Modulation of μ-opioid receptor signal transduction and endocytosis by ADP-ribosylation factor proteins

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Summary

Physiological effects of opioids are mediated through binding to specific G protein-coupled opioid receptors. The μ-opioid receptor (MOPr) is of particular importance for the mediation of both the analgesic and the adaptive effects of clinically relevant opioid drugs. After opioid binding, the ligand-receptor complex is endocytosed via clathrin coated vesicles. Internalized receptors are then either recycled back to the plasma membrane or degraded in the lysosome.

Previous studies have shown that endocytosis of MOPr plays a protective role in the development of tolerance to opioid drugs by facilitating receptor reactivation and recycling. It has been further demonstrated, that the opioid-mediated activation of phospholipase D2 (PLD2) is a prerequisite for MOPr endocytosis and is dependent on small GTPases of ADP-ribosylation factor (ARF) family. However, precise identity of ARF protein (ARF1 or ARF6) as well as the mechanisms involved in opioid-mediated PLD2 activation by ARF proteins are still not clear.

By coexpressing the MOPr and different ARF mutants in human embryonic kidney (HEK) 293 cells and cultured primary cortical neurons, we have identified the ARF6 protein to be involved in the regulation of MOPr endocytosis. This conclusion was based on the two facts: 1) overexpression of dominant negative ARF6 mutant blocked receptor internalization after treatment with potent endocytotic drug DAMGO and 2) receptor endocytosis was increased in the presence of an active, "fast cycling" ARF6 mutant after treatment with morphine, an agonist that is unable to induce MOPr endocytosis by itself. Moreover, siRNA-mediated knock down of endogenous ARF6 protein expression significantly decreased receptor internalization. Presented study also documents that expression of an effector domain mutant of ARF6 which is incapable of activating PLD2 ("PLD-defective" mutant) blocked agonist-induced receptor endocytosis showing that ARF6 function in MOPr trafficking is PLD2-mediated. Analogously, opioid-mediated activation of PLD2 is blocked in the presence of dominant negative ARF6 mutants. Furthermore, we have also shown that ARF6 protein influences the recycling/reactivation of internalized MOPr and thus modulates agonist-induced MOPr desensitization. And finally, we demonstrated the importance of GTP hydrolysis of activated ARF6 protein and full GDP/GTP cycle for the trafficking of internalized MOPr back to the plasma membrane since locking ARF6 in its GTP-bound, active state blocked the recycling of the receptor.

Taken together, these results provide evidence that ARF6 protein regulates MOPr trafficking and signaling via PLD2 activation and hence affects the development of opioid receptor desensitization and tolerance to opioid drugs.

Zusammenfassung

Die physiologischen Effekte von Opioiden werden über die Interaktion mit spezifischen G-Protein-gekoppelten Rezeptoren vermittelt. Für die analgetischen und adaptiven Effekte klinisch relevanter Opioide ist der µ-Opioidrezeptor (MOPr) von besonderer Bedeutung. Nach Opioidbindung wird der Ligand-Rezeptor-Komplex in Clathrin-ummantelten Vesikeln endozytiert. Internalisierte Rezeptoren können nun entweder in reaktiviertem Zustand wieder zur Plasmamembran zurücktransportiert oder in Lysosomen degradiert werden.

Frühere Studien zeigten, dass die Endozytose des MOPr aufgrund einer erleichterten Reaktivierung/Rezyklisierung von Rezeptoren eine protektive Funktion bei der Entwicklung einer Opioidtoleranz besitzt. Es konnte weiterhin gezeigt werden, dass die opioid-vermittelte Aktivierung der Phospholipase D2 (PLD2) eine Voraussetzung für die MOPr Endozytose darstellt und dass diese Aktivierung über kleine GTPasen aus der Familie der ADP-Ribosylierungsfaktoren (ARF) vermittelt wird. Allerdings ist die Identität des an der Aktivierung der PLD2 beteiligten ARF Proteins (ARF1 oder ARF6) wie auch der Mechanismus der opioid-vermittelten PLD2 Aktivierung durch ARF-Proteine noch nicht geklärt.

Durch die Koexpression des MOPr mit verschiedenen ARF Mutanten in humanen embryonalen Nierenzellen (HEK293) und primären cortikalen Neuronen konnten wir zeigen, dass das ARF6 an der Regulation der MOPr Endozytose beteiligt ist. Diese Schlussfolgerung beruht auf zwei Fakten: 1) die Überexpression einer dominant negativen ARF6 Mutante führt zu einer vollständigen Blockade der MOPr Internalisierung nach Behandlung mit dem rezeptor-internalisierenden Agonisten DAMGO; 2) die Rezeptorendozytose nach Behandlung mit dem nicht rezeptor-internalisierenden Agonisten Morphin war in Gegenwart einer aktiven "fast cycling" ARF6 Mutante deutlich erhöht. Zusätzlich führte die Verminderung der endogenen ARF6 Expression mittels siRNA zu einer signifikanten Abnahme der Rezeptorinternalisierung. Die vorliegende Studie zeigt auch, dass die Expression einer ARF6-Mutante, die keine PLD2 Aktivierung auslösen kann ("PLD2-defekte" ARF Mutante), zu einer Blockade der agonisten-induzierten Rezeptorendozytose führt. Dies Ergebnis zeigt, dass die Funktion von ARF6 bei der Regulation des intrazellulären MOPr Transportes über die Aktivierung der PLD2 vermittelt wird. Analog dazu ist die opioid-vermittelte Aktivierung der PLD2 in Gegenwart einer dominant negativen ARF6 Mutante blockiert. Darüberhinaus konnten wir zeigen, dass das ARF6 Protein die Rezyklisierung/Reaktivierung von internalisierten MOPr beeinflusst und somit die die agonisten-induzierte Desensitisierung moduliert. Abschliessend konnte gezeigt werden, dass die GTP-Hydrolyse des aktivierten ARF6 und somit ein kompletter GDP/GTP Zyklus für den

Rücktransport des internalisierten MOPr an die Plasmammembran notwendig ist. So führt die Expression einer GTPase defizienten ARF6 Mutante, die das ARF6 in der GTP-gebundenen aktiven Form hält, zu einem Verlust der MOPr Rezyklisierung.

Zusammenfassend zeigen die vorliegenden Ergebnisse, dass das ARF6 Protein den intrazellulären Transport und die Signaltransduktion des MOPr über die Aktivierung der PLD2 reguliert und dadurch die Entwicklung einer Opioidrezeptor Desensitisierung und Opioidtoleranz beeinflusst.

1. Introduction

Opium, a preparation of the opium poppy *papaver somniferum*, has been used for thousands of years to relieve pain and to alter mood. The oldest records about the use of opium poppy as a "joy plant" are from the ancient Sumerian civilization that existed four thousand years B.C.. Later, the use of opium poppy juice has spread and by the 10^{th} and 11^{th} centuries A.D., the opium trade was firmly established in Europe (*Berridge and Edwards, 1981*).

In 1806, morphine (named after Morpheus, the Greek god of dreams) was isolated by Friedrich Sertürner and later shown to be almost entirely responsible for the analgesic activity of crude opium. Over the next decades, several other alkaloids, such as codeine and papaverine, were isolated. Heroin, the first semi-synthetic opioid, was produced in 1874 and while it was first used in medicine, later it became a popular opioid drug of abuse.

To date, opioids such as morphine are still the best analgesic choice in the treatment of chronic and serious pain, such as cancer pain. However, it is now well recognized that their extensive and long-term use leads to development of physiological tolerance and dependence (see Chapter 1.3.4.), adaptive changes in the nervous system that greatly limit the therapeutic use of opioid drugs (for review see *Taylor and Fleming, 2001*). In addition, opioid drug abuse is still a great problem nowadays. Due to these reasons, one of the major goals in opioid research is to develop drugs or administration strategies that result in effective analgesia without the detrimental adaptive responses.

1.1. Opioids

Extensive research has resulted in many distinct opioids being isolated. Some of them were discovered endogenously and some were synthetically derived. Many of these compounds are still used medically or abused illegally and therefore we could say that opioids today play both beneficial and deleterious role in society.

The term ***opioid*** applies to the chemical substances that have a morphine-like action in the body, including analgesia, sedation, euphoria as well as respiratory depression and antidiarrhea. There are several classes of opioids:

- **Natural opiates**, alkaloids contained in the resin of the opium poppy including morphine and codeine;

- **Semi-synthetic opiates**, created from the natural opioids, such as hydromorphone, hydrocodone, oxycodone, oxymorphone, buprenorphine, diacetylmorphine (heroin) etc.
- **Fully synthetic opioids**, such as fentanyl, pethidine, methadone, tramadol and propoxyphene;
- **Endogenous opioid peptides**, produced naturally in the body, such as β-endorphin, enkephalins, dynorphins and endomorphins.

Although the term ***opiate*** is often used as a synonym for *opioid*, it is more properly limited to the natural opium alkaloids and the semi-synthetics derived from them.

1.2. Opioid receptors

1.2.1. Structure of opioid receptors

Opioids mediate their physiological effects by binding to specific opioid receptors in the central nervous system and in other tissues, mainly in the gastrointestinal tract. In 1973, three groups of researchers independently identified stereospecific binding sites for opioids in mammalian nervous system (*Pert and Snyder, 1973*; *Simon et al., 1973*; *Terenius, 1973*). Later, the begin of the 20th century was marked by cloning of genes encoding three well defined or "classical" types of the opioid receptors: μ (mu), δ (delta) and κ (kappa) opioid receptor (*Kieffer et al., 1992*; *Evans et al., 1992*; *Chen et al., 1993a*; *Minami et al., 1993*; *Fukuda et al., 1993*; *Li et al., 1993*; *Meng et al., 1993*; *Yasuda et al., 1993*; *Wang et al., 1993*). Sequence analysis revealed that these receptors belong to the superfamily of G protein-coupled receptors (GPCRs) and the subfamily of rhodopsin receptors. As shown in Figure 1.1., the μ-, δ- and κ-opioid receptors have seven transmembrane domains of 20-25 hydrophobic residues that form α-helices, three intra- and three extracellular loops, extracellular N-terminus and intracellular C-terminal tail. These receptors are about 60% identical to each other, with the greatest homology found in the transmembrane domains (73-76%) and intracellular loops (86-100%). The lowest homology in amino acid sequence is found in the N-terminus (9-10%), extracellular loops (14-72%) and the C-terminus (14-20%) (*Chen et al., 1993b*; *Law et al., 2000*).

Numerous pharmacological studies have suggested subtypes of the μ-opioid receptor (MOR1) and studies have raised the possibility that some of these might reflect splice variants of the MOR1 gene (*Wolozin and Pasternak, 1981*; *Pasternak, 1993*; *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

were continually identified and characterized (*Pan et al., 1999, 2000, 2005; Pasternak et al., 2004*). However, more recent nomenclature for the μ-opioid receptor is MOPr and therefore this abbreviation has been used in this work.

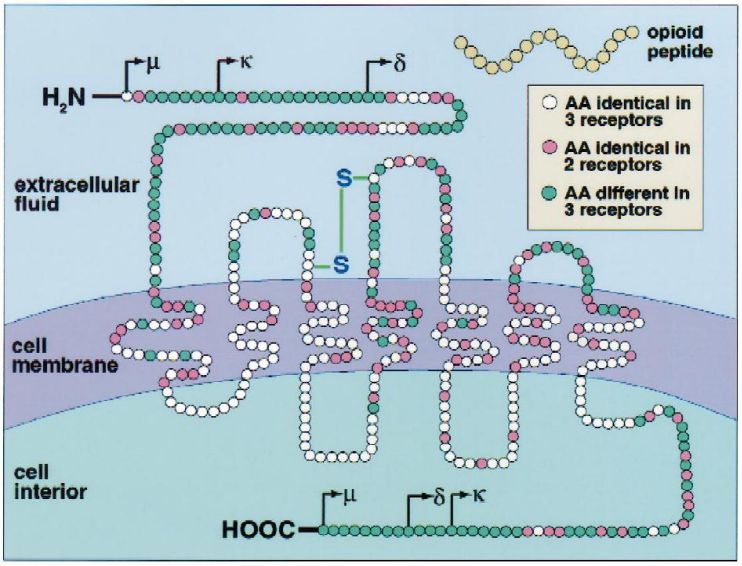


Figure 1.1. Structure of opioid receptors (modified from *LaForge et al., 2000*). Opioid receptors have a central common core composed of seven transmembrane helices connected by three intra- and three extracellular loops. The differences in N-terminal and C-terminal length for each receptor type are shown.

1.2.2. Effector mechanisms of opioid receptors and opioid receptor-evoked cellular responses

As mentioned above, opioid receptors belong to the family of GPCRs. They are prototypical "$G_{i/o}$ coupled" receptors because receptor signaling can be blocked by pertussis toxin (PTX), a bacterial toxin produced by *Bordetella pertussis* that is commonly used as a pharmacological tool to inactivate the α-subunit of $G_{i/o}$ proteins.

The binding of agonist to extracellular domains of opioid receptor induces a conformational change that promotes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the α-subunit of $G_{i/o}$ protein coupled to the receptor. This allows the dissociation of the G protein into G_{α} subunit and $G_{\beta\gamma}$ dimer which carry the signals to their effectors, namely enzymes and/or ion channels. Generally, through coupling to $G_{i/o}$ family of heterotrimeric G proteins, opioid receptors activate a class of inwardly rectifying potassium channels and inhibit certain voltage-sensitive calcium channels. Moreover, acute stimulation of opioid receptors leads to inhibition of the adenylate cyclase (AC) and decrease in production of

cAMP (cyclic adenosine monophosphate) and can activate a number of kinase-mediated signaling cascades, thereby having additional effects on cytoplasmic signaling events and controlling neural gene expression (see *Law et al., 2000* and *Williams et al., 2001* for reviews). The summary of opioid receptor-evoked cellular responses is shown in Figure 1.2.

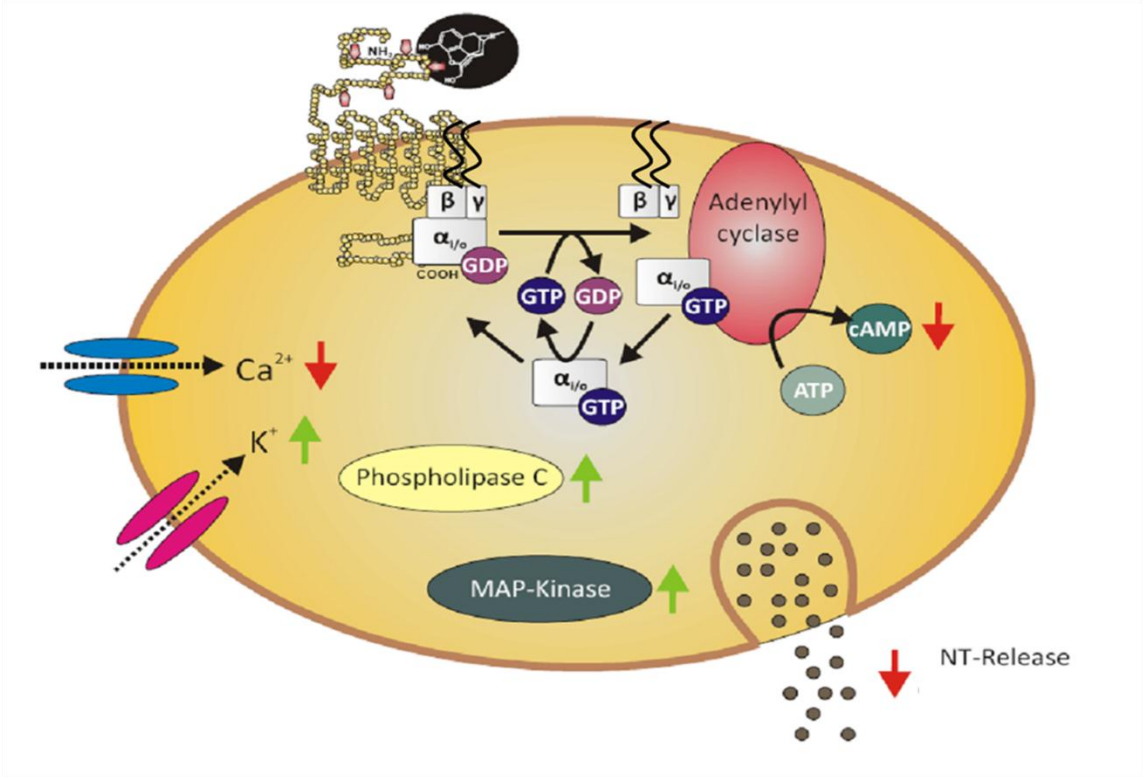


Figure 1.2. Main opioid receptor-evoked cellular responses. Binding of μ-opioid receptor agonists (e.g. morphine) results in the following $G_{i/o}$ protein mediated intracellular effects: inhibition of the adenylate cyclase (AC), inhibition of voltage-dependent Ca^{2+} channels, activation of inwardly rectifying K^+ channels, activation of phospholipase C and activation of MAP kinase. NT stands for neurotransmitter.

The anatomical localization of the μ-opioid receptors in the brain is consistent with known pathways of nociceptive signaling. They are expressed on peripheral nociceptors after inflammation, on spinal cord dorsal horn neurons and on the neurons in the various regions of the brain involved in pain perception and processing. Interruption of nociceptive signaling is the basis of analgesic effects of opioids. However, since the receptors are also expressed in the periphery (including gastrointestinal tract and skin) and not only in pain processing brain regions, activation of opioid receptors triggers not only analgesia but also numerous unwanted effects such as sedation, nausea and vomiting, constipation and respiratory depression, confusion, hallucinations, nightmares, dizziness, dysphoria, hyperalgesia, etc.

On the cellular level, a decrease of the calcium ion (Ca^{2+}) influx induced by activation of μ-opioid receptors on presynaptic neurons leads to a reduction of a neurotransmitter release into the synaptic gap. On the postsynaptic side, activation of μ-opioid receptors enhances the potassium ion (K^+) efflux resulting in a hyperpolarization of postsynaptic neurons. Thus, activation of pre and postsynaptic μ-opioid receptors on spinal cord dorsal horn neurons leads to a decrease of synaptic nociceptive transmission. In such a way, opioids exhibit their analgesic effects. In contrast to the immediate effects on pain transmission, alterations in the cAMP levels are associated with cellular changes that lead to the development of tolerance and physical dependence to opioids.

Although acute opioid treatment induces inhibition of AC and cAMP reduction, it has been shown that chronic opioid treatment followed by opioid withdrawal leads to enhanced AC activity and cAMP accumulation, a phenomenon termed ***AC superactivation***. This has been considered as a cellular hallmark of opioid withdrawal (*Bohn et al., 2000*; *Fin and Whistler, 2001*). However, mechanisms that are responsible for AC superactivation are still controversially discussed (for review see *Liu and Anand, 2001*).

1.2.3. Regulation of μ-opioid receptor activity

Like most GPCRs, the μ-opioid receptor can be regulated by multiple mechanisms including receptor ***desensitization***, ***internalization (endocytosis)***, ***resensitization*** and ***downregulation***.

MOPr-mediated signal transduction is usually rapidly attenuated by process of receptor desensitization. Namely, as shown in Figure 1.3., following agonist treatment, the receptor becomes phosphorylated by G protein-coupled receptor kinases (GRKs) (*Kovoor et al., 1997*; *Pak et al., 1997*; *Wolf et al., 1999*; *Deng et al., 2000*; *Wang, 2000*; *Law et al., 2000*, *Schulz et al., 2004*) or second messenger-regulated protein kinases, such as Ca^{2+}/calmodulin-dependent kinase II (*Mestek et al., 1995*; *Koch et al., 1997, 2000*; *Brüggemann et al., 2000*) and mitogen activated protein (MAP) kinase (*Polakiewicz et al., 1998*; *Schulz and Höllt, 1998*; *Schmidt et al., 2000*). Phosphorylated receptors then associate with β-arrestins and this leads to uncoupling of receptors from heterotrimeric G proteins disrupting their signaling and causing receptor ***desensitization***. In addition, β-arrestins bind to clathrin heavy chain and the β2-adaptin subunit of heterotrimeric AP-2 adaptor complex and therefore physically link and target receptors to clathrin-coated pits and endocytic membranes (for review see *Claing et al., 2002*). Once the plasma membrane is invaginated, the GTPase dynamin wraps around and constricts the necks upon GTP hydrolysis

leading to vesiculation and ***internalization (endocytosis)*** of the receptor. Internalized vesicles soon shed their clathrin coats and fuse with early endosomes. The ligands and receptors are separated in the acidified perinuclear compartment, β-arrestins dissociate and the receptors are either dephosphorylated by phosphatases and recycled back to the plasma membrane or are targeted to lysosomal degradation. This process is called 'post-endocytic sorting' (*von Zastrow et al., 2003*). Dephosphorylation and subsequent recycling of receptors contributes to a reversal of the desensitization state (***resensitization***), which is required for full recovery of cellular signaling potential following agonist removal. It is now well accepted that different opioid agonists induce receptor phosphorylation, desensitization, and internalization to a different extent. However, the molecular mechanisms underlying these differences remain unsolved. Moreover, it is known that after acute stimulation of MOPr with agonists that induce receptor endocytosis, most of the receptor is recycled back to the plasma membrane and a significant degradation of receptor is not detected.

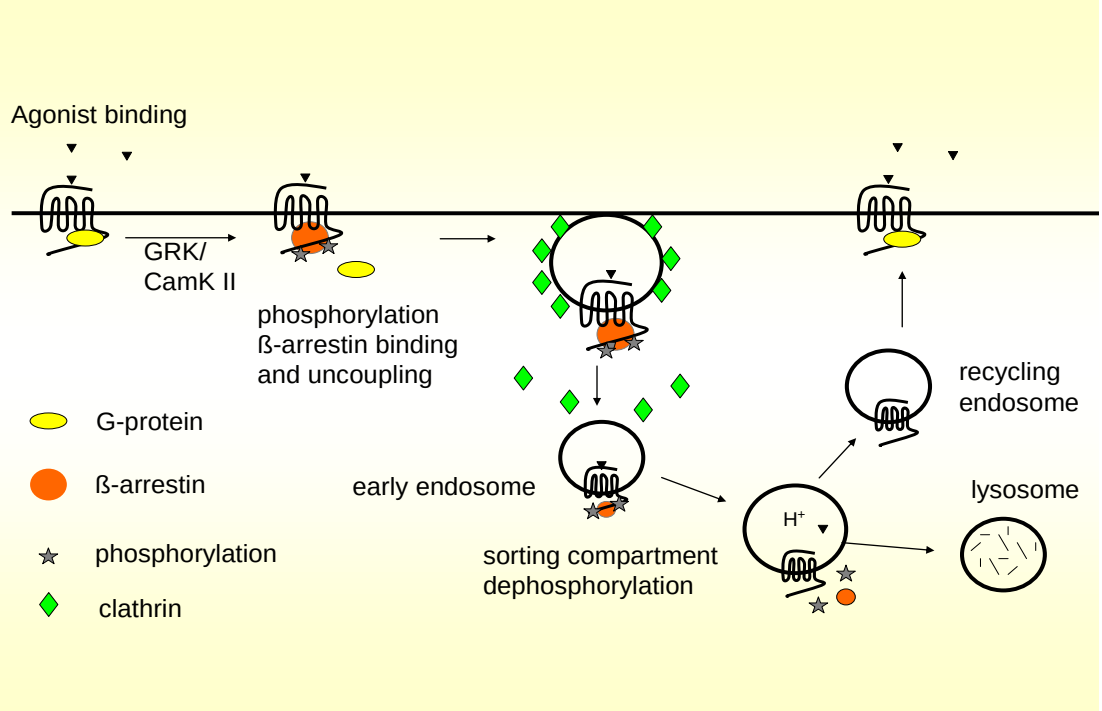


Figure 1.3. Agonist-induced endocytosis and recycling of the μ-opioid receptor. After agonist treatment, receptor is phosphorylated by kinases and uncoupled from G-proteins by β-arrestin binding. β-arrestin also promotes receptor internalization by clathrin recruitment. Following internalization, receptors are sorted in endosome and either dephosphorylated and recycled back to the plasma membrane in reactivated state or targeted to lysosome for degradation. GRK= G protein coupled receptor kinase; CamKII= Ca^{2+} - calmodulin dependent protein kinase II.

Receptor ***downregulation*** refers to a decrease in the total number of receptors present in cells or tissues, which is typically induced over a period of hours to days after prolonged or repeated exposure to opioid agonist (*Tsao and von Zastrow, 2000*). It is a consequence of proteolytic or lysosomal degradation of the internalized receptors and therefore the recovery from downregulation is dependent on new protein synthesis.

1.2.4. Opioid tolerance and dependence

Opioid tolerance, as well as physical or physiological dependence to opioids, develops after prolonged use of opioid drugs, over hours/days to weeks. ***Tolerance*** is a decrease in responsiveness manifested as a loss of response to a given dose of an agonist, or the requirement for an increased dose to achieve the original effect. ***Dependence*** is a different phenomenon, much more difficult to define and measure, which involves two separate components, namely physical and psychological dependence. Physical dependence is associated with a physiological withdrawal syndrome (or abstinence syndrome), manifesting as extreme restlessness and distress. Re-administartion of morphine rapidly abolishes the abstinence syndrome. Drug users who are no longer physically dependent can still show psychological dependence manifested by a strong craving for drugs and relapse.

