Die Rolle der Phospholipase D2 in der agonisten-induzierten Endozytose von Opioid Rezeptoren

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Table of Contents

Table of Contents

1. Introduction

Opiates have been used for pain relieve from ancient times. Its principal component morphine is the first alkaloid purified from plants. Even today, opioid agonists are still extensively used for the treatment of severe pain associated with traumatic injuries, cancer or heart attacks. However, the development of opiate tolerance and dependence severely limits their clinical administration. In the early 1970s, the existence of opioid receptors in the brain was identified by radiolabeled opioid ligands-binding studies (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). Since these first reports, extense pharmacological studies were carried out using a large number of opioid derivatives and provided evidence to divide opioid receptors into three different types, δ, μ, and κ (Gilbert and Martin, 1976; Lord et al., 1977; Martin et al., 1976). During the same time, various endogenous ligands of opioid receptors, enkephalin, β-endorphin and dynorphin, were discovered and isolated (Goldstein et al., 1981; Hughes et al., 1975; Li and Chung, 1976; Tachibana et al., 1982), which were followed by cloning of their precursor proteins, proopiomelanocortin (POMC), prodynophin (PDYN) and proenkephalin (PENK) (Kakidani et al., 1982; Nakanishi et al., 1979; Noda et al., 1982). In the early 1990s, cDNAs encoding three members of the receptor family were cloned, beginning with the mouse δ-opioid receptor (DOR) (Evans et al., 1992; Kieffer et al., 1992, 1994) and followed by cloning of μ-opioid receptor (MOR) (Chen et al., 1993a; Fukuda et al., 1993; Thompson et al., 1993; Wang et al., 1993) and κ-opioid receptor (KOR) (Chen et al., 1993b; Li et al., 1993; Meng et al., 1993; Minami et al., 1993; Nishi et al., 1993).

1.1 Opioid receptors

The cloned δ-, μ-, and κ-opioid receptors are highly homologous (Fig. 1). All three opioid receptors belong to the G protein-coupled receptor superfamily (Childers, 1991; Gilman, 1987), which interacts with heterotrimeric G proteins and spans the cell membrane seven times forming an extracellular amino terminal, three extracellular loops, three intracellular loops and an intracellular carboxyl terminal. Studies conducted on the cloned opioid receptors demonstrated that the amino acids of the three opioid receptors are 65% homologous, that means the other 35% confer type selectivity (Reisine and Bell, 1993). Higher identities are found in the transmembrane regions (73-76%) and the intracellular loops (86-100%). Conversely, the most divergent regions are the extracellular loops and the extracellular amino- and intracellular

carboxyl-terminals (30-40%). In addition to the well-established three types of opioid receptors, an orphan opioid-like receptor (ORL1) has been cloned (Chen et al., 1994). It is also a G-protein coupled receptor and shares 50-60% sequence homology with the other opioid receptors. However, ORL1 receptor has a low affinity to opioid agonists and the non-selective opioid receptor antagonist naloxone. Its pharmacological profile differs greatly from that of the classic opioid receptors. The activation of the ORL1 receptor is considered to mediate the physiological actions of orphanin FQ/nociceptin, such as nociceptive response, locomotion, food intake, cognitive processes and emotional behavior (Henderson and McKnight, 1997; Meunier, 1997).

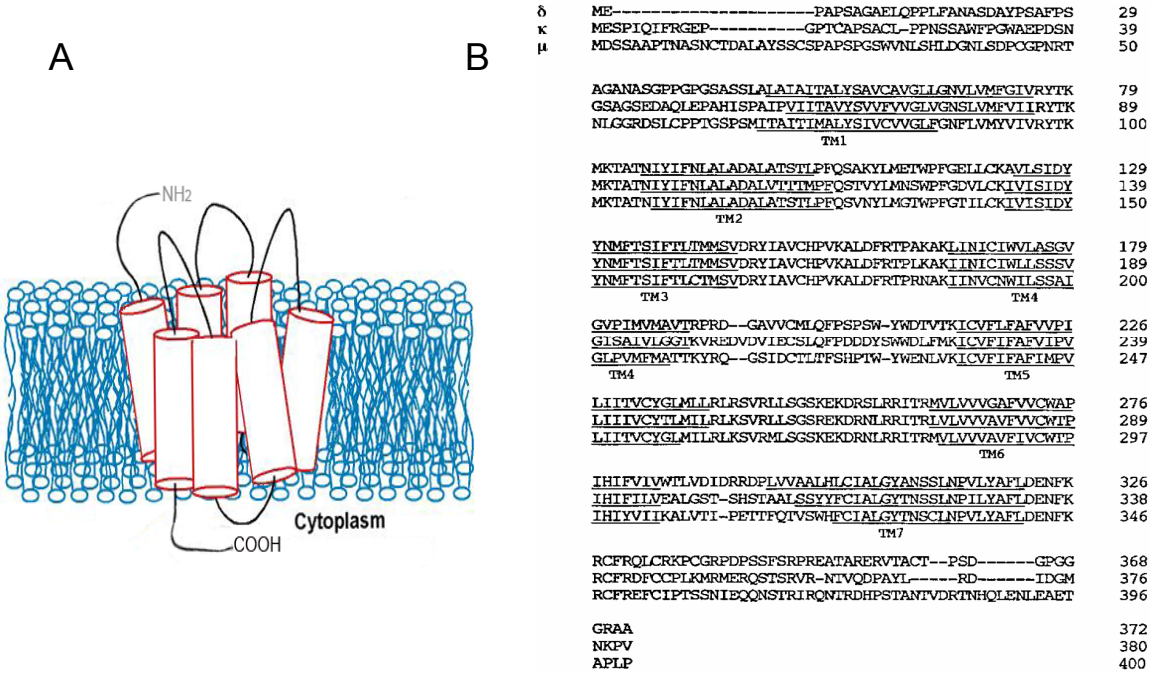


Fig. 1. Structure and amino acid sequence of opioid receptors. A, Structure of opioid receptors as G protein coupled receptors. B, Human δ-, κ-, and μ-opioid receptor amino acid comparison (Knapp et al., 1995).

