solution: 20 mg X-Gal in 1 ml dimethylformamide (DMF).

Z-buffer/X-gal solution:

100ml Z-buffer with 0.27 ml β-mercaptoethanol and 1.67 ml X-α-gal stock solution

PEG-LiAc-TE solution: 40% PEG, 50 mM Tris-EDTA, 100 mM LiAc

Breaking buffer (buffer F):

2% TritonX-100, 1% SDS, 100 mMNaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 8.0

Radioimmune precipitation buffer (RIPA buffer):

50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM disodium pyrophosphate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and the following proteinase inhibitors: 0.2 mM phenylmethyl-sulfonyl

fluoride, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml aprotinin, and 10 µg/ml bacitracin. (proteinase inhibitors were added prior to use)

SDS-sample buffer:

62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 0.005% bromphenol blue, 100 mM DL-dithiotreitol (dithiotreitol was added prior to use)

1 X TPBS: 10 mM Tris, 10 mM phosphate buffer, 137 mM NaCl and 0.05% thimerosal, pH 7.4

1 X PBS: 137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4

1:1 TE/LiAC buffer: 50 mM Tris-EDTA, pH 7.5, 100 mM LiAc

1 X TAE: 40 mM Tris-acetate, 1 mM EDTA

Trypsin/EDTA solution: 0.5 mM EDTA and 0.05% Trypsin

3. Methods

3.1. Yeast two hybrid assay

3.1.1. Principle of the two hybrid assay

Yeast GAL4 protein is a transcriptional activator. It consists of two physically separable, functionally independent domains: one acts as the DNA-binding domain while the other functions as the transcriptional activation domain. In the yeast two hybrid MATCHMAKER system, a bait gene is expressed as a fusion to the GAL4 DNA-binding domain (DNA-BD), while a pray, another gene or cDNA library, is expressed as a fusion to the GAL4 activation domain (AD). When bait protein and pray protein (library protein) interact with each other, the DNA-BD and AD are brought into proximity, this will activate transcription of reporter genes (Figure 3.1). The yeast strain Y187 has one reporter, lacZ, the yeast strain AH109 has three reporters-ADE2, HIS3 and lacZ. After expression of ADE2 (encoding phosphoribosylamino-imidazole-carboxylase) and HIS3 (encoding imidazoleglycerol-phosphate dehydratase) genes, yeast AH109 with (ADE^-, HIS^-) phenotype requires no adenine and histidine in the medium to grow. Lac Z encodes β-galactosidase that can be checked by β -galactosidase assay.

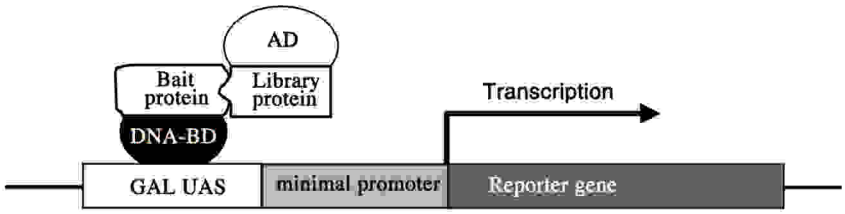


Figure 3.1. The two hybrid principle

3.1.2 Procedure

3.1.2.1. Construction of DNA-BD/bait fusion plasmids

Four baits were used for yeast two hybrid screen: full length of rMOR1, the carboxyl cytoplasmic terminus of rMOR1 (amino acids 340-398), the carboxyl terminus of rMOR1B (amino acids 340-391), and the receptor truncation from the third intracellular

loop of rMOR1 (amino acids 258-398). Using the following primers, these baits were independently subcloned into pGBKT7 vector which contains sequence encoding DNA-BD.

Forward primer for subcloning full length of rMOR1 5´-GTC CCA GAC CAT ATG GAC AGC AGC ACC-3´, reverse primer 5´-AGA TGG TGT GAA TTC CAG TTA GGG CAA-3´. Forward primer for subcloning the carboxyl terminus of rMOR1, 5´-CTT TAC GCC CAT ATG GAT GAA AAC TTC AAG CGA-3´, reverse primer 5´-CTC TAG CTG CAG TTA GGG CAA TGG-3´. Forward primer for subcloning truncation from the third intracellular loop of rMOR1: 5´-TGT TAC GGC CAT ATG ATC TTA CGA CTC AAG AGC-3´. reverse primer 5´-CTC TAG CTG CAG TTA GGG CAA TGG-3´. Forward primer for subcloning the carboxyl terminus of rMOR1B, 5´-CTT TAC GCC CAT ATG GAT GAA AAC TTC AAG CGA-3´, reverse primer 5´-CTA GAT CTG CAG TCA AAA TAA ATC-3´. All forward primers introduced NdeI restriction site, reverse primer for full length of rMOR1 introduced EcoRI restriction site, the other three reverse primers introduced PstI restriction site.

These fusion constructs (pGBKT7-rMOR1, pGBKT7-rMOR1/C-terminus, pGBKT7-rMOR1B/C-terminus, or pGBKT7-rMOR1/C-terminus+3rd loop) were confirmed by double strand DNA sequencing.

3.1.2.2. Library amplification

Commercially available rat brain cDNA library (in E.coli BNN132) was amplified according to manufacturer's protocol. Briefly, 2 µl of the rat brain cDNA library culture were added to 9 ml of LB/Amp (50 µg/ml), and then 175 µl were spread on each of the 100 mm LB/Amp agar (50 µg/ml) plate. Plates were incubated at 30°C for 36-48 h. 5 ml of LB media containing 25% glycerol were added to each of the plate, colonies were scraped into the liquid media with spreading glass rod. The resuspended colonies were pooled in one flask and mixed thoroughly. After incubation at 30°C for 2-4 h with vigorous shaking (200 rpm), the library culture was collected for large scale plasmid preparation.

3.1.2.3. Large scale plasmid preparation

AD-library fusion plasmid was extracted with Qiagen plasmid Maxi kit according to the manufacturer's protocol. Library plasmid DNA was stored at –20°C.

3.1.2.4. Chemical sequential transformation of yeast

First, overnight culture of Yeast AH109 was made for making competent cells. The growth phase yeast (OD_{600}=0.5) was pooled to 50 ml tube, washed with H_2O and resuspended in 6 ml of TE/LiAC buffer. After centrifugation at 200 x g for 5 min, the supernatant was decanted. 2.5 ml H_2O was added to the tube. The cells were put on ice for 30 min. The fresh competent cells were used for transfection.

For transfection, 2 µl (2 µg) of each bait plasmid DNA (pGBKT7-rMOR1, pGBKT7-rMOR1/C-terminus, pGBKT7-rMOR1/C-terminus +3rd intracellular loop or pGBKT7-rMOR1B/C-terminus) and 5 µl (50 µg) herring testis carrier DNA were mixed, and then added into 50 µl yeast competent cells and 300 µl PEG-LiAc-TE solution. After incubation with shaking (200 rpm) at 30°C for 20 min, cells were heat shocked with shaking (200 rpm) for 20 min in 42°C water bath. Cells were put on ice for 1 min and then centrifuged for 3 min at 3000 rpm. The supernatant was decanted and the pellet was resuspended in 250 µl of H_2O. 100 µl of transformation mixture were spread onto 100 mm SD/-W plate followed by incubation at 30°C for 2 days until visible colonies were formed.

Competent cells of Yeast AH109 bait construct transformant were made as described above. 20 µl (20 µg) of the library DNA was mixed with the 70 µl (700 µg) of herring testis carrier DNA. 1 ml yeast competent cells and 6 ml of PEG-LiAc-TE solution were added into the tube containing library and carrier DNA. Transformation was done as described above, 300 µl of transformation mixture was spread onto each of 150 mm SD/-L-W plates followed by incubation at 30°C for 2 days to select the DNA-BD and AD transformant.

The clones grown on SD/-L-W media (low stringency plate) were transferred to 150 mm plate containing SD/-L-W-H-A media (high stringency plate) using colony-lift

technique with filter for selection of ADE2, HIS3 expression. Clones that can grow on high stringency plate were picked up for β-galactosidase assay.

3.1.2.5. β-galactosidase assay

First a filter was presoaked with Z-buffer/X-gal solution. Then a second filter was placed over the surface of the agar plate containing the yeast colonies with ADE2, HIS3 expression. The filter was pressed to pick up colonies from the plate, then submerged in liquid nitrogen for 10 sec to increase the permeability of the cell. After the filter was thawed, it was placed on the first filter presoaked with Z-buffer/X-gal solution. The filters were kept at 30°C. Clones that became blue within 8 h were regarded as positive and picked up for further investigation.

3.1.2.6. Plasmid amplification and mini-preparation from yeast

Single yeast transformant colony was inoculated in 5 ml of SD/-L-W liquid media. It was incubated at 30°C overnight with shaking at 250 rpm. Yeast cells were collected and 200 µl buffer F was added to lyse the cells. Cells were vortexed and resuspended, then moved to a 1.5 ml Eppendorf tube. 0.3 g glass beads (one PCR tube) and 200 µl phenol chloroform isoamylalcohol (25:14:1) were added to lyse the cells. The mixture was vortexed for 1 min, then centrifuged at 20,780 x g for 5 min. Plasmid DNA was precipitated from the supernatant by 15 µl NaAc (3M, pH5.2) and 116 µl isopropanol. The DNA pellet was washed and dissolved in 50 µl H_2O, then stored at -20°C.

3.1.2.7. Rescue AD/library plasmids via electroporation transformation of E. coli

The plasmid DNA isolated from yeast was a mixture of the DNA-BD/bait plasmid and AD/library plasmid. AD vectors contain an ampicillin marker. By transforming the yeast-derived plasmid DNA into E. coli and selecting for transformants that can grow on LB medium containing ampicillin, AD/library plasmid can be rescued.

3.1.2.7.1. Making competent cells

50 ml fresh growth phase culture (OD_{600} = 0.3-0.4) of E. coli XL1 was harvested by

centrifugation for 5 min at 3,800 x g and chilled, cells were washed twice with 15 ml of ice-cold water containing 5% glycerol. The volume of the cells was estimated, glycerol was added to achieve final concentration of 10%. 80 µl competent cells were aliquoted to each microeppendorf tube and stored at –70°C.

3.1.2.7.2. Electroporation

Competent cells were thawed on ice, 1 µl (1 µg) of plasmid DNA is added to each tube containing 80 µl competent cells, cells were transferred to precooled 0.2cm cuvette, Electroporator parameter setting was as the following: voltage, 2.4 kV; capacitor, 25 F; resistor, 200 Ohme; time constant, 5.0 ms.

1ml LB media was added to the cuvette and mixed. 100 µl of the mixture were plated on LB/Amp agar plate, the plate was incubated at 37°C overnight.

3.1.2.7.3. Plasmid amplification and mini-preparation

Single colony of E.coli transformant was inoculated into 3ml LB/Amp media for plasmid amplification. Plasmid is extracted with the Qiagen plasmid buffer set according to the manufacturer's protocol. Extracted plasmid DNA was stored at –20°C.

3.1.2.8. Yeast mating (microtiter plate method)

3.1.2.8.1. Principle

Yeast mating is a convenient method of introducing two plasmids into the same host cells. This can be used to verify that the candidate AD/library protein identified in the yeast two hybrid library screen can activate the reporter genes only in the presence of DNA-BD/bait protein.

pGBKT7-Lam encodes a fusion of the DNA-BD with human lamin C and provides a negative control. Lamin C neither forms complexes nor interacts with most other proteins. pGBKT7-53 encodes the fusion protein of GAL4 DNA-BD and murine p53, and pGADT7-T encodes the fusion protein of AD and SV40 large T-antigen. p53 and large T-antigen interact in yeast two hybrid assay and are used as positive controls.

3.1.2.8.2. Procedure

Yeast strain Y187 was transformed with the following plasmids encoding bait or control: pGBKT7, pGBKT7-rMOR1, pGBKT7-rMOR1/C-terminus, pGBKT7-rMOR1/C-terminus+3rd loop, pGBKT7-rMOR1B/C-terminus and pGBKT7-Lam separately. And yeast strain AH109 was transformed with pGADT7 and DNA-AD-library fusion plasmid identified from cDNA library separately. For each type of plasmid encoding prey to be tested, a single transformant colony was placed in 1 ml YPD medium. For each type of plasmid encoding bait, pGBKT7-Lam or pGBKT7, several transformant colonies were placed in 3 ml YPD media. After vortex, in a 96-well microtiter plate containing 150 µl YPD medium in each well, 30 µl of the cell suspension containing pGADT7 or DNA-AD/library fusion plasmid were put into each well of a vertical column, 20 µl of the cell suspension containing pGBKT7, DNA-BD/bait fusion plasmid or pGBKT7-Lam were put into each well of a horizontal row. In this arrangement, each row represents one type of plasmid. The microtiter plate was put on shaker at 200 rpm, 30°C overnight. 100 µl of mating culture from each well were spread on SD/-L-W plate at 30°C, 3 days was required for the formation of diploid cells, His/Ade and LacZ phenotype of the cotransformants was checked as described above. Yeast strain Y187 transformed with pGBKT7-53 and yeast strain AH109 transformed with pGADT7-T was mated as positive control. The true positives were DNA-AD-library clones exhibiting reporter gene expression only when the DNA-AD-library fusion plasmid was introduced by mating only with the fusion plasmid encoding the DNA-BD/bait protein, but not the others.

3.1.3. Plasmid sequencing

To acquire the nucleotides sequence, DNA-AD/library fusion plasmids identified as true positives were purified with phenol/chloroform and sequenced using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit according to the manufacturer’s protocol.

3.1.4. Analysis of nucleotide sequence

After sequencing, the nucleotide sequences of the cDNA inserts were analyzed with the Standard Nucleotide-nucleotide BLAST search from NCBI gene database. (http://www.ncbi.nlm.nih.gov/blast).

3.2. Coimmunoprecipitation

3.2.1. Principle

Coimmunoprecipitation is designed to detect protein-protein interaction. When a cell is lysed under nondenaturing conditions, many of the protein-protein associations that exist within the intact cell are conserved. First an antibodies specific to the interesting protein is used to immunoprecipitate the interesting protein. If there are any proteins that are stably associated with the interesting protein in vivo, they may also be precipitated. Identification of these proteins can be determined by western blot.

3.2.2. Procedure

3.2.2.1. Plasmid construction

For cloning of the full length gene of synaptophysin (Syp), polymerase chain reaction (PCR) primers were designed based on the sequence of cDNA segment from the library obtained from yeast two hybrid assay. The sequence of forward primer introducing a SalI restriction site is 5´-TAG CTT GTC GAC TAT GGA CGT GGT GAA TCA GCT GGT GGC TGG GGG TCA GTT CCG GGT GGT CAA GGA GCC CCT TGG CTT CGT GAA G-3´, and sequcence of reverse primer introducing a KpnI restriction site is 5´-ACT TCA GGT ACC AGA TTA CAT CTG ATT GGA-3´. Using the pAD/Syp plasmid containing part length of Syp (nucleotides 49-924) as template, the full length gene of Syp was amplified and subcloned into the SalI/Kpn I sites of pCMV-Myc expression vector with T4 DNA ligase to produce pCMV-Myc-Syp. The resultant construct fused the Myc tag in front of the start codon of Syp.

pCMV-Myc-M6a was constructed from pCMV-SPORT6-M6a containing M6a gene

by PCR. Forward primer 5´-GAA GAA GAA TTC CC ATG GAA GAG AAT ATG G-3´ introduced EcoRI restriction site, and the reverse primer 5´-AGA GAA GGT ACC TTA TGT GTA CGC ATT GAG-3´ introduced KpnI restriction site.