It is increasingly evident that opioid-induced tolerance and dependence occur as adaptive changes at multi-levels in the nerve cell, beginning with regulation of opioid receptors themselves and extending to a complex network of direct and indirect modifications of "downstream" signaling machinery. It is known that traditional mechanisms like receptor phosphorylation, G protein uncoupling, receptor downregulation, desensitization, AC superactivation, the amount of effector proteins etc. are implicated in the development of opioid tolerance and dependence, but precise mechanisms involved in this complex phenomena still remain elusive.

To address this complex issue, cellular models of tolerance have been developed (*Taylor and Fleming, 2001*; *Kieffer and Evans, 2002*; *von Zastrow et al., 2003*; *von Zastrow 2004*) and different opinions were created. According to prevailing hypothesis, it was assumed that receptor endocytosis leads to a decrease in receptor signaling by receptor desensitization after prolonged agonist treatment. Moreover, receptor internalization and degradation after agonist treatment results in fewer available receptors at the cell surface and together these events would favor development of cellular tolerance to opioids. However, recent studies have demonstrated that endocytosed μ-opioid receptors are predominantly and rapidly recycled to the cell surface in a reactivated state (*Ferguson et al., 1998*; *Koch et al., 1998, 2001*; *El Kouhen et al., 1999*;*Law et*

al., 2000). These findings led to a revision of the prevailing hypothesis and suggest that μ-opioid receptor endocytosis is an important mechanism in ensuring that desensitized and internalized receptors are rapidly recycled to the cell surface in an active form, maintaining receptor signaling and reducing receptor desensitization and tolerance development (*Koch et al., 1998, 2001*; *Finn and Whistler, 2001*). In this revised model, noninternalizing agonists such as morphine cause an accumulation of desensitized receptors in the plasma membrane, resulting in greater opioid tolerance (*Koch et al., 2001, 2004*; *Schulz et al., 2004*).

1.3. Regulation of MOPr endocytosis

Receptor endocytosis has been in the center of opioid research for a long time since it is an important step in signaling regulation of many GPCR. It also has been implicated in physiological adaptations to opioid agonist treatment (*von Zastrow, 2001*; *von Zastrow et al., 2003)*. Moreover, recently it has been shown that rapid recycling of internalized μ-opioid receptors in a reactivated form back to the plasma membrane counteracts development of opioid tolerance as mentioned above (*Koch et al., 2005*). Thus, regulators of receptor endocytosis and trafficking might play a critical role in the development of opioid tolerance and dependence, together with other mechanisms involved in these complex phenomena. Good candidate molecules for these regulators might be proteins interacting with MOPr.

1.3.1. Phospholipase D2 (PLD2)

Investigation of molecules involved in μ-opioid receptor endocytosis and recycling identified phospholipase D2 (PLD2) as a novel μ-opioid receptor interacting protein (*Koch et al., 2003*). Furthermore, it has been shown that PLD2 is activated by binding of receptor internalizing agonists to MOPr and that this activation is dependent on ADP-ribosylation factor (ARF) protein and essential for receptor endocytosis (*Koch et al., 2003, 2006*). However, the mechanisms involved in PLD2 activation and regulation of receptor endocytosis are still not clear.

PLD2 is a membrane associated phospholipid-specific phosphodiesterase that catalyses hydrolysis of phosphatidylcholine (PC), a major phospholipid in the cell membrane, to membrane-bound phosphatidic acid (PA) and soluble choline (for review see *Liscovitch et al., 2000*). PA has been implicated to have many different functions in signal transduction, vesicle formation, and cytoskeleton dynamics (*Liscovitch and Cantley, 1995*; *Liscovitch et al., 1999*). Beside simple hydrolysis, PLD2 can catalyze transphosphatidylation reactions using short-chain

primary alcohols, e.g. ethanol, as phosphatidyl-group acceptors. Resulting phosphatidylalcohols are not normally found in biological membranes and their formation can serve as a convenient and sensitive marker for PLD activation in cultured cells (***PLD assay***).

Up to now, two mammalian PLDs, PLD1 and PLD2, have been cloned (the structures of the enzymes are shown in Figure 1.4.). Subcellular fractionation studies have demonstrated the presence of PLD1 in intracellular membranes like endoplasmatic reticulum, Golgi and vesicular compartments, whereas PLD2 has been shown to be largely associated with plasma membrane (*Liscovitch et al., 1999*). Both PLD enzymes require phosphatidylinositol-4,5-biphosphate (PIP_2) as a cofactor necessary for enzyme activity and proper membrane targeting. Other main regulators of PLD activity are protein kinase C (PKC) and small GTPases of the ARF and Rho families (*Liscovitch et al., 2000*; *Exton, 2002*; *Hiroyama and Exton, 2005*).

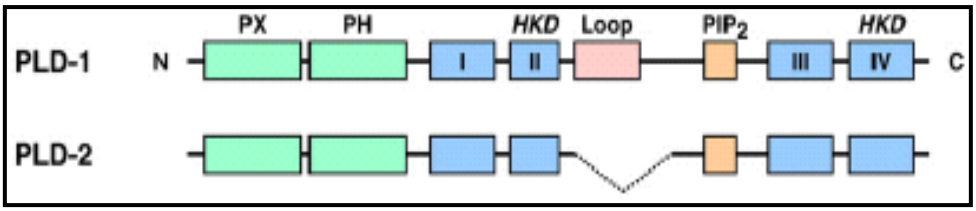


Figure 1.4. Domain structure of PLD isoforms. Both enzymes have four conserved sequences (I-IV), two of which contain catalytic HKD motif (where H is histidine, K is lysine and D is aspartate) which is conserved in numerous enzymes of phospholipid metabolism. Pleckstrin homology (PH) and phox homology (PX) domains present in tandem at N-terminus are implicated in phospholipid and protein binding (Frohman et al., 1999; Exton, 2002). N-terminally to domain III is a well conserved basic sequence that binds PIP_2. PLD1 is distinguished from PLD2 by a loop region that seem to contribute to the regulation of PLD1 activity.

Beside its important role in regulation of various cellular processes such as exocytosis, secretion and cytoskeletal reorganization, it has been shown that PLD2 can be regulated by a number of GPCRs including VPAC 1 and 2 receptors and PAC1 receptor (*McCulloch et al., 2001*), metabotropic glutamate receptors (*Shinomura et al., 2000*; *Kanumilli et al., 2002*; *Bhattacharya et al., 2004*), m1-m4 muscarinic receptors (*Sandmann et al., 1991*; *Mitchell et al., 2003*), the endothelin receptor (*Ambar and Sokolovsky, 1993*), the α_2-adrenergic receptor (*MacNulty et al., 1992*), the D2 dopamine receptor (*Senogles, 2000*), the somatostatin sstr2 receptor (*Cheng et al., 2005*), the 5HT2A-receptor (*Johnson et al., 2006*), the cannabinoid receptor isoform 1 (*Koch et al., 2006*) and the μ- and δ-opioid receptor (*Koch et al., 2003, 2006*). Recent studies implicated an involvement of receptor-mediated PLD2 activation in the regulation of receptor endocytosis (*Bhattacharya et al., 2004*; *Koch et al., 2006*; *Shen et al., 2001*; *Du et al.,*

2004). In addition, our group has previously shown that activation of PLD2 is a key step during the induction of agonist-mediated endocytosis and recycling of the MOPr affecting the development of opioid tolerance (*Koch et al., 2003, 2004*). However, the mechanism by which opioid receptors stimulate PLD2 activity has not been well established.

What is the role of PLD2 in endocytosis of GPCRs? Most cellular responses following enzyme activation are mediated by the PC hydrolysis product, PA. Besides being a 2^{nd} messenger, PA can be further metabolized to other bioactive lipids, such as lysophosphatidic acid (LPA) and diacylglycerol (DAG). It can alter physical and chemical properties of the plasma membrane (pH, charge, etc.) to assist formation of membrane curvature and to help formation of vesicles together with other acidic phospholipids. In addition, PA can affect both cellular localization and activity of various proteins (*Jenkins and Frohman, 2005*), like phosphatidylinositol-4-phosphate-5-kinase (PIP5K), an enzyme that synthesizes PIP_2 which is a PLD cofactor. Moreover, PIP_2 regulates the clathrin-dependent endocytosis by membrane recruitment of dynamin, GTPase responsible for fission of budding vesicles from membrane and components of the AP-2 adaptor complex. Therefore, opioid-activated PLD2 migth have a multiple roles in MOPr endocytosis and signaling.

1.3.2. ADP-ribosylation factor (ARF) proteins

Previous work demonstrated that PLD2 is predominantly activated by ARFs and not by PKC (*Exton, 2002*; *Hiroyama and Exton, 2005*; *Koch et al., 2003*). The ARF proteins belong to the Ras superfamily of small GTPases (for review see *D'Souza-Schorey and Chavrier, 2006*). These low molecular mass proteins (~20 kDa) are myristoylated at the second glycine (Gly) residue of the N-terminus, and this lipid modification seems to be important for the tethering of ARF proteins to membranes (*Amor et al., 1994*). Like other GTP-binding proteins, ARFs cycle between their active (GTP-bound) and inactive (GDP-bound) conformations. Hydrolysis of bound GTP is mediated by GTPase-activating proteins (GAPs), whereas the exchange of GDP for GTP is mediated by guanine nucleotide exchange factors (GEFs). In the recent years, an increasing number of these ARF regulators has been identified (*Donaldson and Jackson, 2000*).

Based on amino acid sequence identity, the six mammalian ARF proteins are categorized into three classes. Class I ARF proteins (ARF1, ARF2, and ARF3) regulate trafficking in the secretory pathway and in endosomes (*Bonifacino and Glick, 2004*). However, very little is known about the functions of class II ARFs 4 and 5. ARF6, which is the sole member of class III ARF

proteins, is thought to regulate actin cytoskeleton arrangement and endosomal trafficking at the cell surface (*D'Souza-Schorey et al., 1995*; *Peters et al., 1995*).

From six members of the ARF family of small GTPases, ARF1 and ARF6 are the best described. Both molecules are important components of the molecular machinery that regulates membrane trafficking along endocytic and biosynthetic pathways and are involved in activation of lipid-modifying enzymes like PLD and PIP5K. Since their GTP-bound, active conformations are very similar (*Pasqualato et al., 2001*), it seems that *in vivo* the specificity of ARF1 or ARF6 for their downstream effectors is regulated by their distinct localizations in the cell. ARF1 is localized mainly to the Golgi complex where it regulates the assembly of different types of 'coat' complexes onto budding vesicles, whereas ARF6 is associated to the plasma membrane and involved in regulation of plasma membrane/endosome trafficking as well as actin cytoskeleton rearrangements as mentioned above.

However, it has been shown that ARF1 can be also recruited to the plasma membrane upon activation of some GPCRs (*Mitchell et al., 2003*). Moreover, both ARF1 and ARF6 proteins have been reported to interact with different GPCRs and to be involved in regulation of their trafficking and signaling events (*Mitchell et al., 2003*; *Mitchell et al., 1998*; *Robertson et al., 2003*; *Johnson et al., 2006*). The authors proposed a conserved NPxxY motif (where N is asparagine, P is proline, x is any amino acid and Y is tyrosine) found in C-terminal part of most of GPCRs as a possible ARF binding site. The receptors that do not have an NPxxY motif, such as metabotropic glutamate receptors, have been demonstrated to activate PLD2 in an ARF-independent but PKC-dependent way (*Bhattacharya et al., 2004*). Since the NPxxY motif is present also in MOPr, this data suggest that a direct binding of ARF protein(s) can be involved in receptor mediated activation of PLD2. However, previous studies revealed that for a coimmunoprecipitation of MOPr and ARF protein the presence of PLD2 seemed to be important (*Koch et al., 2003*). Thus, it is reasonable to assume that ARF binds directly to PLD2 rather than to MOPr, but it can not be excluded that an interaction with PLD2 induces a conformational change of receptor which is necessary to facilitate ARF binding to MOPr. Having this in mind, it can be suggested that there is some kind of functional "ternary complex" formed between MOPr, PLD2 and ARF protein upon opioid treatment and formation of this complex seems to be important for opioid-mediated PLD2 activation and MOPr endocytosis. However, the identity of ARF protein (ARF1 or ARF6) as well as the precise interactions in this hypothetical "ternary complex" remain to be investigated.

To study cellular effects of ARF proteins, different mutants are described. As mentioned above, ARFs cycle between their inactive, GDP-bound state and their active, GTP-bound state.

Therefore the function of these proteins is largely investigated by using dominant negative and constitutively active mutants which are thought to be "locked" in GDP- and GTP-bound states, respectively. However, some recent studies have shown that in the case of ARF6 these "classical" mutants, namely dominant negative ARF6/T27N and constitutively active ARF6/Q67L, show some artifacts *in vivo* (*Macia et al., 2004*; *Santy, 2002*; *Klein et al., 2006*). Namely, it was demonstrated that ARF6/T27N mutant has a high tendency to lose its nucleotide and to denature *in vitro* (*Macia et al., 2004*) and thus, is no longer located in the plasma membrane where ARF6 normally resides. To overcome these problems, another dominant negative ARF6 mutant, ARF6/T44N was generated, which has a 30-fold decreased affinity for GTP in comparison to the wild type protein and, importantly, is properly located in the plasma membrane *in vivo* (*Macia et al., 2004*). This mutant has been suggested to be a better choice for investigation of blocking ARF6 function *in vivo*. On the other hand, regarding active ARF6 mutants, the "fast cycling" ARF6/T157N mutant was found to induce phenotypes that have been previously attributed to ARF6 activation without the toxic effects demonstrated after "classical" constitutively active ARF6/Q67L mutant expression (*Santy, 2002*; *Klein et al., 2006*). This mutant "cycles" i.e. binds GTP and releases GDP more quickly than the wild type protein and therefore has an enhanced activity *in vivo*. Moreover, unlike ARF6/Q67L mutant that is "locked" in its GTP-bound form, "fast cycling" ARF6/T157N retains a full cycle of GTP binding, hydrolysis and release which is necessary for proper function of ARF6 and therefore is suggested to represent better the ARF6 active form *in vivo* (*Santy, 2002*; *Klein et al., 2006*).

1.4. The aim of the present research project

Agonist-induced endocytosis is an important regulatory and signaling event for G protein-coupled receptors (for review see *von Zastrow, 2001*). For the mu-opioid receptor, the investigation of molecular mechanisms regulating this process is of clinical importance because MOPr endocytosis counteracts the development of tolerance to opioid drugs by facilitating the reactivation of desensitized receptors (*Koch et al., 2005*).

In search of molecular players involved in MOPr endocytosis, our group has recently identified phospholipase D2 as a MOPr interacting protein (*Koch et al., 2003*). This ubiquitously expressed and plasma membrane located enzyme was previously reported to be activated by a great variety of hormones, neurotransmitters, growth factors, cytokines and stimulation of various GPCRs (reviewed in *Liscovitch et al., 2000*). We have further demonstrated that the opioid-

mediated activation of PLD2 is ARF-dependent and is a prerequisite for MOPr endocytosis. However, it is still not known which ARF protein (ARF1 or ARF6) is involved in opioid-mediated PLD2 activation and what are the mechanisms of ARF function in MOPr trafficking and signaling.

ARF1 and ARF6 are the best described members of ARF family of small GTPases. Both molecules have been reported to interact with different GPCRs and to be involved in regulation of their trafficking and signaling as mentioned above. Although very similar in structure, these proteins differ in their effectors and downstream signaling pathways. Thus, the knowledge of whether MOPr trafficking is regulated by ARF1 or ARF6 might provide new insights into MOPr-mediated signaling pathways and may lead to the identification of further regulatory proteins involved in the modulation of MOPr trafficking and signaling.

Therefore, the aim of the present study is to determine which ARF protein, ARF1 or ARF6, is involved in opioid-mediated PLD2 activation and migth be a part of a hypothetical "ternary complex" which is formed between MOPr, PLD2 and ARF protein. We also investigated the main molecular mechanisms of ARF function in MOPr trafficking and signaling.

The major goal of this thesis is to understand the mechanisms of endocytosis and trafficking of the μ-opioid receptor and to further investigate the role of the key players involved in regulation of these processes, mainly phospholipase D2 and ARF protein(s). As outlined before, opioid drugs such as morphine are well known for their ability to produce potent analgesia as well as such unwanted side effects like tolerance, physical dependence, respiratory suppression and constipation. Better understanding of MOPr pharmacology and signaling will contribute to the development of new opioid drugs and therapeutic approaches that will be able to overcome the problems of negative side effects in clinical applications of opioids and make them more useful in treatment of severe pain.

2. Materials and Methods

2.1. Materials

2.1.1. Lab instruments and equipment

Item	**Company**
UV-visible Spectrophotometer	Pharmacia Biotech, Germany
Expert Plus Microplate Reader	ASYS, Austria
Leica TCS-NT laser-scanning confocal microscope	Leica Microsystems, Germany
PTC-0200 DNA Engine PCR mashine	MJ Research, Inc. USA
Electrophoresis power supply	Bio-Rad
Gel electrophoresis system	Bio-Rad
Semi-dry Transfer Cell and Western blot system	Bio-Rad
Flasks, plates and dishes for cell culture	Greiner Bio-One, Frickenhausen, Germany

2.1.2. Kits and enzymes

Product	**Company**
Endonucleases (Restriction enzymes)	New England Biolabs
Taq DNA polymerase & PCR Kit	Promega
T4 DNA ligase	New England Biolabs
Pfu DNA polymerase	Fermentas
Oligonucleotides (Primers)	Metabion International AG, Germany
Plasmid Mini Kit, Plasmid Midi Kit, PCR purification Kit, Gel Extraction Kit	Qiagen, Germany
Cyclic AMP (^{3}H) assay system	Amersham Biosciences, Braunschweig, Germany

2.1.3. Molecular weight markers

DNA and protein molecular weight markers	**Company**
GeneRuler™ 1kb DNA Ladder	Fermentas
Precision Plus Protein™ Standards	Bio-Rad

2.1.4. Plasmids

Plasmid	**Company or kindly provided by:**
pEAK10:HA-MOPr pcDNA3:T7-MOPr	from Dr. T. Koch (IPT, Magdeburg, Germany)
pcDNA3.1:PLD2	from Dr. S. Ryu (Pohang, South Korea)
pCMV5:HA-ARF6/T44N, pCMV5:HA-ARF6/N48I, pCMV5:HA-ARF6/T157N	from Dr. J. Jaworski (IIMBC, Warsaw, Poland)
pXS:HA-ARF1/T31N	from Dr. R. Mitchell (CIP, Edinburgh, UK)
pGEM-T easy vector	Promega, Medison, USA
pcDNA3.1 c-myc-pcDNA3.1	Invitrogen, Karlsruhe, Germany

2.1.5. Bacterial and eukaryotic cell lines

Cells	**Company**
E. coli XL1 blue	Promega
Human embryonic kidney (HEK) 293 cells	German Collection Of Microorganisms and Cell Cultures, Braunschweig, Germany
African green monkey kidney fibroblast (COS-7) cells	Clontech

2.1.6. Cell culture media, antibiotics and reagents for mammalian cells

Item	**Composition**
HEK 293 cell culture medium	Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Basel, Switzerland) and 10% fetal calf serum (FCS) (Bachem, Heidelberg, Germany)
COS-7 cell culture medium	DMEM, 10% FCS, 2 mM L-glutamine (Lonza, Basel, Switzerland), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich)

Poly-L-lysin	50 μg/ml poly-L-lysin (Sigma-Aldrich) in sterile PBS, pH 7.4
Tripsin/EDTA	0.5 mM ethylenediaminetetraacetic acid (EDTA) and 0.05% Trypsin
UltraMEM	Cambrex Bio Science Verviers S.p.r.l., Verviers, Belgium
Neurobasal	Gibco (Invitrogen)
HBSS	Hank´s balanced salt solution, Cambrex Bio Science Verviers S.p.r.l., Verviers, Belgium
G418	PAA Laboratories GmbH, Pasching, Germany
Puromycin, Penicillin and Streptomycin	Sigma-Aldrich

2.1.7. Culture media and additives for bacteria

Culture medium	**Composition**
LB-medium	20 g LB Broth Base (Invitrogen) / 1000 ml H2O
LB-Agar	15 g Select Agar (Invitrogen) / 1000 ml LB-medium
Ampicillin	Sigma-Aldrich

***** All media were autoclaved at 121°C for 15 minutes. Antibiotic was added additionally after cooling down LB-agar media to 50-55ºC.

2.1.8. Drugs and other chemicals

Product	**Company**
Rhodamine-phalloidin	Invitrogen, Karlsruhe, Germany
Lipofectamine™ 2000	Invitrogen, Karlsruhe, Germany
Protein A-agarose beads	Amersham Biosciences, Braunschweig, Germany
Triton-X 100	Merck, Darmstadt, Germany
DPX mounting media	Fluka, NeuUlm, Germany
ABTS solution	Roche Molecular Biochemicals
Leupeptin, Pepstatin A, Aprotinin, Dithiotreitol	Sigma-Aldrich
HEPES	Serva, Heidelberg, Germany
DNase	Sigma-Aldrich
Bovine serum albumin (BSA)	Serva, Heidelberg, Germany
Enhanced chemiluminiscence detection system	Amersham Biosciences, Braunschweig, Germany

Ammonium persulfate (APS)	Sigma-Aldrich
30% acrylamide mix	Carl Roth Gmbh & Co
TEMED	Serva, Heidelberg, Germany
Morphine [(D-Ala^2,NMe-Phe^4,Gly-ol^5)-enkephalin (DAMGO)	Fagron GmbH & Co, KG, Barsbuettel, Germany Bachem, Heidelberg, Germany
[^{3}H]DAMGO	NEN, Koeln, Germany
Naloxone	Pfitzer/Goedecke, Freiburg, Germany
Forskolin	Applichem, Darmstadt, Germany
phorbol 12-myristate 13-acetate (PMA)	Biomol International
N-myristoylated (2-13) ARF6 peptide	Calbiochem, Darmstadt, Germany
Agarose	Biozym
[1,2,3-^{3}H]glycerol (1μCi/ml; specific activity 40 Ci/mmol)	American Radiolabeled Chemicals, St. Louis, MO

2.1.9. Antibodies

2.1.9.1. Primary antibodies for Western blot and immunostaining

Antibodies	**Species**	**WB dilution**	**IF dilution**	**Company**
anti-T7 antibody	mouse	1:1000	1:1000	Novagen, Darmstadt, Germany
anti-HA serum	rabbit		1:300	Gramsch Laboratories, Schwabhausen, Germany
anti-PLD2 antibody	mouse		1:500	Invitrogen, Karlsruhe, Germany
anti-c-myc antibody	mouse		1:500	BD Biosciences, Heidelberg, Germany
anti-ARF6 antibody	mouse	1:100		Santa Cruz Biotechnology, USA
anti-Rab11 antibody	goat		1:100	Santa Cruz Biotechnology, USA
anti-HA antibody	rat	1:1000		Roche Applied Science, Mannheim, Germany
anti-actin antibody	rabbit	1:500		Santa Cruz Biotechnology, USA
anti-actin antibody	mouse	1:10000		Sigma-Aldrich

2.1.9.2. Secondary antibodies for Western blot and immunostaining

Antibodies	Species	Dilution	Company
Anti-rabbit IgG, cyanine 3.18-conjugated (Cy3)	goat	1:1000	Dianova, Germany
Anti-rabbit IgG, Alexa Fluor™ 488 and 647 conjugated	goat	1:1000 and 1:500	Molecular Probes, Invitrogen
Anti-goat IgG, Alexa Fluor™ 647 conjugated	donkey	1:500	Molecular Probes, Invitrogen
Anti-rabbit IgG, peroxidase-conjugated	goat	1:5000	Amersham Biosciences, Braunschweig, Germany
Anti-mouse IgG, peroxidase-conjugated	sheep	1:5000	Amersham Biosciences, Braunschweig, Germany
Anti-rat IgG, peroxidase-conjugated	goat	1:5000	Amersham Biosciences, Braunschweig, Germany

2.1.10. Buffers and Solvents

Zamboni's fixative:

4% paraformaldehyde and 0.2% picric acid in phosphate buffer, pH 6.9

Radioimmunoprecipitation (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 phenylmethylsulfonyl 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 dithiotreitol (dithiotreitol was added prior to use)

1 x TPBS (Tris/phosphate-buffered saline):

10 mM Tris, 10 mM phosphate buffer, 137 mM NaCl and 0.05% thimerosal, pH 7.4

1 x PBS (phosphate-buffered saline):

137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4

1 x PBS/Tween 20:

137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4 and 0.01% Tween 20

1 x TAE (Tris-acetate-EDTA) buffer:

40 mM Tris, 0.2 mM acetic acid, 1 mM EDTA, pH 7.6

2.2. Methods

2.2.1. cDNA cloning into expression vectors

In the following experiments, gene subcloning was carried out using standard molecular cloning protocols or according to the manufacturer's instructions. Briefly, genes or DNA fragments of interest were amplified by polymerase chain reaction (PCR). Following 1% agarose gel electrophoresis in TAE buffer, the fragments were purified by the PCR Purification Kit. The fragments were then subjected to appropriate enzymatic digestion (2 hours or overnight at 37ºC) and ligated with T4 ligase to the pre-digested vector. The ligations were performed at room temperature for 1.5 hour. To select the positive clones, obtained constructs were transformed into freshly generated *E. coli* XL1 Blue competent cells by heat-shock transformation for subsequent DNA mini-prep isolation. Extracted DNA from isolated clones was incubated with restriction enzymes and the positive clones showing the insert with the expected molecular size were identified by 1% agarose gel electrophoresis. The identity of these clones was subsequently confirmed by sequence analysis (Seqlab Göttingen). For mammalian cell transfection, DNA with high concentration and purity was prepared using the Plasmid Midi Kit. The DNA concentration was determined by spectrophotometrical quantification at 260 nm.