The cloned μ-opioid receptor is more sensitive to morphine than other opioid receptors, and endomorphins may be its endogenous agonists. Enkephalins bind to the δ-opioid receptor with great affinity, and therefore are considered to be endogenous δ-opioid receptor agonists. Dynorphins bind to κ-opioid receptors and therefore function as endogenous κ-opioid receptors ligands. β-endorphin was found to have a similar affinity to bind with μ- and δ-opioid receptors. The ORL1 receptor is responsive to the novel peptide orphanin FQ or named nociceptin (Meunier et al., 1995; Reinscheid et al., 1995).

1.2 Signal Transduction

As mentioned above, the opioid receptors belong to G protein-coupled receptor family. G proteins are heterotrimeric proteins, consisting of α, β and γ subunits. The activation of G protein-coupled receptors by agonist results in the dissociation of GDP from the α subunit, followed by association of GTP with the open nucleotide binding site. The binding of GTP to the α subunit induces a conformational change that results in dissociation of the heterotrimer into α and βγ subunits. Both the GTP-bound α subunit and the combined βγ subunits can initiate distal steps in the signaling pathway. These signals are terminated when the endogenous GTPase of the α subunit hydrolyze the bound GTP to GDP. The α subunit/GDP complex then reassociates with the βγ subunits to form heterotrimeric G protein again. Opioid receptors are prototypical Gi/o-coupled receptors because opioid signals are efficiently blocked by pertussis toxin that ADP-ribosylates and inactivates the α subunits of Gi/o proteins (Connor and Christie, 1999).

1.2.1 Regulation of adenylyl cyclase activity

The inhibitory coupling of opioid receptors to the adenylate cyclase has been studied in transformed cell lines and in brain tissues. In early time the opioid receptors in NG108-15 cells had been identified as δ-type (Chang et al., 1981). In this cell line, the δ-opioid receptor agonist DADLE inhibited cAMP production. The inhibition was reversed by the nonselective opioid antagonist naloxone (Costa et al., 1985). Pertussis toxin blocked the inhibition of adenylyl cyclase by opioids in NG108-15 cells (Burns et al., 1983; Law et al., 1985), suggesting that the inhibitory effects on cAMP production is mediated through the activation of the Gi/o protein. δ-selective inhibition of cAMP production has also been verified in human δ-opioid receptor-transfected cell lines where forskolin-stimulated cAMP production was inhibited by the agonist DPDPE and this DPDPE-mediated inhibition was antagonized by naltrindol (Malatynska et al., 1995). In addition, δ-selective agonists have also been reported to inhibit basal cAMP levels in rat brain regions (Unterwald et al., 1993). The cloned μ-, and κ-opioid receptors expressed in COS or CHO cell lines also mediate the inhibition of adenylate cyclase (Chen et al., 1993a; Chen et al., 1993b; Childers, 1991). These inhibitory effects of opioid agonist on the adenylate cyclase are blocked by pertussis toxin, suggesting that the μ- and κ-opioid receptors are also coupled to Gi/o protein to exert their inhibitory effects. Studies with Gα-specific antibodies suggested that G_{i2} mediates the δ-opioid receptor inhibition of adenylyl cyclase (McKenzie and Milligan, 1990).

The Gz protein, a PTX-insensitive member of the Gi subfamily, can also potently inhibit cAMP accumulation upon receptor activation (Wong et al., 1992). Detailed examination of opioid-induced inhibition of adenylyl cyclase in NG108-15 cells, which are known to coexpress the δ-opioid receptor and Gz, revealed a small but significant inhibitory component that cannot be completely abolished by PTX (Selley et al., 1998).

On the other hand, chronic opioid treatment can also produces a paradoxical enhancement of adenylate cyclase activity, thus increasing cyclic AMP accumulation when the action of the inhibitory receptor is terminated. This phenomenon, by which chronic activation of Gi/o coupled receptors leads to an increase of cAMP level, is so called "cAMP overshoot" or "adenylyl cyclase superactivation". Opioid-induced adenylyl cyclase superactivation was shown to be mediated by the βγ subunits of G protein (Avidor-Reiss et al., 1996; Steiner et al., 2005), and protein kinases including tyrosine kinase and protein kinase C converging at Raf-1 protein kinase (Varga et al., 2002; Varga et al., 2003).

1.2.2 Regulation of ion channels

Calcium channels. All three opioid receptors have the ability to inhibit different types of calcium channels (Acosta and Lopez, 1999; Gross et al., 1990; Hamra et al., 1999), and thus influence the release of neurotransmitters and modulate the function of several protein kinase families.

Potassium channels. Another cellular event, which is thought to be important for the reduction of cellular excitability and inhibition neurotransmitter release by opioids, is the potassium conductance. The activation of opioid receptors have been shown to increase an inwardly potassium conductance (Alreja and Aghajanian, 1993; Grudt and Williams, 1993; Jiang and North, 1992; North et al., 1987).

1.2.3 Mitogen-activated protein kinases (MAPK)

There are at least three sets of mammalian MAP kinase modules: the extracellular-signal-regulated kinases (ERKs), the Jun N-terminal kinases (JNKs), and the p38 kinases. Mitogenic signals from GPCRs are often transmitted along the ERK pathway. Stimulation of the ERK1/2 by opioids was first demonstrated with the μ-opioid receptor in recombinant CHO cells (Li and Chang, 1996). The stimulation showed ligand selectivity, agonist dose-dependency, and PTX sensitivity. Similarly, when expressed in Rat-1 fibroblasts, the δ-opioid receptor can stimulate the phosphorylation and activation of ERK1/2 (Burt et al., 1996). The activation of

ERK1/2 was shown to occur through the Gβγ subunits in a Ras-dependent manner (Fukuda et al., 1996). Apart from linking opioid receptor activation to mitogenesis, stimulation of the MAP kinase cascade may be required for other aspects of opioid signaling. For example, desensitization of μ-opioid receptors may involve MAP kinase (Polakiewicz et al., 1998; Schmidt et al., 2000). Little is known with regard to the involvement of JNK or p38 kinase in opioid signaling. Recently p38 MAP kinase was demonstrated to be activated through the μ-opioid receptor by [D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin (DAMGO) but not by morphine, regulating μ-opioid receptor endocytosis (Mace et al., 2005).