3.2.2.2. Cell culture and transfection

HA-rMOR1 cells (HEK 293 cells stably expressing rMOR1 with a N-terminal HA epitope tag) were maintained in Dulbecco's modified Eagle´s medium supplemented with 10% fetal calf serum and 1.25 µg/ml puromycin in a humidified atmosphere containing 10% CO_2.

Transient transfection of HA-rMOR1 cells with pCMV-Myc-Syp or M6a was performed using lipofectamine according to the manufacturer's protocol. Briefly, the day prior to transfection, 1.5×10^6 HA-rMOR1 cells were seeded in 100 mm plate without antibiotics so that they were about 80% confluent before transfection. For each plate, quantities of 20 µg of pCMV-Myc-Syp or pCMV-Myc-M6a plasmid DNA and 20 µl lipofectamine were used. 48 h after transfection, cells were subjected to immunoprecipitation.

To generate cell lines stably coexpressing HA-rMOR1 and PLD2, HA-rMOR1 cells were transfected with pcDNA3-PLD2 using effectene and selected in the presence of 1 µg/ml puromycin and 500 µg/ml G418. The whole pool resistant cells were used without selection of individual clones. Receptor and PLD2 expression was monitored using immuno-cytochemical method.

3.2.2.3. Immunoprecipitation and western blot analysis

3.2.2.3.1. Detection of interaction of rMOR1 with various proteins

48 h after transfection, cells were incubated in OPTIMEN medium for 30 min. Then incubated with 6 ml of 5 mM dithiobis-(succinimi-dylpropionate) in 10 mM Hepes/PBS for 20 min at room temperature. Subsequently, cells were lysed using 1 ml of RIPA buffer at 4°C for 45 min with gentle shaking. After centrifugation at 40,000 × g for 1 h, the supernatant was collected. The receptor proteins were then immunoprecipitated with 50 µl

of protein A agarose beads preloaded with 5 μg anti-HA antibodies at 4ºC for 5 h. Beads were washed five times with RIPA buffer, and immunoprecipitates were eluted from the beads with 100 μl of SDS-sample buffer at 60°C for 20 min. The receptor or potential interacting protein in each sample was separated with regular 8% or 12% SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred from the gel to nitrocellulose membrane by electroblotting. Then membranes were incubated with either mouse monoclonal anti-Myc or affinity-purified rabbit anti-HA antibody at a concentration of 1 μg/ml for 12 h at 4°C, followed by detection using an enhanced chemiluminescence detection system.

3.2.2.3.2. Immunoprecipitation of PLD2 and rMOR1

Cells stably coexpressing PLD2 and HA-rMOR1 were plated onto poly-L-lysine-coated 150-mm dishes and grown to 80% confluence. Cells were then washed twice with PBS and harvested into ice-cold lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, 10 mM iodoacetamide, and the following proteinase inhibitors: 0.2 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mg/ml aprotinin, 10 mg/ml bacitracin). Cells were swollen for 15 min on ice and homogenized. The homogenate was spun at 500 × g for 5 min at 4°C to remove unbroken cells and nuclei. Membranes were then pelleted at 20,000 × g for 30 min at 4 °C, and pelleted membranes were lysed in HEPES detergent buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 3 mM EGTA, 4 mg/ml b-dodecylmaltoside, 10 mM iodoacetamide, and proteinase inhibitors as above) for 1 h on ice. The lysate was centrifuged at 20,000 × g for 30 min at 4 °C.

For immunoprecipitation, the resulting supernatant was loaded on 100 μl of protein A-agarose beads preloaded with 10 μg of anti-HA antibodies. Beads were washed with detergent buffer, and absorbed proteins were eluted into 200 μl of SDS-sample buffer at 60°C for 20 min. After SDS-polyacrylamide gel electrophoresis and electroblotting, membranes were incubated with 1 μg/ml rabbit anti-human PLD2 antibody or anti-HA

antibody for 12 h at 4°C. Immunoreactive bands were visualized by using an enhanced chemiluminescence detection system.

3.3. Immunocytochemistry

3.3.1. Coexpression of HA–rMOR1 and rMOR1 interacting proteins

Using PCR method, Myc-Syp was subcloned from pCMV-Myc-Syp to pcDNA3 vector harboring G418 resistance marker to generate Myc-Syp stable expressing cell lines. To perform this subclone, forward primer of 5´-CGC GGG AAG CTT ATG GCA TCA ATG CAG AAG-3´ was used to introduce Hind III restriction site, and the reverse primer 5´-CGC GGT TCT AGA TTA CAT CTG ATT GGA GAA-3´ was used to introduce XbaI restriction site. Transfection of HA-rMOR1 cells with pcDNA3.1-Myc-Syp was done with lipofectamine according to the manufacturer´s protocol. Cells were selected in the presence of 1.25 μg/ml puromycin and 500 μg/ml G418.

3.3.2. Coexpression of rMOR1-GFP and rMOR1 interacting proteins

Using the forward primer 5´-GAC TTG TTG CTG CAG CTT CAC ACC ATG GAC-3´ to introduce Pst I restriction site, and the reverse primer 5´-TGT GAG ACC AAG CTT GGG CAA TGG AGC AGT-3´ to introduce Hind III restriction site and delete the stop condon, the cDNA of rMOR1 was amplified using pRc/CMV-rMOR1 as template and subcloned into humanized pGFP-N_3 expression vector to produce pGFP-N_3-rMOR1. The resultant construct fused the GFP tag to the carboxyl terminal tail of rMOR1. Transient co-transfection of HEK 293 cells with pGFP-N_3-rMOR1 and pCMV-Myc-M6a was done using lipofectamine according to the manufacturer´s protocol. About 20 h after transfection, cells were subjected to immunocytochemical study.

3.3.3. Staining

Cells were grown on poly-L-lysine-treated coverslips for 24 or 48 h and then untreated or exposed to 1 μM naloxone or 1μM DAMGO as indicated. Cells were fixed with 4% paraformaldehyde and 0.2% picric acid in phosphate buffer, pH 6.9, for 40 min at room temperature. Then cells were washed several times with TPBS. Specimens were then

incubated for 3 min in 50% and 100% methanol respectively. Subsequently washed several times in TPBS and then preincubated with TPBS containing 3% normal goat serum for 2 h at room temperature. For single immunofluorescence, detection of cells coexpressing GFP-rMOR1 and Myc-M6a, cells were incubated with mouse anti-Myc antibody at a concentration of 1 μg/ml in TPBS and 1% normal goat serum overnight. Bound primary antibody was detected with cyanine 3.18 (Cy3)-conjugated anti-mouse antibodies. For double immunofluorescence, cells coexpressing HA-rMOR1 and Myc-Syp, cells were incubated with a mixture of mouse monoclonal anti-Myc and affinity-purified rabbit anti-HA antibodies at a concentration of 1 μg/ml. Bound primary antibodies were detected with a mixture of cyanine 3.18 (Cy3)-conjugated anti-mouse and cyanine 5.18 (Cy5)-conjugated conjugated anti-rabbit secondary antibodies. Cells were then dehydrated with enthanol, cleared in xylol and permanently mounted in DPX. Specimens were examined using a Leica TCS-NT laser-scanning confocal microscope, equipped with a krypton/argon laser. GFP was imaged with 488 nm excitation and 530 nm emission filter, Cyanine 3.18 was imaged with 568 nm excitation and 570–630 nm band pass emission filters, and cyanine 5.18 with 647-nm excitation and 665-nm long pass emission filters.

3.4. BRET assay

3.4.1 Principle of BRET assay

Bioluminescence Resonance Energy Transfer (BRET) is a proximity assay based on non radiative transfer of energy between a bioluminescent donor (Rluc) and a fluorescent acceptor (GFP) that allows real time monitoring of protein-protein interaction in living cells. For BRET principle, see Figure 3.2.

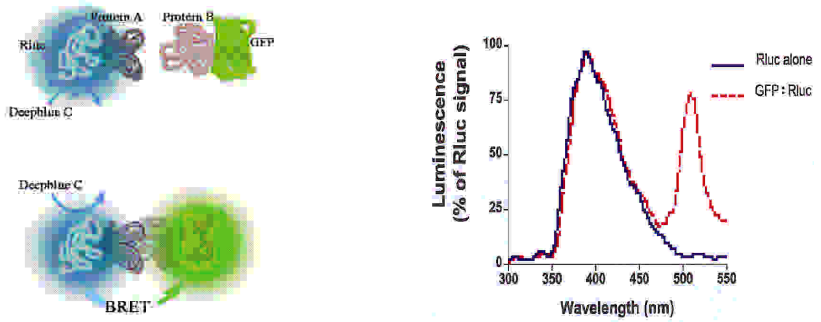


Figure 3.2. BRET principle. Resonance Energy Transfer occurs when part of the energy from DeepBlueC (DBC)-bound Rluc is transferred to GFP, which in turn, emits green light. If Rluc and GFP are not in close proximity (upper left panel), energy is not efficiently transferred and only the blue light emitted by the Rluc/DBC reaction is detected. When Rluc and GFP are brought into close proximity (lower left panel), by means of a specific biological interaction between Protein A and Protein B (fused to Rluc and GFP, respectively), energy is efficiently transferred from DBC-bound Rluc to GFP resulting in the production of green light. The BRET signal is determined by measuring the ratio of green light (515nm) over blue light (410nm) (right panel).

3.4.2. Procedure

3.4.2.1. Donor and acceptor plasmid construction

Construction of donor plasmid: Using Pst I and Hind III enzymes, the cDNA for rMOR1 was cut out from pGFP-N_3-rMOR1 and then subcloned into Pst I and Hind III sites of humanized pRluc-N_3 to produce pRluc-N_3-rMOR1. The resultant construct fused the Rluc tag to the C-terminus of rMOR1. rMOR1B coding sequences without stop codon were amplified from pcDNA3.1-rMOR1B by PCR using the following sense and antisense primers harboring unique Pst I and Kpn I sites, respectively. Sense primer: 5´-AAG CTT CTG CAG ATG GAC AGC AGC ACC GGC-3´; antisense primer: 5´-TAT ATG GGT ACC CAA AAT AAA TCT ATT TTC-3´. Purified PCR products were digested and subcloned into the Pst I and Kpn I sites of humanized pRluc-N_3 encoding a Rluc tag downstream to the Kpn I insertion site, which produce pRluc-N_3-rMOR1B.

Construction of acceptor plasmid: The M6a or Syp gene was cut out from pCMV-Myc-M6a or pCMV-Myc-Syp and subcloned into the EcoR I and Kpn I sites of pGFP-C_3 expression vector to produce pGFP-C_3-M6a or pGFP-C_3-Syp. These resultant constructs fused the GFP tag to the N terminus of M6a or Syp.

3.4.2.2 Co-transfection of HEK 293 cells with donor and acceptor plasmid

The day prior to transfection, HEK-293 cells were seeded in a 24-well plate, cells were 90% confluent before transfection. Cells were transfected with donor plasmid and acceptor plasmid using lipofectamine according to the manufacturer's protocol.

3.4.2.3 BRET detection

48 h post-transfection, cells were detached with PBS/0.5mM EDTA and resuspended in PBS containing 0.1% glucose and 2 µg/ml aprotinin. 40 µl of cells were transferred to 96-well white Optiplate at a density of 10^5 cells/well. 10 µl of Deep Blue C was added at a final concentration of 5 µM. The BRET signal were determined using Fusion™ Universal Microplate Analyzer using the following settings: Mode: dual wavelength; Read length: 1 second per wavelength; PMT: 1100 volts; Gain: 100; Filter pair: 410/80 nm (Rluc emission) and 515/30 nm (GFP emission). The BRET signal is determined by the following formula.

$$\text{BRET signal} = \frac{\text{(emission at 515 nm)-(background emission at 515 nm)}}{\text{(emission at 410 nm)-(background emission at 410 nm)}}$$

3.5. Radioligand binding assay

Binding studies were performed on membranes prepared from stably transfected cells. For whole cell binding, 10^6 cells were incubated with 2.5 nM [^{3}H]DAMGO for 40 min at 25 °C in 50 mM Tris-HCl, pH7.8. Cells were collected on GF 10 glassfiber filters and unbound ligand was removed by extensive washes with 50 mM Tris-HCl, pH7.8. The radioactivity on the filters was determined by liquid scintillation counting. Specific binding was calculated by subtracting nonspecific binding from total binding. Nonspecific binding was determined as radioactivity bound in the presence of 1 µM unlabeled DAMGO. Results were calculated as fmol bound radioligand per mg of protein, measured by the Lowry method. The binding characteristics of the receptor in cell expressing rMOR1 alone or coexpressing rMOR1 and PLD were determined by saturation binding assays on

membranes prepared from transfected HEK 293 cells. The dissociation constant (K_D) and number of [^{3}H]DAMGO binding sites (Bmax) were calculated by Scatchard analysis using at least seven concentrations of labeled DAMGO in a range from 0.3 to 9 nM.

3.6. Detection of PLD activity

3.6.1 Principle

PLD hydrolyses the distal phosphodiester bond in phospholipids such as phosphotidylcholine (PC). A phosphatidyl-enzyme intermediate is transiently formed which is normally hydrolysed by water, generating phosphatidic acid (PA). Primary short chain alcohol ethanol can substitute for water in a competing transphosphatidylation reaction resulting phosphatidylethanol (PEt)(Figure 3.3.). This reaction (thick arrow pointing to the left) occurs at the expense of the hydrolytic reaction (thin arrow pointing to the right), decreasing PA formation. Phosphatidylalcohols are metabolically stable and would accumulate in cells upon PLD activation. Because cellular phosphatidylalcohol level is normally extremely low, its accumulation upon PLD activation is readily detectable and can be used as a marker of PLD activation.