2.2.2. Cell culture, transfection and generation of stable cell lines

2.2.2.1. Human embryonic kidney (HEK) 293 cells

HEK293 cells were maintained in DMEM medium supplemented with 10% FCS in a humidified incubator with an atmosphere containing 10% CO_2, at 37ºC. All transfections were done using Lipofectamine™ 2000 according to manufacturer's instructions. For HEK293 stable cell line expressing T7-MOPr, the cells were transfected with pcDNA3:T7-MOPr plasmid containing G418 resistance gene. Stable transfectants were then selected in the presence of 1mg/ml G418. For generation of stable cell line coexpressing HA-MOPr and PLD2 that was used for PLD2 assay, HEK293 cells were first transfected with pEAK10:HA-MOPr plasmid containing puromycin resistance and stable transfectants were selected in the presence of 1μg/ml puromycin. The cells were then subjected to second round of transfection with pcDNA3.1:PLD2 plasmid and selected in the presence of 1μg/ml puromycin and 500 μg/ml G418. The whole pool of resistant cells was used without selection of individual clones. Receptor and/or PLD2 expression was monitored using receptor ligand binding assays, PLD assays, Western blot analysis and confocal microscopy as described below.

Transient transfection of T7-MOPr-expressing HEK293 cells with HA-ARF mutants was done using Lipofectamine™ 2000 in 6-well plates in reverse manner. Suspension of cells (approximately 80% confluence) in DMEM/10% FCS was mixed with DNA-Lipofectamine 2000 complexes prepared according to manufacturer's instructions prior to plating. After 5-6 h, medium was changed with fresh DMEM/10% FCS. 24 h after transfection, cells were seeded for further experiments. Confocal microscopy analysis revealed that about 50% of the cells expressed both proteins.

2.2.2.2. African green monkey kidney fibroblast (COS-7) cells

COS-7 cells were were maintained in DMEM medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with an atmosphere containing 5% CO_2, at 37ºC. Transient co-transfection of these cells with pcDNA3:T7-MOPr and HA-ARF6 mutants was done using Lipofectamine™ 2000 in 6-well plates in reverse manner as described above. 24 h after transfection, the cells were seeded for further experiments.

2.2.2.3. Primary neuronal cell cultures

Neuronal cultures were prepared from rat cortex of E17 Sprague Dawley rat embryos (Charles River). Dissection medium consisted of Hans balanced salt solution (HBSS) with 20 mmol/L HEPES, pH 7.3. The cerebral cortex was dissected and then incubated for 15 min at 37ºC in dissection medium containing 0.05% (w/v) trypsin and 0.5 mmol/L EDTA. Trypsinization was terminated using dissection medium containing 0.5 mg/ml trypsin inhibitor, 0.24 mg/ml DNase and 3 mg/ml BSA. The tissue was rinsed with dissection medium, triturated, centrifuged and resuspended in dissection medium. All animal procedures were approved by Otto-von-Guericke University, Magdeburg.

About $3x10^5$ freshly prepared cortical cells were seeded onto poly-L-lysine-treated coverslips in 12-well plates and grown for 4 days in Neurobasal medium supplemented with 2% B-27, 0,5 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured at 37ºC and 5% CO_2 in a humidified incubator. On the 4^{th} day after seeding the medium was removed and saved in a tube at 37ºC to be used again after transfection. The cells were then transfected with peak10:HA-MOPr plasmid alone or together with pcDNA3.1:c-myc-ARF6/T44N or pcDNA3.1:c-myc-ARF6/T157N plasmid using Lipofectamine™ 2000 according

to manufacturer's instructions. 5-6 h after transfection medium was changed and used (conditioned) medium was put back onto cells. Immunocytochemistry was done 48 h after transfection.

2.2.3. RNA interference (RNAi)

2.2.3.1. Principle

RNA interference (RNAi) is the process of mRNA degradation that is induced by double-stranded RNA (dsRNA) in a sequence-specific manner. In the laboratory, RNAi is designed and used for specifically silencing the expression of any gene for which sequence is available in order to investigate the influence of specific gene knock-down on some cellular processes.

First, the dsRNAs that were introduced into the cell by different mechanisms get processed into 20-25 nucleotide long small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer (initiation phase) (Figure 2.1.). Alternatively, siRNAs can be introduced into the cell directly. Then, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (effector phase). Knock down of the specific gene expression is usually detected on RNA level (by RT-PCR) or protein level (by Western blot analysis).

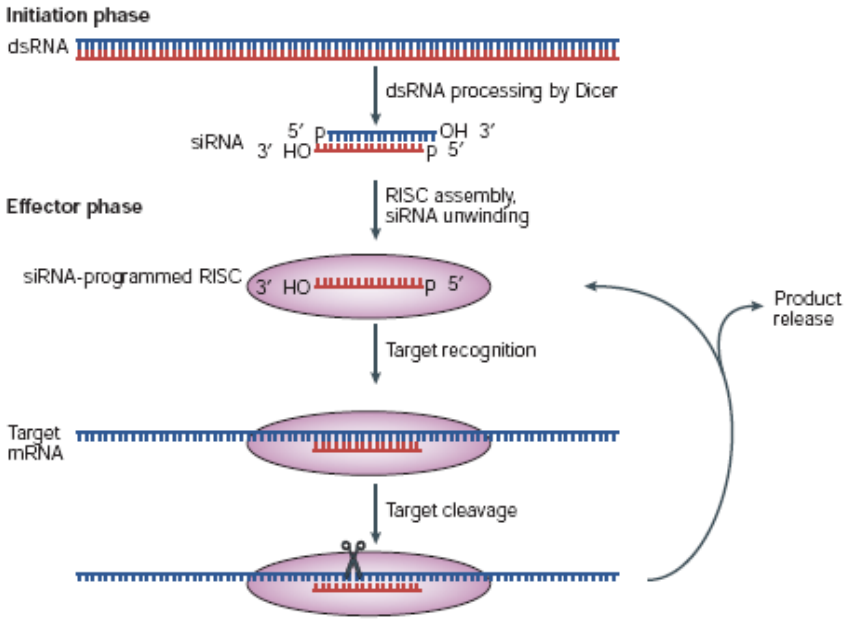


Figure 2.1. The Mechanism of RNA interference. See text for details.

2.2.3.2. Procedure

To design ARF6-specific siRNA duplexes, the nucleotide sequence of human ARF6 gene was screened for unique 21-nucleotide sequence starting with two adenosines (AA) and containing a G/C ratio of 30-50%. The following 21-nucleotide sequence was chosen corresponding to the position 244-265 on the human ARF6 mRNA relative to the start codon: 5'-AAGGUCUCAUCUUCGUAGUGG-3'. Similarly, the following sequence targeting ARF1 gene was used as a control: 5'-ACCGTGGAGTACAAGAACA-3'. The sequences were compared with the human genome data base using BLAST and no homology was found with other genes. Non-specific control sequence was 5'-AGGUAGUGUAAUCGCCUUGTT-3'. All sequences were manufactured by Eurofins MWG Operon, Ebersberg, Germany and their efficiency in protein knock down was tested by Western blot analysis as described. Briefly, due to initial problems in detection of endogenous ARF proteins with commercially available antibodies that we tested, first siRNA efficiency tests were done with overexpression of HA-tagged ARF proteins. Namely, HEK293 cells stably expressing T7-MOPr were transiently co-transfected with wild type HA-ARF1 and HA-ARF6 in combination with two different concentrations of indicated siRNAs. 48 h after the transfection, the cells were lysed and lysate was directly tested in Western blot analysis using rat anti-HA antibody (1:1000). The efficiency of ARF6 siRNA to knock down the expression of endogenous ARF6 protein was then reconfirmed without overexpression of ARF6 protein using anti-ARF6 antibody (1:100). For quantitative assays, the transfection of T7-MOPr-expressing HEK293 cells with appropriate siRNAs was done in 6-well plates with Lipofectamine^{TM} 2000 according to manufacturer's instructions. 24 h after transfection the cells were seeded in 48-well plates and one day later tested in ELISA assay.

2.2.4. Radioligand binding assay

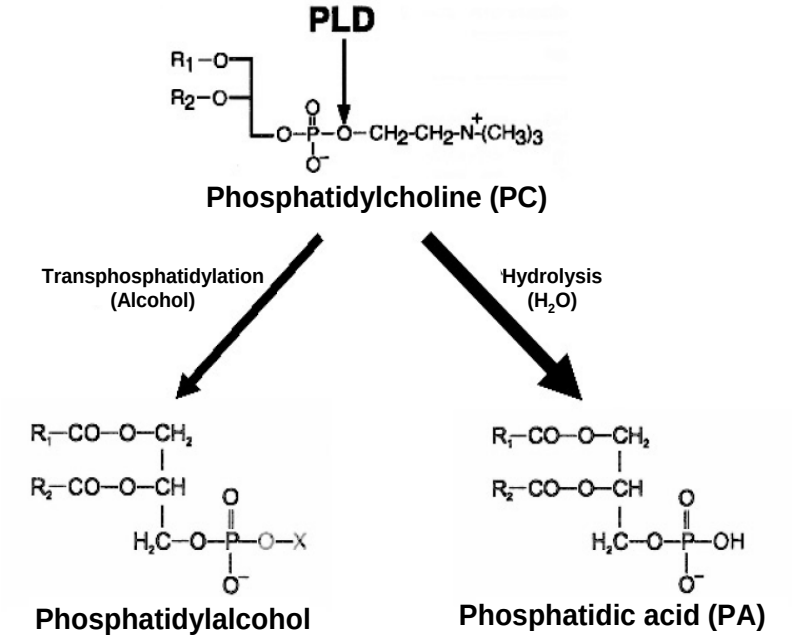
The binding characteristics of the receptor in cells expressing MOPr alone or together with PLD2 were determined by saturation binding assays on membranes prepared from stably transfected HEK293 cells. For whole cell binding, 10^6 cells were incubated with at least six different concentrations of [^{3}H]DAMGO in a range from 0.3 to 9 nm for 40 min at 25ºC in 50 mM Tris-HCl, pH 7.8. Cells were collected on GF 10 glass-fiber filters and unbound ligand was removed by extensive washing with 50 mM Tris-HCl, pH 7.8. The radioactivity of 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 unlabelled DAMGO. Results were calculated as fM bound radioligand per mg of protein, measured by Lowry method. The dissociation constant (K_D) and number of [^{3}H]DAMGO binding sites (B_{max}) were calculated by Scatchard analysis.

2.2.5. Transphosphatidylation reaction – PLD assay

2.2.5.1. Principle

PLD catalyzes hydrolysis of the distal phosphodiester bond in phospholipids such as phosphatidylcholine (PC), the most abundant phospholipid in biological membranes. A phosphatidyl-enzyme intermediate that is transiently formed is normally hydrolysed by water, generating phosphatidic acid (PA). Primary short chain alcohol ethanol can substitute water in a competing transphosphatidylation reaction giving rise to phosphatidylethanol (PtdEtOH) (Figure 2.2.). This reaction occurs at the expense of the hydrolytic reaction decreasing PA formation. Phosphatidylalcohols are metabolically stable and would accumulate in cells upon PLD activation. Since cellular phosphatidylalcohol levels are extremely low, its accumulation upon PLD activation is readily detectable and can be used as a marker of PLD activation.



<u>Figure 2.2.</u> Hydrolysis and transphosphatidylation of phosphatidylcholine by phospholipase D2. X- the rest of alcohol, e.g. C_2H_5 for ethanol**.**

2.2.5.2. Procedure

HEK293 cells stably coexpressing MOPr and PLD2 were transiently transfected with indicated ARF mutants as described above and seeded into 6 cm dishes. 24 h after transfection, the cells were kept for 24 h in serum-free UltraMEM medium containing [1,2,3-^{3}H]glycerol (1μCi/ml; specific activity 40 Ci/mmol) in order to label phospholipids. The cells were then treated for 30 min at 37ºC with indicated substances (1μM DAMGO, 1μM morphine or 1μM PMA) in serum-free UltraMEM containing 2% ethanol. After stimulation, the cells were extracted in 2.5 ml of ice-cold methanol/water mixture (3:2, v/v). Subsequently, 1.5 ml chlorophorm and 0.35 ml H_2O were added giving the final ratio methanol:chlorophorm:water = 10:10:9, v/v/v. The lower, lipid phase was separated by thin layer chromatography. Individual phospholipids were stained with iodine, identified by standards and spots corresponding to PtdEtOH, PA and PC were isolated and subjected to liquid scintillation counting. PLD activity was calculated as percentage [^{3}H]- PtdEtOH of the total cellular PC concentration and then shown as the percent of the control values.

2.2.6. Quantitative analysis of receptor internalization and recycling by Enzyme-linked Immunosorbent Assay (ELISA)

24 h after transfection with ARF mutants or adequate siRNAs, T7-MOPr-expressing HEK293 cells were seeded in DMEM medium supplemented with 10% FCS and grown onto poly-L-lysine-treated 48-well plates overnight. In experiments with N-myr-ARF6, DMEM medium was also supplemented with 25 μM N-myr-ARF6 peptide. To estimate endocytosis, the cells were specifically surface labeled with T7-antibody (1:1000) in UltraMEM for 1.5 h at 4°C and after washing stimulated with indicated agonists for 30 min at 37°C. After fixation, the cells were incubated with anti-mouse peroxidase-conjugated secondary antibody (1:5000) for 2h at room temperature. Plates were developed with 150 μL of ABTS solution per well. After 20-30 min, 100 μL of the substrate solution from each well was transferred to a 96-well plate. Color reaction was analyzed at 405 nm using an Expert Plus Microplate Reader. During the assay, the cells were kept on 4ºC for 30 min (4ºC control), treated with indicated agonists in UltraMEM for 30 min at 37°C or not treated (37ºC control). Constitutive endocytosis in the absence of agonist was calculated as percentage loss of surface receptors in 37ºC control to 4ºC control. Agonist-induced receptor endocytosis was calculated by subtraction of constitutive endocytosis from total

endocytosis that was detected in agonist-treated samples in comparison to 4ºC control (100%), or total endocytosis was shown as indicated.

To measure recycling, after 30 min of DAMGO treatment the cells were washed with warm media to remove the agonist and then incubated for further 30 min at 37°C in the presence of 1 μM receptor antagonist naloxone in order to block residual DAMGO-stimulated endocytosis of MOPr. After fixation of cells, surface receptors were detected as described in this section. In these experiments, 37ºC control that was taken as 100% for calculation of receptor endocytosis in agonist-treated samples. The recycling of internalized receptor was estimated as the percentage of recovered surface receptors from endocytosed receptors.

2.2.7. Immunocytochemistry

2.2.7.1. HEK293 cells

24 h after transfection, HEK293 cells coexpressing T7-MOPr and various HA-ARF mutants were seeded on poly-L-lysine-coated coverslips and grown overnight. After surface labeling of receptors with T7-antibody (1:1000) for 1.5 h at 4°C, the cells were washed and stimulated or not with indicated agonists at 37°C for 30 min and then fixed with Zamboni's fixative for 30 min at room temperature. After washing several times with TPBS, the cells were then permeabilized by 3 min subsequent incubation with 50% and 100% methanol. To visualize cells transiently transfected with ARF mutants, cells were further incubated with 1 μg of affinity purified rabbit anti-HA antibody for 1.5 h at room temperature. Bound primary antibodies were detected using anti-mouse cyanine 3.18-conjugated secondary antibody and/or anti-rabbit Alexa 488-conjugated secondary antibody. Cells were permanently mounted in DPX and examined using Leica TCS-NT confocal microscope. Images were recorded digitally and processed using ImageJ NIH (National Institute of Health) and Adobe Photoshop CS (version 9.0 CS2).

2.2.7.2. COS-7 cells

24 h after transfection, COS-7 cells coexpressing HA-MOPr and indicated ARF6 mutants were seeded on poly-L-lysine-coated coverslips and grown overnight. In these experiments different combinations of triple immunostaining were done as indicated. In some cases, MOPr receptors were first surface labeled with anti-HA serum (1:300) for 20 min at room temperature and then the cells were washed and stimulated or not with 10 μM DAMGO at 37°C for 30 min. In recycling experiments, after stimulation the agonist was washed away and the cells were treated

for further 30 min (during receptor recycling) with 1μM receptor antagonist naloxone in order to block residual DAMGO-stimulated endocytosis of MOPr. After fixing with Zamboni's fixative for 30 min at room temperature, the cells were permeabilized with 5 min incubation with 0.3% Triton X-100 in PBS at room temperature. Blocking was done by 1 h incubation with 1% NGS at room temperature. Subsequently, HA-ARF6 mutants or Rab11 protein were immunostained with primary antibodies (1 μg of affinity purified rabbit anti-HA antibody or anti-Rab11 antibody, 1:100) and then visualized by 1.5 h incubation with a mixture of appropriate secondary antibodies at room temperature. Finally, actin cytoskeleton was detected by 20 min incubation with rhodamine-phalloidin according to manufacturer's instructions. Cells were permanently mounted in DPX and examined using Leica TCS-NT confocal microscope.

2.2.7.2.1. Transferrin trafficking-"pulse-chase" assay

In COS-7 cells co-transfected with HA-MOPr and "GTP-locked" ARF6 mutant a previously described "pulse-chase" assay (*Tsao and von Zastrow, 2000*; *Tulipano et al., 2004*) was done. Here the degree to which a "pulse" of internalized MOPr was accessible to a subsequent "chase" of endocytosed transferrin was estimated.

24 h after transfection, COS-7 cells coexpressing HA-MOPr and "GTP-locked" ARF6 mutant were seeded on poly-L-lysine-coated coverslips and grown overnight. First, MOPr was surface labeled with anti-HA serum like described above and then the cells were stimulated or not with 10 μM DAMGO for 30 min at 37°C to drive internalization of antibody-labeled receptor. After washing, the cells were incubated with UltraMEM containing 5 μg/ml Alexa Fluor-conjugated transferrin for 20 min at 37°C. These conditions label both early and recycling endosomes (*Dunn et al., 1989*). The cells were then fixed and permeabilized and antibody-labeled receptor as well as actin cytoskeleton were detected like described above.

2.2.7.3. Neuronal cultures

Immunocytochemistry was done 48 h after transfection of primary cultured corical neurons with HA-MOPr and c-myc-ARFs. First, MOPr was surface labeled with anti-HA serum (1:300) for 20 minutes at room temperature. The cells were then washed with UltraMEM, treated or not with 10 μM DAMGO or 10 μM morphine for 30 minutes at 37ºC, fixed with Zamboni's fixative and permeabilized with methanol like described above. After blocking with 10% NGS in PBS for 1 h, ARFs were immunostained with c-myc antibody (1:500) in 3% NGS for 1.5 h at

room temperature. The proteins were visualized after 1.5 h incubation at room temperature with mixture of appropriate secondary antibodies using Leica TCS-NT confocal microscope.

For quantification of subcellular fluorescent density, NIH Image 1.62 software was used as previously described (*Schröder et al., 2009*). Briefly, cytosolic fluorescent intensity was substracted from whole cell fluorescent intensity to obtain surface fluorescent intensity. Fluorescent intensity values were divided per surface unit (pixel) to obtain densities. Ratios of cytoplasmic (D_f cyto) versus surface (D_f surf) fluorescence densities were calculated to normalize data across neurons examined. A value of 1.0 results from equal densities of MOPr in the cytoplasm and at the cell surface.

For desensitization studies, the newly prepared cortical cells were transfected with the catalytically inactive (K758R) PLD2 mutant (nPLD2) (*Koch et al., 2006*) after 4 days in culture using Lipofectamine 2000 according to manufacturer's instructions. Two days later cAMP levels were determined as described below under "Materials and Methods" (2.2.9.). As a control, untransfected cells were assayed after 6 days in culture.

2.2.8. Western blot analysis

Depending on the experiment, 24 or 48 h after transfection of HEK293 cells stably expressing T7-MOPr with indicated plasmids or siRNAs, the cells were washed one time with PBS and RIPA buffer was added (1.5 mL per flask or 250 µL per well of 6-well-plate). The cells were swollen for 15 min on ice and then homogenized and scraped into eppendorf tube. After 30-40 min of gentle shaking at 4ºC, samples were centrifuged at 18000 rpm for 1h at 4ºC, the supernatant was collected and either used immediately or aliquoted and frozen at -80ºC. Before loading onto gel, samples were prepared by adding appropriate amounts of SDS-sample buffer (plus freshly added DTT) and then cooked for 5 min at 95ºC.

Proteins were separated using one-dimensional 8% or 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under fully denaturing and reducing conditions (Laemmli system). Gels were allowed to run at voltage of 100-150 mV in an electrophoresis chamber (Bio-Rad) filled with 1 x Laemmly buffer. Subsequently, proteins were electrotransfered from polyacrylamide gels to nitro-cellulose membranes (Amersham Biosciences) using Semi-dry Transfer Cell and Western blot system (Bio-Rad). After 1-1.5h blocking in 5% milk in PBS/Tween 20, the membrane was washed three times with PBS/Tween 20 for 3 minutes. Blots were incubated at 4°C overnight with the primary antibody diluted in PBS/Tween 20 or in 5% milk in PBS/Tween 20, depending on the antibody. After five times washing with PBS/Tween 20

for 5 min each time, the membrane was then submerged in appropriate secondary antibody (depending on the nature of the primary antibody) for 90 minutes at room temperature in PBS/Tween 20 or in 5% milk in PBS/Tween 20. After washing the blot as describe above, immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system.

2.2.9. Determination of receptor desensitization by measurement of cAMP accumulation

The cells were seeded at a density of approximately $1x10^5$ per well and grown onto poly-L-lysine-treated 24-well plates overnight. For testing ARF6 mutants, experiment was done 48h after transfection. On the day of the assay, the cells were preincubated at 37ºC with UltraMEM containing either 1 μM DAMGO or 1 μM morphine up to 2 hours to induce receptor desensitization. After washing with DMEM, the cells were incubated for another 15 min with DMEM containing either 5 μM or 25 μM forskolin, for neuronal cultures and HEK293 cells, respectively, or combination of forskolin and the previously used agonist. After washing the cells one time with ice-cold PBS, the intracellular cAMP was extracted immediately with 0.5 ml of ice-cold HCl/ethanol (1 volume of 1N HCl/100 volumes of ethanol, stored at -20ºC). The supernatant was transferred into a 1.5 ml tube and then evaporated by vacuum centrifugation. The residue of cAMP was frozen at -20ºC or the extracted cAMP content was determined using a commercially available cAMP (^{3}H) radioassay kit. Maximum agonist-induced inhibition of cAMP accumulation without agonist preincubation has been defined as 100%. Receptor desensitization was measured as the decreased ability of the agonist to inhibit forskolin-stimulated adenylate cyclase activity after extended agonist pretreatment.

2.2.10. Data analysis

Statistic analysis of the data was done by one-way ANOVA followed by Bonferroni test or Student's t test as indicated using GraphPad Prism 4.0 software.

3. Results

3.1. Overexpression of the dominant negative ARF6 mutant (DN-ARF6) decreases agonist-induced MOPr endocytosis in HEK293 cells

Previous work from our group demonstrated that opioid-mediated activation of phospholipase D2 (PLD2) is dependent on ADP-ribosylation factor (ARF) proteins (*Koch et al., 2003*). Since PLD2 activation is essential for μ -opioid receptor (MOPr) endocytosis (*Koch et al., 2003, 2006*), we wanted to investigate in this study whether MOPr endocytosis is regulated via ARF proteins and to elucidate which ARF protein is involved, ARF1 or ARF6.

To answer these questions, first we generated HEK293 cell line stably expressing T7-tagged full length MOPr as described in "Materials and Methods" section. Stable transfectants were selected in the presence of 1 mg/ml G418 and the whole pool of resistant cells was used without selection of individual clones. Confocal analysis shown on Figure 3.1.a reveals a membrane expression of MOPr in untreated cells with very few intracellular vesicles containing constitutively internalized receptor. However, 30 min treatment of cells with 1μM of the receptor internalizing agonist DAMGO [(D-Ala^2,NMe-Phe^4,Gly-ol^5)-enkephalin] induced massive endocytosis of antibody-labeled MOPr to clusters of intracellular vesicles, as expected. This cell line stably expressing T7-MOPr alone was used in further experiments as a control.

To investigate the effects of ARF1 and ARF6 on MOPr trafficking, next we transiently transfected T7-MOPr expressing HEK293 cells with HA-tagged dominant negative ARF mutants DN-ARF1 (ARF1/T31N; *Dascher and Balch, 1994*) or DN-ARF6 (ARF6/T44N; *Macia et al, 2004*). These mutants are commonly used to block ARF function since they cannot exchange GDP to GTP and therefore are trapped in their GDP-bound, inactive state. 48 h after transfection, the cells were treated or not with 1 μM DAMGO for 30 min at 37ºC and the effects of ARF mutants on MOPr endocytosis were analyzed using immunocytochemistry and quantitative ELISA assay.

Protein expression of the HA-tagged ARF mutants was determined by immunostaining using anti-HA antibody which revealed that in all experiments about 50% of the cells were transfected and expressed HA-epitope. When investigating the effects of ARF mutants on MOPr endocytosis, only the cells that show ARF immunostaining were examined (Figure 3.1.b). Data show that blocking ARF6 but not ARF1 function significantly impaired agonist-induced MOPr endocytosis (Figure 3.1.b). It can be seen that in cells co-transfected with MOPr and DN-ARF6 mutant most of the receptor stayed in the plasma membrane after DAMGO treatment and much

less receptor was internalized in comparison to the control cells expressing MOPr alone (Figure 3.1.a) or to the adjacent cell that is not co-transfected with the mutant (Figure 3.1.b, see ARF immunostaining). On the other hand, overexpression of DN-ARF1 had no significant effect on MOPr endocytosis (Figure 3.1.b, upper panels).