1.3 Phosphorylation and Desensitization

Agonist-induced opioid receptor phosphorylation was first shown with the δ-opioid receptor (Pei et al., 1995). Studies with the δ-opioid receptor and μ-opioid receptor suggested that the agonist-induced phosphorylation is most likely mediated via G-protein coupled receptor kinases (GRKs) (Guo et al., 2000; Kovoor et al., 1997; Pei et al., 1995; Zhang et al., 1996). Expression of the dominant negative mutant of beta-adrenergic receptor kinase-1 (note: GRK2) or overexpression of GRK2 resulted in the attenuation or potentiation of agonist-dependent phosphorylation of the opioid receptors. It was also demonstrated that other protein kinases such as Ca^{2+}/calmodulin-dependent kinase II (Koch et al., 1997; Mestek et al., 1995) and mitogen activated protein (MAP) kinase (Polakiewicz et al., 1998; Schmidt et al., 2000) are involved in the phosphorylation of opioid receptors. ERK1/2 could probably mediate the agonist-dependent phosphorylation of the δ-opioid receptor at position Thr361 which is a putative MAP kinase phosphorylation site. It is apparent that the major phosphorylation sites are at the carboxyl tails of opioid receptors. Deletion of the last 31 amino acids of the δ-opioid receptor resulted in the abolition of both GRK- and PKC-mediated agonist-dependent phosphorylation of the receptor (Zhao et al., 1997). Truncation of the mouse δ-opioid receptor DOR344T also blocked the ability of DPDPE to induce phosphorylation of the receptor (Murray et al., 1998). The phosphorylation of δ-opioid receptor was shown to be hierarchical, with Ser363 acting as the critical primary phosphorylation site (Kouhen et al., 2000). Among those Ser/Thr residue phosphorylation sites at the carboxyl tails of μ-opioid receptor, in the absence of the agonist, a basal phosphorylation of Ser363 and Thr370 was observed, whereas DAMGO-induced receptor phosphorylation occurs at Thr370 and Ser375 residues (El

Kouhen et al., 2001).

Exposure of opioid receptors to opiates causes decreased receptor sensitivity to the drugs, in which the ability of receptors to modulate second messengers is reduced (Law et al., 1983; Nomura et al., 1994; Mestek et al., 1995). Desensitization of GPCR signaling in most mammalian cells involves agonist-mediated receptor phosphorylation, followed by recruitment of arrestins and the sequestration of the arrestin-bound receptors (Bohm et al., 1997; Bohn et al., 2004; Bohn et al., 1999; Yu et al., 1997). The mutation of putative phosphorylation sites to Alanine could block or significantly attenuate agonist-induced μ-opioid receptor desensitization (Deng et al., 2000; Schulz et al., 2004; Wang et al., 2002). Desensitization of the δ-opioid receptor was also shown to correlate with the phosphorylation of the receptor protein in the SKN-BE cells (Hasbi et al., 1998). DPDPE-induced receptor desensitization can be blocked with the dominant negative mutants of GRKs (Pei et al., 1995). Overexpression of GRK2 in HEK293 cells could accelerate the DPDPE-induced δ-opioid receptor desensitization (El Kouhen et al., 1999). Mutation of the last four Thr and Ser residues at the C-terminus of the δ-opioid receptor to Ala would block the GRK- and arrestin-mediated desensitization (Kovoor et al., 1997). It was demonstrated that DPDPE-induced rapid receptor desensitization, as measured by adenylyl cyclase activity, and receptor internalization are intimately related to the phosphorylation of Thr(358) and Ser(363) at the C-terminal of receptor, with Thr(358) being involved in the receptor internalization (Kouhen et al., 2000). However, only phosphorylation might not be sufficient for rapid δ-opioid receptor desensitization in all cases. Law et al reported that deltorphin II-induced desensitization of the δ-opioid receptor involves cellular events in addition to receptor phosphorylation. The rapid desensitization of the delta-opioid receptor requires both the phosphorylation and internalization of the receptor (Law et al., 2000).

1.4 Role of internalization/β-arrestin in signaling

Agonist-induced opioid receptor internalization was initially demonstrated in cultured neuroblastoma cells (Moses and Snell, 1983). The internalization of opioid receptors is thought to involve clathrin-coated vesicles (Chu et al., 1997; Gaudriault et al., 1997; Hasbi et al., 2000; Keith et al., 1996; Law et al., 1999). Agonist-activated opioid receptors are rapidly concentrated in clathrin-coated pits, which subsequently undergo dynamin-dependent fission from the plasma membrane and then fuse with early

endosomes (Chu et al., 1997; Keith et al., 1996). This process is regulated by a highly conserved mechanism, involving phosphorylation of the agonist-bound receptors by G protein-coupled receptor kinases and association of the receptors with β-arrestins. Upon receptor activation, β-arrestins translocate to the cell membrane and bind to the agonist-occupied receptors. These events terminate receptor signaling by preventing receptor interaction with heterotrimeric G proteins. Morphine, in agreement with its inability to phosphorylate the opioid receptors and recruit β-arrestin, is unable to promote opioid receptors internalization in transfected cells (Keith et al., 1996; Kramer and Simon, 2000). The carboxyl-terminus and the third intracellular loop regions of δ-opioid receptor exhibit high affinity to both β-arrestin1 and β-arrestin2 (Cen et al., 2001). It is also suggested that a Thr residue in the second intracellular loop region may serve as an additional β-arrestin binding site in the opioid receptor (Celver et al., 2001). Binding of β-arrestin leads to physical separation of the receptor from the G proteins and promote endocytosis by physically linking receptors to the clathrin-containing coated vesicles. β-arrestin can interact with the β-subunit of AP-2 through their C terminal domains (Mousavi et al., 2004). AP-2 is the clathrin adaptor protein that seems to be involved in nearly all stages of clathrin-coated vesicle formation. The interaction of β-arrestin with AP-2 is the essential targeting step recruiting the receptor to coated pits. Interactions of β-arrestin with both constitutively produced and signal-induced phosphoinositides also contribute to the incorporation of activated receptors into clathrin-coated pits. The formation of endocytic clathrin-coated pits and vesicles involves a complex series protein-protein and protein-lipid interactions. To form a free clathrin-coated vesicle from the plasma membrane, amphiphysin functions as a linker between dynamin and clathrin coats. Dynamin, acting as a GTPase, provide force to induce the scission of forming free vesicles, which are soon fused with early endosomes.