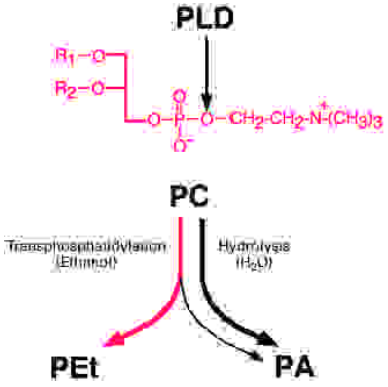


Figure 3.3. Phospholipase D-catalysed reactions

3.6.2 Procedure

HEK 293 cells coexpressing rMOR1 and PLD2 were kept in serum-free OPTIMEM containing [1,2,3-^{3}H]glycerol (1 µCi/ml; specific activity 40 Ci/mmol) for 24 h in order to

label phospholipids. Cells were then exposed to serum free medium containing drugs and 2% ethanol. The following drugs were added as aqueous solutions resulting in final concentrations of 1 µM DAMGO, 1 µM phorbol-12- myristate-13-acetate (PMA). After 30 min of drug treatment, cells were extracted in 2.5 ml of ice-cold methanol/water (3:2, v/v). Subsequently, 1.5 ml chloroform and 0.35 ml H_2O were added, and the lipid phase was separated by thin layer chromatography using the lower phase of methanol/chloroform/water (10:10:9, v/v/v). Individual phospholipids were stained with iodine, identified by standards, and spots corresponding to phosphatidylethanol (PtdEtOH), PA, and PC were isolated and subjected to liquid scintillation counting. PLD activity was expressed as percent [^{3}H]PtdEtOH of the total cellular PC concentration.

3.7. Data analysis

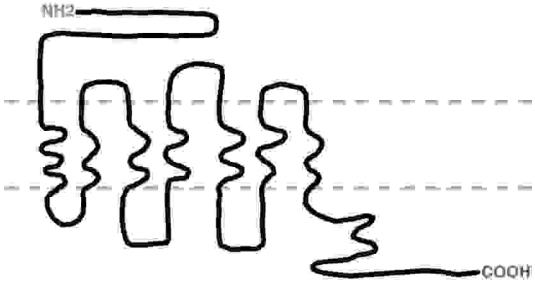
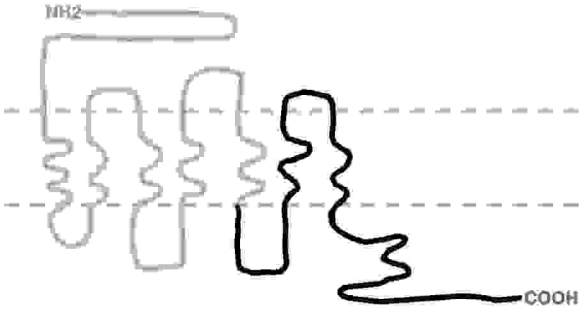
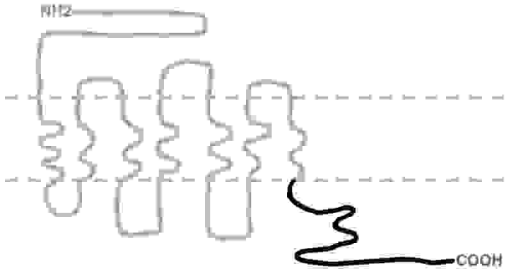
Data from ligand binding, BRET assays and PLD assay were analyzed by nonlinear regression curve fitting using GraphPad Prism 3.0 software.

4. Results

4.1. Search for proteins interacting with the mu opioid receptor

To identify proteins interacting with rMOR1 and rMOR1B, a yeast two hybrid system was used. The full length of rMOR1, the 3rd intracellular loop plus the carboxyl terminus of rMOR1, and the carboxyl termini of rMOR1 and rMOR1B were used as baits to screen a rat brain cDNA library. Over 1200 positive clones were analyzed. Most of these clones were genes from mitochondria and ribosomes which normally do not interact with membranous receptors under physiological conditions. Potentially interesting clones includes one clone encoding the NH_2-terminal amino acids 116–226 of phospholipase D2 (representing exon 4-8), one clone encoding amino acids 108-278 of membrane glycoprotein M6a, two clones encoding amino acids 17-307 and amino acids 81-307 of synaptophysin. The following proteins listed in table 4.1 showed specific interaction verified by yeast mating. Note that 1) synaptophysin interacts with full length of rMOR1, with the 3rd intracellular loop plus the cytoplasmic carboxyl terminus of rMOR1, but not with the cytoplasmic carboxyl termini of the receptors, indicating the 3rd intracellular loop of the receptor is the important site for the interaction. 2) PLD2 (amino acids 116-226) interacts with the carboxyl termini of rMOR1 and rMOR1B. 3) Glycoprotein M6a (M6a) (amino acids 108-287) interacts with the carboxyl terminus of rMOR1B but not with that of rMOR1. 4) Heat shock cognate protein 70 (amino acids 421-646) interacts with the carboxyl terminus of rMOR1.

Some proteins interacting with the cytoplasmic carboxyl terminus of the receptor failed to activate reporter gene while using the full length of the receptor as bait, this indicates the possibility that the protein complex containing the whole receptor is too large to be translocated through the nuclear pore to start transcription.

BAIT	PREY
Full length of rMOR1:  **MDSSTGPGNTSDCSDPLAQASCSPAPGSWLNLSHVDGNQSDPCGLNR TGLGGNDSLCPQTGSPSMVTAITIMALYSIVCVVGLFGNFLVMYVIVR YTKMKTATNIYIFNLALADALATSTLPFQSVNYLMGTWPFGTILCKIV ISIDYYNMFTSIFTLCTMSVDRYIAVCHPVKALDFRTPRNAKIVNVCN WILSSAIGLPVMFMATTKYRQGSIDCTLTFSHPTWYWENLLKICVFIF AFIMPVLIITVCYGLMILRLKSVRMLSGSKEKDRNLRRITRMVLVVV AVFIVCWTPIHIYVIIKALITIPETTFQTVSWHFCIALGYTNSCLNPVLY AFLDENFKRCFREFCIPTSSTIEQQNSTRVRQNTREHPSTANTVDRTN HQLENLEAETAPLP**	**Synaptophysin (Syp)** (amino acids 17-307), (amino acids 81-307) Gamma-glutamyltranspeptidase-like protein **Phospholipase D2 (PLD2)** (amino acids 116–226)
C-terminus and the third intracellular loop of rMOR1:  **RLKSVRMLSGSKEKDRNLRRITRMVLVVVAVFIVCWTPIHIYVIIKAL ITIPETTFQTVSWHFCIALGYTNSCLNPVLYAFLDENFKRCFREFCIPT SSTIEQQNSTRVRQNTREHPSTANTVDRTNHQLENLEAETAPLP**	**Synaptophysin** (amino acids 17-307), (amino acids 81-307)
C-terminus of rMOR1:  **DENFKRCFREFCIPTSSTIEQQNSTRVRQNTREHPSTANTVDRTNHQ** **LENLEAETAPLP**	**PLD2** (amino acids 116-226) **Heat shock cognate protein 70 (Hsc70)** (amino acids 421-646) Protein similar to interferon regulatory factor 3 Nuclear receptor binding factor 1

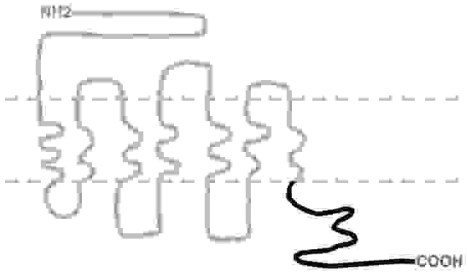
C-terminus of rMOR1B:	**PLD2** (amino acids 116-226)
 **DENFKRCFREFCIPTSSTIEQQNSTRVRQNTREHPSTANTVDRTNHQ** **KIDLF**	**Glycoprotein M6a** (amino acids 108-287) Cadherin Protein similar to DKF2P586G1722 Carboxypeptidase E Chaperonin subunit 6 a Neuron-specific enolase Rhotekin Interferon-gamma receptor 10 days neonate head cDNA N-terminal Asn amidase Cytoplasmic gamma isoform of actin Actin-related protein Adult male hippocampus cDNA Autoantigen ssb Dehydrooratate dehydrogenase Complement component1 q subcomponent

Table 4.1. Identification of the mu opioid receptor interacting proteins by yeast two hybrid assay. The table shows the proteins that potentially interact with different baits of rMOR1 or rMOR1B after yeast mating analysis. The amino acid sequences of baits are shown in the left column. The sequence difference at the C terminus between rMOR1 and rMOR1B are underlined.

The receptor interacting proteins resulting from the yeast two hybrid assay exhibit a wide variety of functions. For example, rhotekin is a partner of small GTPase Rho that can inhibit Rho GTPase activity; Complement component1 q subcomponent might be involved in the cross-talk between the opioid system and the immune system. Hsc70 is an ATPase involved in uncoating of clathrin coated vesicle; synaptophysin interacts with dynamin which regulates the fission of the endocytotic vesicle; M6a is a membrane glycoprotein abundant in the central nervous system (CNS) neurons, which may be implicated in the trafficking of the mu opioid receptor; PLD2 catalyzes the production of phosphatidic acid (PA), which participates in vesicle formation. Because we are specifically interested in proteins modulating opioid receptor trafficking, our work first focused on these proteins which might regulate the internalization and recycling of the mu opioid receptor.

4.2. Synaptophysin

The interactions resulting from yeast two hybrid assays were verified by coimmunoprecipitation and bioluminescence resonance energy transfer (BRET) assays in mammalian cells, where proteins are more likely to be in their native conformation and of correct posttranslational modification.

4.2.1. Coimmunoprecipitation of synaptophysin and rMOR1

To confirm the interaction between rMOR1 and synaptophysin (Syp) in HEK 293 cells, we first carried out coimmunoprecipitation studies. rMOR1 was tagged with the HA epitope tag (HA-rMOR1), synaptophysin was tagged with the Myc epitope tag (Myc-Syp) both at the amino terminus. Differently epitope tagged rMOR1 and synaptophysin were co-expressed in HEK 293 cells. Expression of HA-rMOR1 and Myc-Syp was examined by directly immunoblotting lysates from these cells with antibodies against HA and Myc tag, respectively. A rMOR1 band migrating at about 75 KD and a synaptophysin band migrating at about 37 kD were detected (Figure 4.1, lane 1 and 5, lysate). For coimmunoprecipitation, HA-rMOR1 receptors were precipitated from the lysates of HA-rMOR1 stably expressing cells and cells coexpressing HA-rMOR1 and Myc-Syp using anti-HA antibodies. The resulting precipitates were immunoblotted with antibodies directed against Myc epitope tag. As shown in Figure 4.1 (lane 2, IP HA), Myc tagged synaptophysin migrating at about 37 KD was detected in immunoprecipitates from HEK 293 cells coexpressing HA-rMOR1 and Myc-Syp, suggesting that rMOR1 is physically associated with synaptophysin in vivo. In immunoprecipitates from HA-rMOR1 or Myc-Syp expressing control cells, no Myc-Syp was detected (Figure 4.1, lane 3 and 4, IP HA, respectively), indicating the specific interaction between rMOR1 and synaptophysin.

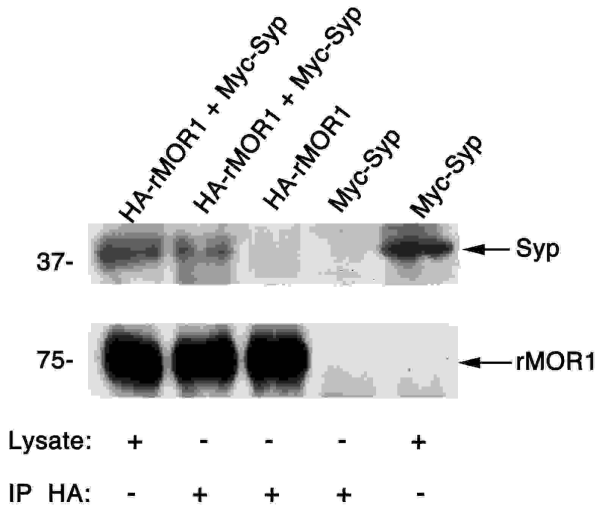


Figure 4.1. Interaction of rMOR1 and synaptophysin in HEK 293 cells detected by coimmunoprecipitation. Cell lysate proteins from HEK 293 cells coexpressing HA-rMOR1 and Myc-Syp (HA-MOR1+Myc-Syp) were extracted and either immunoblotted directly (lane 1, lysate) or immunoprecipiated using anti-HA antibodies (lane 2, IP HA). Cell lysate proteins from HEK 293 cells expressing HA-rMOR1 alone were extracted and immunoprecipiated using anti-HA antibodies (lane 3, IP HA). Lysate proteins from HEK 293 cells overexpressing Myc-Syp alone were extracted and either immunoblotted directly (lane 5, lysate) or immunoprecipitated using anti-HA antibodies (lane 4, IP HA). Note that synaptophysin were coimmunoprecipitated with the mu opioid receptor only from cells coexpressing HA-rMOR1 and Myc-Syp but not from cells expressing HA-rMOR1 or Myc-Syp alone. The positions of molecular mass markers are indicated on the left (in kDa). Arrows point to synaptophysin, rMOR1. Two additional experiments gave similar results.

4.2.2. Analysis of interaction of rMOR1 and synaptophysin by BRET

Detergent solubilization of cells during coimmunoprecipitation studies could promote artificial aggregation of hydrophobic proteins such as transmembraneous proteins. We therefore analyzed the interaction of the mu opioid receptor with synaptophysin in living HEK 293 cells using BRET. This technique is a proximity assay based on non radiative transfer of energy between a bioluminescent donor (Rluc) and a fluorescent acceptor (GFP) that allows real time monitoring of protein-protein interaction in living cells.

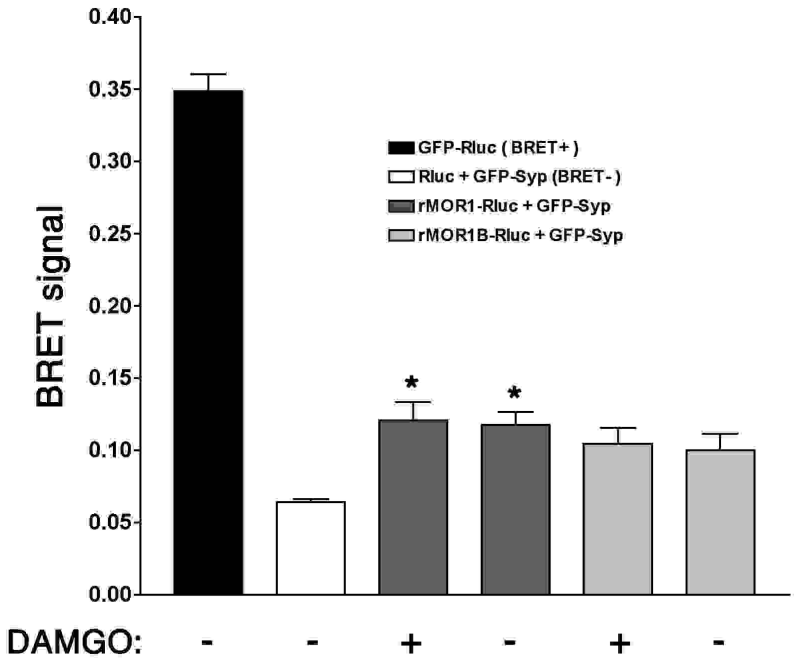


Figure 4.2. The interaction of the mu opioid receptor and synaptophysin in living HEK 293 cells detected by BRET. HEK 293 cells were transiently cotransfected with Rluc, rMOR1-Rluc or rMOR1B-Rluc in combination with GFP-Syp. A transfection with GFP-Rluc fusion protein was used as positive control. Cells were harvested 48 h post-transfection. The energy transfer was initiated by addition of 5 µM Deep Blue C coelenterazine, and BRET was assessed in a Fusion™ Universal Microplate Analyzer as described under "Method". For those treated with DAMGO, cells were exposed to DAMGO (1 µM final concentration) for 50 minutes in 37°C prior to BRET assay. Note that the full length of synaptophysin interacts with rMOR1 in living cells, but not with rMOR1B. No significant difference was detected when the cells were stimulated with 1 µM DAMGO. Data are presented as mean ± S.E. of 3 independent experiments performed in triplicate. Asterisk indicates significant difference ($p<0.05$) between cells coexpressing rMOR1-Rluc and GFP-Syp and cells coexpressing Rluc and GFP-Syp as determined using one way ANOVA followed by Bonferroni test.