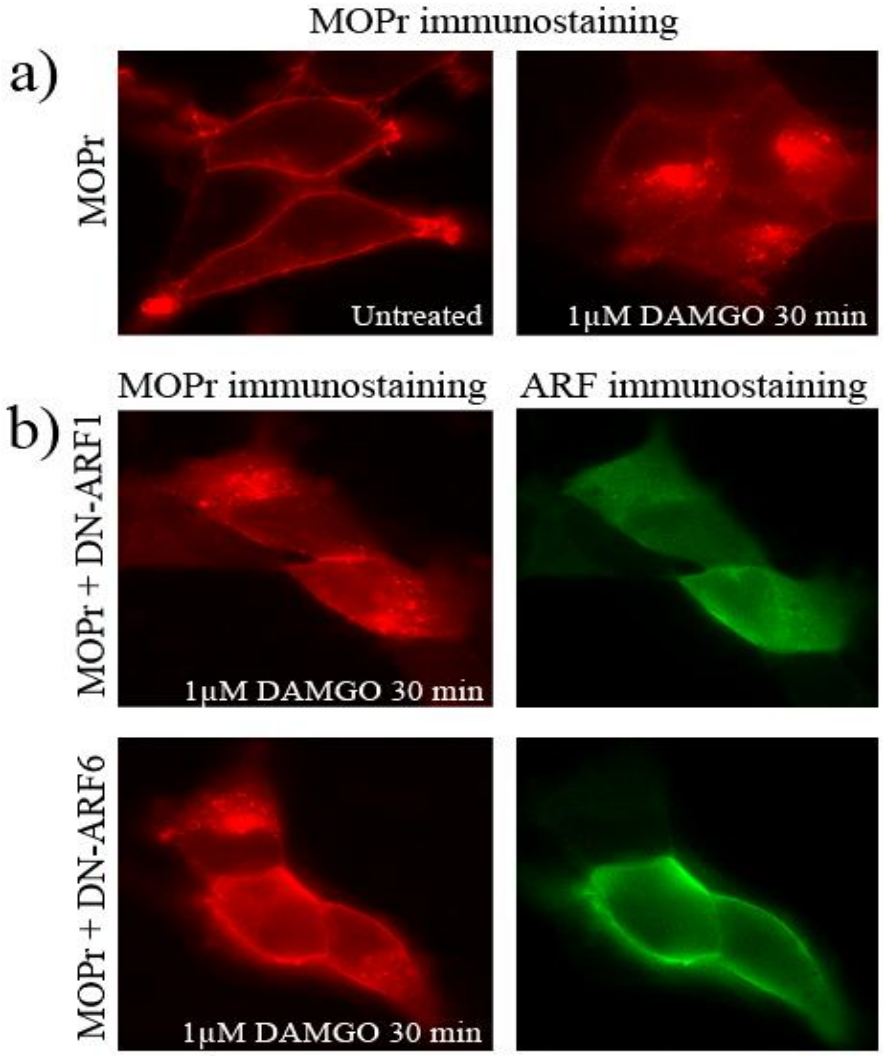


Figure 3.1. Reduction of DAMGO-induced MOPr endocytosis by DN-ARF6 overexpression in HEK293 cells. Control HEK293 cells stably expressing T7-tagged MOPr alone (a) or cells coexpressing T7-tagged MOPr and HA-tagged DN-ARF1 or DN-ARF6 mutant (b) were treated or not with 1 μM DAMGO for 30 min at 37ºC. After immunostaining, the distribution of MOPr and ARFs was examined by fluorescent microscopy as described under "Materials and Methods". Representative images from three independent experiments are shown. Note that in cells coexpressing MOPr and DN-ARF6 only cells that are transfected with ARF6 mutant show decreased endocytosis.

To confirm and further analyze the effects of DN-ARF1 and DN-ARF6 mutants on agonist-induced and basal (constitutive) endocytosis of MOPr, quantitative ELISA assay was done as described in "Materials and Methods". The results revealed that blocking ARF6 function resulted in ~50% decrease in the DAMGO-induced MOPr endocytosis (from ~30% to almost ~15%) (Figure 3.2.a). This only partial inhibition of MOPr endocytosis by co-expression of the DN-ARF6 mutant might be due to the transient transfection efficacy of only about 50%. In all experiments, basal (constitutive) endocytosis in untreated cells was unaffected by expression of both ARF1 and ARF6 mutants (Figure 3.2.b), suggesting that binding of agonist to the MOPr is necessary for the effects of ARF protein to occur. These data demonstrated that ARF6 and not ARF1 protein is involved in regulation of agonist-induced MOPr endocytosis since blocking ARF6 function by overexpression of DN-ARF6 mutant significantly decreased receptor internalization after DAMGO-treatment.

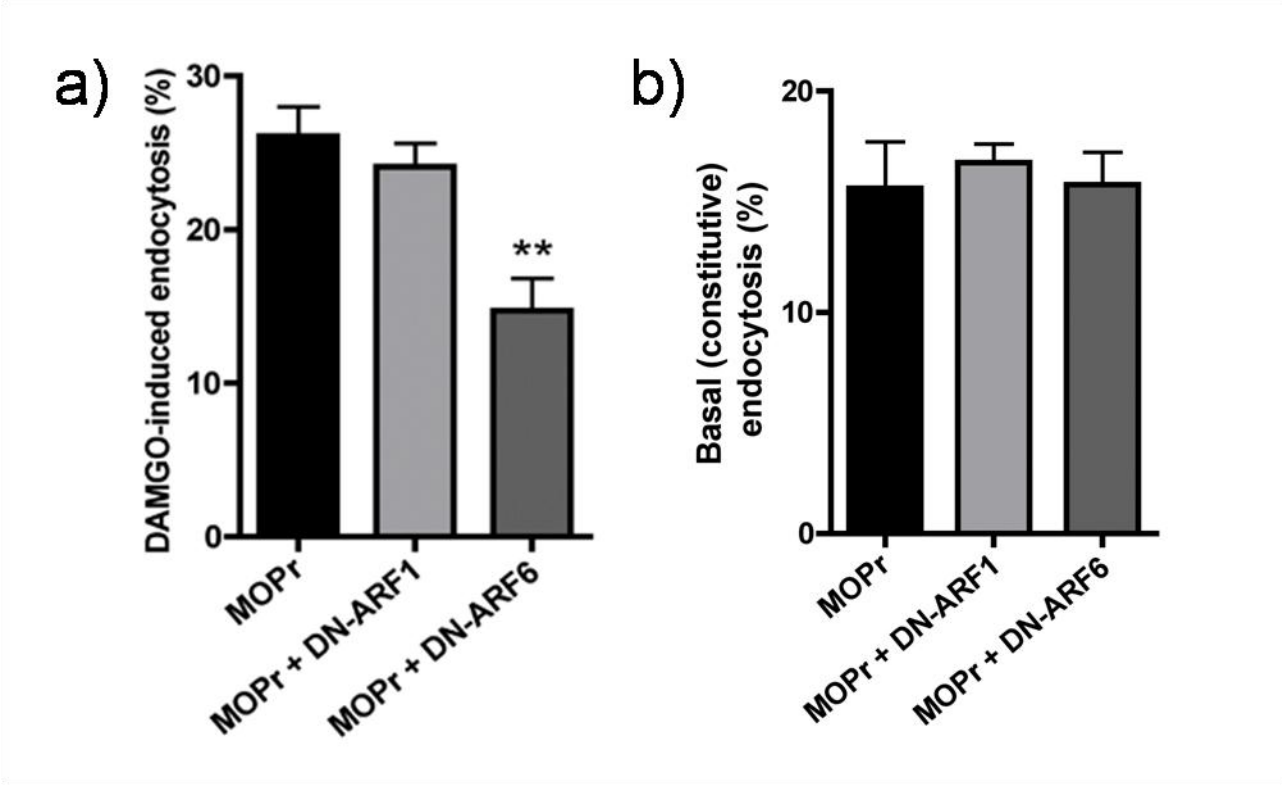


Figure 3.2. Quantitative analysis of MOPr endocytosis in HEK293 cells transfected with DN-ARF1 and DN-ARF6 mutants. Quantification of (a) DAMGO-induced and (b) basal (constitutive) MOPr endocytosis was done by ELISA assay as described under "Materials and Methods". DAMGO-induced endocytosis was calculated by subtracting basal (constitutive) endocytosis from total endocytosis that was measured after DAMGO treatment and quantified as the percent loss of cell surface receptors. Data are presented as means ± SEM of 4-6 independent experiments performed in triplicate. ** $p<0.01$ versus MOPr alone was calculated by one-way ANOVA followed by Bonferroni test.

3.2. Knocking down the expression of endogenous ARF6 by siRNA decreases agonist-induced MOPr endocytosis in HEK293 cells

To confirm the results from previous section, we did similar experiments in HEK293 cells stably expressing T7-MOPr after knocking down the endogenous ARF1 and ARF6 protein expression using siRNA technology. Western blot analysis shown on Figure 3.3.a demonstrates the efficiency of siRNAs that were used. Due to the initial problems in detection of endogenous ARF proteins with commercially available antibodies that we tested, Western blot analysis was first done in cells overexpressing HA-tagged wild type ARFs as described in "Materials and Methods" section. Detection was done from cell lysate directly using anti-HA antibody. 48 h after co-transfection of cells with wild type HA-ARFs and indicated concentrations of appropriate siRNAs, dose-dependent reduction of ARF protein level was detected. Further analysis revealed that cells transfected with 150 pM and 300 pM ARF6 siRNA decreased ARF6 expression for ~50% and ~80%, respectively, compared to the control cells transfected with wild type HA-ARF6 alone. For ARF1, the reduction of protein expression with the same concentrations of ARF1 siRNA were ~70% and ~95%, respectively. Representative Western blots from three separate experiments are shown on Figure 3.3.a. These data show that the siRNAs used show high efficiency in knocking down the expression of appropriate proteins.

To confirm that ARF6 protein is endogenously expressed in HEK293 cells and to be sure that used ARF6 specific siRNA can knock down the expression of endogenous ARF6 protein as well, we repeated again similar experiment without overexpression of ARF6 protein. Although previous studies have demonstrated the endogenous expression of ARF6 in HEK293 cells (*Houndolo et al., 2005*; *Cotton et al., 2007*), the amount of the protein seems to be quite low and from all commercially available anti-ARF6 antibodies that we tested, in our hands only one turned out to be specific and sensitive enough to detect the endogenous levels of ARF6 protein in this cell line. HEK293 cells stably expressing T7-MOPr were transfected or not (control) with 150 pM and 300 pM ARF6 specific siRNA or 150 pM and 300 pM of non-specific siRNA control as indicated (Figure 3.3.b). 48 h after transfection the cells were lysed and tested in Western blot analysis using ARF6 specific mouse monoclonal antibody (1:100, Santa Cruz Biotechnology, Inc). It can be seen that although the endogenous levels of ARF6 are quite low, they can be clearly decreased with used ARF6 specific siRNA in concentration-dependent manner, as expected (Figure 3.3.b). Further analysis showed that transfection of cells with 150 pM ARF6 specific siRNA resulted in ~50% decrease in ARF6 protein expression, while 300 pM ARF6 specific siRNA decreased the amount of endogenous ARF6 protein for ~70%. As expected, both

concentrations of non-specific siRNA control were without effect on the level of endogenous ARF6 in this experiment, confirming again the specificity of ARF6 siRNA that we used.

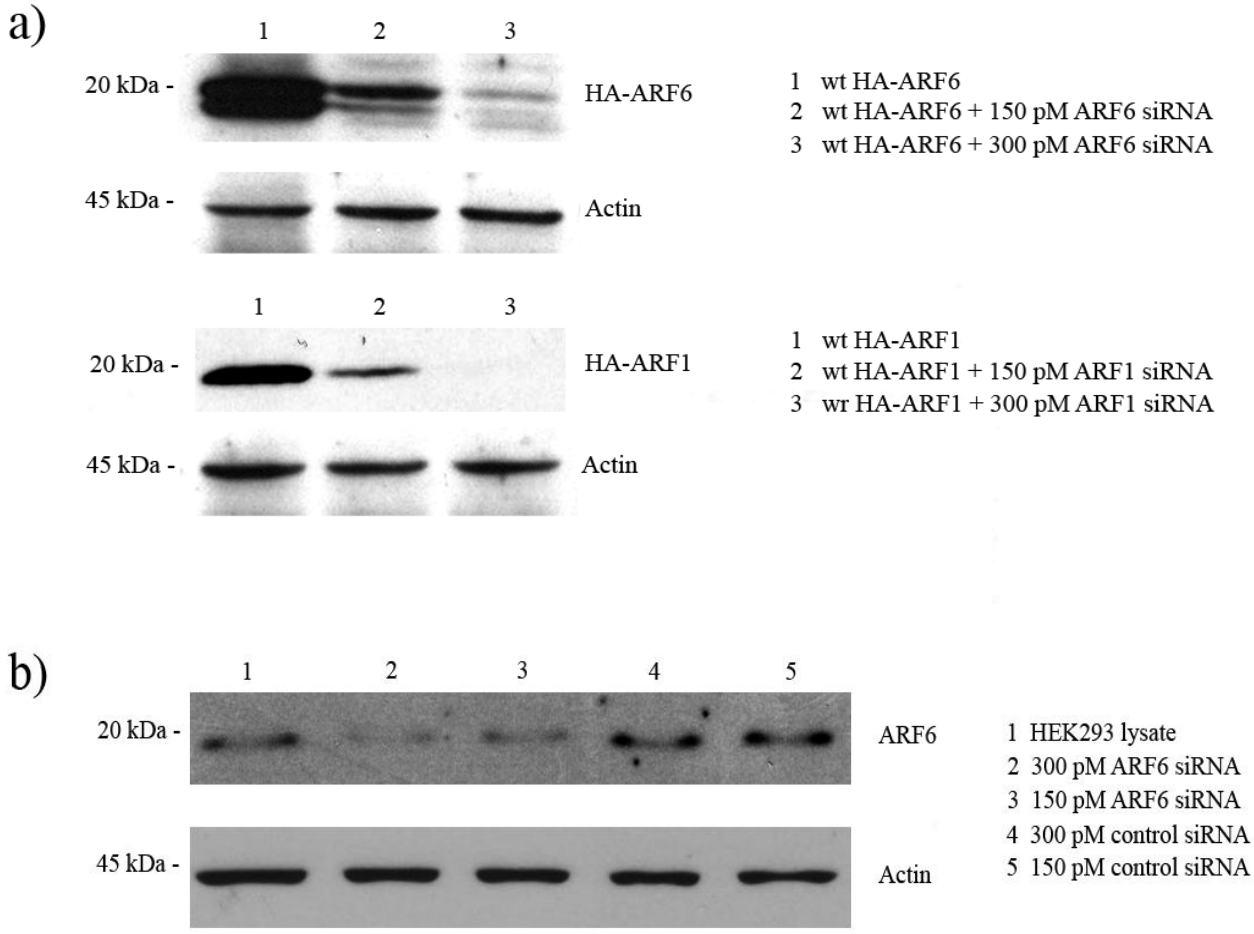


Figure 3.3. Western blot analysis of efficiency of ARF1 and ARF6 specific siRNAs. HEK293 cells stably expressing T7-tagged MOPr were transfected with a) HA-tagged wild type ARF1 or ARF6 protein alone or together with indicated concentrations of appropriate siRNAs or b) only with indicated siRNAs in two different concentrations. 48 h after transfection, the cells were lysed as described in "Materials and Methods" and the cell lysate was blotted directly using anti-HA antibody (a) or anti-ARF6 antibody (b). Shown are representative Western blots from three independent experiments. Concentration-dependent reduction of protein levels was detected (see text for details). Actin immunodetection of the same blot was done in order to show similar loading levels in all lines.

Finaly, we tested the effects of endogenous ARF protein knock down on DAMGO-induced MOPr endocytosis. These experiments were done without ARF protein overexpression and HEK293 cells stably expressing T7-MOPr were transfected or not (control) only with 150

pM or 300 pM of indicated siRNAs. Quantitative analysis on Figure 3.4. shows that siRNA-mediated knock down of the endogenous ARF6 expression was able to decrease DAMGO-induced MOPr endocytosis in concentration-dependent manner. This was not the case for ARF1 specific siRNA as well as for non-specific siRNA control in both concentrations tested, confirming our suggestion that ARF6 and not ARF1 protein is involved in regulation of agonist-induced MOPr endocytosis.

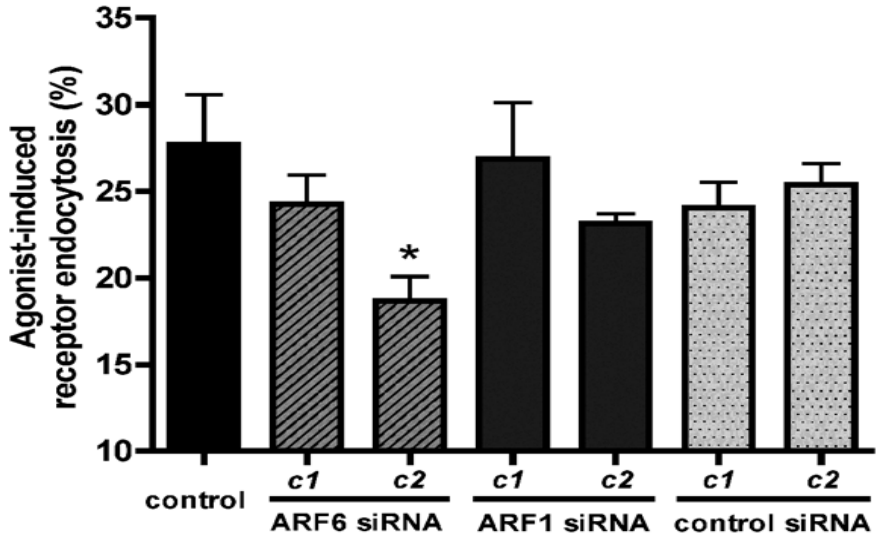


Figure 3.4. Effects of siRNA-mediated protein knock down of endogenous ARF1 and ARF6 protein on DAMGO-induced MOPr endocytosis in HEK293 cells. HEK293 cells stably expressing T7-tagged MOPr were transfected with indicated siRNAs in two different concentrations (*c1*=150 pM and *c2*=300 pM). 48 h after transfection, quantitative analysis of DAMGO-induced MOPr endocytosis was done by ELISA assay as described under "Materials and Methods". Data are presented as means ± SEM of 4-6 independent experiments performed in triplicate. * $p<0.05$ versus MOPr alone (control) was calculated by one-way ANOVA followed by Bonferroni test.

3.3. Overexpression of DN-ARF6 decreases agonist-induced MOPr endocytosis in cultured primary cortical neurons

Next, we tested these findings in cultured primary cortical neurons in order to assure that the effect of ARF6 on the agonist-induced endocytosis of MOPr was not an artifact of the HEK293 cell model system. Neuronal cultures were prepared from rat cortex of E17 Sprague Dawley rat embryos. After 4 days *in vitro*, the cells were co-transfected with HA-MOPr and c-myc-DN-ARF6 plasmids as described (see "Materials and Methods"). 48 h later, the cells were

treated or not with 10 μM DAMGO, immunostained and then examined under the confocal microscope. As expected, control cells expressing MOPr alone had much more endocytotic vesicles with internalized receptors after DAMGO treatment than cells expressing both MOPr and DN-ARF6 (Figure 3.5.a). Moreover, more receptor is retained in the plasma membrane after blocking ARF6 function. Quantitative analysis was done by subcellular fluorescent density measurements as described in "Materials and Methods" section. In these experiments, basal (constitutive) endocytosis in untreated cells was also unaffected (Figure 3.5.b, quantitative data) like in the HEK293 cell model system (see Figure 3.2.b) and therefore not shown by immunocytochemistry.

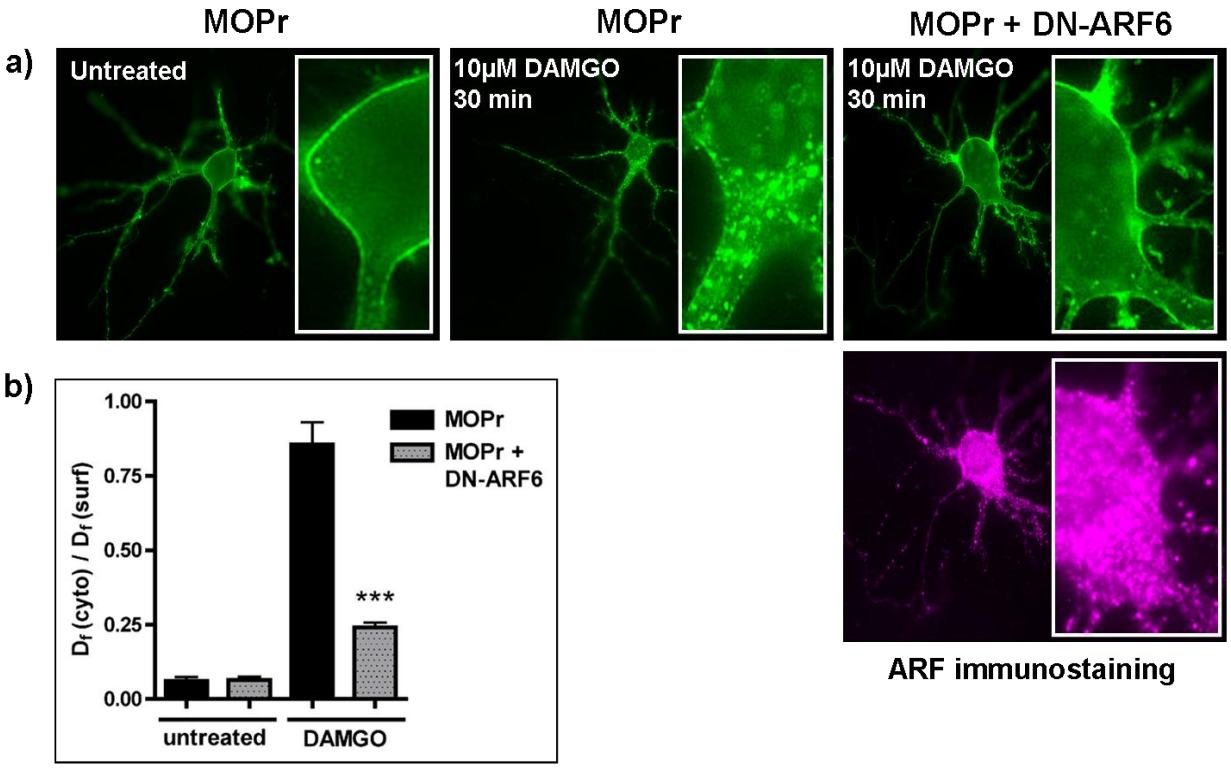


Figure 3.5. Coexpression of DN-ARF6 mutant decreases DAMGO-induced MOPr endocytosis in transfected cortical neurons. a) Newly prepared rat cortical neurons (E17) were transfected with HA-tagged MOPr alone (left panels) or HA-tagged MOPr and c-myc-tagged DN-ARF6 in combination (rigth panel). 48 h after transfection, the cells were treated or not with 10 μM DAMGO for 30 min at 37ºC. After immunostaining, labeled receptors (green) and DN-ARF6 mutant (magenta) were detected as described in "Materials and Methods" section. Shown are representative images from several independent experiments. b) Quantitative analysis of MOPr internalization: the values are expressed as ratio of cytoplasmic (D_f cyto) versus surface (D_f surf) fluorescent densities (n=10 per treatment) (see "Materials and Methods" section for details).

Moreover, it can be seen that by quantitative analysis we detected much higher block of endocytosis here than in experiments with HEK293 cells co-transfected with MOPr and DN-ARF6 mutant (see Figure 3.2.a for comparison). The reason for this is the fact that in this case only the cells transfected with DN-ARF6 were used for calculation by subcellular fluorescent density measurement since they could be clearly identified under the microscope by ARF immunostaining.

3.4. Expression of active ARF6 increases morphine-induced MOPr endocytosis in HEK293 cells and cultured primary cortical neurons

Since blocking of ARF6 function leads to decrease in the amount of internalized MOPr after DAMGO treatment, we investigated next whether MOPr endocytosis can be increased by expression of an active ARF6 mutant. Besides the classical, constitutively active ARF6 mutant GTP-ARF6 (ARF6/Q67L; *Peters et al, 1995*) that is "locked" in its GTP-bound state, another active "fast cycling" ARF6 mutant FC-ARF6 (ARF6/T157N; *Santy 2002*; *Klein et al., 2006*) was recently characterized that can bind GTP and release GDP more quickly than the wild type ARF6. As mentioned in the "Introduction" section, several studies have shown that this mutant represents better the real active state of ARF6 small GTPase in the cells and prefer FC-ARF6 over "GTP-locked" ARF6/Q67L mutant (*Santy 2002*; *Klein et al., 2006, 2008*). Therefore we used FC-ARF6 mutant in this set of experiments to investigate the effects of active ARF6 on agonist-induced MOPr endocytosis.