β-arrestins serve as important adaptors that link receptors to the clathrin-dependent internalization pathway. However, accumulating evidence indicates that beta-arrestins also function as scaffold proteins that interact with several cytoplasmic proteins and link GPCRs to intracellular signaling pathways. β-arrestin is reported to be involved in the activation of ERK1/2 kinase cascades by some GPCRs (Tohgo et al., 2003). Heterodimerization of μ-opioid receptor with δ-opioid receptor was described to lead to a constitutive recruitment of β-arrestin2 to the receptor complex resulting in changes in

the spatio-temporal regulation of ERK1/2 signaling, indicating that μ-opioid receptor-δ-opioid receptor heterodimers are in a conformation conducive to β-arrestin-mediated signaling. Destabilization of this conformation by cotreatment with MOR and DOR ligands leads to a switch to a non-β-arrestin-mediated signaling (Rozenfeld and Devi, 2007). Recent work has also revealed that, beta-arrestin appears to play important roles in cell growth, apoptosis and modulation of immune functions by mediating regulation of transcription. In response to activation of certain GPCRs, beta-arrestins translocate from the cytoplasm to the nucleus and associate with transcription cofactors such as p300 and cAMP-response element-binding protein (CREB) at the promoters of target genes to promote transcription. They also interact with regulators of transcription factors, such as IκBα and MDM2, in the cytoplasm and regulate transcription indirectly (see Ma and Pei, 2007 for review). Applying beta-arrestin2 knockout mice, Bradaia et al. found that beta-Arrestin2, interacting with phosphodiesterase 4, regulates synaptic release probability and presynaptic inhibition by opioids (Bradaia et al., 2005). Beta-arrestins were also shown to bind and direct the activity of several nonreceptor tyrosine kinases in response to seven-transmembrane receptor stimulation (Shenoy and Lefkowitz, 2005). Thus as indicated by these novel functions of the internalization adaptor arrestin, receptor internalization which originally characterized as negative regulation process of G-protein-coupled receptor (GPCR) signaling, on the other hand, is also a signal transduction process.

Etorphine or DAMGO promotes rapid internalization of μ-opioid receptor, whereas morphine fails to promote significant receptor internalization. Consistent with these findings, μ-opioid receptor is phosphorylated at a low level in the absence of agonist, and receptor phosphorylation is significantly enhanced in the presence of etorphine or DAMGO (Arden et al., 1995; Whistler et al., 1999), whereas morphine was observed to promote phosphorylation of μ-opioid receptor to a lesser extent than opioid peptides and certain other alkaloid agonists (Arden et al., 1995; Yu et al., 1997; Zhang et al., 1998). The mutation of agonist-induced phosphorylation site serine375 to alanine in the carboxyl terminal significantly attenuates μ-opioid receptor internalization (El Kouhen et al., 2001; Schulz et al., 2004). The carboxyl terminal of the δ-opioid receptor was shown to have a critical role in receptor internalization. Examination of a series of chimeric mutant κ/δ receptors, revealed that at least two receptor domains, including the highly divergent carboxyl-terminal cytoplasmic tail, determine the type of the

endocytic mechanism of δ-opioid receptor (Chu et al., 1997). The transfected CHO cells expressing mouse δ-opioid receptor lacking the C-terminal 15 or 37 amino acids exhibit a substantially slower rate of internalization. Furthermore, the cells expressing receptors with point mutations of any of Ser/Thr between Ser344 and Ser363 in the C-terminal tail exhibit a significant reduction in the rate of receptor internalization (Trapaidze et al., 1996). However, there are also confusing data whether receptor phosphorylation is absolutely required in agonist-induced δ-opioid receptor endocytosis, especially in carboxy-terminal truncated δ-opioid receptor studies. Murray et al. reported that the DOR344T receptor, a functional and truncated mutant δ-opioid receptor which is missing phosphorylation sites located in the carboxyl-terminal cytoplasmic domain, when expressed in CHO cells could not undergo endocytosis. However, the same DOR344T truncated receptor expressed in HEK293 cells exhibited rapid ligand-induced internalization with the similarly rapid kinetics as full-length δ-opioid receptor in the absence of phosphorylation (Murray et al., 1998). Whistler et al. further addressed this question by examining the endocytic trafficking of a series of mutant version of the δ-opioid receptor in stably transfected HEK293 cells (Whistler et al., 2001). They confirmed that for agonist-induced endocytosis of truncated mutant δ-opioid receptors that lack the distal carboxyl-terminal cytoplasmic domain containing sites of regulatory phosphorylation, receptor phosphorylation is not required. However, phosphorylation is required for endocytosis of the full-length receptors. Mutation of all serine/threonine residues located in the distal carboxyl-terminal tail domain of full-length receptor to alanine created functional mutant receptors that exhibit no detectable agonist-induced endocytosis. So they put forward the hypothesis that the distal portion of the carboxyl-terminal cytoplasmic domain functions as an endocytic 'brake' mechanism that is released by phosphorylation.

Recently the enhanced green fluorescent protein (EGFP) was knocked into δ-opioid receptor gene and mice expressing a functional DOR –EGFP C-terminal fusion in place of native DOR were produced. In DOR-EGFP animals, drug treatment triggered receptor endocytosis that correlated with the behavioral response. Mice with internalized receptors were insensitive to subsequent agonist administration, providing evidence that δ-opioid receptors receptor sequestration limits drug efficacy *in vivo* (Scherrer et al., 2006).