To assess the interaction of synaptophysin with rMOR1 or rMOR1B, fusion constructs linking Rluc to the receptor carboxyl terminus and GFP linking to the amino terminus of synaptophysin were cotransfected in HEK 293 cells. The transfer of energy between the two partners was assessed following the addition of Deep Blue C. The BRET signal is determined by calculating the ratio of the light emitted by the GFP-Syp over the

light emitted by the receptor-Rluc. As shown in Figure 4.2, a very high BRET signal was obtained with the GFP-Rluc fusion protein (positive control), whereas a very small signal was obtained when GFP-Syp was coexpresed with soluble Rluc (negative control). A significant BRET signal was obtained in cells coexpressing rMOR1-Rluc/GFP-Syp indicating that the interaction between rMOR1 and synaptophysin also occurs in living cells; A tendency for interaction between rMOR1B and synaptophysin was also seen, which, however, did not show statistical significance. No significant changes were detected in the measured rMOR1-Rluc/GFP-Syp or rMOR1B-Rluc/GFP-Syp BRET levels when the cells were stimulated with 1 µM DAMGO, indicating the interaction is agonist independent.

4.2.3. Colocalization of rMOR1 and synaptophysin

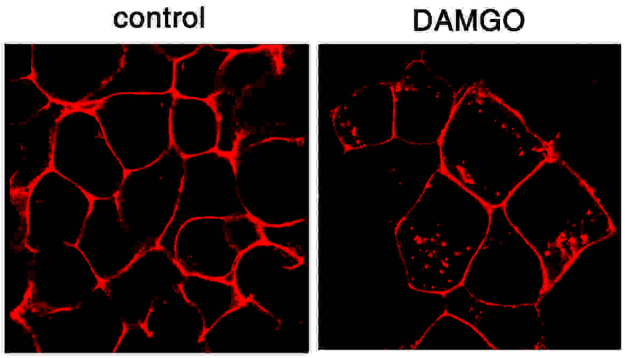


Figure 4.3. Localization of the mu opioid receptor in HEK 293 cells expressing HA-rMOR1 alone. HEK 293 cells expressing HA-rMOR1 were either not treated (left) or treated with 1µM DAMGO for 10 min (right). Cells were subsequently fixed, subjected to double immunofluorescent staining using rabbit anti-HA antibodies. Shown are representative results from one of three independent experiments performed in duplicate.

Previous studies showed that synaptophysin interacts with dynamin, which regulates the fission of endocytotic vesicle. To investigate how synaptophysin influences rMOR1 trafficking, Myc tagged synaptophysin and HA tagged rMOR1 were overexpressed in HEK 293 cells and the subcellular distribution of HA-rMOR1 and Myc-

Syp was examined using double immunofluorescence and confocal microscopy. In cells expressing HA-rMOR1 alone, HA-rMOR1 was localized in the cell membrane; Receptor endocytosis could be observed after DAMGO treatment for 10 minutes (Figure 4.3).

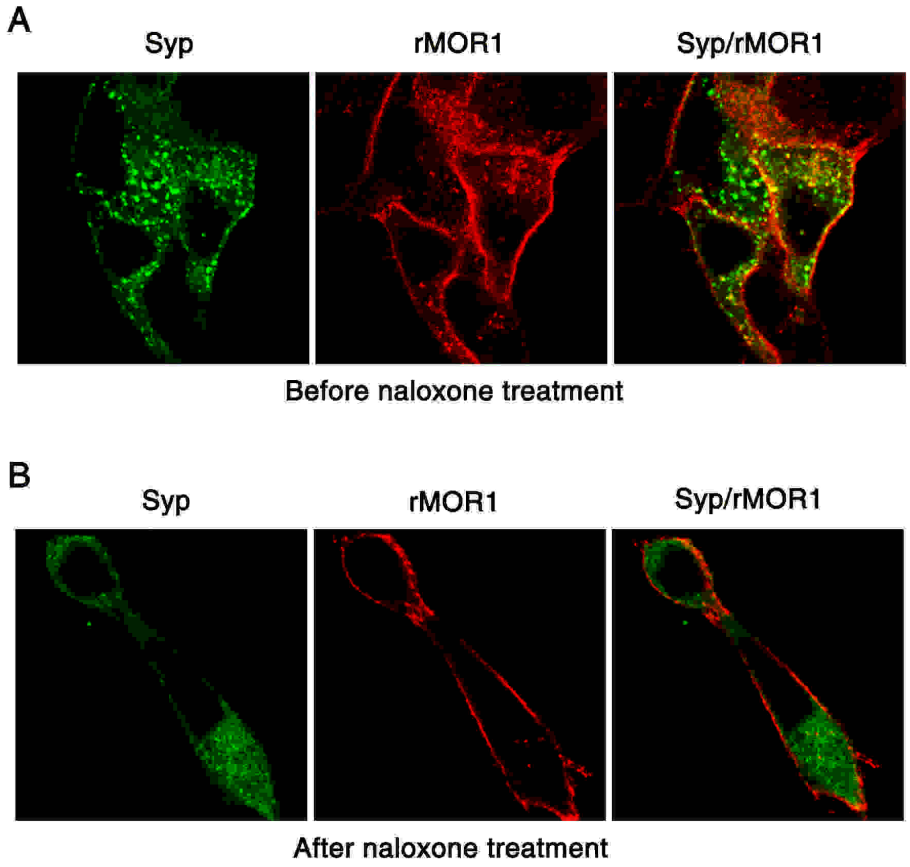


Figure 4.4. Cytosolic localization of the mu opioid receptor in Myc-synaptophysin/HA-rMOR1 coexpressing HEK 293 cells. HEK 293 cells coexpressing HA-rMOR1 and Myc-synaptophysin were either not treated (A) or treated with 1μM naloxone for 17 hours (B). Cells were subsequently fixed, subjected to double immunofluorescent staining using a mixture of rabbit anti-HA and mouse anti-Myc antibodies, and examined by confocal microscopy. Note that synaptophysin (green) is expressed in the cytosol, and in the meantime, without antagonist treatment, rMOR1 (red) is expressed both cytosolically and membranously bound, colocalization between rMOR1 and synaptophysin (yellow) can be detected. After naloxone treatment, rMOR1 is confined to the plasma membrane. Shown are representative results from one of three independent experiments performed in duplicate.

As shown in Figure 4.4 A, HA-rMOR1 was both cytosolically and membraneously localized with the coexpressed Myc-Syp in HEK 293 cells. To test the possibility that there is a constitutive internalization of rMOR1 in cells expressing synaptophysin, rMOR1-Syp expressing cells were incubated in the presence of 1 μM naloxone for 17 hours. The results in Figure 4.4 B revealed that rMOR1 was confined to the plasma membrane after antagonist treatment, indicating that synaptophysin facilitates the constitutive internalization of rMOR1.

4.3. Membrane glycoprotein M6a

4.3.1. Coimmunoprecipitation of rMOR1 and M6a

To detect an interaction between the mu opioid receptor and membrane glycoprotein M6a in HEK 293 cells, we carried out coimmunoprecipitation studies. rMOR1 was labelled with HA epitope tag, M6a was marked with Myc epitope tag (Myc-M6a) both at the amino terminus (HA-rMOR1). The differently tagged rMOR1 and M6a were coexpressed in HEK 293 cells. Expression of HA-rMOR1 and Myc-M6a was examined by directly immunoblotting lysates from these cells with antibodies against HA and Myc tag, respectively. A rMOR1 band migrating at about 75 KD and a M6a band migrating at about 30 kD were detected (Figure 4.5, lane 1 and 5, lysate). For coimmunoprecipitation, HA-rMOR1 receptors were precipitated from the lysates of HA-rMOR1 stably expressing cells and cells coexpressing HA-rMOR1 and Myc-M6a using anti-HA antibodies. The resulting precipitates were immunoblotted with antibodies directed against Myc epitope tag. As shown in Figure 4.5 (lane 2, IP HA), Myc tagged M6a migrating at about 30 KD was detected in immunoprecipitates from HEK 293 cells coexpressing HA-rMOR1 and Myc-M6a, suggesting that rMOR1 is physically associated with M6a in vivo. In immunoprecipitates from HA-rMOR1 or Myc-M6a expressing control cells, no Myc-M6a

was detected (Figure 4.5, lane 3 and 4, IP HA, respectively), indicating the specific interaction between rMOR1 and M6a.

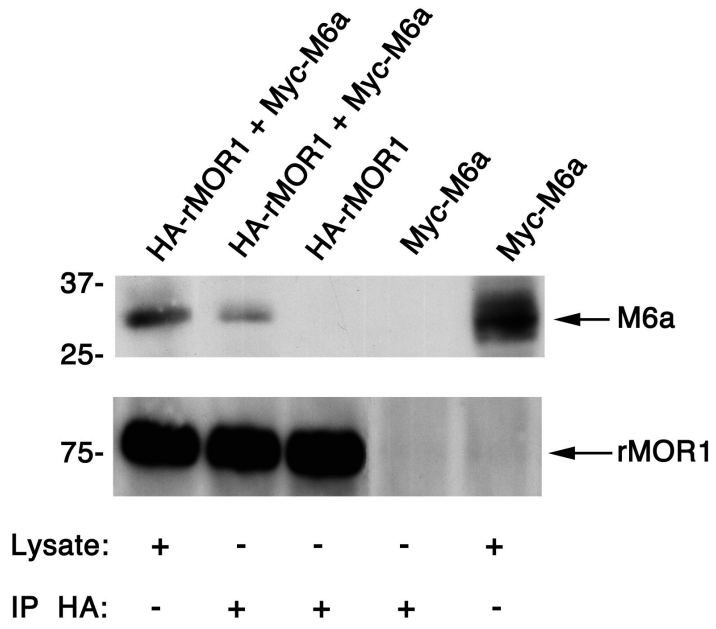


Figure 4.5. Interaction of rMOR1 and membrane glycoprotein M6a detected in HEK 293 cells by coimmunoprecipitation. Cell lysate proteins from HEK 293 cells coexpressing HA-rMOR1 and Myc-M6a (HA-rMOR1+Myc-M6a) were extracted and either immunoblotted directly (lane 1, lysate) or immunoprecipiated using anti-HA antibodies (lane 2, IP HA). Cell lysate proteins from HEK 293 cells stably expressing HA-rMOR1 alone (HA-rMOR1) were extracted and immunoprecipiated using anti-HA antibodies (lane 3, IP HA). Lysate proteins from HEK 293 cells transiently overexpressing Myc-M6a alone (Myc-M6a) were extracted and either immunoblotted directly (lane 5, lysate) or immunoprecipitated using anti-HA antibodies (lane 4, IP HA). The resulting immunoprecipitates were electrophoretically separated, transferred to nitrocellulose membrane and detected with anti-Myc or anti-HA antibodies. Note that M6a were coimmunoprecipitated with the mu opioid receptor only from cells coexpressing HA-rMOR1 and Myc-M6a but not from cells expressing HA-rMOR1 or Myc-M6a alone. The positions of molecular mass markers are indicated on the left (in kDa). Arrows point to M6a or rMOR1. Two additional experiments gave similar results.

4.3.2. Analysis of the interaction of rMOR1 and M6a by BRET

To provide further evidence that the mu opioid receptor interacts with membrane glycoprotein M6a, we also analysed M6a/rMOR1 or M6a/rMOR1B interaction in living cells using BRET.

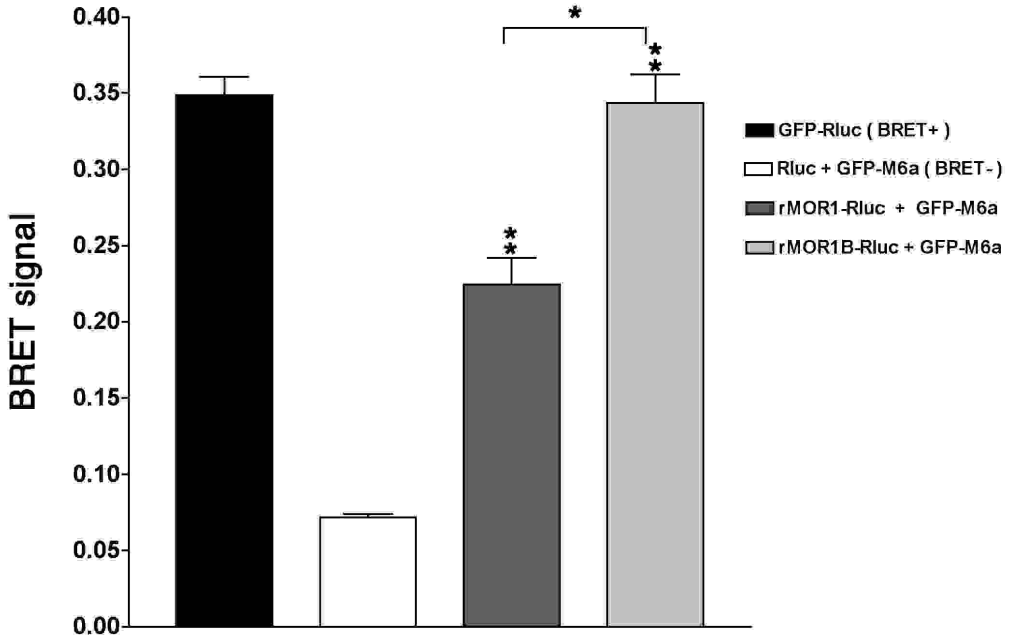


Figure 4.6. The interaction of M6a with rMOR1 or rMOR1B exists in living HEK 293 cells detected by BRET. HEK 293 cells were transiently cotransfected with GFP-M6a in combination with soluble Rluc, rMOR1-Rluc or rMOR1B-Rluc. A treatment of Rluc-GFP fusion protein was used as positive control. Cells were harvested 45 h post-transfection. The energy transfer was initiated by addition of 5 µM Deep Blue C, and BRET signal was assessed in a Fusion™ Universal Microplate Analyzer as described under "Method". Note that 1) Significant BRET signal was detected in cells coexpressing rMOR1-Rluc and GFP-M6a, rMOR1B-Rluc and GFP-M6a compared with control cells overexpressing Rluc and GFP-M6a. 2) Significant difference in BRET signal was detected between cells expressing rMOR1-Rluc and cells expressing rMOR1B-Rluc when they were cotransformed in combination with GFP-M6a. The results represent mean ± S.E. of 3 independent experiments performed in triplicate. Asterisk indicates a significant difference ($P<0.01$), double asterisks indicate significant difference ($P<0.001$) analyzed by one way ANOVA followed by Bonferroni test.