HEK293 cells stably expressing T7-MOPr were transiently transfected or not (control) with FC-ARF6 mutant and 48 h after transfection MOPr endocytosis was investigated by immunocytochemistry and quantitative ELISA assay. Our results revealed that coexpression of FC-ARF6 mutant did not enhance the DAMGO-induced MOPr internalization significantly (Figure 3.6.a, quantitative data), presumably because DAMGO induces maximum endocytosis itself. Therefore we also treated the cells with 1 μM morphine, an agonist that is known to activate the MOPr without promoting efficient receptor endocytosis. Quantitative data on Figure 3.6.a show that the constitutive endocytosis (without agonist treatment) was slightly increased in the untreated cells coexpressing MOPr and FC-ARF6 mutant. However, it is clear that only after stimulation with morphine MOPr endocytosis is significantly increased to more than 2-fold higher level than in control cells expressing MOPr alone. This suggests that a conformational change of the receptor induced by binding of an agonist is needed for the observed increase in

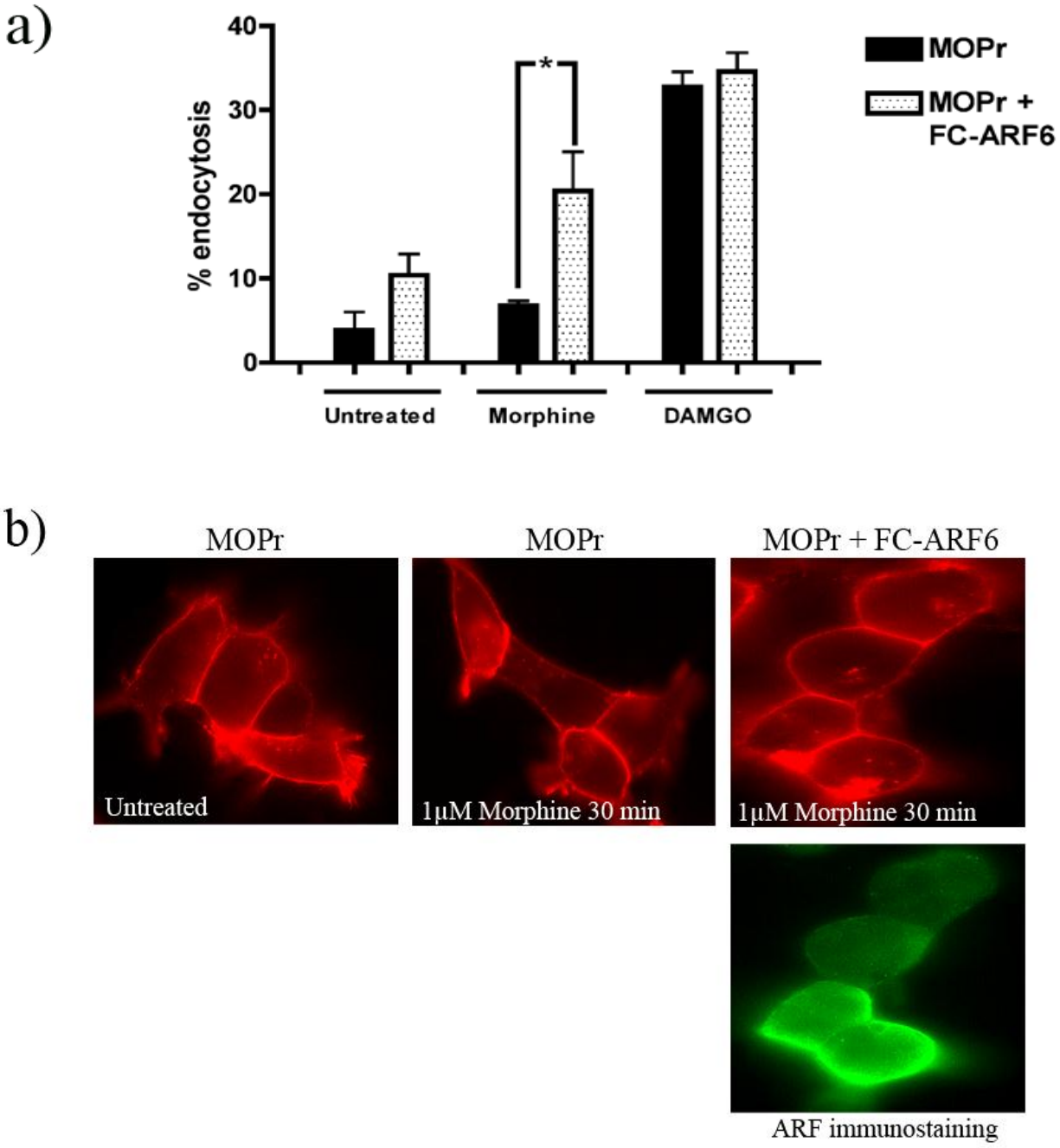


Figure 3.6. Expression of active FC-ARF6 mutant increases morphine-induced MOPr endocytosis. a) Control HEK293 cells stably expressing T7-tagged MOPr alone (left panels) or cells coexpressing T7-tagged MOPr and HA-tagged FC-ARF6 mutant (rigth panel) were treated with 1 μM morphine for 30 min at 37ºC. After immunostaining, the distribution of MOPr (upper panels) and FC-ARF6 (lower panel) was examined by fluorescent microscopy. Representative images from several independent experiments are shown. b) MOPr endocytosis after DAMGO and morphine treatment was quantified by ELISA assay as described under "Materials and Methods". Receptor endocytosis was calculated as the percent loss of cell surface receptors. Data are presented as means ± SEM of three independent experiments performed in triplicate. * $p<0.05$ versus MOPr alone (control) was calculated by Student's t test.

endocytosis in the presence of FC-ARF6. Moreover, the amount of internalized receptor after morphine treatment seemed to be dependent on the expression level of FC-ARF6 mutant, since cells with a high expression of FC-ARF6 showed more MOPr internalization than cells with a lower expression of this mutant (Figure 3.6.b, right panels). Similar results were obtained from primary cultured cortical neurons transfected with HA-MOPr and c-myc-FC-ARF6 (quantitative analysis, Figure 3.7.). Data show that expression of active "fast cycling" ARF6 mutant significantly increases morphine-induced MOPr endocytosis in this cellular system as well. These results further support our hypothesis and show that ARF6 protein plays a role in regulation of agonist-induced MOPr endocytosis.

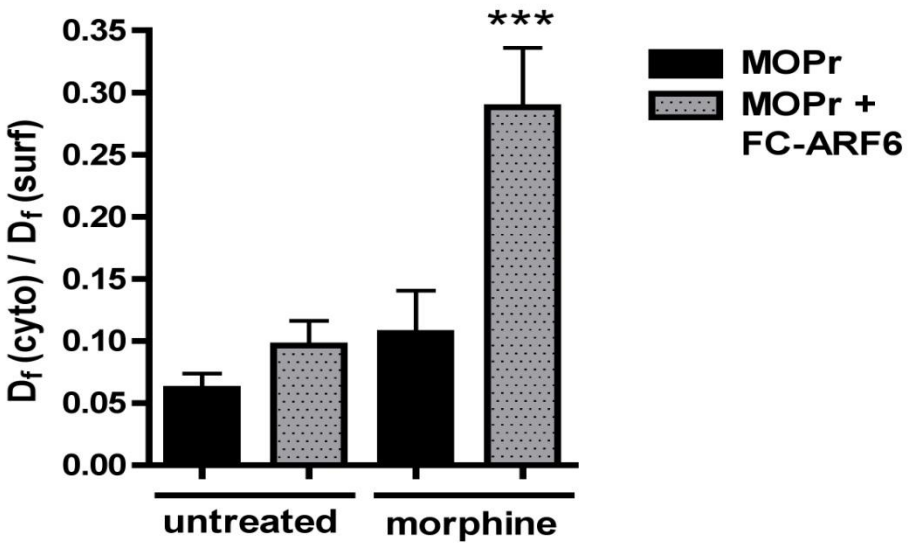


Figure 3.7. Quantification of effect of active FC-ARF6 mutant on morphine-induced MOPr endocytosis in cultured primary cortical neurons. Quantitative analysis of MOPr internalization in transfected primary cultured cortical neurons: the values are expressed as ratio of cytoplasmic (D_f cyto) versus surface (D_f surf) fluorescent densities (n=10 per treatment) (see "Materials and Methods" section for details).

3.5. ARF6 function in the regulation of MOPr endocytosis is PLD2-mediated

Next we investigated the molecular mechanisms by which ARF6 might facilitate MOPr internalization. Our previous studies have shown that opioid-mediated activation of PLD2 is ARF-dependent and essential for the induction of MOPr internalization (*Koch et al., 2003, 2006*). Namely, blocking PLD2 activity either by primary alcohols (such as 1-butanol) or expression of a nonfunctional N-terminal fragment of PLD2 significantly decreased MOPr endocytosis (*Koch et*

al., 2003, 2004). Moreover, expression of a catalytically inactive PLD2 mutant (K758R) was shown to block the agonist-induced MOPr endocytosis (*Koch et al., 2006*). Therefore, it is reasonable to suggest that the observed decrease of MOPr endocytosis by blocking ARF6 function is due to an impaired PLD2 activation.

Thus, to elucidate if ARF6 regulation of MOPr endocytosis is mediated via PLD2 activation, we first tested if a PD-ARF6 (ARF6/N48I, *Vitale et al., 2002*) which is unable to activate PLD ("PLD-defective") would also impair DAMGO-induced MOPr endocytosis. PD-ARF6 mutant has been created and characterized as selectively incapable of activating PLD, whereas all other ARF6 functions remain unaffected in this protein. Namely, this mutant could be activated by ARNO and inactivated by Git1, an ARF6 GEF and a GAP, respectively, and remained able to stimulate PIP5K but not PLD. Therefore, PD-ARF6 mutant is a useful molecular tool for investigation of downstream effectors of ARF6 and has been already used to show that PLD plays a role in endosomal membrane recycling (*Jovanovic et al., 2006*).

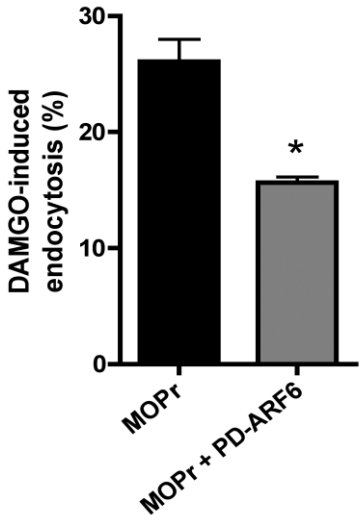


Figure 3.8. PD-ARF6 mutant overexpression reduces DAMGO-mediated MOPr endocytosis. HEK293 cells stably expressing T7-tagged MOPr were transiently transfected or not (control) with PD-ARF6 mutant. After 30 min of 1 μM DAMGO treatment, agonist-induced receptor endocytosis was determined by quantitative ELISA assay as described under "Materials and Methods". Data are presented as means ± SEM of 3-5 independent experiments performed in triplicate. * $p<0.05$ versus control (MOPr alone) was calculated by Student's t test.

As Figure 3.8. shows, coexpression of PD-ARF6 mutant together with MOPr in HEK293 cells decreased receptor internalization to the levels comparable to those obtained after blocking ARF6 function by DN-ARF6 mutant overexpression (see Figure 3.2.a for comparison). Since PD-ARF6 mutant is selectively incapable of activating PLD, whereas all other ARF6 functions

remain unaffected, it can be suggested that the main negative effects of blocking ARF6 function in regulation of MOPr endocytosis are due to impaired activation of PLD2 and not due to the blocking of other ARF6 effector functions.

It is also known that protein kinase C (PKC) is another pathway of PLD2 activation *in vivo*, besides ARF proteins. Thus, we further tested if ARF6 regulation of MOPr endocytosis is mediated via PLD2. Previous sections revealed that expression of DN-ARF6 or PD-ARF6 mutants decreases agonist-induced MOPr endocytosis. If these effects are due to blocked PLD2 activation, heterologous activation of PLD2 via PKC should restore DAMGO-induced MOPr endocytosis to the control levels in HEK293 cells coexpressing MOPr and DN-ARF6 or PD-ARF6 mutant. Indeed, Figure 3.9.a-c shows that after 30 min co-stimulation of cells with 1 μM DAMGO and 0,1 μM PKC-activator PMA (phorbol 12-myristate 13-acetate), MOPr endocytosis in cells expressing both ARF6 mutants is rescued to the levels similar to those seen in the control cells. PMA treatment together with DAMGO had no significant effect on agonist-induced endocytosis in control cells expressing MOPr alone. Moreover, we show that this effect is not due to the increased PKC activity itself since PMA treatment alone did not induce significant MOPr endocytosis in both control cells and cells expressing ARF6 mutants. These results additionally demonstrate that the decrease in DAMGO-induced MOPr endocytosis detected after DN-ARF6 expression might be due to a block of PLD2 activation.

We also tested N-myristoylated ARF6 peptide (N-myr-ARF6) which consists of the N-terminal region (amino acids 2-13) of ARF6 protein in endocytosis experiments. This cell-permeable synthetic peptide has been previously reported to block PLD activity (*Caumont et al., 1998*; *Le Stunff et al., 2000*). Figure 3.10. shows that overnigth treatment of T7-MOPr-expressing cells with N-myr-ARF6 leads to significant and concentration-dependent reduction of DAMGO-induced receptor endocytosis. Confocal analysis shows a retention of the receptor in the plasma membrane of HEK293 cells expressing T7-MOPr which were treated with N-myr-ARF6 peptide (Figure 3.10.a), meaning that less receptor is internalized in these cells in comparison to the control cells. Moreover, quantitative analysis on Figure 3.10.b shows that effect was present with both agonist concentrations tested (1 μM and 10 μM DAMGO) demonstrating strong inhibition. This experiment also confirmed our previous findings showing that blocking PLD2 activity significantly decreases agonist-induced MOPr endocytosis and stands in line with our data demonstrating that the function of ARF6 protein in regulation of MOPr endocytosis might be PLD2 mediated.

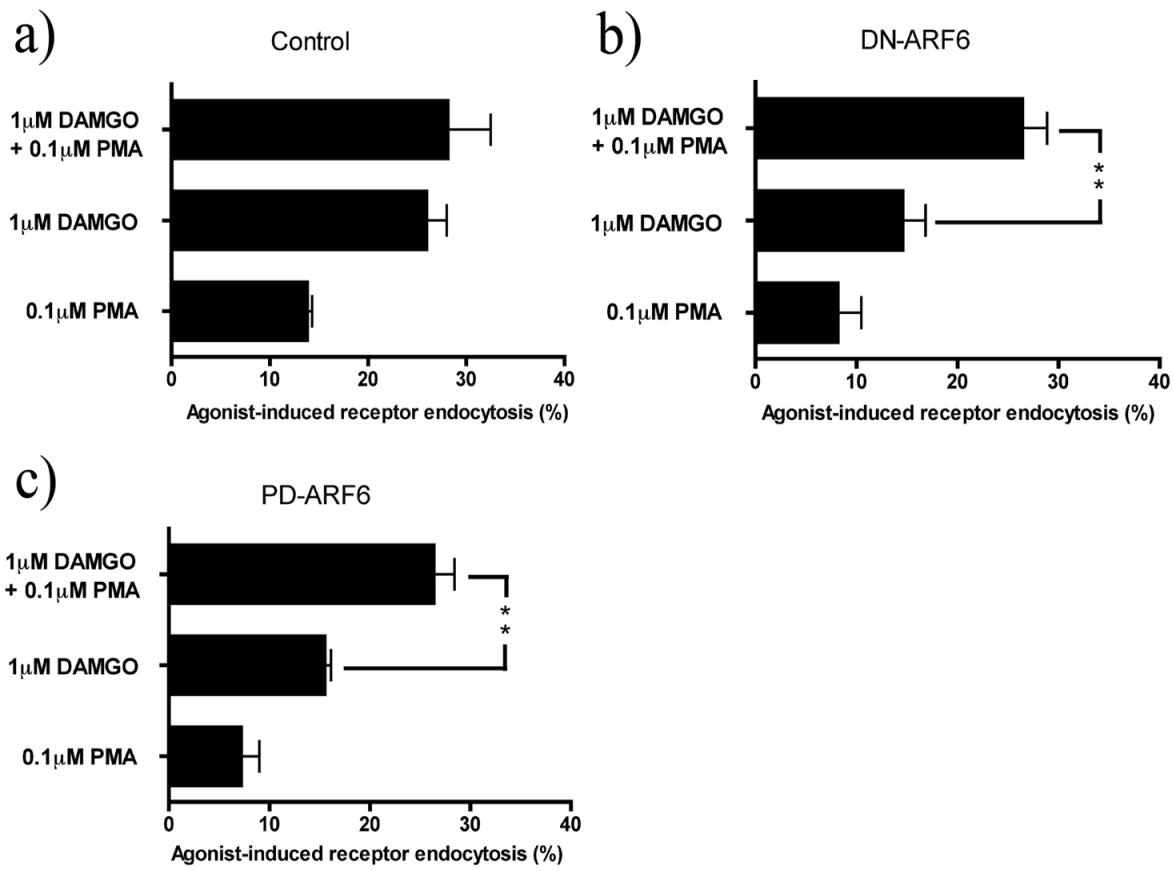


Figure 3.9. Effect of PMA-induced PLD2-activity on the DAMGO-mediated MOPr endocytosis in the presence of ARF6 negative mutants. HEK293 cells stably expressing T7-tagged MOPr were transiently transfected or not (control) with DN-ARF6 or PD-ARF6 mutant. 48 h after transfection surface receptors were labeled with T7-antibody and then the cells were treated for 30 min with the substances indicated. Receptor endocytosis was determined by quantitative ELISA assay as described under "Materials and Methods". Note that PMA treatment together with DAMGO rescued MOPr endocytosis in the cells expressing DN- and PD-ARF6 mutants to the levels similar to DAMGO-induced endocytosis in control cells expressing MOPr alone. In control cells, DAMGO + PMA treatment had no significant effect on MOPr endocytosis as well as PMA treatment alone in all cells used. Data are presented as means ± SEM of three independent experiments performed in triplicate. ** $p<0.01$ was calculated by one-way ANOVA followed by Bonferroni test.

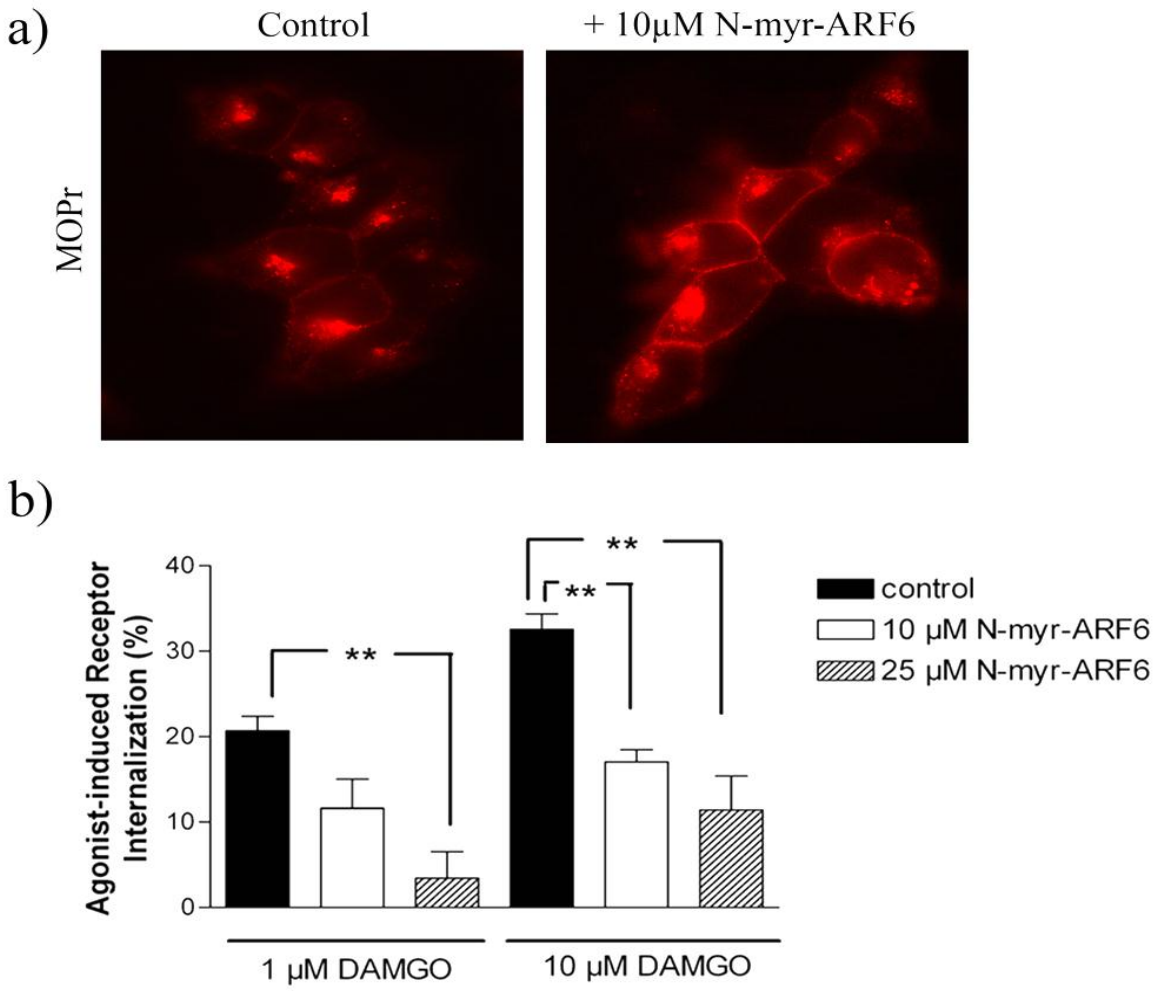


Figure 3.10. N-myr-ARF6 peptide effects on DAMGO-induced MOPr endocytosis. T7-MOPr expressing cells were incubated or not (control) overnight with indicated concentrations of N-myr-ARF6 peptide. The cells were treated with 1μM DAMGO (a) or 1 μM and 10 μM DAMGO (b) for 30 min and then immunocytochemistry (a) or quantitative analysis of endocytosis by ELISA (b) was done as described in "Materials and Methods" section. Note clear retention of receptor in the plasma membrane in cells treated with N-myr-ARF6 (a) as well as significant reduction of endocytosis with both concentrations of peptide and agonist tested (b).

3.6. Role of ARF6 in opioid-mediated PLD2 activation

Having in mind that both PMA as well as N-myr-ARF6 are unspecific substances that might induce various and multiple cellular responses that are not only dependent on PLD2 activation, the following experiment was done in order to investigate the role of ARF6 in opioid-mediated PLD2 activation. As a direct evidence that ARF6 protein modulates PLD2 activity, we measured opioid-mediated PLD2 activation using PLD assay in the presence of different ARF6

mutants. Evidence for an endogenous PLD2 expression and PLD activity in HEK293 cells has been provided in numerous publications (*Rümenapp et al., 1997*; *Meier et al., 1999*; *Voss et al., 1999*). However, PLD2 levels in wild type HEK293 cells were below the detection limit of the transphosphatidylation assay used for measuring PLD activity. Therefore we first generated HEK293 cell line stably co-expressing HA-tagged MOPr and PLD2 as described under "Materials and Methods". Briefly, HEK293 cells stably expressing HA-MOPr were generated and then subjected to the second round of transfection with plasmid containing PLD2 gene. Stable transfectants were selected in the presence of puromycin and G418 antibiotics and the whole pool of resistant cells was used without selection of individual clones.

MOPr and PLD2 expression was monitored by ligand binding experiments, Western blot and immunocytochemical analysis which showed membrane expression of both proteins, as expected. As shown in Table 1, saturation binding experiments (n=3) revealed no substantial differences between HA-MOPr and HA-MOPr-PLD2 expressing cells with respect to their affinities (K_D) to [^{3}H]DAMGO and their numbers of binding sites (B_{max}).

Stable cell line	K_D **(nM)**	B_{max} **(fM/mg)**
HA-MOPr	1±0.1	967±110
HA-MOPr-PLD2	1.4±0.4	938±107

Table 1. Functional properties of HA-MOPr and HA-MOPr-PLD2 stable cell lines. The K_D and B_{max} for the binding of [^{3}H]DAMGO to MOPr in stable cell line expressing N-terminally HA-tagged MOPr with or without co-expression of PLD2 in HEK293 cells were determined by Scatchard analyses as described in "Materials and Methods" section. Values shown are the means ± SEM of 3 independent experiments.

The generated HEK293 cell line stably co-expressing MOPr and PLD2 was transiently transfected with indicated ARF mutants (DN-ARF1, DN-ARF6, PD-ARF6 and FC-ARF6) using Lipofectamine™ 2000 according to manufacturer's instructions. 24 h after transfection, the medium was changed into serum-free medium containing [1,2,3-^{3}H]glycerol and the cells were kept in this medium overnight in order to label the newly synthesized phospholipids. On the day of the assay, the cells were stimulated with the serum-free medium containing 2% ethanol and 1 μM agonists DAMGO or morphine or 0.1 μM PKC activator PMA for 30 min and individual phospholipids phosphatidylethanol (PtdEtOH), PA and PC were isolated as described (see "Materials and Methods"). The relative PLD2 activity was calculated as percentage [^{3}H]-

PtdEtOH of the total cellular PC concentration and then shown as the percent of the control values.