1.5 Down-regulation

Down-regulation of opioid receptors is characterized by a decrease in the total number of receptors in a cell. Opioid receptor down-regulation may result from a proteolytic degradation of internalized receptors and/or a decrease of newly synthesized receptors. The receptor degradation caused by agonist exposure was intensively studied. The down-regulation of many GPCRs involves the clathrin-coated vesicle pathway (Krupnick and Benovic, 1998). The fate of the opioid receptor types after agonist-mediated internalization is quite different. After agonist-induced endocytosis, the internalized δ-opioid receptor are preferentially targeted to protein degradation pathways and undergoes quick and significant down-regulation in recombinant cell lines (Malatynska et al., 1996), neuroblastoma cells (Afify et al., 1998) and intact brain tissues (Tao et al., 1988), while the μ-opioid receptor is preferentially recycled to the cell membrane (Afify et al., 1998; Koch et al., 1998). Recycling of internalized opioid receptors to the plasma membrane is associated with dephosphorylation of receptors, counteracting receptor down-regulation (Schulz et al., 2004; Tsao and von Zastrow, 2000). The role of the carboxyl tail in directing opioid receptor trafficking has been established. C-terminal domain of δ-opioid receptor has a fundamental role in receptor down-regulation. Exchange of C-terminal tail between the μ and δ-opioid receptors led to reciprocal changes in the kinetics of receptor down-regulation (Afify et al., 1998). Truncation of last 37 amino acids from the C-terminus of the mouse δ-opioid receptor prevented DADLE-mediated down-regulation in transfected CHO cells, and the cytoplasmic tail residue Thr353 in the receptor was identified as the crucial residue, since when it was mutated to an Ala, the down-regulation of the mutant receptor was blocked (Cvejic et al., 1996). Transfer of a putative recycling motif of the μ-opioid receptor (Tanowitz and von Zastrow, 2003) or β2-adrenergic receptor (Gage et al., 2001) was shown to redirect the chimeric δ-opioid receptor to the recycling pathway. Down-regulation of δ-opioid receptor was demonstrated to involve predominantly lysosomal degradation in neuroblastoma cells (Ko et al., 1999; Shapira et al., 2001). Whistler et al (Whistler et al., 2002) have identified a protein that binds preferentially to the cytoplasmic tail of δ-opioid receptor and which is G protein-coupled receptor-associated sorting protein (GASP) that targets the mouse δ-opioid to the lysosomal pathway in HEK293 cells. Disruption of the interaction between the δ-opioid receptor and the GASP by receptor mutation or overexpression of a dominant negative

fragment of GASP inhibited receptor trafficking to lysosomes and promoted receptor recycling.

1.6 Phospholipase D

The phospholipase D (PLD) is a ubiquitous enzyme present in organism ranging from viruses to bacteria, yeast, plants and animals. PLD hydrolyze phosphatidylcholine (PC) to yield phosphatidic acid (PA) and free choline. In mammalian cells, there are two major subtypes of PLDs (Fig. 2) (Exton, 1999; Frohman et al., 1999; Liscovitch et al., 2000). In line with a role for PLD enzymes in different cellular tasks, PLD1 and PLD2 show a diverse subcellular distribution. PLD1 is found throughout the cell, but primarily localizes to intracellular compartments, including the Golgi apparatus, endoplasmic reticulum, late endosomes, and the perinuclear region (Colley et al., 1997; Freyberg et al., 2001; Lucocq et al., 2001), whereas PLD2 is most often reported to distribute to the plasma membrane (Colley et al., 1997; Du et al., 2004; Liscovitch et al., 1999). Mammalian PLD activity can be regulated by many factors including phosphoinositides, protein kinase C, ADP-ribosylation factor and Rho GTPase (Frohman et al., 1999). PLD activity also has been demonstrated to respond to a number of GPCRs, including metabotropic glutamate receptors (Bhattacharya et al., 2004; Kanumilli et al., 2002; Shinomura et al., 2000), m1-m4 muscarinic receptors (Mitchell et al., 2003; Sandmann et al., 1991), the endothelin receptor (Ambar and Sokolovsky, 1993), the a2-adrenergic receptor (MacNulty et al., 1992), and D2 dopamine receptor (Senogles, 2000).

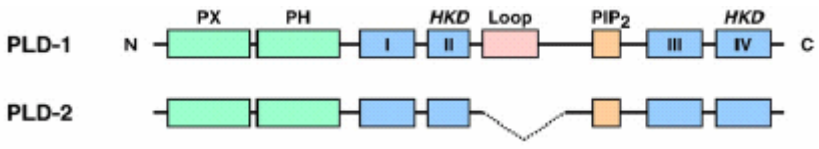


Fig. 2. Domain structure of PLD isoforms. The PLD isoforms PLD1 and PLD2 contain N-terminal PX and PH domains and the highly conserved domains I–IV. The domains II and IV contain HKD sequence motifs that are necessary for catalytic activity. N-terminal to domain III is a well conserved basic sequence that binds PIP2. PLD1 is distinguished by a loop region that seem to contribute to the regulation of PLD1 activity.

The PLD-produced PA can be converted into DAG by the family of enzymes known as phosphohydrolases (PAPs or PPHs), which are highly active in vivo (Brindley and Waggoner, 1996; Sciorra and Morris, 1999). The isoform PAP2b co-localizes to PLD2-enriched membrane domains, thus possibly contributing to a rapid turnover of