Fusion constructs linking Rluc to the receptor carboxyl terminus and GFP linking to the amino termini of M6a were cotransfected in HEK 293 cells. The transfer of energy between the two partners was assessed following the addition of Deep Blue C. As shown in Figure 4.6, a high BRET signal was obtained with the GFP-Rluc fusion protein (positive control), whereas a very small signal was obtained when GFP-M6a was coexpressed with soluble Rluc (negative control). A significantly high BRET signal was obtained in cells coexpressing rMOR1-Rluc/GFP-M6a indicating the interaction between rMOR1 and M6a also occurs in living cells. A significantly high BRET signal was also seen in cells coexpressing rMOR1B-Rluc/GFP-M6a indicating that the interaction between rMOR1B and M6a. A significant difference of BRET signal between rMOR1 and rMOR1B expressing cells was found, indicating that M6a probably plays a different role in different subtype of the mu opioid receptors.

4.3.3. The colocalization and cointernalization of rMOR1 and M6a

GFP was linked to the carboxyl tail of rMOR1 to facilitate the observation of the receptor in living cells.

As shown in Figure 4.7, in cells stably expressing rMOR1-GFP (left panel), receptor was localized in cell membrane (upper panel, control). A clear receptor internalization could be observed after 1μM DAMGO treatment for 2 h (lower panel). For cells transiently expressing Myc-M6a (right panel), M6a were localized both in the membrane and endosome like structure before and after DAMGO treatment.

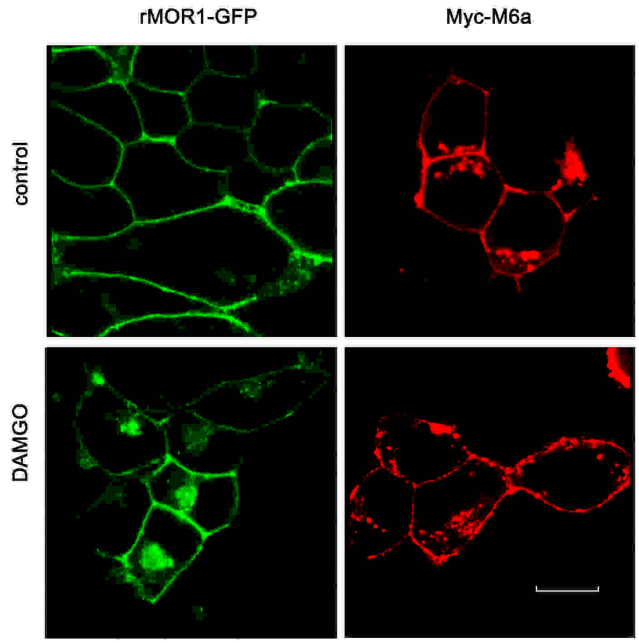


Figure 4.7. Subcellular distribution of rMOR1-GFP and Myc-M6a in HEK 293 cells overexpressing rMOR1-GFP or Myc-M6a. HEK 293 cells stably expressing rMOR1-GFP (left panel) and HEK 293 cells transiently transfected with Myc-M6a (right panel) were either not exposed (upper pannel, control) or exposed to 1 µM DAMGO (lower pannel, DAMGO) for 2 hours at 37°C, 10% CO_2 cell incubator. Cells were subsequently fixed, and Myc-M6a was labeled using anti-Myc antibody. The subcellular distribution of the receptor and M6a were examined by confocal microscopy. As shown in the left panel, rMOR1 was localized on the cell membrane, after agonist treatment, receptor endocytosis could be observed. As shown in the right panel, Myc-M6a was localized both on the cell membrane and endosome like structure inside of the cells. After DAMGO treatment, the localization of M6a did not change. Scale bar, 16 µM. Shown are representative results from one of three independent experiments performed in duplicate.

rMOR1-GFP and Myc-M6a were coexpressed in HEK 293 cells to investigate the influence of M6a on the subcellular localization of rMOR1. As shown in Figure 4.8 left and middle upper panel, rMOR1-GFP and Myc-M6a were localized in both plasma membrane and endosome like structures. As the predicted result from BRET assay, rMOR1-GFP and Myc-M6a showed constitutive colocalization in HEK 293 cells (Figure 4.8, right upper panel). To test the possibility that physical association would promote cointernalization of rMOR1 and M6a after agonist exposure, rMOR1-GFP and Myc-M6a

coexpressing cells were incubated in the presence of 1 μM DAMGO for 2 hours. The results in Figure 4.8 (lower panel) showed that most of rMOR1 was internalized. Interestingly, M6a was co-internalized with rMOR1 and was confined to endocytotic vesicles.

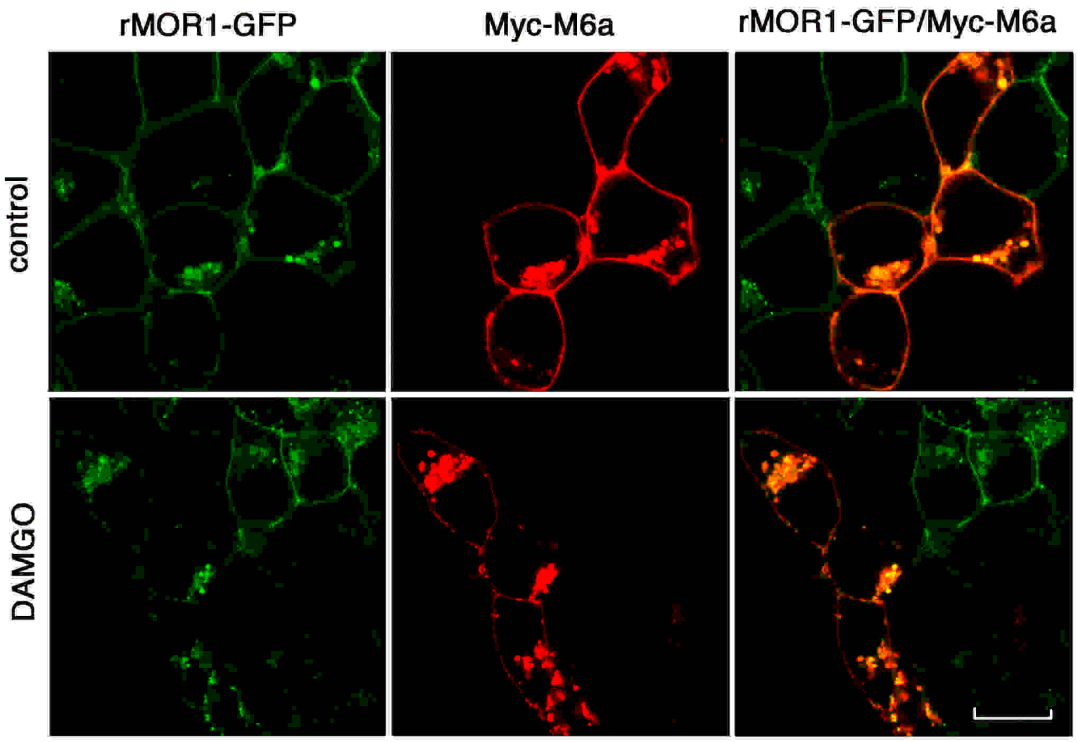


Figure 4.8. Subcellular distribution of rMOR1-GFP and Myc-M6a in HEK 293 cells coexpressing rMOR1-GFP and Myc-M6a. HEK 293 cells stably expressing rMOR1-GFP were transiently transfected with Myc-M6a, and either not exposed (upper pannel) or exposed (lower pannel) to 1 μM DAMGO for 2 hours at 37°C. Myc-M6a was labeled using anti-Myc antibody. The subcellular distribution of receptor protein and M6a were examined by confocal microscopy. Left and middle panel showed the subcellular distribution of rMOR1-GFP or Myc-M6a, respectively. Right panel showed their merged image. Note, in both treated and untreated cells, rMOR and M6a showed strict colocalization. In untreated cells, rMOR1 and M6a were confined mostly to the cytoplasma membrane, and also to endosome like structure inside of the cell membrane. In DAMGO treated cells coexpressing rMOR1-GFP and Myc-M6a, both rMOR and M6a were internalized and confined to endocytotic vesicles. The rMOR1-GFP is shown in green, M6a in red, and overlay in yellow. Scale bar, 16 μM. Shown are representative results from one of three independent experiments performed in duplicate.

4.4. Phospholipase D2 (PLD2)

4.4.1 Coimmunoprecipitation of rMOR1 and PLD2

To detect an interaction between rMOR1 and PLD2 in HEK 293 cells, we carried out coimmunoprecipitation studies. Expression of HA-rMOR1 and PLD2 was examined by directly immunoblotting lysates from these cells with specific antibodies against HA tag and PLD2, respectivly (Figure 4.9, lane 1 and 5, lysate). For coimmunoprecipitation, HA-rMOR1 receptors were precipitated from lysates of HA-rMOR1 expressing cells and cells coexpressing HA-rMOR1 and PLD2 using anti-HA antibodies. The resulting precipitates were immunoblotted with antibodies directed against PLD2. As shown in Figure 4.9 (lanes 2 and 3, IP HA), PLD2 was detected in immunoprecipitates from cells coexpressing HA-rMOR1 and PLD2, suggesting that rMOR1 is physically associated with PLD2 in vivo. Surprisingly, after agonist treatment we observed a decrease in the amount of coimmunoprecipitated PLD2 in HA-rMOR1 and PLD2 coexpressing cells (Figure 4.9, lane 3, IP HA). In immunoprecipitates from HA-rMOR1 expressing control cells no PLD2 was detected, which might be due to the low basal PLD2 expression level (Figure 4.9, lane 4, IP HA). In cells overexpressing PLD2 alone, no PLD2 was immunoprecipitated with HA-rMOR1, indicating the specificity of the detected interaction between rMOR1 and PLD2.

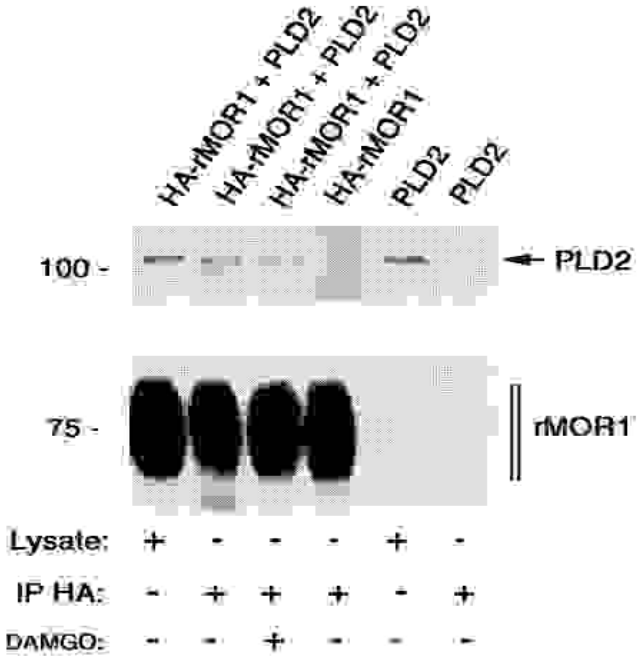


Figure 4.9. Interaction of rMOR1 and PLD2 in HEK 293 cells detected by coimmunoprecipitation. Membrane proteins from HEK 293 cells stably coexpressing HA-rMOR1 and PLD2 (HA-rMOR1+PLD2) were extracted and either immunoblotted directly (lane 1, lysate) or immunoprecipiated using anti-HA antibodies (lane 2, IP HA). Membrane proteins from HA-rMOR1 and PLD2 expressing HEK 293 cells pretreated with DAMGO for 30 min (HA-rMOR1+PLD2/DAMGO) were extracted and immunoprecipitated using anti-HA antibodies (lane 3, IP HA). Membrane proteins from HEK 293 cells expressing HA-rMOR1 alone (HA-rMOR1) were extracted and immunoprecipiated using anti-HA antibodies (lane 4, IP HA). Membrane proteins from HEK 293 cells expressing PLD2 alone (PLD2) were extracted and either immunoblotted directly (lane 5, lysate) or immunoprecipitated using anti HA antibodies (lane 6, IP HA). The resulting immunoprecipitates were electrophoretically separated, transferred to nitrocellulose and detected with anti-PLD and anti-HA antibodies. Note that PLD2 were coimmunoprecipitated with the mu opioid receptor only from cells coexpressing HA-rMOR1 and PLD2 but not from cells expressing HA-rMOR1 or PLD2 alone. The positions of molecular mass markers are indicated on the left (in kDa). Arrows point to PLD2 and rMOR1. Densitometric measurements revealed that in HA-rMOR1 and PLD2 coexpressing HEK 293 cells 68.8% of the expressed PLD2 protein were coimmunoprecipitated with the mu opioid receptor, whereas after agonist stimulation 35.6% of the expressed PLD2 were coimmunoprecipitated with the mu opioid receptor. Two additional experiments gave similar results.

4.4.2 rMOR1 stimulates PLD2 activity

Since PLD2 activation has been previously described for various G protein-coupled receptors, the association of rMOR1 and PLD2 indicated that agonist stimulation of the rMOR1 might activate PLD2. Therefore, we stably expressed rMOR1 and full length PLD2 in HEK 293 cells. rMOR1 and PLD2 expression was monitored by ligand binding experiments, Western blot and immunocytochemical analyses.

As seen in table 4.2, saturation binding experiments (n = 4–5) revealed no substantial differences between rMOR1 and rMOR1-PLD2 expressing cells with respect to their affinities (K_D) to [^{3}H]DAMGO. The number of binding sites (B_{max}) in rMOR1-PLD2 coexpressing cells was lower than that in cells expressing rMOR1 alone, this might be due to the lower expression level of the receptor in rMOR1 and PLD2 coexpressing cells.

Cell type	K_D (nM)	B_{max}(fmol/mg)
rMOR1	1.4±0.3	1329±399
rMOR1-PLD2	1.4±0.4	938±107

Table 4.2. Functional properties of opioid receptor in rMOR1 or rMOR1-PLD stably expressing HEK 293 cells. The K_D and B_{max} for the binding of [^{3}H]DAMGO to receptors in both cell lines were determined by Scatchard analysis using at least seven concentrations of labeled DAMGO in a range from 0.3 to 9 nM. Non-specific binding was determined as radioactivity bound in the presence of 1 μM unlabeled DAMGO. Values shown are the mean±S.E. from at least four experiments.