As shown in Figure 3.11., treatment with the μ-agonist DAMGO resulted in a 2.5-fold increase in the PLD2 activity, whereas incubation with the noninternalizing agonist morphine failed to induce activation of PLD2, which is in line with our previous observations (*Koch et al., 2003*). As mentioned above, it is known that besides ARFs, PKC is another pathway of PLD2 activation *in vivo*. Indeed, activation of PKC by PMA promoted an almost 5-fold increase in the PLD2 activity, indicating a comparable PLD2 expression level in all tested MOPr expressing cells with and without co-expression of ARF mutants. Furthermore, DAMGO-mediated activation of PLD2 could be completely blocked in MOPr-PLD2 cells co-expressing the DN-

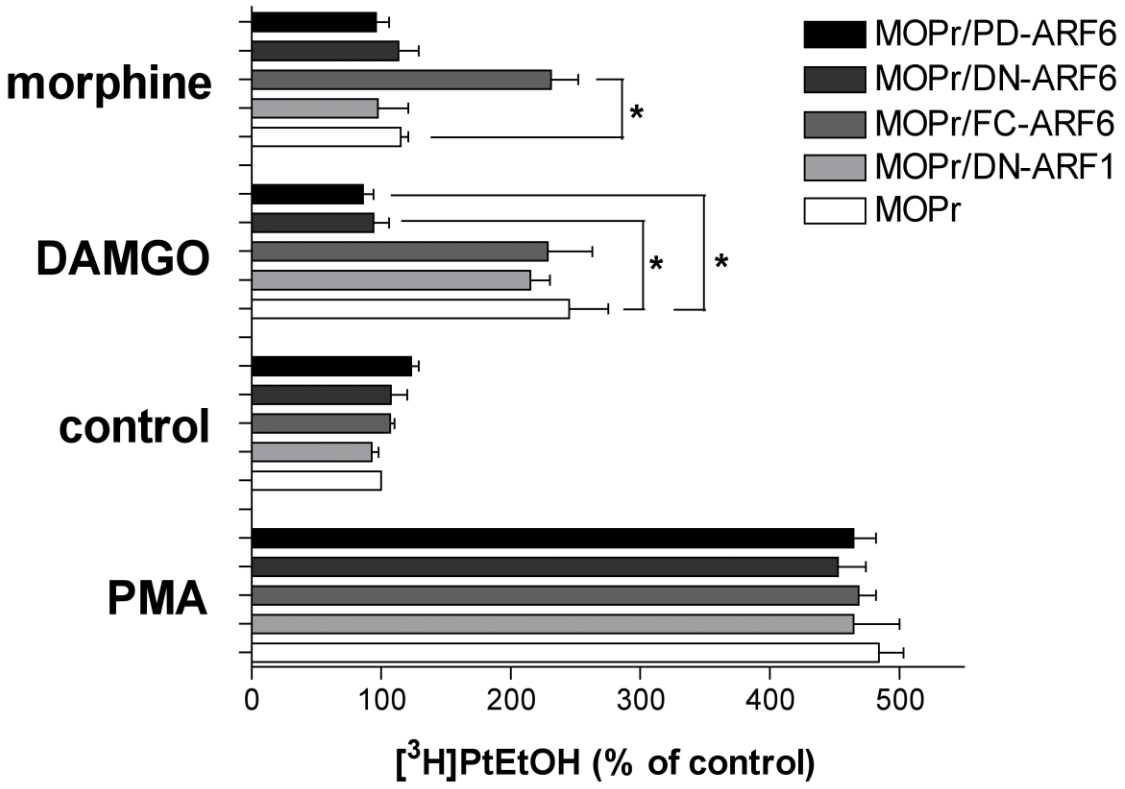


Figure 3.11. Effect of coexpression of ARF1 or ARF6 mutants on the opioid-induced PLD2 activation measured by transphosphatidylation assay. MOPr-PLD2 expressing HEK293 cells were transfected with dominant negative ARF1 or ARF6 mutants as well as with "fast cycling" and "PLD-defficient" ARF6 mutant and then treated or not with 0.1 μM PMA, 1 μM DAMGO or 1 μM morphine for 30 min as indicated. Then, PLD2 activation was determined as described above (see also "Materials and Methods"). Values represent means ± SEM of three independent experiments performed in triplicate. * $p<0.05$ versus MOPr alone (control) was calculated by one-way ANOVA followed by Bonferroni test.

ARF6 or PD-ARF6 mutants, but not by co-expression of the DN-ARF1 mutant (Figure 3.11.). On the other hand, co-expression of FC-ARF6 mutant together with MOPr and PLD2 increased PLD2 activation after morphine treatment almost 2-fold over the control levels, as expected. Altogether, our results demonstrated that opioid-induced PLD2 activation is ARF6 mediated and PLD assay in cells expressing different ARF6 mutants directly implicated ARF6 protein in this process.

3.7. Blocking ARF6 function impairs MOPr recycling

ARF6 protein has been widely implicated in the regulation of plasma membrane/endosome trafficking (*D'Souza-Schorey and Chavrier, 2006*). Therefore we investigated next if ARF6 protein also plays a role in trafficking of internalized MOPr to the plasma membrane and thus is involved in regulation of receptor recycling. T7-MOPr expressing HEK293 cells were transfected with negative ARF6 mutants, DN- and PD-ARF6, or with active FC-ARF6 mutant. After 30 min of DAMGO treatment, agonist was removed and the cells were treated with the MOPr antagonist 1 μM naloxone for further 30 min to avoid residual DAMGO-stimulated endocytosis and to keep recycled receptor in the plasma membrane. As shown in Figure 3.12.a, the coexpression of FC-ARF6 mutant only slightly increased the amount of recycled MOPr, indicating that ARF6 protein activation is not the rate-limiting step in this process. However, recycling was strongly decreased after blocking ARF6 function with both DN- and PD-ARF6 mutant expression (Figure 3.12.a). Similar reduction of the amount of recycled receptor after DAMGO removal was detected after overnight incubation of MOPr expressing cells with a myristoylated synthetic peptide N-myr-ARF6, a blocker of PLD activity and a very potent inhibitor of DAMGO-induced MOPr endocytosis as well (see Figure 3.12.a). These results show that ARF6 protein also regulates MOPr recycling.

In order to retest our data, these findings were confirmed after knocking down the expression of endogenous ARF6 protein using siRNA technology (Figure 3.12.b). ARF6 specific siRNA significantly impaired MOPr recycling already in very low concentration (c=150 pM), suggesting that trafficking of internalized receptor back to the plasma membrane is very sensitive to proper ARF6 function. As expected, ARF1 siRNA as well as non-specific siRNA control had no significant effects on the amount of recycled receptor (Figure 3.12.b). Altogether, these results implicate both ARF6 and PLD2 proteins in the regulation of MOPr recycling. Together with previous data mentioned above, our experiments suggest that ARF6 protein has an important role in regulation of MOPr trafficking both from and back to the plasma membrane and this function is mediated via modulating PLD2 activity.

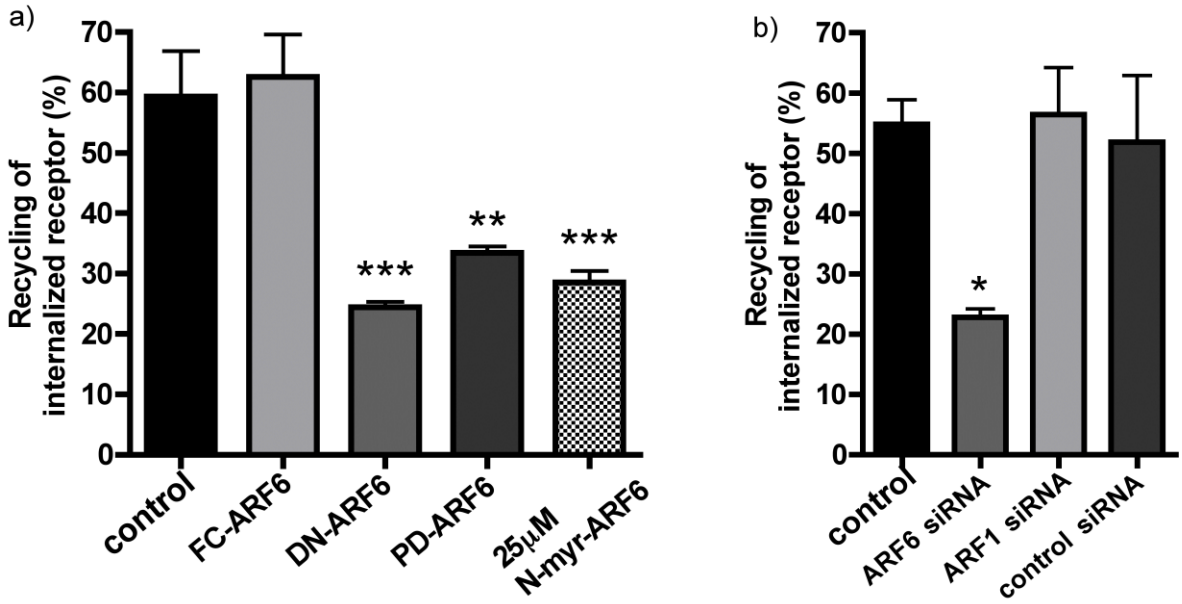


Figure 3.12. Reduction of MOPr recycling rate by blocking ARF6 function. Control HEK293 cells stably expressing T7-tagged MOPr alone were transfected or not with a) HA-tagged ARF6 mutants (FC-ARF6, DN-ARF6 and PD-ARF6) or b) 150 pM indicated siRNAs. In experiment with N-myr-ARF6, the cells were kept overnight in DMEM medium supplemented with 25 μM peptide prior to experiment. 48 h after transfection, the receptor was surface labeled with T7-antibody and the cells were stimulated for 30 min with 1 μM DAMGO at 37ºC. Agonist was then removed and the cells were incubated at 37ºC for further 30 min with 1 μM naloxone. After fixation, the amount of endocytosed receptors was determined by quantitative ELISA as described in "Materials and Methods". The recycling rate was estimated as a percentage of recovered surface receptors to endocytosed receptors. Data are presented as means ± SEM of 3-4 independent experiments performed in triplicate. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus MOPr alone (control) was calculated by one-way ANOVA followed by Bonferroni test.

3.8. ARF6 influences agonist-induced MOPr desensitization

Presented data clearly demonstrate that ARF6 small GTPase regulates agonist-induced MOPr endocytosis as well as receptor recycling via regulating the activity of PLD2 enzyme. Since receptor trafficking strongly influences agonist-induced receptor desensitization, it was reasonable to assume that ARF6 protein will also affect the rate of MOPr desensitization. To test this, we treated the control HEK293 cells expressing MOPr alone and cells co-expressing MOPr and either PD-ARF6 or FC-ARF6 mutant with receptor internalizing agonist DAMGO or noninternalizing agonist morphine for 2 h (Figure 3.13.). Receptor desensitization was measured as the decreased ability of the agonist to inhibit forskolin-stimulated adenylate cyclase activity

after extended agonist pretreatment as described in "Materials and Methods" section. Figure 3.13.a shows that 2 h exposure of receptor to internalizing agonist DAMGO led to receptor desensitization of about 25% in control cells. However, after overexpression of PD-ARF6 mutant, which is defective in PLD2 activation, the DAMGO-induced receptor desensitization was drastically increased (>50%). These results are in line with our previous findings showing that inhibition of PLD2 leads to decreased receptor internalization and reactivation and therefore to a stronger MOPr desensitization (*Koch et al., 2004*).

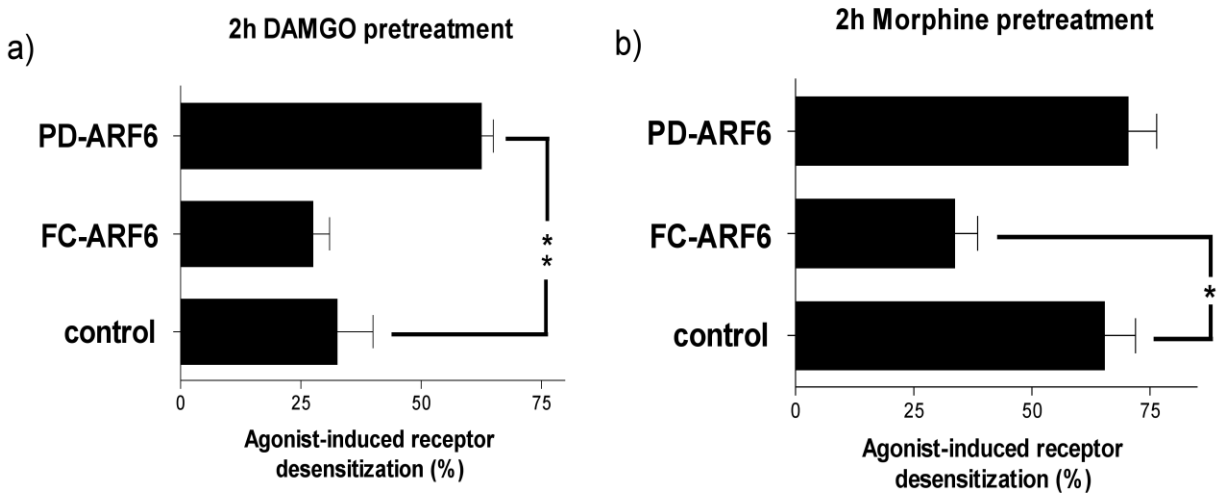


Figure 3.13. Effects of expression of ARF6 mutants on agonist-induced MOPr desensitization. HEK293 cells expressing MOPr with and without coexpression of indicated ARF6 mutants were treated with 1 μM DAMGO or 1 μM morphine for 2 h. After washing, the cells were treated with forskolin or combination of forskolin and previously used agonist for another 15 min and cAMP levels were determined as described under "Materials and Methods". Receptor desensitization was measured as the decreased ability of the agonist to inhibit forskolin-stimulated adenylate cyclase activity after extended agonist pretreatment. Values represent mean ± S.E.M. of 3-4 independent measurements performed in duplicate. * $p<0.05$, ** $p<0.01$ compared with control cells expressing MOPr alone (one-way ANOVA followed by Bonferroni test).

Furthermore, as Figure 3.13.b shows, co-expression of active FC-ARF6 mutant with MOPr did not influence DAMGO-induced receptor desensitization significantly, since PLD2 is already activated by DAMGO treatment as we have shown in this study (see Figure 3.11.) and previous studies (*Koch et al., 2003, 2006*). However, morphine can not induce receptor endocytosis/recycling and therefore leads to a stronger receptor desensitization compared to the internalizing agonist DAMGO (Figure 3.13.a,b). Since morphine can not activate PLD2 (*Koch et*

al., 2003, 2006), blocking the activity of this enzyme by PD-ARF6 mutant expression does not influence morphine-induced receptor desensitization. However, after PLD2 activation by FC-ARF6 mutant, receptor internalization, reactivation and recycling after morphine treatment are enhanced and MOPr desensitization is decreased compared to control cells (Figure 3.13.b). Therefore, by influencing PLD2-mediated processes like receptor endocytosis and reactivation/recycling, ARF6 protein modulates agonist-induced MOPr desensitization as well. Altogether, our findings indicate that ARF6 protein via PLD2 activation plays an important role in regulation of MOPr trafficking and signaling, processes implicated in tolerance and dependence to opioid drugs.

3.9. Desensitization of endogenous MOPr in primary cultured neurons

Since MOPr and PLD2 are endogenously expressed in many brain regions including cerebral cortex (*Choi et al., 2004*; *Liao et al., 2005*; *Mansour et al., 1995*; *Taki et al., 2000*), we investigated next whether the DAMGO-induced MOPr desensitization is affected by the inhibition of PLD2 activity in cultured primary cortical neurons. For this experiment neuronal cultures were prepared from rat cortex of E17 Sprague Dawley rat embryos as described under "Materials and Methods" section. To inhibit the function of endogenously expressed PLD2, catalytically inactive PLD2 mutant (nPLD2) which has the point mutation K758R was used. Two days after transfection with nPLD2, cortical cells were treated with 1 μM DAMGO (up to 2 h) and receptor desenzitization was measured as described above. Compared to untransfected control cells, nPLD2 expressing cortical neurons exhibited a significantly increased receptor desensitization after both 1 h and 2 h of DAMGO pretreatment (Figure 3.14.). This might be due to the impaired receptor endocytosis and recycling/reactivation of the MOPr after blocking endogenous PLD2 activity. These findings confirm the important regulatory role of PLD2 identified in our HEK293 cellular model system in the endocytosis and desensitization of the endogenously expressed MOPr in neuronal cells.

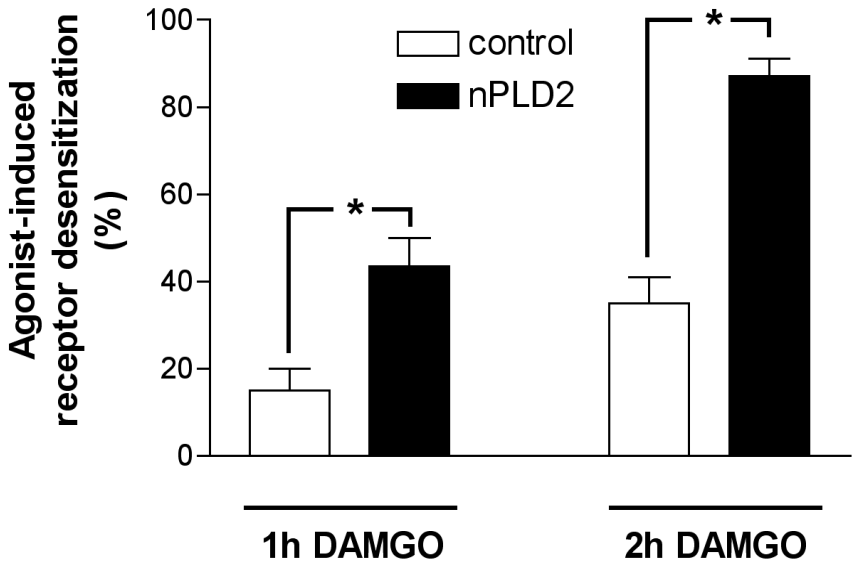


Figure 3.14. Desensitization of the MOP receptor in cultured primary cortical neurons. Newly prepared primary cortical cells (E17) were either kept in culture for 6 days (control) or transfected with the catalytically inactive (K758R) PLD2 mutant (nPLD2) on day 4 and kept for two additional days in culture before agonist treatment. Then the cells were exposed to 1 μM DAMGO for the indicated time periods. After washing, the cells were treated with 5 μM forskolin or forskolin plus DAMGO for 15 min, and cAMP levels were determined as described under "Materials and Methods". The maximum inhibition of intracellular cAMP accumulation in cells without agonist pretreatment was defined as 100%. Values represent mean ± SEM of 3-4 independent measurements performed in duplicate. *$p<0.01$ compared with the desensitization of the control cells (Student's t test).

3.10. Role of ARF6 GDP/GTP cycle in MOPr endocytosis/recycling

Presented results demonstrate that ARF6 protein plays an important role in regulation of MOPr endocytosis, desensitization/signaling and recycling. Moreover, our data show that these effects of ARF6 are mediated via PLD2, a phospholipid modifying enzyme that has a crucial role in these processes as we have shown previously (*Koch et al., 2003, 2004, 2006*).

As already mentioned, ARF6 cycles between its GTP-bound and its GDP-bound states like other small G proteins, and these states are considered to represent ARF6 active and inactive conformation, respectively. Due to the reasons mentioned before (see Chapter 1.4.2.), the "fast cycling" ARF6/T157N mutant was used in our previous work to investigate the effects of active ARF6 mutant overexpression of MOPr endocytosis (see Chapter 3.4.). However, we wanted to investigate next how important is a full GDP/GTP cycle for ARF6 regulation of MOPr

endocytosis and recycling. To do this, first we tested and compared the following ARF6 active mutants in both MOPr endocytosis and recycling experiments:

a) "GTP-locked" ARF6/Q67L mutant (GTP-ARF6) that can not hydrolyze GTP and

b) "fast cycling" ARF6/T157N mutant (FC-ARF6) that can undergo spontaneous GDP/GTP exchange.

HEK293 cells stably expressing T7-tagged MOPr were transiently transfected with HA-tagged GTP-ARF6 or FC-ARF6 mutant. For endocytosis measurements, 48 h after transfection the receptor was specifically surface labeled with T7-antibody and then the cells were treated or not with 1 μM receptor internalizing agonist DAMGO for 30 min at 37ºC. After agonist treatment, the cells were fixed and the amount of internalized receptor was quantified by ELISA as described. As Figure 3.15.a shows, both mutants had no significant effects on DAMGO-induced MOPr endocytosis. We detected ~25% of agonist-induced receptor internalization in both control cells expressing MOPr alone and cells coexpressing MOPr and GTP-ARF6 or FC-ARF6 mutant. This result suggests that both ARF6 active mutants act similarly in the cells and GTP hydrolysis seems not to be a crucial step for ARF6 function in the regulation of MOPr endocytosis.

Next we tested receptor recycling using the same cellular model system and methodological approach. In these experiments, after 30 min of 1 μM DAMGO stimulation the agonist was removed and after washing the cells were treated for further 30 min with 1 μM naloxone before fixation. As mentioned before, this MOPr antagonist was used in order to avoid residual DAMGO-stimulated endocytosis and to keep recycled receptor in the plasma membrane. Figure 3.15.b shows that expression of FC-ARF6 mutant did not influence receptor trafficking to the plasma membrane significantly. After 30 min agonist washout, ~60% of receptor was recycled in both control cells expressing MOPr alone and cells co-expressing MOPr and FC-ARF6 mutant. Surprisingly, expression of GTP-ARF6 mutant strongly decreased the amount of recycled MOPr for ~50% (from ~60% to ~30%, Figure 3.15.b). A similar level of reduction of MOPr recycling was detected after expression of dominant negative ARF6 mutant DN-ARF6 (see Figure 3.12.a for comparison), suggesting that GTP-ARF6 mutant, although active, exhibits a negative phenotype in MOPr recycling assays. This is in line with previous studies that have reported that GTP-ARF6 mutant acts similarly like dominant negative mutant in some experiments, showing negative effects although it is regarded to be constitutively active (*D'Souza-Schorey et al., 1995*; *Radhakrishna and Donaldson, 1997*; *Altschuler et al., 1999*; *Claing et al., 2001*; *Hashimoto et al., 2004*). Therefore, GTP hydrolysis and ARF6 inactivation seem to be an essential step for proper function of ARF6 in MOPr trafficking back to the plasma

membrane. Moreover, in our experiments HEK293 cells transfected with GTP-ARF6 mutant got round-shaped, detached from the dish and had decreased survival rate after transfection in comparison to the cells transfected with FC-ARF6 mutant. These findings are also in line with the previous reports showing that locking ARF6 in its GTP-bound form alters cell morphology and impairs cell viability (*Brown et al., 2001*; *D'Souza-Schorey et al., 1998*; *Peters et al., 1995*).

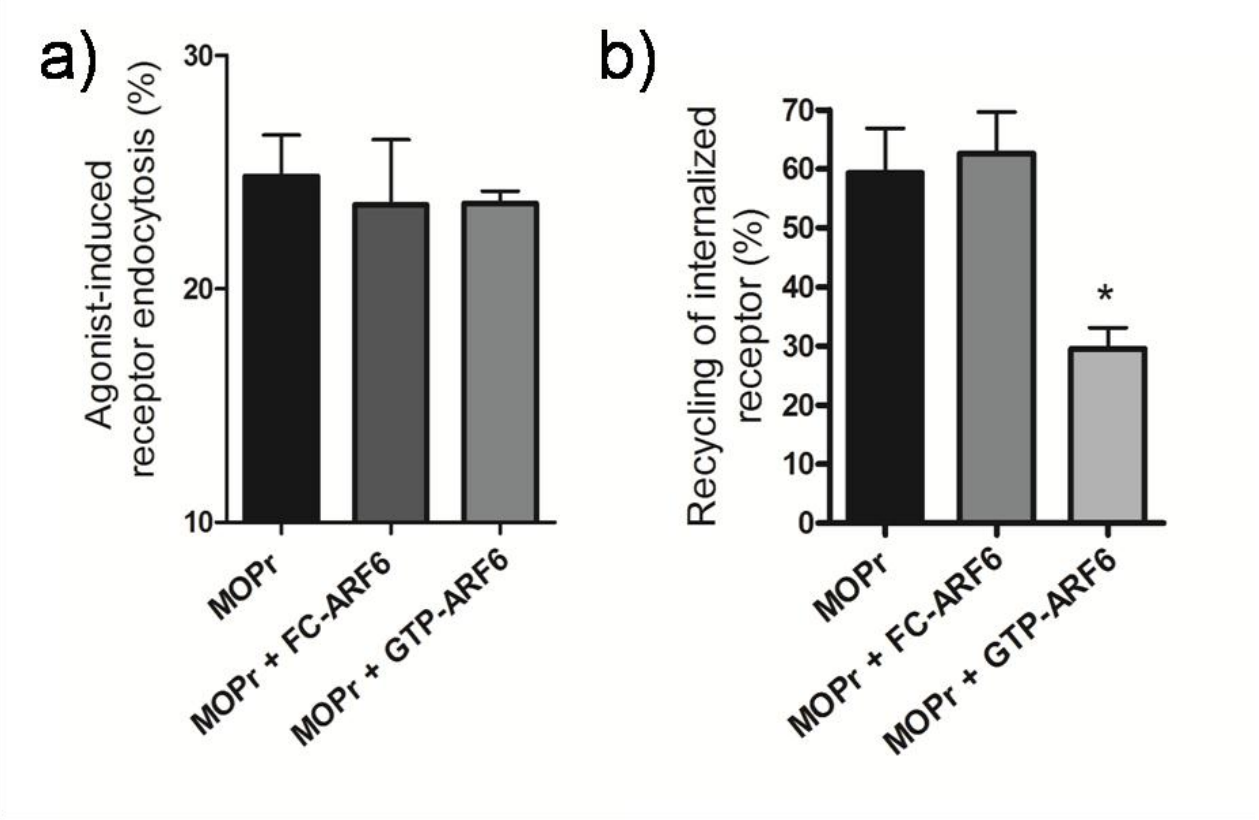


Figure 3.15. Quantitative analysis of MOPr endocytosis and recycling in HEK293 cells co-transfected with MOPr and FC-ARF6 or GTP-ARF6 mutant. Control HEK293 cells stably expressing T7-tagged MOPr alone or cells coexpressing T7-tagged MOPr and indicated HA-tagged ARF6 mutants were incubated for 1,5 h at 4ºC with T7-antibody to label surface receptors. For endocytosis quantification (Fig. 3.15.a), the cells were treated with 1μM DAMGO for 30 min at 37ºC to induce receptor internalization and then fixed. In recycling experiments (Fig. 3.15.b), after DAMGO stimulation the agonist was washed away and the cells were treated with 1μM naloxone for further 30 min as described under "Materials and Methods". Receptor internalization was quantified as the percent loss of cell surface receptors. The recycling rate was estimated as a percentage of recovered surface receptors to endocytosed receptors. Data are presented as means ± SEM of three independent experiments performed in triplicate. * $p<0.05$ versus MOPr alone (control) was calculated by one-way ANOVA followed by Bonferroni test.