PLD-produced PA to DAG (Sciorra and Morris, 1999). PA can also be deacylated by phospholipase A (PLA) to form lysoPA (LPA). Activation of PLD results in the increase of both PA and DAG. The produced DAG can be reconverted to PA via phosphorylation by diacylglycerol kinases (DGKs) (Topham and Prescott, 1999). The regulation of PA and DAG appears to be tightly controlled via the activities of PPH and DGK. The comprehensive studies on the lipids hydrolyzed and produced by PLD clearly defined the fatty acid composition of these lipids (Pettitt et al., 1997; Pettitt et al., 2001). The acyl chains which were derived from PLD2 activation were palmitate (16:0), stearate (18:0) and oleate (18:1). The DAG produced by PAP dephosphorylation of PA, whose diacyl composition is primarily of the monounsaturated and/or saturated species, is different from polyunsaturated DAG produced via phospholipase C cleavage of PI4,5P_2 (Pettitt et al., 1997). The *in vivo* specificity of PLD for PC was limited to mainly monounsaturated or saturated acyl chains as determined by analysis of the phosphatidylbutanol formed, of which the diacyl profile was comparable to that of endogenous PC (Pettitt et al., 2001), indicating that PLD seems to hydrolyze PC with either monounsaturated and/or saturated acyl chains forming PA of the same diacyl composition. As a long studied lipid second messenger, DAG has been shown to activate lipid-dependent kinases such as the classical and most novel PKC families (Newton, 1997). However, the more saturated forms of DAG produced from PA have been suggested not to activate PKC in vivo (Hodgkin et al., 1998; Pettitt et al., 1997), though a report showed that a metabotropic glutamate receptor associated to phospholipase D might influence translocation of PKC subtype ε in a calcium-independent manner (Pastorino et al., 2000). Therefore the possible role of these DAG derived from PA remains to be determined. The signaling function of PA and DAG is strongly dependent on their fatty acid contents. Also PA was described to be only active when it is formed from PC or contains predominantly saturated fatty acids (Hodgkin et al., 1998; Jones et al., 2000).

PLD can play multiple roles in cellular function. PA generated via PLD has been linked to a variety of events of intracellular signal processes through quenching of PA synthesis by 1-butanol, which has been proven useful and is widely used to investigate the involvement of PLD enzymes in cell physiology. One of the important effects of PA (Jenkins et al., 1994; Moritz et al., 1992), mainly the saturated/monounsaturated PA *in vivo* (Jones et al., 2000), is the stimulation of type I PI4P 5-kinase, to produce

PI4,5P2. PA can also bind to Raf-1 kinase which causes its translocation to plasma membranes and promote the activation of ERK1/2 mitogen activated protein kinase (Ghosh et al., 2003; Rizzo et al., 1999; Rizzo et al., 2000). In addition, PLD has also been implicated in survival pathways such as anti-apoptosis and cell proliferation (Klein, 2005). In *Dictyostelium* it was reported to be essential for actin localization and actin-based motility (Zouwail et al., 2005). Recently, PLD and its product PA have been found to be involved in vesicular trafficking, secretion and receptor endocytosis. It was demonstrated to regulate insulin release in pancreatic beta-cells (Hughes et al., 2004), macrophage phagocytosis (Iyer et al., 2004; Kusner et al., 1999), and B-Cell antigen receptor trafficking (Snyder and Pierce, 2006). PLD2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis (Du et al., 2004), epidermal growth factor endocytosis (Shen et al., 2001). Our group also found phospholipase D2 modulates agonist-induced μ-opioid receptor endocytosis (Koch et al., 2003).

1.7 Aims of the present research

As demonstrated above, opioid receptors internalization is an important regulatory event of opioid signaling. Previously our group has found that a μ-opioid receptor interacting protein, PLD2, is critical for agonist-induced μ-opioid receptor endocytosis (Koch et al., 2003). Thus we raised and addressed the following questions:

1. Does PLD2 associate with δ-opioid receptor, and what is the role of PLD2 activity in δ-opioid receptor endocytosis?

To answer these questions, we investigated the relation between PLD2 enzyme activity and δ-opioid receptor agonist stimulation. The physiological interaction of PLD2 with δ-opioid receptor was also studied. The function of PLD2 on δ-opioid receptor endocytosis was investigated by analyzing the effects of coexpressing PLD2, and the inhibition of PLD2 activity by coexpressing a catalytically inactive PLD2 mutant and applying the specific PLD inhibitor.

2. If PLD2 could regulate δ-opioid receptor endocytosis, what would be the mechanism of PLD2 for the regulation of both δ- and μ-opioid receptor endocytosis?

To uncover the mechanism of PLD2 which regulates opioid receptors endocytosis, the further metabolism of PA, the product of PLD2 phosphodiesterase activity, was specially focused on. Once the downstream molecule PA-derived DAG, which might mediate the function of PLD2 in opioid receptors endocytosis, was identified, its

further involvement in the clathrin-dependent endocytosis pathway was investigated. Then the further functional relation between PLD2 and p38 kinase, a potential regulator of clathrin-dependent endocytosis pathway, was established by analyzing the involvement of PLD2-PA-DAG pathway in the opioid receptors-mediated p38 kinase activation.

2 Materials

2.1 Instruments

UV-visible Spectrophotometer (Pharmacia Biotech, Germany)

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

Gene Pulser II and Pulse Controller Plus (Bio-Rad, USA) for Electroporation

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

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

TRI-CARB 1900 TR Liquid Scintillation Analyzer (Packard, USA)

Expert Plus Microplate Reader (ASYS, Austria)

Electrophoresis power supply (Bio-Rad)

Gel electrophoresis system (Bio-Rad)

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

2.2 Kits

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

MinElute Reaction Cleanup Kit (QIAGEN, Germany)

Cyclic AMP (^{3}H) assay system (Amersham Biosciences, Braunschweig, Germany)

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

2.3 Chemicals and reagents

DPDPE (Bachem, Heidelberg, Germany)

DAMGO (Bachem, Heidelberg, Germany)

Morphine (Synopharm, Barsbüttel, Germany)

Naloxone (Tocris)

[1,2,3-^{3}H]glycerol (American Radiolabeled Chemicals, St. Louis, MO)

[^{3}H]Naltrindol (NEN, Köln, Germany)

1-butanol (Merck, Darmstadt, Germany)

Propranolol (Calbiochem)

R59949 (Calbiochem)

Chelerythrine chloride (Sigma, ST. Louis, USA)

Calphostin C (Calbiochem)

RHC80627 (Calbiochem)

DOG (1-palmitoyl 2-oleoyl-sn-glycerol) (Calbiochem)

Sucrose (MP Biomedicals, France)

Filipin (Sigma, ST. Louis, USA)

SB203580 (Calbiochem)

Forskolin (Biotrend, Köln, Germany)

LipofectamineTM 2000 (Life technologies, Invitrogen)

Enhanced chemiluminescence detection system (Amersham Biosciences)

DPX mountant for histology (resinous mounting media) (Fluka, NeuUlm, Germany)

Protein A-agarose beads (Amersham Biosciences, Sweden)