HEK 293 cells coexpressing rMOR1 and PLD2 were kept in serum free media containing [1,2,3-^{3}H]glycerol for 24 h in order to label phospholipids. Cells were then exposed to serum free medium containing drugs and 2% ethanol. Individual phospholipids phosphatidylethanol (PtdEtOH), PA, and PC were isolated. PLD activity was expressed as percent [^{3}H]PtdEtOH of the total cellular PC concentration. As shown in Figure 4.10, treatment with the mu opioid receptor selective agonist DAMGO, a modified form of

endogenous opioid peptide enkephalin, led to a time dependent increase in the PLD2 activity with a maximum (3 fold increase in activity) after 30 min, whereas an incubation for 30 min with morphine, an opioid alkaloid which does not induce the receptor endocytosis, failed to induce activation of PLD2. The observed DAMGO induced PLD2 activation was opioid receptor mediated, because it could be completely blocked by the opioid antagonist naloxone. Activation of PKC by PMA also promoted a 4.5 fold increase in PLD2 activity, which was not blocked by naloxone (data not shown).

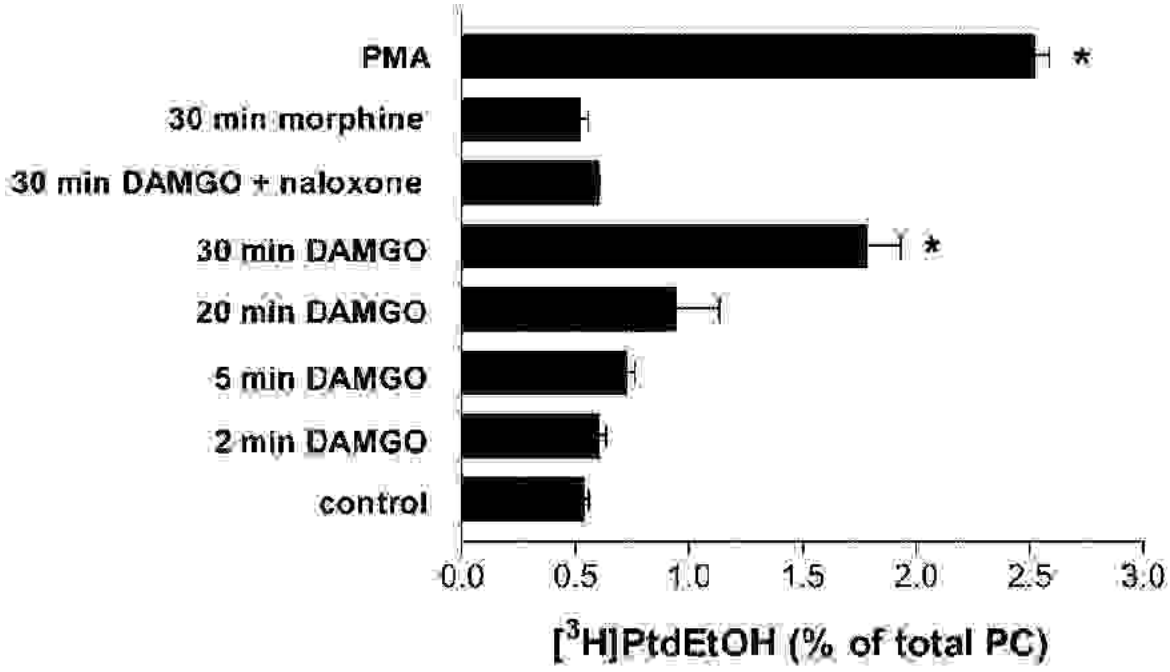


Figure 4.10. Opioid receptor mediated PLD2 activation in HEK 293 cells. HEK 293 cells stably coexpressing rMOR1 and PLD2 were incubated in serum free medium containing 2% ethanol and exposed or not exposed to either 1 µM DAMGO, 1 µM morphine, or 1 µM PMA in the presence or absence of 1 µM naloxone for the indicated time periods. PLD activity was determined as described under "Methods". The control condition was assayed with 2% ethanol for 30 min. Note that 1) DAMGO and PMA but not morphine promoted an obvious increase in PLD2 activity. 2) DAMGO mediated stimulation of PLD2 activity was time dependent and completely blocked by naloxone. Values represent means ± S.E. of triplicate determinations from three independent experiments. Asterisks indicate significant difference ($p<0.05$) compared with ethanol treated control cells as determined using ANOVA followed by Bonferroni test.

5. Discussion

By yeast two hybrid technique, a series of proteins were found to be interacting with the mu opioid receptor. Our attention first focused on these proteins which might modulate opioid receptor trafficking like Hsc70, synaptophysin, membrane glycoprotein M6a and PLD2.

5.1. Heat shock cognate protein 70

Using the mu opioid receptor carboxyl terminus as bait, we identified a novel mu opioid receptor binding partner, heat shock cognate protein 70 (Hsc70) in a yeast two hybrid screen. This specific interaction was confirmed by yeast mating. However, in coimmunoprecipitation experiment and by BRET analysis, we failed to detect a clear interaction between rMOR1 and Hsc70. The reason might be that a ternary protein interaction is involved. A third protein might stabilize the interaction between the receptor and Hsc70. It is possible that HLJ 1, a member of Hsp40 family might be such a protein, since it binds to the carboxyl terminus of the mu opioid receptor (Ancevska et al., 2002). It is well known that Hsp40 family members, complexed with Hsc70, are chaperones involved in protein folding and play a role in protein trafficking (Kelley, 1999).

Hsc70, a constitutively expressed member of the heat shock protein family, is known to play a role in coat disassembly in receptor mediated endocytosis. Hsc70 is recruited to clathrin triskelions in clathrin coated vesicles and its ATPase activity is stimulated by auxilin (Holstein et al., 1996; Barouch et al., 1997), thus the clathrin uncoating reaction occurs (Ungewickell et al., 1995). For a model of the interaction of rMOR1 and Hsc70 and the proposed role of Hsc70 on rMOR1 trafficking, see Figure 5.1.

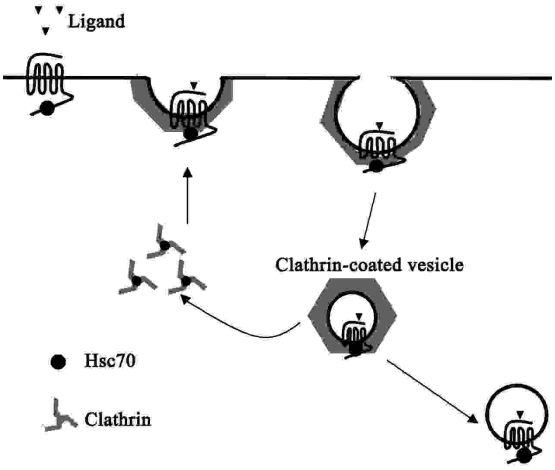


Figure 5.1. Hsc70 interacts with rMOR1 and its proposed role in rMOR1 trafficking. Hsc70 interacts with the carboxyl terminus of rMOR1, it is involved in releasing clathrin from newly budded clathrin coated vesicles.

5.2. Synaptophysin

Synaptophysin is a major integral membrane glycoprotein that is localized in the presynaptic vesicles of neurons. As shown in Figure 5.2, the structure of synaptophysin consists of four transmembrane domains (TMD) with the amino and carboxyl termini in the cytosol, an N-terminal glycosylation site (Leube et al., 1987). Using yeast two hybrid technique, we identified a truncated synaptophysin starting from the first intravesicular loop (amino acids 81-307) which interacts with the third intracellular loop of rMOR1.

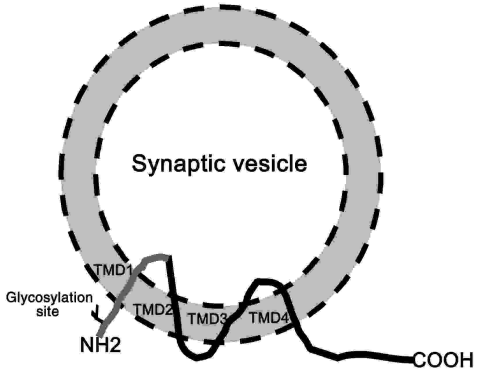


Figure 5.2. The structure of synaptophysin. Rat synaptophysin has four transmembrane domains, Segment starting from the first intravesicular loop (shown in bold) binds to the 3rd intracellular loop of rMOR1.

There is a report showing presynaptic localization of the mu opioid receptor within the striatopallidal projection systems, as evidenced by rMOR1 colocalized with presynaptic marker protein synaptophysin in the spiny neurons that project to the globus pallidus and ventral pallidum (Olive et al., 1997). Other investigators have also found the mu opioid receptors to be presynaptic on enkephalinergic terminals in the striatum and nucleus accumbens (Svingos et al., 1995, 1996; Guttenberg et al., 1996).

Synaptophysin may be implicated in the endocytosis of the opioid recptor in the presynaptic plasma membrane. Receptor mediated endocytosis is thought to be initiated by the formation of ß-arrestin/ß2-adaptin/clathrin complexes. The reactions subsequent to clathrin coating include rapid fission of the budding vesicle, which depends essentially on dynamin, an ATPase that acts as a molecular scissor in sequestrating the newly formed clathrin coated vesicle away from the plasma membrane and into the cytosol (Hinshaw, 2000; Slepnev and De Camilli, 2000). Recently, the carboxyl cytoplasmic tail of synaptophysin has been shown to interact with dynamin (Daly and Ziff, 2002). Disruption of the synaptophysin-dynamin interaction results in an inhibition of synaptic vesicle endocytosis (Daly et al., 2000). These data are consistent with a model in which the interaction between synaptophysin and dynamin can facilitate the process of endocytosis.

Our data showed that in HA tagged rMOR1 and Myc-tagged synaptophysin coexpressing HEK 293 cells, rMOR1 was colocalized both on the membrane and cytosol with synaptophysin. There are two possible explanations for this: 1. After expression of rMOR1 from trans-Golgi complex, it interacts with the cytosolically localized synaptophysin, this interaction traps rMOR1 in the cytosol, prevents its transportation to cell membrane. 2. Membranous rMOR1 interacts with synaptophysin, which can form a complex with dynamin, thus facilitating the constitutive endocytosis of rMOR1. To test the latter hypothesis, we treated the cells with the opioid receptor antagonist naloxone, which is known to fix the receptor in a conformation that is unable to be endocytosed. After naloxone treatment, rMOR1 was retained in the membrane, indicating constitutive

endocytosis. This is in line with a model that the association of rMOR1 with synaptophysin plays a role in rMOR1 endocytosis by targeting dynamin to the presynaptic membrane, dynamin then wraps around the necks of budding vesicles, membrane fission is facilitated, leading to an enhanced clathrin mediated endocytosis (Figure 5.3).

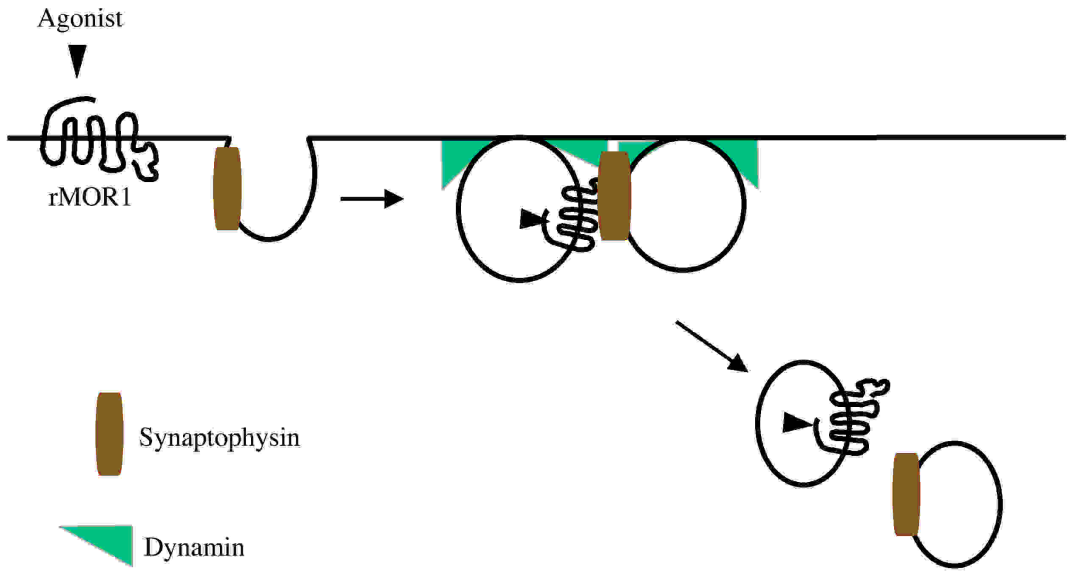


Figure 5.3. Model of the proposed role of synaptophysin-rMOR1 interaction in rMOR1 endocytosis. Binding of dynamin to synaptophysin, which interacts with the third intracellular loop of rMOR1 enhances endocytosis of rMOR1 by facilitated membrane fission.

5.3. Membrane glycoprotein M6a

M6a is a membrane glycoprotein with high abundance in neurons in the central nervous system (CNS). It has four transmembrane domains with its amino and carboxyl termini placed inside of the cytoplasmic membrane. M6a protein possesses two potential phosphorylation sites for PKC (Yan et al., 1993, Mukobata et al., 2002). There is a report showing that M6a is functioning as a Ca^{2+} channel involved in neuronal differentiation (Mukobata et al. 2002). Addition of anti-M6 antibody to living neurons in monolayer cultures of mouse cerebellum causes a disruption of the normal extension of neurites (Lagenaur et al., 1992). Till now, the detailed function of M6a is still obscure.

The present study demonstrates that M6a interacts with the mu opioid receptor. The direct interaction is supported by several lines of evidence: 1) The yeast two hybrid screen of a rat brain cDNA library with C-terminus of rMOR1B as bait led to the finding of a cDNA clone encoding the carboxyl part of neuronal membrane glycoprotein M6a (amino acids 108-278), 2) In coimmunoprecipitation experiments, M6a was specifically pulled down with full length of rMOR1. 3) M6a showed a strong positive BRET signal with Rluc linked both rMOR1 and rMOR1B.

In yeast mating, the carboxyl part of M6a showed reporter gene activation when it was mated with the carboxyl tail of rMOR1B but not with the carboxyl tail of rMOR1, the reason for this might be that the interaction between M6a and rMOR1 is weaker than that with rMOR1B so that it can not be detected in the yeast two hybrid assay. This hypothesis is supported by BRET analysis in living HEK 293 cells, the BRET signal between M6a and rMOR1B are stronger than that between M6a and rMOR1.

It is known that M6a is expressed at high levels in the hippocampus, especially in the granule cells of the dentate gyrus (Yan et al., 1996). There is a report showing that rMOR1 immunoreactivity is prominent in the same region (Arvidsson et al., 1995). Given the frequent coexpression of rMOR1 and M6a in granule cells in hippocampus, a direct interaction of the two proteins under physiological conditions is possible.