3.11. "GTP-locked" ARF6 mutant (GTP-ARF6) induces formation of the actin-rich vacuolar structures

In the previous section we demonstrated that overexpression of GTP-ARF6 mutant impairs MOPr recycling but not receptor endocytosis. Therefore we looked next what could be the mechanism of the observed effects. It has been previously reported that expression of GTP-ARF6 induces formation of a large vacuolar clusters in the cells that are rich in actin and PIP_2 (*D'Souza-Schorey et al., 1995*; *Brown et al., 2001*; *Radhakrishna and Donaldson 1997*). Since these structures are endosomally derived, it can be suggested that they might be implicated in MOPr trafficking and connected with decreased recycling rate that we detected in the cells expressing GTP-ARF6 mutant.

Thus, we first investigated the effect of expression of GTP-ARF6 mutant on the actin cytoskeleton in HEK293 cells. For these experiments, commercially available rhodamine-phalloidin was used (see "Materials and Methods"). This fluorescently labeled phallotoxin is isolated from *Amanita phalloides* mushroom and specifically binds to and stabilizes F-actin. Rhodamine-phalloidin staining revealed that HEK293 cells transfected with GTP-ARF6 mutant got round-shaped, as noticed before, and indeed developed actin-rich structures (Figure 3.16.a). However, due to dramatic morphological changes of HEK293 cells transfected with GTP-ARF6 mutant, these cells were very hard to image under the microscope and therefore we decided to use COS-7 cell line for further experiments. These cells are bigger and more flat than HEK293 cells and in the literature represent more commonly used cell model system for investigation of actin cytoskeleton rearrangements. It could be seen that COS-7 cells transfected with GTP-ARF6 mutant lost most of the actin stress fibers that are present throughout the control (untransfected) cells and clearly developed actin-rich accumulations, similar to those seen in transfected HEK293 cells (Figure 3.16.b). Figure 3.16.c shows a closer look to these structures revealing that these are indeed clusters of vacuoles with strong actin staining in the membrane. These structures were formed approximately 8-10 hours after transfection, consistently with the time of GTP-ARF6 expression (data not shown) and by their existence the transfected cells could be clearly identified under the microscope. On the other hand, in COS-7 cells transfected with FC-ARF6 mutant no vacuolar structures were detected (Figure 3.16.b, lower panels). These results show that generation of vacuolar structures enriched in actin molecules in our experiments is not an artifact of cell transfection and is specificaly due to the expression of "GTP-locked" ARF6 mutant.

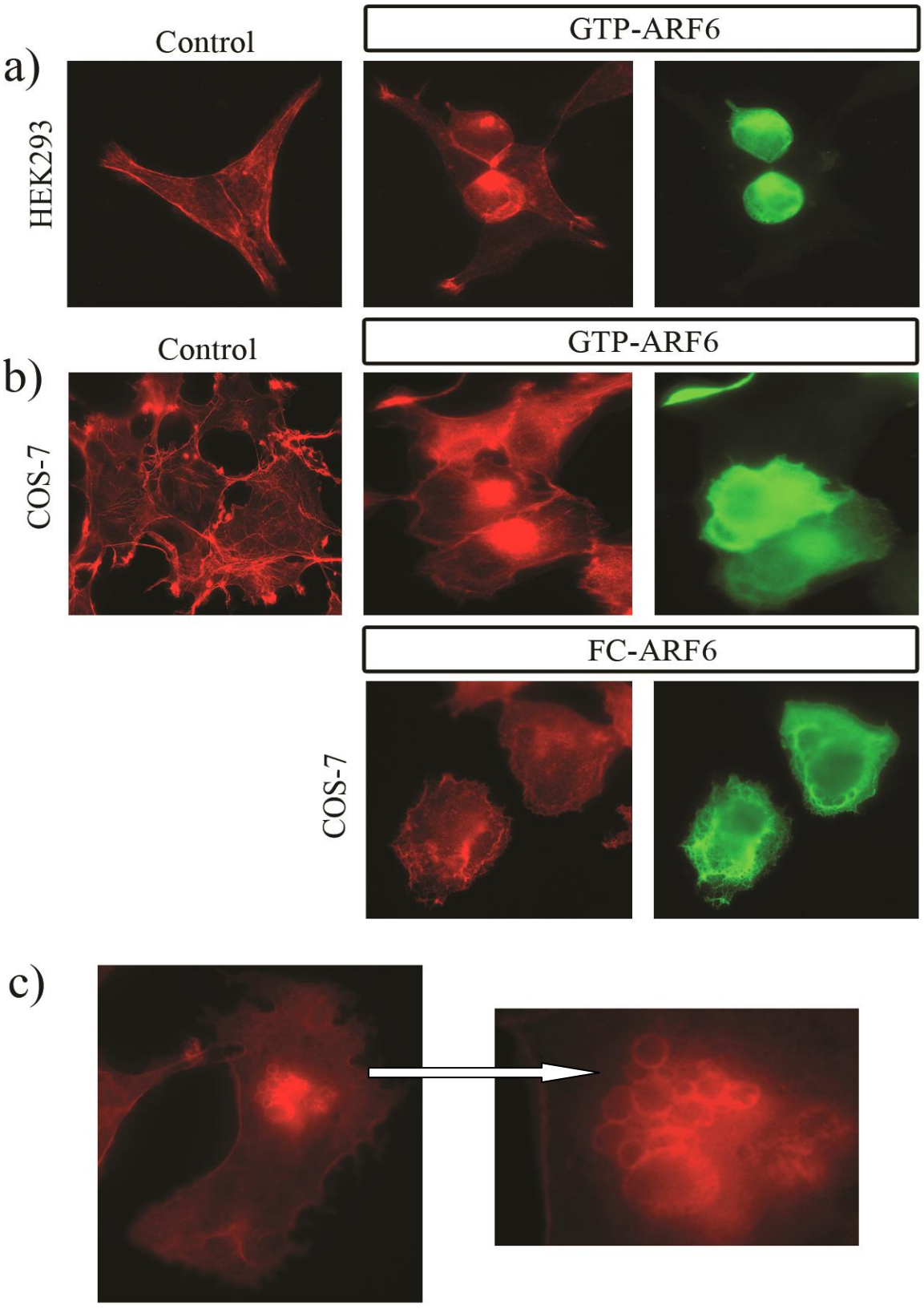


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Figure 3.16. Overexpression of GTP-ARF6 mutant induces formation of actin-rich vacuolar structures. HEK293 cells (Fig. 3.16.a) or COS-7 cells (Fig. 3.16.b) were transfected or not (control) with indicated ARF6 mutants (HA-ARF immunostaining is shown in green). 48 h after transfection, the cells were stained for F-actin using rhodamine-phalloidin (shown in red) as described under "Materials and Methods". Note the loss of actin stress fibers and formation of actin-rich clusters of vesicles in cells transfected with GTP-ARF6 mutant. FC-ARF6 mutant induced no dramatic changes of actin cytoskeleton or cluster formation (Fig. 3.16.b, lower panels). c) Closer look to actin-rich cluster of vesicles in COS-7 cell transfected with GTP-ARF6 mutant.

3.12. Endocytosed MOPr gets "trapped" in actin-rich vacuolar compartment induced by GTP-ARF6 mutant overexpression in COS-7 cells

Next we investigated how MOPr trafficking in COS-7 cells is affected by transfection with GTP-ARF6 mutant. Since in the literature COS-7 cells are not commonly used as a cell model system for investigation of receptor trafficking, we made some preliminary tests first in order to be sure that MOPr expressed in COS-7 cells follows the same endocytosis and recycling pattern like in HEK293 cellular model or neuronal cells. MOPr belongs to a group of G protein-coupled receptors that are endocytosed via clathrin-coated pits and inside the cells it can be found colocalized with transferrin receptor (TfR) which is used as a marker for clathrin-dependent endocytosis. In COS-7 cells transfected with HA-MOPr, we could also detect extensive colocalization of internalized HA-tagged MOPr with TfR (Figure 3.17.) demonstrating clathrin-dependent route of MOPr internalization in this cell model. Following MOPr internalization and sorting, most of the receptors are recycled back to the plasma membrane in reactivated form and significant downregulation of receptor is not detected after short agonist treatment. Indeed, as shown on the Figure 3.18., MOPr is almost completely recycled back to the plasma membrane of control COS-7 cells expressing MOPr alone in recycling experiments (30 min of 1 μM DAMGO-induced internalization, agonist washout and 30 min of treatment with 1 μM receptor antagonist naloxone). Moreover, the residual vesicles that can be seen in the cells are positive for Rab11, a small GTPase that is a marker for recycling endosomes (Fig. 3.18.a, blue immunostaining). These results confirmed that trafficking behaviour of MOPr expressed in COS-7 cells resembles trafficking in other cell model systems including neuronal cells which are physiological environment for opioid receptors.

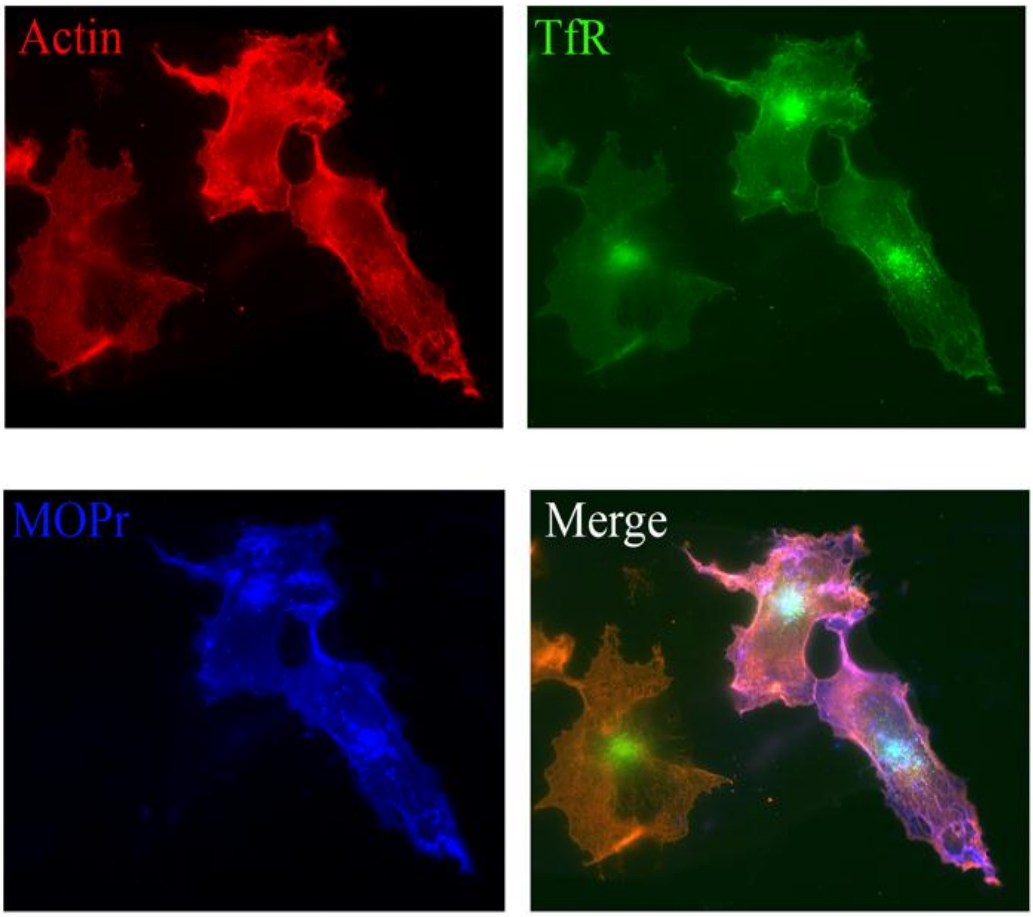

Figure 3.17. Colocalization of internalized HA-MOPr and transferrin receptor (TfR) demonstrates clathrin-dependent endocytosis of MOPr in transfected COS-7 cells. COS-7 cells were transfected with HA-MOPr and 48 h after transfection "pulse-chase" assay was done as described in "Materials and Methods" section. Note massive colocalization of internalized MOPr receptor and TfR receptor which is used as a marker for clathrin-dependent endocytosis.

Then we investigated how expression of “GTP-locked” ARF6 mutant influences the recycling of internalized MOPr. It can be seen on Figure 3.19. that situation is dramatically different in cells transfected with this mutant which could be clearly identified by formation of actin-rich clusters. Namely, most of the receptor stayed inside the transfected cells after agonist washout and during recycling, and strikingly, colocalized extensively with actin-rich structures induced by the expression of GTP-ARF6 mutant. Moreover, Figure 3.19. shows an adjacent cell that is transfected with HA-MOPr but not with GTP-ARF6 mutant and has no enlarged endosomes or changes in actin cytoskeleton. It can be seen that this cell shows similar pattern of MOPr recycling like control cells transfected only with MOPr (see Figure 3.18. for comparison).

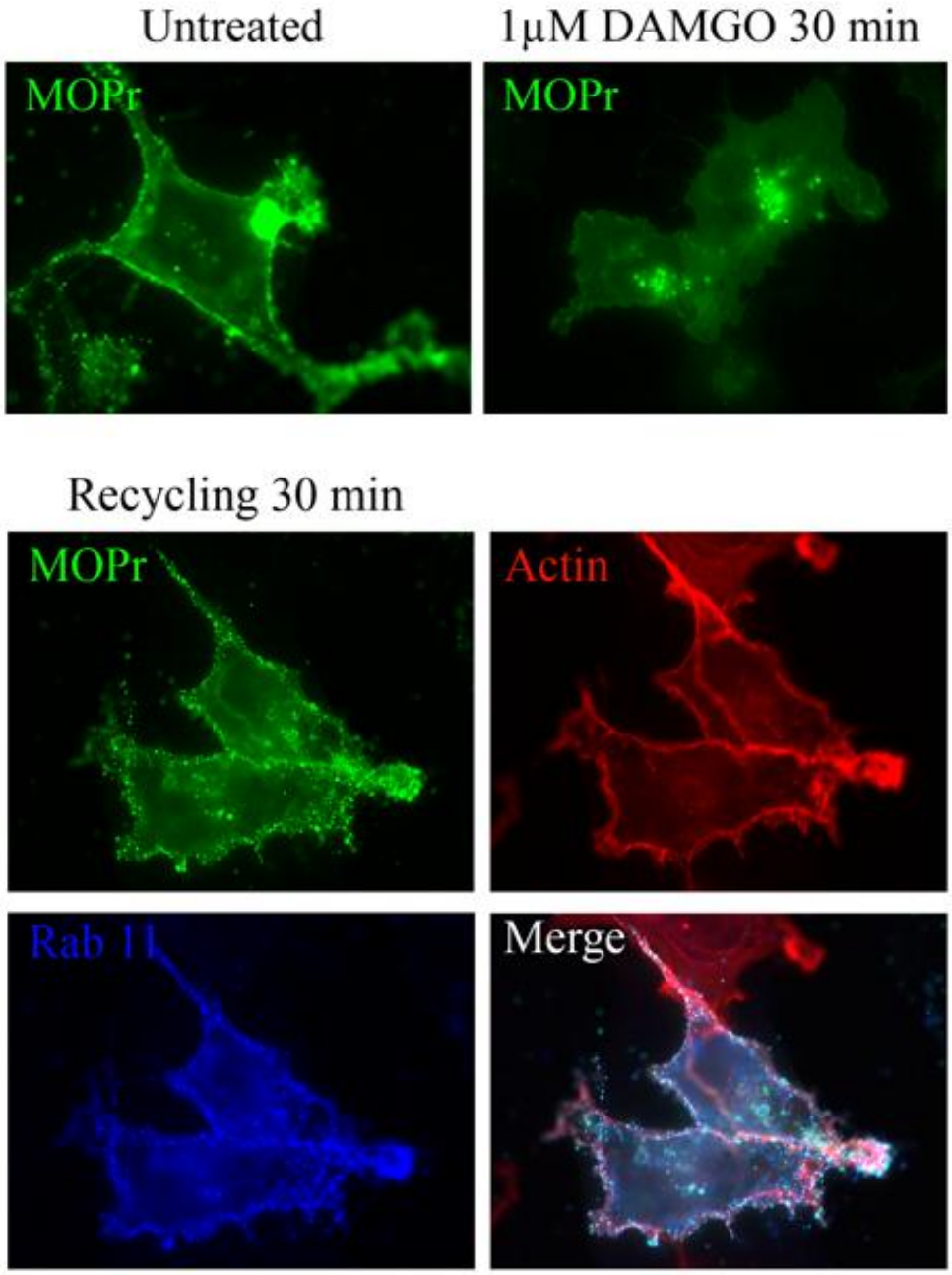

Figure 3.18. Trafficking of HA-MOPr in transfected COS-7 cells. COS-7 cells were transfected with HA-MOPr as described in "Materials and Methods". 48 h after transfection, the cells were (a) treated with 1 μM DAMGO for 30 min to induce receptor endocytosis or (b) after 30 min of treatment with 1 μM DAMGO the agonist was washed away and the cells were treated for another 30 min with1 μM receptor antagonist naloxone (receptor recycling). Note that the most of receptor is internalized after DAMGO treatment (a) and could be seen in endocytotic vesicles inside the COS-7 cells. Moreover, internalized MOPr recycled almost completely to the plasma membrane after 30 min DAMGO-free interval (recycling) (b).

These experiments show that when GTP hydrolysis of ARF6 protein is blocked by GTP-ARF6 mutant overexpression, endocytosed MOPr seems to get "trapped" in actin-rich vacuolar structures that are formed in the cells and can not recycle back to the plasma membrane. This is in correlation with our quantitative data from HEK293 cells (Fig. 3.15.b) that show a decrease in MOPr recycling after overexpression of GTP-ARF6 mutant. Therefore, these results suggest that GTPase activity and full cycle of GTP/GDP exchange of ARF6 protein are important for the trafficking of endocytosed MOPr back to the plasma membrane during receptor recycling.

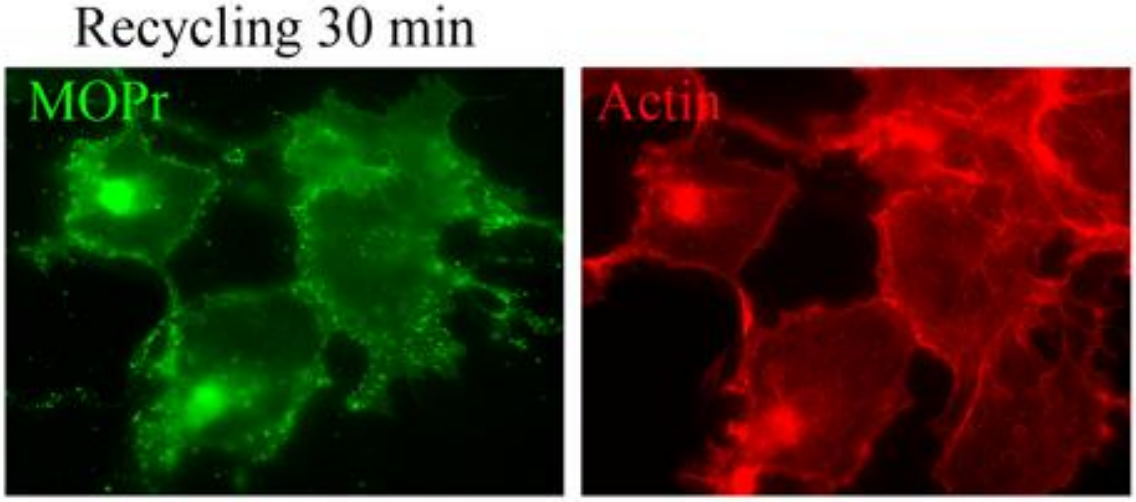

Figure 3.19. Recycling of internalized HA-MOPr is blocked in COS-7 cells transfected with GTP-ARF6 mutant. COS-7 cells were co-transfected with HA-MOPr and GTP-ARF6 mutant as described in "Materials and Methods". 48 h after transfection, the cells were first treated with 1 μM DAMGO for 30 min to induce receptor endocytosis and then the agonist was washed away and the cells were treated for another 30 min with1 μM receptor antagonist naloxone (receptor recycling). Note that in the cells that are co-transfected with HA-MOPr and GTP-ARF6 mutant (in which actin-rich structures are formed) the most of internalized receptor stayed inside the cells and extensively colocalized with actin-rich clusters of vesicles. Moreover, in adjacent control cell that is transfected only with MOPr, internalized receptor recycled almost completely to the plasma membrane after 30 min DAMGO-free interval.

4. Discussion

The investigation of molecular mechanisms regulating endocytosis of MOPr is of clinical importance because MOPr endocytosis counteracts the development of tolerance to opioid drugs by facilitating the reactivation of desensitized receptors (*Koch et al., 2005*). Therefore, the present study investigated the role of two molecular players involved in regulation of MOPr trafficking and signaling, namely PLD2 and ARF proteins.

In the first part of the present study, reported data demonstrate that opioid-induced PLD2 activation and subsequent receptor endocytosis is mediated via ARF6 and not ARF1 protein. This conclusion is based on the following observations: 1) dominant negative mutants or siRNA knock down of ARF6 but not of ARF1 inhibit agonist-induced MOPr endocytosis; 2) active, "fast cycling" mutant of ARF6 increases the morphine-induced MOPr endocytosis; 3) blocking PLD2 activation by the PLD-defective ARF6 mutant inhibits agonist-induced MOPr endocytosis; 4) opioid-mediated activation of PLD2 is blocked in the presence of dominant negative or PLD-deficient ARF6 mutants. Further, we demonstrated that blocking of ARF6 function impairs MOPr recycling as well and that ARF6 protein also affects the receptor desensitization/resensitization via regulating MOPr trafficking. Second part of this study demonstrates the importance of GTP hydrolysis of activated ARF6 protein and full GDP/GTP cycle for the trafficking of internalized MOPr back to the plasma membrane since locking ARF6 in its GTP-bound, active state blocks recycling of the receptor.

4.1. ARF6 protein regulates MOPr endocytosis via PLD2 activation

PLD is an ubiquitous enzyme that catalyzes the hydrolysis of PC, the major phospholipid of membranes, to PA and choline. PA has been implicated to have many different functions in signal transduction, vesicle formation, and cytoskeleton dynamics (*Liscovitch and Cantley, 1995*; *Liscovitch et al., 1999*). There are two mammalian PLD isoforms, PLD1 and PLD2, differing in their cellular localization and regulation. PLD1 is located in the cytosol, Golgi apparatus, nucleus and plasma membrane, while PLD2 seems to be largely found on the plasma membrane (*Liscovitch et al., 1999*).

PLDs can be activated by ARFs, Rho, PIP_2 and protein kinase C (*Liscovitch et al., 2000*; *Exton, 2002*; *Hiroyama and Exton, 2005*). Additionally, PLD activity has been shown to be

regulated by a number of GPCRs as mentioned before (see Chapter 1.4.1.). Using a yeast two-hybrid screening, our group has shown interaction between cytoplasmatic C-terminal part of MOPr and N-terminal part of PLD2 (*Koch et al., 2003*). Moreover, these studies demonstrated that PLD2 can be activated via MOPr and that opioid-mediated activation of PLD2 is a key step during the induction of agonist-mediated endocytosis and recycling of the receptor affecting the development of opioid tolerance (*Koch et al., 2003, 2004*).

PLD2 is predominantly activated by ARF family of small GTPases and not by PKC (*Exton, 2002*; *Hiroyama and Exton, 2005*; *Koch et al., 2003*). Therefore ARF proteins might be involved in regulation of receptor trafficking and signaling via modulating PLD2 activity. As mentioned before, both ARF1 and ARF6 members of ARF family of small GTPases seem to be promising candidates for this function. This led us to examine whether ARF1 or ARF6 protein is involved in the μ-opioid receptor-mediated PLD2 activation and receptor trafficking.