ABTS solution (Roche Molecular Biochemicals)

Ammonium persulfate (Sigma)

30% acrylamide mix (Carl Roth GmbH & Co)

HEPES, TEMED (Serva)

2.4 Bacterium and eukaryotic cell line

E. coli XL1 (Promega)

Human embryonic kidney HEK 293 cell (ATCC CRL 1573)

2.5 Enzymes

All endonucleases from New England Biolab

Taq DNA polymerase, T4 ligase from Promega

RNase A from Sigma

2.6 plasmids

pcDNA3.1-DOR from Dr. Manuela Pfeiffer (IPT, Magdeburg Univ., Germany)

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

pEAK10 expression vector (Edge Bio Systems, Gaithersburg, MD)

pGEM-T easy vector (Promega, Madison, USA)

pcDNA3.1 (Invitrogen)

2.7 Mediums and Antibiotics

DMEM with 4.5g/L Glucose and L-Glutamine (Cambrex, Belgium)

Ultra MEM (Modified Eagle's Minimum Essential Medium) (Cambrex, Belgium)

LB media and LB-Agar media (Gibco)

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

Poly-L-lysine (PAA Laboratories GmbH, Pasching, Germany)

Hygromycin B (PAA Laboratories GmbH, Pasching, Germany)

Puromycin, Ampicillin, Penicillin and Streptomycin (Sigma)

Zeocin (Life technologies, Invitrogen)

G418 (Gibco)

2.8 Antibodies

Mouse anti-Myc monoclonal antibody (Clontech)

Rabbit anti-HA serum (IPT, Magdeburg Univ., Germany)

Mouse anti-HA antibody (Sigma)

All fluorescent conjugated second antibodies (Jackson ImmunoResearch, PA)

HRP -conjugated anti-rabbit antibody (Amersham Biosciences)

Phospho-p38 MAPK (Thr180/Tyr182) Rabbit mAb (Cell signaling)

Phospho-Akt (Ser473) Rabbit mAb (Cell signaling)

Mouse anti-β-actin antibody (Sigma, ST. Louis, USA)

Peroxidase-conjugated second antibodies (Jackson ImmunoResearch, PA)

2.9 Buffers and solvents

Zamboni's fixative:

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

Radioimmune precipitation buffer (RIPA buffer):

50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM disodium pyrophosphate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and the following proteinase inhibitors: 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 10 μg/ml bacitracin. (Proteinase inhibitors were added just prior to use)

SDS-sample buffer:

62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 0.005% bromphenol blue, 10% 2-mercaptoethanol. (2-mercaptoethanol was added prior to use)

Blotting buffer: 50 mM Tris-HCl, 40 mM glycine, 0.0375% SDS, 20% methanol

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

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

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

3 Methods

3.1 Gene subclone

In the following experiments, the related PCR, restriction endonuclease cleavage, ligation, transformation by calcium chloride or electroporation, plasmid preparation, purification and identification were carried out using standard protocols according to *Molecular Clone* or manufacturer's instruction.

Epitope tagging and cloning of cDNA

A HA epitope tag sequence MYPYDVPDYA was added to the NH_2 terminus of the mouse δ-opioid receptor by polymerase chain reaction. Then this tagged δ-opioid receptors gene was subcloned to pEAK10 expression vector. PLD2 expressing vector pcDNA3.1-PLD2 and PLD1b expressing vector pcDNA3.1-PLD1b were acquired from Dr. S. Ryu (Pohang, South Korea). Using Hind III, BamH I sites, a myc tag was further introduced at the N-terminus of the PLD2 in the same vector by PCR method. The catalytically inactive (K758R) PLD2 mutant (nPLD2) was constructed by PCR-mutagenesis and subcloned into the pcDNA3 expression vector.

Plasmid sequencing

To confirm the plasmid constructions, all subclones were sequenced using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit according to the manufacturer's protocol.

3.2 Cell culture and generation of stable cell lines

Generation of stable cell lines expressing δ-opioid receptor and/or PLD2, PLD1b or catalytically inactive PLD2 mutant (nPLD2)

HEK293 cells were maintained in DMEM supplemented with 10% FCS in a humidified incubator with an atmosphere containing 10% CO2, at 37°C. To generate δ-opioid receptor expressing cell line, HEK293 cells were transfected with pEAK10-HADOR plasmid by the calcium phosphate precipitation method and stable transfectants were selected with 1.25 μg/ml puromycin. Then HADOR stably expressing cells were subjected to a second round of transfection with pcDNA3.1-PLD2, pcDNA3.1-PLD1b or pcDNA3-nPLD2 plasmids using liptofectamime 2000 according to manufacturer's protocol to generate the HADOR and PLD2, PLD1 or mutant PLD2 (K758R) coexpressing cell lines. Stable transfectants was selected with 1.25 μg/ml puromycin and 500 μg/ml G418 in DMEM medium containing 10% fetal calf serum. Receptor and/or PLD2 expression of the stable

transfectants were monitored and selected with confocal microscopy, quantitative ELISA and radioligand binding assay. Stably transfected multi-clones in similar receptor density were used for the further studies.

Calcium-phosphate-mediated transfection of HEK293 cells

24 h before transfection, exponentially growing cells were harvest by trypsinization and replated at a density of about 6 x 105 cells in 5 ml complete growth medium (DMEM with 10% FCS) in 6 cm dishes. Incubate the cultures for 20-24 h at 37°C in a humidified incubator with an atmosphere containing of 10% CO_2. Change medium 2~4 h before transfection. For one transfection in 6 cm-dish, the calcium phosphate-DNA coprecipitate was prepared as follows: 8 µg of purified plasmid DNA, 25 µl of 2.5 M $CaCl_2$ and suitable amount of H_2O were added to a final volume of 250 µl. Then an equal volume of 2 x HEPES-buffered saline (280 mM NaCl, 50 mM HEPES, 1.5 mM Na2HPO4, pH 7.05) was mixed to 1 volume of this 2 x calcium-DNA solution. The solution was quickly vortexed and allowed to stay for 20 min at RT. Hereafter this calcium phosphate-DNA suspension was dropped immediately into the medium over the cell monolayer. After shaking the plate gently to mix the medium, cells were incubated for 16-18 h at 35°C in a humidified incubator with an atmosphere containing of 3% CO2. Then medium was change to complete growth medium and cells were further incubated for 1-2 days at 37°C in a normal condition (10% CO2, 37°C) before experiments or selection.