We found that in untreated HEK 293 cells, overexpressed rMOR1 showed strict colocalization with M6a not only in the membrane, but also in vesicles of the cytosol. This result is surprising, as rMOR1 is normally found mainly in the plasma membrane. Thus, it appears that M6a enhanced the basal rate of endocytosis of rMOR1. M6a might change the property of the endocytotic vesicle. It may form pores in the vesicles as suggested by the inability of endosomal acidification inhibitor monensin to inhibit rMOR1 recycling (unpublished data from our group). M6a also promotes endocytosis when it is expressed alone, indicating that M6a acts as an enhancer of endocytotic vesicle formation.

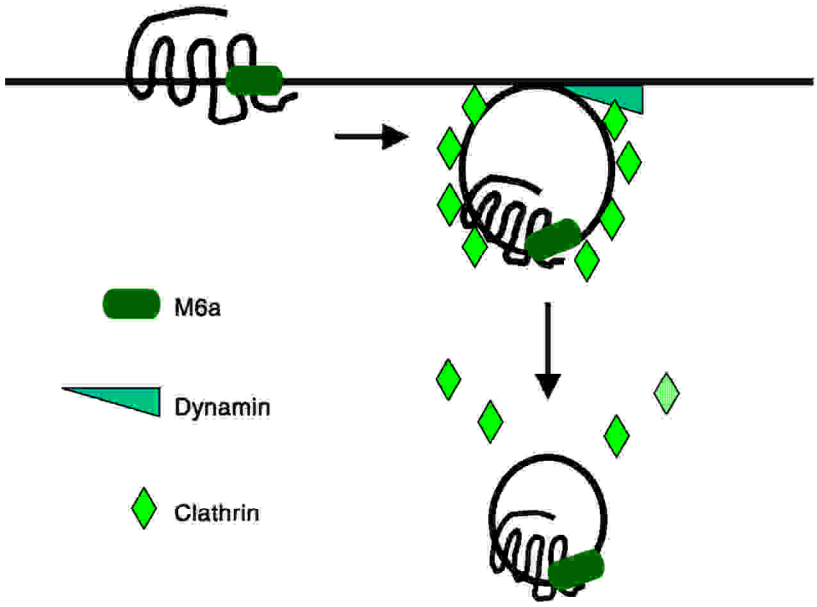


Figure 5.4. Model of the proposed role of M6a-rMOR1 interaction in rMOR1 endocytosis. M6a, which might act as an enhancer of formation of endocytotic vesicle, cointernalizes with rMOR1 thus facilitats the basal endocytosis of the receptor.

Following the endocytosis of the mu opioid receptor after DAMGO treatment, M6a was cointernalized and most of the original membranous M6a was located in the vesicles in the cytosol. DAMGO treatment induces a conformational change of the complex of the receptor and M6a, causing the internalization of both M6a and the opioid receptor. It is reported that M6a acts as a nerve growth factor-gated Ca^{2+} channel in neuronal differentiation (Mukobata et al., 2002). The depletion of the functional M6a located on the membrane after opiates treatment attenuates Ca^{2+} influx in neuronal cells exposed to NGF stimulation. This might explain the inhibitory effect of opiates on neuronal differentiation. Further characterization is needed to support this hypothesis.

5.4. Phospholipase D2

5.4.1. Interaction of rMOR1 and PLD2

A yeast two hybrid screen of a rat cDNA library using the full length of rMOR1 as bait identified a clone encoding the NH_2 terminal amino acids 116–226 of PLD2. This NH_2 terminal fragment (representing exons 4–8) of the PLD2 harbors a major part of the phosphoinositide-binding Phox homologous (PX) domain (amino acids 63–192) of the enzyme (Figure 5.5). The fact that Phox homologous (PX) domain of the PLD2 interacts with the carboxyl terminus of the opioid receptor indicates a dual functional role of PX domain in phosphoinositide binding and regulation of opioid receptor activity.

PLD is a widely distributed phospholipid-specific diesterase that hydrolyzes phosphatidylcholine (PC) to phosphatidic acid (PA) and choline and is assumed to play an important function in cell regulation (Morris et al., 1997; Liscovitch and Cantley, 1994).

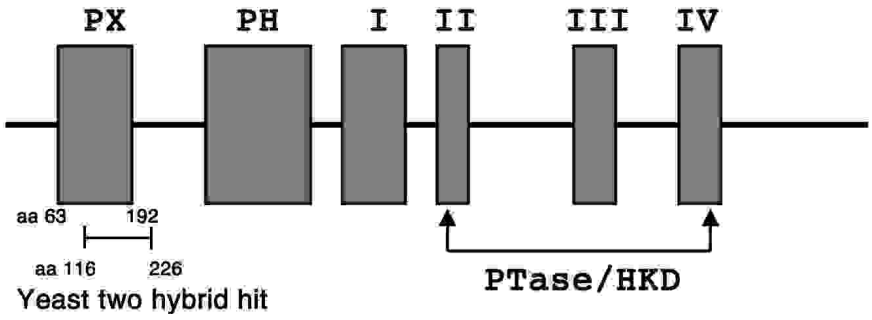


Figure 5.5. Domain structure of rat PLD2. Rat PLD2 has four conserved domains that have been designated domains I-IV (Morris et al., 1996; Sung et al., 1997). PX domain is identified in the N-terminal region. Following the PX domain there is a PH domain which is capable to bind phosphoinositide (PI) (Sciorra et al., 1999). Domains II and IV contain a short sequence motif ($HXKX_4DX_6GG/S$) termed HKD or the phosphatidyltransferase motif. The highly conserved histidine, lysine, aspartate, glycine and serine residues in the HKD/PT motif play an important role in catalysis (Sung et al., 1997; Gottlin et al., 1998). Structure domains are depicted as boxes.

PX domains are known to be phosphoinositide-binding motifs found in a number of signaling and adapter proteins. Proteins containing PX doamins (such as sorting nexins) have been shown to interact with the EGF receptor, the platelet-derived growth factor (PDGF) receptor, the insulin receptor and the long form of the leptin receptor (for a review, see Xu et al., 2001). Untill now it was not clear whether PX domains directly interact with the receptors or PI binding is required for the interaction with membranous receptor. Here for the first time, we show that PX domain containing protein PLD2 is associated with a member of GPCR family, namely the mu opioid receptor, and PX domain is involved in a direct interaction with the receptor.

There is no indication that the PX domain is important for the catalytic activities of PLD (for a review, see Xu et al., 2001). It remains, however, possible that other physiological regulation of PLDs (such as translocation) may be dependent on the interaction of phosphoinositides (PIs) with the PX domain. For instance, the PX domain of PLD1 can be phosphorylated by protein kinase C (Kim et al., 1999). This phosphorylation may influence the PI interaction, which in turn controls membrane association. Our findings suggest that the interaction of the PX domain of PLD2 with rMOR1, activates PLD2 resulting in increased PA synthesis, which in turn affects the trafficking of the receptor.

The specific interaction between rMOR1 and PLD2 was confirmed by coimmunoprecipitation experiments and shown to be constitutive and not inducible by agonist treatment. 68% of the expressed PLD2 was coimmunoprecipitated with rMOR1 under basal conditions, whereas only 35% of the PLD2 was coimmunoprecipitated upon agonist stimulation. Since by the technique used predominantly membrane attached proteins are immunoprecipitated, it is possible that in the absence of agonist both rMOR1 and PLD2 are located on the cell membrane, whereas after agonist treatment the receptor is internalized without PLD2. This hypothesis was verified by confocal microscopy studies (Koch et al., 2003).

5.4.2. Activation of PLD2 by DAMGO stimulation

Stimulation of PLD activity has been observed for numerous G protein-coupled receptors including the VPAC 1 and 2 (for vasoactive intestinal polypeptide) receptors, PAC1 (for pituitary adenylate cyclase-activating peptide) receptor (McCulloch, 2001), metabotropic glutamate receptors (Shinomura et al., 2000; Kanumilli et al., 2002), m1-m4 muscarinic receptors (Sandmann et al, 1991), the endothelin receptor (Ambar and Sokolovsky, 1993), the α2-adrenergic receptor (MacNulty et al., 1992), and the D2 dopamine receptor (Senogles, 2000). After demonstrating the physical interaction of PLD2 with rMOR1, it was further shown that the stimulation of the mu opioid receptor with DAMGO activates PLD2. Interestingly, morphine, an opioid alkloid which fails to induce receptor internalization, does not activate PLD2. This indicates that PLD2 activation is related to internalization of the mu opioid receptor.

There are reports showing that pathways leading to PLD activation include protein serine/threonine kinases, e.g. protein kinase C, small GTPases, e.g. ADP-ribosylation factor (ARF), RhoA and Ral, phosphatidylinositol 4,5-bisphosphate (PIP2), and tyrosine kinases (Kiss, 1996; Natarajan et al., 1996; Exton, 1998). The NPXXY motif, which is highly conserved within the seventh transmembrane domain of many G protein-coupled receptors including the mu opioid receptor, was previously demonstrated to represent a specific ARF binding site implicated in dopamine receptor mediated PLD activation (Mitchell et al., 1998). Therefore, it is reasonable to assume that ARF binds to the the receptor and facilitates the activation of PLD2, which binds to the carboxyl tail of rMOR1. Later coimmunoprecipitation studies revealed that ARF binds to the rMOR1-PLD2 signaling complex. Further studies showed that the DAMGO-mediated PLD2 activation was inhibited by brefeldin A, an inhibitor of ARF, indicating that opioid receptor mediated activation of PLD2 is ARF dependent (Koch et al., 2003).

5.4.3. Function of PLD2 activation

Two mammalian PLDs (PLD1 and PLD2) have been identified. Subcellular fractionation studies have demonstrated the presence of PLD1 in intracellular membranes, e.g. ER, Golgi, and vesicular compartment (Colley et al., 1997; Sung et al., 1999), whereas PLD2 was largely associated with the plasma membrane (Kodaki and Yamashita, 1997). PA formation by PLD1-mediated hydrolysis of phosphatidylcholine has been reported to be required for the transport of vesicles from the Golgi complex (Ktistakis et al., 1995, 1996). On the other hand, PLD2 is known to be associated with the EGF receptor (Slaaby et al., 1998). Interestingly, EGF receptor endocytosis is impaired when PLD2 activity is inhibited suggesting a role for PLD2 in receptor trafficking (Shen et al., 2001). It is likely that PLD2 plays a similar role in receptor trafficking as PLD1 in vesicle transport from Golgi complex.

Specifically, PLD2 might influence membrane topology and vesicle formation in the following aspects:

1) PLD2 converts phosphatidylcholine to PA, which could activate phosphatidylinositol-4-phosphate-5-kinase leading to the production of phosphatidylinositol-4,5-bisphosphate (PIP2) (Moritz et al., 1992; Honda et al., 1999). PIP2 could further activate PLD2, increase PA production and thus provide a positive feedback.

2) Both PA and PIP2 could bind to coat proteins and facilitate vesicle budding (Jones et al.,1999; Ktistakis et al., 1996; Roth et al., 1999). The generation of PA by PLD2 results in a significant change in both charge and pH at the membrane. The association of proteins with the PA-enriched membrane regions could initiate the changes in membrane topology that could ultimately result in the generation of an endocytic vesicle. For example, one important step in the initiation of clathrin-mediated endocytosis is the interactions of ß-arrestin with the AP-2 complex, which is necessary for the initial

targeting of receptors to coated pits (Laporte et al., 2002). It has been further demonstrated that the plasma membrane translocation of AP-2 is facilitated by the acidic phospholipid-enriched membrane resulting from phosphatidic acid (PA) production by ARF-activated PLD2 (Liscovitch and Cantley, 1995; De Camilli et al., 1996).

3) Dynamin, a protein which is involved in the scission of the endocytotic vesicle, is modulated by the acidic phospholipids (Liscovitch and Cantley, 1995).

In conclusion, our experiments showed that opioid receptor is physically associated with phospholipase D2, and DAMGO treatment led to PLD2 activation while morphine, which failed to induce the mu opioid receptor endocytosis, did not activate PLD2. These data strongly suggests that PLD2 is an enhancer of agonist induced receptor endocytosis.

For a model of opioid receptor-mediated activation of PLD2 and its proposed role in vesicle formation, see Figure 5.6.

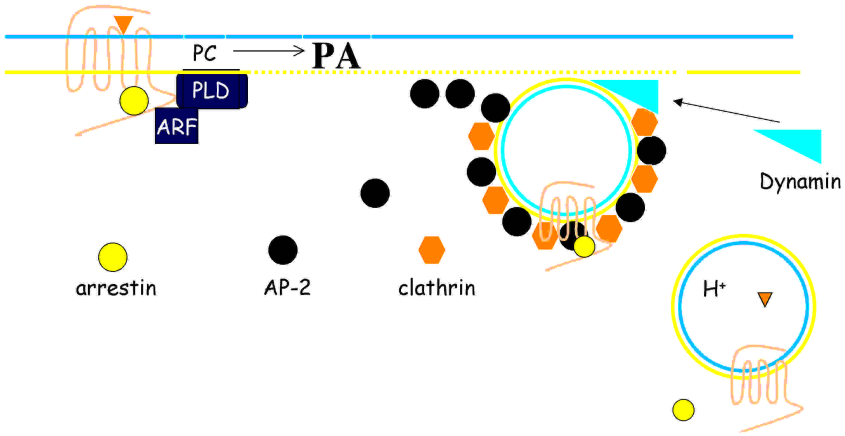


Figure 5.6. Model of opioid receptor-mediated activation of PLD2 and its proposed role in vesicle formation. After agonist binding to rMOR1, ARF is recruited to the receptor, activating PLD2 which interacts with the carboxyl tail of rMOR1. The resultant production of PA by ARF-activated PLD2 changes the physical properties of the cytosolic face of the membrane. PA may also activate PIP2 synthesis by activating PI 4-phosphate 5 kinases in a positive feedback loop (not shown for simplicity). The acidic phospholipids-enriched membrane facilitates recruitment of adaptor proteins AP-2 (shown as dark circles), where applicable, to plasma membrane and endosomes. This causes budding of the coated vesicle. The scission of vesicle neck is mediated by dynamin (wedge), itself modulated by acidic phospholipids (Liscovitch and Cantley, 1995).

In conclusion, Figure 5.7 summaries the possible function of the rMOR1 interacting proteins found in this study. After agonist binding, PLD2 interacting with the carboxyl tail of rMOR1 is activated, the resultant PA production facilitates vesicle formation; M6a cointernalizes with rMOR1, enhancing the receptor endocytosis; Synaptophysin recruits dynamin for sequestrating the vesicle away from the membrane; Hsc70 is required for sheding clathrin coats from clathrin coated vesicles.