The data reported here provide evidence that opioid-induced PLD2 activation and subsequent receptor endocytosis is mediated via ARF6 and not ARF1 protein. This conclusion is based on several experiments. First, overexpression of dominant negative ARF6 and not ARF1 mutant induced significant decrease in DAMGO-mediated MOPr endocytosis in HEK293 cell model system (Figure 3.1. and 3.2.) as well as transfected cortical neurons (Figure 3.5.). Moreover, knocking down of endogenous ARF6 protein expression in HEK293 cells by siRNA technology gave similar results (Figure 3.3. and 3.4.). On the other hand, overexpression of active "fast cycling" ARF6 mutant significantly increased MOPr endocytosis in both cellular systems used after treatment with morphine, an agonist that does not induce receptor endocytosis itself (Figure 3.6. and 3.7.). Finally, several lines of evidence show that ARF6 protein has a role in opioid-mediated PLD2 activation which is essential for MOPr internalization. Namely, overexpression of mutant which is selectively incapable of activating PLD decreases MOPr endocytosis and this block can be bypassed by heterologous activation of PLD2 via PKC pathway using PMA treatment (Figure 3.8.). Moreover, direct measurement of PLD activity using transphosphatidylation reaction showed that DAMGO-induced PLD2 activity is decreased in the presence of negative ARF6 mutants while activating ARF6 by "fast cycling" ARF6 mutant overexpression increases PLD2 activation after morphine treatment, as expected (Figure 3.11.).

Altogether, these findings are in agreement with other publications showing that ARF6 protein can activate PLD2 *in vivo* (*Hiroyama and Exton, 2005*), that ARF6 mediates the somatostatin-induced PLD-activity (*Grodnitzky et al., 2007*) and that ARNO, a guanine nucleotide exchange factor for ARF6, can increase PLD activity (*Santy and Casanova, 2001*). In addition, our results are consistent with previous findings demonstrating that ARF6 and not

ARF1 regulates the internalization of other GPCRs, such as ß2-adrenergic receptor, endothelin receptor and m2 muscarinic receptor (*Claing et al., 2001*; *Houndolo et al., 2005*). In summary, several studies indicate that ARF6 can increase PLD activity and GPCR endocytosis *in vivo* and the present findings point to PLD2 as the target of ARF6 *in vivo*.

It is interesting to mention that ARF6 seems to have a role in receptor internalization through multiple endocytic pathways. Namely, knock down of ARF6 protein expression using siRNAs revealed that ARF6 regulates the internalization of most GPCRs, irrespective of receptor's route of entry (*Houndolo et al., 2005*). As such, receptors internalized through the clathrin, the caveolae, and the clathrin- and caveolae-independent pathway require ARF6 activity. Moreover, it has been shown that activation of ARF protein occurs upon agonist activation of a number of different GPCRs, including the β_2-adrenergic, m3 muscarinic acetylcholine, fMet-Leu-Phe, H1 histamine, gonadotropin releasing hormone, and B2 bradykinin receptors (*Bornancin et al., 1993*; *Rümenapp et al., 1995*; *Houle at al., 1995*; *Mitchell et al., 1998*). Therefore the investigation of whether treatment of cells expressing MOPr with opioid agonists also induces ARF6 activation and determination if there is a difference between internalizing and noninternalizing MOPr agonists regarding this ability gives space for further research. Since we have shown previously that there is a correlation between the ability of agonist to induce receptor endocytosis and to activate PLD2, it would be interesting to see if there is a parallel between these findings and the ability of agonists to activate ARF6.

According to a model, activated ARF6 would then have the effect on phospholipid metabolism by activating PLD as well as type I PIP5K (*Honda et al., 1999*; *Krauss et al., 2003*). PA, the product of PLD activity, could change the physical (e.g. charge, pH) and chemical properties of the plasma membrane in order to facilitate vesicle budding and in turn functions as a cofactor in the activation of PIP5K (*Martin, 2001*). Therefore it is possible that synergistic effect of ARF6 on PIP5K as well as on PLD activity can lead to a large increases in PIP_2 at the cell periphery which has been shown to have a role in regulation of clathrin-dependent endocytosis (*Wenk and De Camilli, 2004*). Indeed, ARF6 binds to and activates PIP5KIγ, leading to the recruitment of clathrin coats in synaptic vesicle preparations (*Krauss et al., 2003*). Moreover, ARF6-GTP and PIP_2 function synergistically to recruit AP-2 onto liposomes as well, pointing towards the role of ARF6 in coated pit assembly (*Paleotti et al., 2005*). Therefore, the mentioned scenario gives a broader view on the role that ARF6 and opioid-mediated PLD2 activation might have in clathrin-dependent internalization of MOPr.

4.2. Recycling of endocytosed MOPr is regulated via ARF6 protein

Further, we demonstrated that blocking ARF6 function impairs MOPr recycling. This conclusion was drown out from experiments showing that overexpression of ARF6 negative mutants as well as siRNA-mediated knock down of endogenous ARF6 protein expression in HEK293 cells stably expressing HA-MOPr significantly decreased the amount of recycled MOPr receptor after agonist washout (Figure 3.12.). A requirement for ARF6 in endosome recycling was first documented in Chinese Hamster Ovary (CHO) cells, in which the expression of a dominant negative ARF6 mutant blocked the recycling of endosomal ligands (*D'Souza-Schorey et al., 1998*). Furthermore, EFA6, a guanine nucleotide exchange factor for ARF6, regulates constitutive endosomal recycling to the cell surface through a PLD2-dependent pathway (*Padron et al., 2006*). In addition, in the absence of PLD2, the transferrin receptors are mistargeted to the slow recycling pathway (*Padron et al., 2006*). These findings are consistent with a proposed functional role of ARF6 in the endosomal sorting processes (*Naslavsky et al., 2003; 2004*). In fact, there is compelling evidence that ARF6-regulated delivery and insertion of recycling endosomal membranes at the cell surface requires PLD activity (*Padron et al., 2006*; *Jovanovic et al., 2006*) and is mediated by the vesicle-tethering exocyst complex (*Prigent et al., 2003*). It has been demonstrated that ARF6 interacts with sec10, a subunit of the exocyst complex that localizes to the recycling endosomes and is redistributed to the cell surface after ARF6 activation. ARF6 bound to sec10 might activate PLD2, producing PA and subsequently increase the PIP_2 level on vesicles during tethering. We also previously observed that blocking of PLD2-mediated PA synthesis impairs not only the endocytosis but also the recycling/resensitization of MOPr (*Koch et al., 2004*), indicating the important role of PA in the regulation of endosomal trafficking.

Interestingly, in the literature ARF6 protein has been mostly implicated in the recycling of proteins that are internalized via clathrin-independent pathway. These molecules are integral plasma membrane proteins that lack cytoplasmic AP-2 and clathrin-sorting sequences, including the IL2 receptor α subunit, MHC class I, and glycosylphosphatidylinositol (GPI)-anchored proteins (*Naslavsky et al., 2003*; *Radhakrishna and Donaldson, 1997*). ARF6-labelled recycling tubules in these cells exhibit minimal overlap with early endosomes and radiate from the juxtanuclear cell region to the cell periphery. However, there are studies showing that the traffic of plasma membrane proteins that either lack the clathrin-dependent sorting signals or that are mediated by classic clathrin-dependent pathway converge in common recycling endosomes (*Naslavsky et al., 2003*). Indeed, internalized β1 integrin in HeLa cells that recycles to the surface

in an ARF6-dependent manner colocalizes with internalized transferrin (marker for clathrin-dependent endocytosis and early endosome compartment), the small GTPase Rab11 (marker for recycling endosome) and MHC class I molecules in recycling endosomes (*Powelka et al., 2004*). Together with necessity for PLD2 activity in recycling which has been shown previously and mentioned above, these facts might explain the role of ARF6 in regulation of MOPr recycling since this receptor is internalized via clathrin-dependent pathway. Moreover, in the second part of this study we have shown the necessity for GTP hydrolysis of active ARF6-GTP for proper MOPr recycling to the plasma membrane which is discussed below (see Chapter 4.4.).

4.3. ARF6 protein has influence on MOPr desensitization

It is well known that peptide agonists, such as DAMGO, and many opioid alkaloids induce rapid endocytosis of MOPr in a number of cell types. By contrast, the alkaloid drug morphine is weak in promoting receptor internalization. Since receptor trafficking (endocytosis and recycling) is an important regulator of agonist-induced receptor desensitization, the significant differences in DAMGO- and morphine-mediated receptor trafficking should lead to differences in receptor desensitization as well. In our study, Figure 3.13. shows that 2 h treatment with morphine leads to significant MOPr desensitization which occurs at a much higher rate compared with that after 2 h DAMGO treatment. Numerous studies have demonstrated that agonist-induced GPCR endocytosis contributes to functional resensitization of signal transduction by promoting dephosphorylation and recycling of reactivated receptors to the plasma membrane (*Ferguson and Caron, 1998*; *Lefkowitz,1998*; *Koch et al., 1998, 2005*; *Schulz et al., 2004*). Therefore, the explanation for the differences in DAMGO- and morphine-induced desensitization could be the inability of morphine to induce PLD2 activation, MOPr internalization and recycling which lead to receptor resensitization.

In support of this receptor recycling theory, it has been demonstrated that the endocytotic efficacies of various opioid drugs are negatively correlated with their ability to cause receptor desensitization in HEK293 cells (*Koch et al., 2005*) and that opioid drugs with high endocytotic efficacies induced less opioid tolerance than non-internalizing agonists in rats (*Grecksch et al., 2006*). Thus, blocking ARF6-mediated MOPr endocytosis and recycling should also affect the receptor desensitization/resensitization. In fact, blocking ARF6-mediated PLD2 activation resulted in a significantly higher MOPr desensitization after treatment with the internalizing agonist DAMGO (Figure 3.13.). On the other hand, overexpression of an active ARF6 mutant facilitates MOPr endocytosis/recycling and reduces the MOPr desensitization after treatment with

the agonist morphine, which is normally incapable of activating PLD2 and thus does not induce MOPr endocytosis (Figure 3.13.). These findings support the hypothesis that MOPr endocytosis counteracts the development of opioid receptor desensitization and tolerance (*Koch et al., 2005*). Furthermore, they are in line with previous studies from our group demonstrating that PLD2 activity is important for the regulation of MOPr desensitization and resensitization in HEK293 cells stably expressing MOPr (*Koch et al., 2004*). In the presented work, we also reconfirmed these results in cultured cortical neuronal cells endogenously expressing MOPr by showing that expression of catalytically inactive (K758R) PLD2 mutant (nPLD2) increased receptor desensitization after both 1 h and 2 h of DAMGO pretreatment, as expected (Figure 3.14.). Together with other data from neuronal cells , these experiments were done in order to assure that effects that we see are not an artifact of HEK293 cell model system and that the main components and mechanisms identified in transfected HEK293 cells are closely related to those seen in more physiological context, i.e. in neuronal cells. Therefore we can suggest that an ARF6-mediated PLD2 activation after opioid treatment might play a role in development of tolerance and dependence to opioid drugs, adaptive changes in the brain that greatly limit the therapeutic use of opioids.

4.4. GTP hydrolysis of ARF6 is necessary for efficient MOPr recycling

Like other small G proteins, ARF6 cycles between its GTP-bound and GDP-bound states, which are considered to represent ARF6 active and inactive conformation, respectively. This implies that a GTPase-defective mutant locked in a GTP-bound form would reproduce the functions controlled by activated ARF6. However, it has long been appreciated for many G proteins that GTP hydrolysis as well is necessary to elicit the full biological response, suggesting that the completion of the full GTP/GDP cycle is important (reviewed in *Klein et al., 2006*). Therefore, it appears that, in many cases, to recapitulate the biological functions of small G proteins, mutants that cycle between their GTP- and GDP-bound forms, so called “fast cycling” mutants, are more representative of the small G protein natural activities.

In the same study by Klein and colleagues (*Klein et al., 2006*), the authors compared “fast cycling” ARF6 mutant (ARF6/T157N) and classical “GTP-locked” ARF6 mutant (ARF6/Q67L) and established the necessity for completion of the ARF6 GTP/GDP cycle for recycling of MHC class I molecules to the plasma membrane. Moreover, ARF6/Q67L mutant has been previously shown to block the recycling of non-clasically internalized receptors (*Radhakrishna and Donaldson, 1997*; *Naslavsky et al., 2003*). Namely, after expression of this mutant, internalized

molecules accumulated in an intracellular compartment caused by a block in fusion of the endocytic vesicles with the classical early endosome compartment (*Klein et al., 2006*; *Naslavsky et al., 2003*). On the other hand, "fast cycling" ARF6/T157N mutant did not block recycling of the receptors suggesting that ARF6-GTP inactivation, i.e. catalytic activity of certain ARF6 GAP protein(s) is necessary for the proper recycling of MHC class I molecules to the plasma membrane (*Klein et al., 2006*).

By using "fast cycling" ARF6 mutant and classical "GTP-locked" ARF6 mutant (GTP-ARF6), we also tested how important is full GTP/GDP cycle for ARF6 regulation of MOPr endocytosis and recycling. Our results show that both mutants act similarly in the endocytosis assay, but, however, in recycling assay only the GTP-ARF6 mutant was strongly inhibitory (Figure 3.15.). This is in line with the mentioned study (*Klein et al., 2006*) and demonstrates that GTP hydrolysis of activated ARF6-GTP seems to be a necessary step for recycling of MOPr as well. However, it is interesting to mention that previous studies mostly implicated ARF6 in regulation of endocytosis and recycling of receptors that are internalized via clathrin-independent pathways, which is not the case for MOPr. In fact, a novel plasma membrane recycling pathway regulated by ARF6 has been described (*Radhakrishna and Donaldson, 1997*) which is distinct from transferrin-positive endosomes. However, MOPr belongs to a group of GPCRs that are internalized via clathrin-coated pits, as demonstrated in COS-7 cell model system by colocalization of endocytosed MOPr with transferrin receptor which is used as a marker for clathrin-dependent endocytosis (Figure 3.17.). Therefore our data from these experiments together with data showing a block of MOPr recycling after overexpression of negative ARF6 mutants of siRNA knock down of endogenous ARF6 expression suggest that ARF6 might have a broader role in cargo trafficking than previously described.

Further investigation revealed that overexpression of "GTP-locked" ARF6 mutant induces dramatic changes in cytoskeleton organization and formation of actin- and PIP_2-rich large vacuolar clusters in transfected cells (Figure 3.16.). These clusters are enlarged endosomes that have been detected in both HEK293 and COS-7 cell model system after transfection with GTP-ARF6 mutant (Figure 3.16.) and have been described previously (*D'Souza-Schorey et al., 1995*; *Brown et al., 2001*; *Radhakrishna and Donaldson, 1997*). However, since expression of GTP-ARF6 mutant induced dramatic changes of HEK293 cells morphology making a precise investigation impossible, the following experiments were done in COS-7 cells as a model system. Immunocytochemical analysis of MOPr recycling in COS-7 cells transfected with "GTP-locked" ARF6 mutant revealed that internalized MOPr actually gets "trapped" in formed actin-rich structures (Figure 3.19.) and is unable to recycle back to the plasma membrane. Obtained results

were intriguing since previous work reported similar findings again only for molecules internalized by clathrin-independent mechanism (*D'Souza-Schorey et al., 1995*; *Brown et al., 2001*; *Radhakrishna and Donaldson 1997*).

But how and why are actin-rich structures formed in cells overexpressing "GTP-locked" ARF6 mutant? It is thought that these enlarged endosomes are formed by stimulated homotypic fusion of ARF6- and PIP_2-containing endosomes (*Naslavsky et al., 2003*; *Brown et al., 2001*). The authors hypothesized that ARF6-containing early endosome must undergo ARF6 inactivation (through GTP hydrolysis) and probably removal or modification of PIP_2 before becoming competent to acquire early endosomal antigen 1 (EEA1) molecules by heterotypic fusion with "classical" early endosome labeled with EEA1, Rab5 and transferrin molecules, all of which are used as a markers for this cellular compartment. Interestingly, a similar enlargement of early endosomes was previously described for constitutively active Rab5/Q79L mutant also (*Stenmark et al., 1994*). However, the authors in the mentioned study have shown that sequestration of internalized cargo into actin-rich vesicles induced by "GTP-locked" ARF6 mutant overexpression occurs before fusion with "classical" Rab5-containing early endosome (*Naslavsky et al., 2003*). Moreover, since PLD2 is a downstream effector of activated ARF6, locking ARF6 in its active, GTP-bound state migth lead to PLD2 superactivation and extensive production of PA. Further, this phospholipid can increase the formation of PIP_2 over PIP5K by activating this enzyme. Finally, this cascade could promote homotypic fusion and be a possible mechanism of formation of observed enlarged endosomes.

Altogether, we can say regarding this part of the study that additional work is needed in order to investigate the observed effects in more details and to characterize the nature of actin-rich enlarged endosomes induced by GTP-ARF6 mutant overexpression using appropriate markers for different cellular compartments. Moreover, more precise analysis and comparison of the steps that occur during internalization of MOPr in cells expressing "GTP-locked" ARF6 mutant should be done. This would clarify in which step from early endosome to recycling endosome the trafficking of internalized MOPr is blocked and give us some hints about the mechanisms that might be involved.

Finally, we can generally summarize what is known about the role of small GTPase ARF6 in endocytosis by the following cartoon modified from recent review by *D'Souza-Schorey and Chavrier, 2006* (Figure 4.1.). Through its effect on PIP5K and PLD and the production of PIP_2, activated ARF6-GTP can facilitate both clathrin-dependent as well as clathrin-independent internalization of proteins. ARF6-regulated recruitment of adaptor protein AP-2 and other molecules promotes clathrin-dependent endocytosis. Moreover, our previous work as well as this

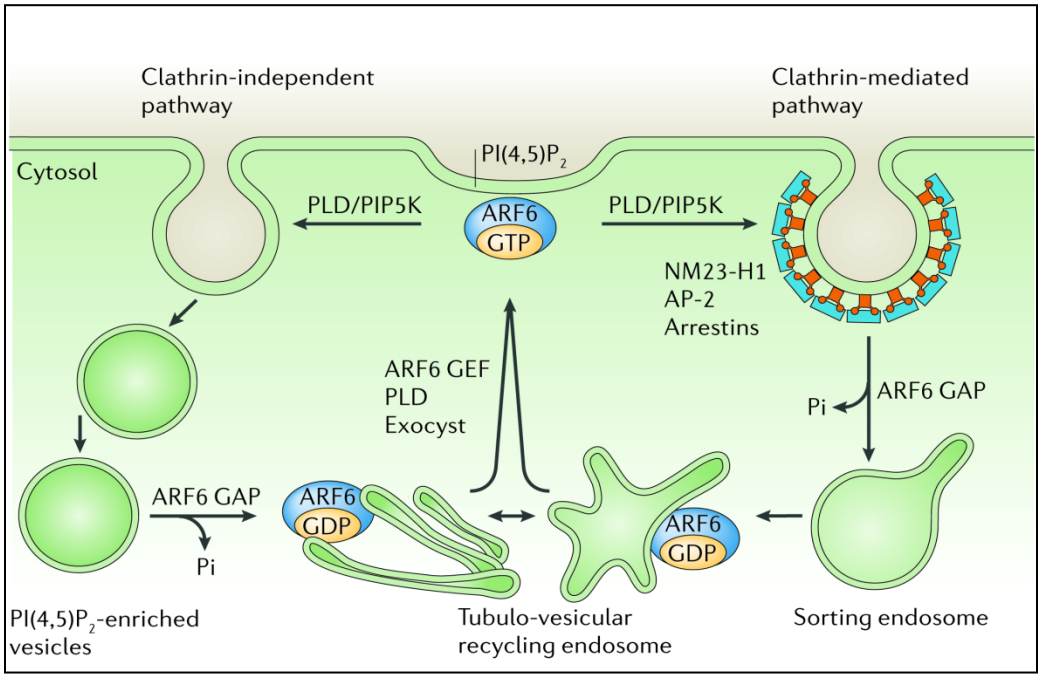

Figure 4.1. The role of ARF6 in clathrin-dependent and clathrin-independent endocytic pathways (modified from *D'Souza-Schorey and Chavrier, 2006*). See text for details.

study demonstrates that ARF6-mediated PLD2 activation and PA production are a key step in agonist-induced internalization of MOPr. On the other hand, ARF6 activation has been also linked to the dissociation of arrestin molecules to facilitate receptor internalization (*Mukherjee et al., 2000*; *Claing et al., 2001*). After internalization of proteins, ARF6-GTP hydrolysis through specific GAPs seems to be required for further trafficking along each pathway, whereas the activation of ARF6 through specific GEFs promotes the recycling and subsequent fusion of an endosomal membranes with the plasma membrane. In this step again activity of PLD has been shown to be crucial since ARF6-regulated membrane recycling is mediated in part by the vesicle-tethering exocyst complex and PLD activation. Therefore our data from the presented study showing a role of ARF6 in opioid-mediated PLD2 activation and trafficking of MOPr are in line with previous findings and presented scenario. The fuctional interaction and the interplay between ARF6, PLD2 and MOPr are required in multiple steps during receptor endocytosis, recycling and signaling and are documented in this work.

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6. Abbreviations

A

AC - adenylate cyclase

AP-2 – adaptor protein 2

APS - ammonium persulfate

ARF – ADP-ribosylation factor

ARNO - ADP-ribosylation factor nucleotide-binding site opener

B

BSA –bovine serum albumin

C

CamKII - Ca^{2+} - calmodulin dependent protein kinase II

cAMP - cyclic adenosine monophosphate

CHO cells- Chinese hamster ovary cells

COS-7 cells- African green monkey kidney fibroblast cells

D

DAG - diacylglycerol

DAMGO - [D-Ala^2,NMe-Phe^4,$Gly\text{-}ol^5$]-enkephalin

DMEM - Dulbecco's Modified Eagle's Medium

DNA - deoxyribonucleic acid

DN-ARF1 – dominant negative mutant of ARF1

DN-ARF6 – dominant negative mutant of ARF6

dsRNA – double stranded ribonucleic acid

DTT - dithiothreitol

E

EEA1 – early endosomal antigen 1

ECL - enhanced chemiluminescence

EDTA - ethylenediaminetetraacetic acid

EFA6 - exchange factor for ARF6

ELISA - enzyme-linked immunosorbent assay

F

FC-ARF6 – "fast cycling" ARF6 mutant

FCS – fetal calf serum

G

GAP - GTPase-activating protein

GDP – guanosine diphosphate

GEF - guanine nucleotide exchange factor

Git1 - G protein-coupled receptor kinase-interacting protein 1

Gly - glycine

GPCR – G-protein coupled receptor

GPI - glycosylphosphatidylinositol

GRK - G protein-coupled receptor kinase

GTP – guanosine triphosphate

GTP-ARF6 – "GTP locked" ARF6 mutant

H

HA antigen – hemagglutinin antigen

HBSS - Hank´s balanced salt solution

HEK293 cells – Human embryonic kidney 293 cells

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

L

LPA – lysophosphatidic acid

M

MAP kinase – mitogen activated protein kinase

MHCI – major histocompatibility complex class I

MOPr – μ-opioid receptor

N

NGS – normal goat serum

NIH – National Institute of Health

N-myr-ARF6 – N-myristoylated ARF6 peptide

nPLD2 – catalytically inactive (K758R) PLD2 mutant

NT - neurotransmitter

P

PA- phosphatidic acid

PBS – phosphate buffered saline

PC – phosphatidyl choline

PCR – polymerase chain reaction

PD-ARF6 – PLD-deficient ARF6 mutant

PH – pleckstrin homology

PIP_2 – phosphatidylinositol-4,5-biphosphate

PIP5K - phosphatydilinositol-4-phosphate-5-kinase

PKC - protein kinase C

PLD2 – phospholipase D2

PMA - phorbol 12-myristate 13-acetate

PtdEtOH - phosphatidylethanol

PTX - pertussis toxin

PX - phox homology

R

RIPA buffer - radioimmunoprecipitation buffer

RISC - RNA-induced silencing complexe

RNA – ribonucleic acid

RNAi – RNA intertference

S

SDS - sodium dodecyl sulfate

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM - standard error of the mean

siRNA - small interfering ribonucleic acid

T

TAE - Tris-acetate-EDTA

TEMED - tetramethylethylenediamine

TfR – transferrin receptor

TPBS – Tris/phosphate-buffered saline

U

UV – ultraviolet

7. Curriculum Vitae

Name:	Marija
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2006 - 2010	Doctoral theses. Otto-von-Guericke University, Institute of Pharmacology and Toxicology, Magdeburg, Germany.
2003-2004	Diploma study at the Institute of Biological Research "Sinisa Stankovic", Belgrade, Serbia
1999- 2004	Faculty of Chemistry Department of Biochemistry University of Belgrade, Serbia
1995 -1999	High School (*Gimnasium*), Belgrade, Serbia
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8. Scientific publications:

Schroeder H., Wu D-F., Seifert A., **Rankovic M.**, Schulz S., Hoellt V., and Koch T. (2009), Allosteric modulation of metabotropic glutamate receptor 5 affects phosphorylation, internalization and desensitization of the mu-opioid receptor. *Neuropharmacology 56, 768-778*.

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9. Erklärung

Ich erkläre, dass ich die der Naturwissenschaftlichen Fakultät der Otto-von-Guericke- Universität zur Promotion eingereichte Dissertation mit dem Titel

” Modulation of μ-opioid receptor signal transduction and endocytosis by ADP-ribosylation factor proteins “

im Institut für Pharmakologie und Toxikologie der Otto-von-Guericke-Universität Magdeburg mit Betreuung durch Prof. Dr. med. Volker Höllt ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe. Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden. Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Naturwissenschaftlichen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Magdeburg, 22.06.2010

(Dipl.-Bioch. Marija Rankovic)