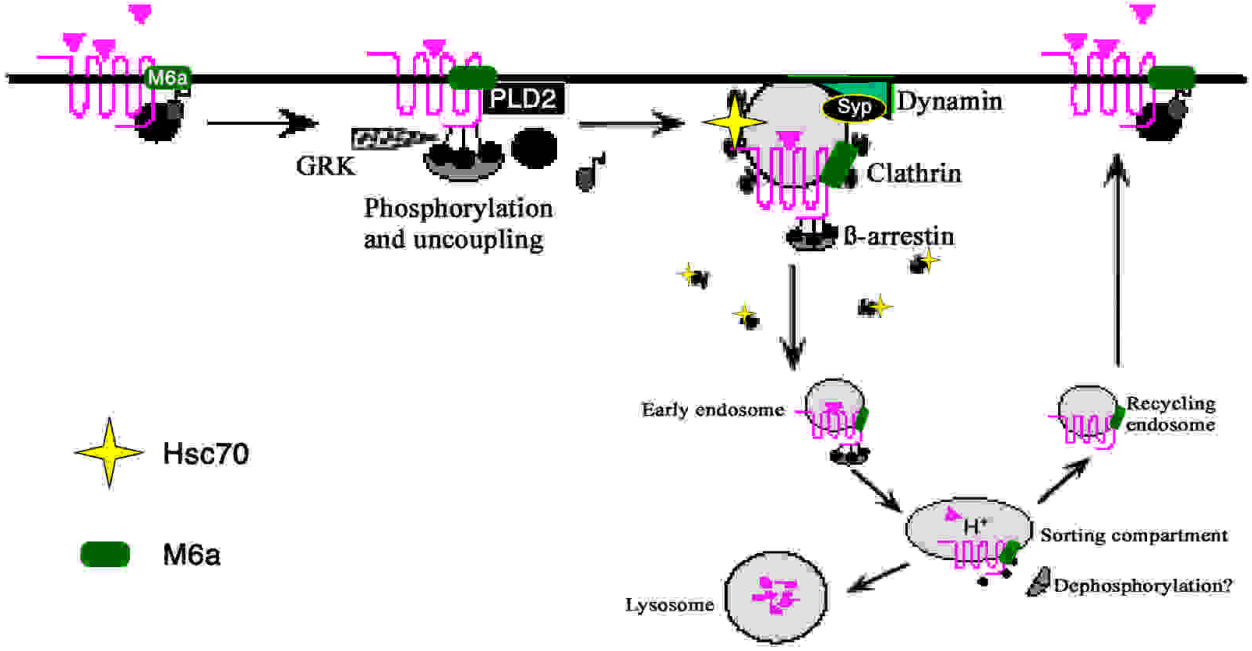


Figure 5.7. Model of the proposed role of rMOR1 interacing proteins in the mu opioid receptor trafficking.

6. Summary

Opioid receptor desensitization and endocytosis are critical steps in opioid tolerance. After opioid binding, the receptor is phosphorylated by G protein-coupled receptor kinases (GRKs) or second messenger kinases. The binding of ß-arrestins to the phosphorylated receptor leads to receptor uncoupling from G-proteins, which causes receptor desensitization. The ligand-receptor complex is internalized via clathrin coated vesicles. These vesicles soon shed their clathrin coat and become endosomes. The receptor is either recycled to the plasma membrane or degraded in the lysosome with the ligand.

To identify components of this endocytotic machinery, we searched for the mu opioid receptor (MOR1) interacting proteins that could modulate trafficking and signaling of the receptor. We used different portions of the mu opioid receptors as baits to screen a rat brain cDNA library by a yeast two hybrid assay. A series of proteins were identified which might be involved in the receptor trafficking: like heat shock cognate protein 70, synaptophysin, membrane glycoprotein M6a and phospholipase D2. In the yeast two hybrid screen Hsc70 interacts with the carboxyl terminus of MOR1; Synaptophysin binds to the 3rd intracellular loop of MOR1; M6a interacts with the carboxyl terminus of splice variant MOR1B; PLD2 (PX domain) interacts with the carboxyl termini of MOR1 and MOR1B.

The interaction between MOR1 and the three of the above four proteins (PLD2, M6a, synaptophysin) was confirmed in HEK 293 cells by Bioluminescence Resonance Energy Transfer (BRET) assay and/or coimmunoprecipitation experiment. The interaction was proved to be agonist-independent.

Furthermore, the putative functions of these proteins on MOR1 trafficking were investigated. As the major integral membrane glycoprotein in the presynaptic vesicles of neurons, synaptophysin is known to interact with dynamin. Coexpression of MOR1 and synaptophysin resulted in constitutive internalization of the receptor. These results suggest that the association of MOR1 with synaptophysin plays a role in MOR1 trafficking by targeting dynamin to the presynaptic membrane.

M6a is a membrane glycoprotein with high abundance in the central nervous system (CNS) of unclear function. After coexpression in HEK 293 cells, MOR1 showed strict colocalization with M6a both in the membrane and in endosome-like structures. The basal endocytotic rate of MOR1 was enhanced by M6a. M6a was cointernalized with the receptor after opioid agonist DAMGO treatment.

DAMGO which promotes receptor internalization activates PLD2 in HEK 293 cells coexpressing MOR1 and PLD2. In contrast, morphine, an opioid alkaloid which fails to induce receptor internalization, does not activate PLD2. This leads to the hypothesis that the ability of agonist to induce endocytosis is linked to their ability to activate PLD2. It was further demonstrated that after agonist binding, PLD2 is stimulated by MOR1 in an ARF-dependent manner. The resultant acidic phospholipid-enriched membrane facilitates the budding and scission of clathrin coated vesicles.

In conclusion, the identified proteins should help to elucidate the molecular mechanisms of opioid receptor tolerance.

Die Desensitisierung und Endozytose von Opioidrezeptoren sind wichtige Schritte bei der Enstehung der Opioidtoleranz. Nach Agonistenbindung wird der Opioidrezeptor zunächst durch G-Protein-gekoppelte Rezeptorkinasen (GRKs) oder “second-messenger“ Kinasen phosphoryliert. An den phosphorylierten Rezeptor binden ß-Arrestine, die zu einer G-Protein-Entkopplung und damit zur Desensitisierung des Rezeptors führen. Der Liganden-Rezeptor-Komplex wird in clathrin-ummantelten, endozytotischen Vesikeln internalisiert. Die internalisierten Rezeptoren können entweder in reaktiviertem Zustand wieder zur Membran zurücktransportiert, oder in Lysosomen degradiert werden.
Um neue regulatorische Proteine zu identifizieren die in die endozytotische Maschinerie des µ-Opioidrezeptors (MOR1) eingebunden sind, sollte nach µ-opioidrezeptor-interagierenden Proteinen gesucht werden, die den Transport und die Signaltransduktion des Rezeptors modulieren können. Hierfür wurde mittels Yeast-Two-Hybrid-Technik eine Ratten-cDNA-Bibliothek mit verschiedenen Teilen des µ-Opioidrezeptorproteins auf mögliche Protein-Protein-Interaktionen getestet. Es konnten eine Reihe von Proteinen identifiziert werden, die möglicherweise an der Regulation des Rezeptortransportes beteiligt sein könnten. Zu ihnen gehören das Hitzeschockprotein 70 (hsc70), Synaptophysin, das Glykoprotein M6a und die Phospholipase D2. Es konnte mittels Yeast-Two-Hybrid-Technik gezeigt werden, dass das Hsc70-Protein mit dem C-Terminus des MOR1 interagiert, Synaptophysin an die 3. intrazelluläre Schleife des MOR1 bindet, M6a mit dem C-Terminus der µ-Opioidrezeptorspleißvariante MOR1B assoziiert ist und die Phospholipase D2 (PLD2) an die C-Termini von MOR1 und MOR1B binden kann. Von den vier genannten Proteinen konnte die Interaktion dreier Proteine (PLD2, M6a und Synaptophysin) mit dem µ-Opioidrezeptor mittels Koimmunopräzipitation und Bioluminscence Resonance Energy Transfer (BRET)-Technik in transfizierten menschlichen embryonalen Nierenzellen (HEK293-Zellen) bestätigt werden. Die bestätigten Protein-Protein Interaktionen waren nachweislich agonisten-unabhängig.
Im weiteren wurde die mögliche Funktion der interagierenden Proteine in Bezug auf den Rezeptortransport näher untersucht. Von dem Synaptophysin, dem häufigsten membran-integrierten Glykoprotein in präsynaptischen Vesikeln von Neuronen, ist bekannt, dass es mit Dynamin interagieren kann. Die Koexpression des MOR1 mit Synaptophysin in HEK293-Zellen führte zu einer verstärkten konstitutiven Rezeptorinternalisierung. Dieses Ergebnis deutet darauf hin, dass die Assoziation des MOR1 mit dem Synaptophysin eine wichtige Rolle beim Transport des Dynamins in präsynaptische Membranen spielen könnte.
Das membranständige Glykoprotein M6a findet man in hoher Expression im zentralen Nervensystem, seine Funktion ist jedoch unklar. Nach Koexpression in HEK293 Zellen, zeigt MOR1 eine strikte Kolokalisation mit M6a in der Plasmamembran und in endosomen-ähnlichen Strukturen. Es konnte gezeigt werden, dass in Gegenwart von M6a die basale endozytotische Rate des MOR1 erhöht war. Darüberhinaus kointernalisierte das Glykoprotein M6a mit MOR1 nach Agonisteninkubation (DAMGO).
Der µ-Opioidrezeptoragonist DAMGO, der zu einer Induktion der Rezeptorendozytose führt, aktivierte die PLD2 in PLD2/MOR1-koexprimierenden HEK293 Zellen. Im Gegensatz dazu führte Morphin, das keine Rezeptorendozytose auszulösen vermag, zu keiner PLD2-Aktivierung. Dies führte zu der Hypothese, dass die Fähigkeit des Agonisten Rezeptorendozytose auszulösen mit der Fähigkeit zur PLD2-Aktivierung gekoppelt ist. Es konnte im weiteren gezeigt werden, dass die PLD2-Aktivierung durch den MOR1 nach Agonistengabe ARF-abhängig erfolgt. Die aus der PLD2-Aktivierung resultierende Anreicherung der Membran mit sauren Phospholipiden erleichtert die Bildung und das Abschnüren von endozytotischen Vesikeln.
Abschließend kann man sagen, dass die identifizierten Proteine helfen sollten, die molekularen Mechanismen der Opioidtoleranz weiter aufzuklären.

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8. Abbreviation

AC	Adenylate cyclase
AD	Activation domain
ANOVA	Analysis of variance
ATCC	American Type Culture Collection (Rockville, MD)
BLAST	Basic Local Aligment Search Tool
BRET	Bioluminescence Resonance Energy Transfer
CaM	Ca^{2+}/calmodulin
cAMP	Adenosine 3′,5′-cylic-monophosphate
cDNA	Complemetary deoxyribonucleic acid
DAMGO	[D-Ala ^{2}, N-Me-Phe ^{4},-Gly ^{5}-ol] enkephalin
DNA	Deoxyribonucleic acid
DMEM	Dulbecco′s Modified Eagle Medium
DNA-BD	DNA-binding domain
DSP	Dithiobis-(succinimi-dylpropionate)
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
g	gram or gravity
G418	Geniticin
GDP	guanosine 5′-diphosphate
GFP	Green fluorescent protein
GFP^{2}	A mutant of the green gluorescent protein
G protein	Guanine nucletide-binding protein
GPCR	G protein-coupled receptor
GRK	G protein receptor kinase
GTP	guanosine 5′-triphosphate
HA	Influenza hemagglutinin-HA-epitope (YPYDVPDYA)
HA-rMOR1	HA epitope-tagged rat mu opioid receptor isoform 1
HEK 293	Human embryonic kidney 293
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
kb	Kilobase
kD	kiloDalton

M	Molar
M6a	Neuron specific membrane glycoproteins M6a
MAP	Mitogen activated protein
Myc	Epitope tag (sequence: MASMQKLISEEDL)
OD_{600}	optical density at 600nm
PA	phosphatidic acid
PBS	Phosphate-buffered saline
PC	phosphatidylcholine;
PCR	Polymerase Chain Reaction
PEt	phosphatidylethanol
PLD	Phospholipase D
PMA	Phorbol-12- myristate-13-acetate
Rluc	Renilla luciferase
rMOR1	Rat mu opioid receptor isoform 1
rMOR1B	Rat mu opioid receptor isoform 1B
rpm	rounds per minte
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
Syp	Synaptophysin
TAE	Tris-Acetate-EDTA buffer
X-gal	5-bromo-4-chloro-3indolyl-ß-D-galactoside
YPD	yeast/peptone/dextrose (medium)

9. Appendix

9.1 Curriculum vitae

Name: **Yingjian Liang**

Birthday: Oct. 16, 1973

Marital status: single

Citizenship: China

Education & Experience:

1990.9—1995.7 Bachelor student of clinical medicine
Dalian Medical University, Dalian, China.

1995.8—1997.8 Clinical physician
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Magdeburg, 16.12.04

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Yingjian Liang

9.2 Publications and presentations

During my PhD studies, I have contributed to some articles as well as conference posters listed as below:

Articles:

Koch T., Brandenburg L.O., Schulz S., **Liang Y.**, Klein J., Höllt V. (2003) ARF-dependent phospholipase D2 activation is required for agonist-induced mu-opioid receptor endocytosis. *J Biol Chem* 278: 9979-9985.

Koch T, Brandenburg L.O., **Liang Y**, Schulz S, Helmut S, and Höllt V. (2003) Inhibition of phosphalipase D2 activation delayed agonist-induced mu-opioid receptor desensitization. *J Neurochem* In press

Brandenburg L.O., Koch T., Schulz S., **Liang Y.**, Kahl E., Grosseheilmann S., Klein J., Höllt V. (2002) Role of PLD2 in the agonist-mediated endocytosis of the mu opioid receptor. *Naunyn-Schmiedeberg´s Archives of Pharmacology* 365 R15

Conference Posters:

Liang Y., Han Z., Brandenburg L., Koch T., Kroslak T., Höllt V. Identification of novel proteins interacting with mu-opioid receptor. The 32nd International Narcotic Research Conference. Helsinky, Finland. 2001.

Koch T., Brandenburg L.O., Schulz S., **Liang Y.**, Höllt V. Mu-opioid receptor-mediated activation of phospholipase D2 enhances receptor endocytosis in HEK293 cells. The 33rd International Narcotic Research Conference. Monterey, USA. 2002

Liang Y., Wu D., Koch T., Höllt V. Heat shock cognate protein 70 (Hsc70) and synaptophysin interact with the mu opioid receptor. The 34th International Narcotic Research Conference. Perpignan, France. 2003.

Wu D., **Liang Y**., Koch T., Höllt V. Glycoprotein M6a interacts with the rMOR1: An analysis by Bioluminescence Resonance Energy Transfer (BRET2). The 34th International Narcotic Research Conference. Perpignan, France. 2003.

Koch T., Brandenburg L. O., Schulz S., **Liang Y**., Höllt V. Role of phospholipase D2 in the agonist mediated desensitization and resensitization of the mu opioid receptor. The 34th International Narcotic Research Conference. Perpignan, France. 2003

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