3.3 Coimmunoprecipitation and Immunoblot

Cells were seed into 10 cm dishes and grown to 80% confluence. Then cells were lysed on ice with 1 ml of ice-cold RIPA buffer (50 mM Tris-HCl, 150mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% Na-Deoxychlorat, 0.1% SDS and proteinase inhibitors) for 30 min, then were transfered to 2-ml tube and further lysed with shaking for 1-2 h at 4°C.. 100 µl of protein A agarose beads for each sample were washed with RIPA buffer and preloaded with 10 µg rabbit anti-HA antibodies for 4-6 h at 4°C. Then they were washed four times before use. After the cell lysate was centrifuged at 14,000 rpm for 1 h, the supernatant was collected and subjected to immunoprecipitation with 100 µl protein A-agarose beads preloaded with 10 µg rabbit anti-HA antibodies or was treated with wheat germ lectin beads to purify the glycoproteins at 4°C overnight. Beads were washed five times with RIPA buffer. Then the proteins on the beads were eluted to 100

μl of SDS-sample buffer at 60°C for 20 min and subjected to 10% SDS-polyacrylamide gel electrophoresis.

Immunoblotting was carried out according to standard procedures. After being separated by SDS-PAGE, proteins were transferred from the gel to nitrocellulose membrane by electroblotting, the membranes were blocked with 5% non-fat milk and incubated with 1 μg/ml mouse monoclonal anti-myc antibody or affinity-purified anti-HA antibody at 4°C overnight, followed by detection using enhanced chemiluminescence detection system.

3.4 Immunocytochemistry

Cells expressing DOR or coexpressing PLD2 and DOR were grown on poly-L-lysin coated coverslips overnight. After being washed with Ultra MEM once, cells were treated or not treated as indicated in text in serum-free Ultra MEM. The cells were then fixed with 4% paraformaldehyde and 0.2% picric acid in PB for 40 minutes at room temperature and washed three times with TPBS. Subsequently the cells were permeablized with 50% methanol and 100% methanol for each 3 minutes, and blocked with 1% and 3% normal goat serum at room temperature for 3 minutes and 1h respectively. Subsequently cells were incubated with rabbit anti-HA antibody and/or mouse anti-Myc antibody at a concentration of 0.5 mg/ml in TPBS containing 1% normal goat serum overnight. Bound primary antibodies were detected with corresponding cyanine 2.18 (Cy2)- and/or 3.18 (Cy3)-conjugated second antibodies (1:400 or 3.75 μg/ml). Cells were then dehydrated with ethanol from concentration 70% to 100%, cleared in xylol and permanently mounted in DPX. Cells were examined using a Leica TCS-NT laser scanning confocal microscope equipped with a krypton/argon laser. Cyanine 2.18 was imaged with 488 nm excitation and 530 nm emission filters, and cyanine 3.18 was imaged with 568 nm excitation and 570-630 nm band pass emission filters. To observe the trafficking of surface DOR and/or PLD2, cells were firstly surface-labeled with rabbit anti-HA antibody at a final concentration of 1:1000 at 4°C for 1.5 h. Cells were subsequently treated as shown in text, and then fixed normally. The bound primary antibodies were detected as described above.

3.5 Radioligand binding assay

The binding characteristics of the δ-opioid receptor were determined by saturation binding assays on membranes prepared from stably transfected HEK293 cells expressing HA-DOR with and without PLD2. Dissociation constant (K_D) and number

of [3H]Naltrindol binding sites (B_{max}) were calculated by Scatchard analysis using at least seven concentrations of radioligand in a range from 0.025 to 25 nM. Nonspecific binding was determined as radioactivity bound in the presence of 1 μM unlabeled Naltrindol. Radioligand binding assay is sensitive to calculate the receptor amount. By using the ratio of specific bound radioligand per mg protein, the number of [3H]Naltrindol binding sites in membrane binding assay reflects the amount of total δ-opioid receptor.

3.6 PLD Activity Assay

PLD activity was determined using a transphosphatidylation assay. HEK293 cells coexpressing DOR and PLD2 were seeded at a density of $1–1.5\times10^6$ per 6 cm-dish and grown for 24 h. After washing once the cells were kept in serum-free OPTI MEM containing [1,2,3-^{3}H]glycerol (1 μCi/ml; specific activity 40 Ci/mmol) for 24 h in order to label phospholipids. Cells were then exposed to serum-free medium containing drugs as indicated and 2% ethanol. The following drugs were added as aqueous solutions resulting in final concentrations of 1 μM DPDPE, 1 μM morphine, 5 μM chelerythrine chloride, or 10 μM naloxone. After 30 min of drug treatment, cells were washed with cold PBS and extracted in 2.5 ml of ice-cold methanol/water (3:2, v/v). Subsequently, 1.5 ml chloroform and 0.35 ml H2O were added, and the lipid phase was separated by centrifuge. The lower phase of methanol/chloroform/water (10:10:9, v/v/v) was collected, evaporated by vacuum and redissolved in methanol/chloroform. Individual phospholipids were separated by thin layer chromatography and stained with iodine, identified by standards, and spots corresponding to phosphatidylethanol (PtdEtOH), PA, and PC were isolated and subjected to liquid scintillation counting. PLD activity was expressed as percent [3H]PtdEtOH of the total cellular PC concentration. Then the relative PLD activity to control of each experiment was calculated.

3.7 Quantitative Analysis of Receptor Internalization

To quantitatively measure receptor internalization, cells were seeded at a density of 2.5×105 per well and grown overnight in poly-L-lysine-treated 24-well plates. Cells were washed and treated differently as indicated in the text in Ultra MEM at 37°C. After washing with ultra MEM, HA-tagged cell surface receptors were labeled by incubation with rabbit anti-HA serum (1:2000) in Ultra MEM at 4°C for 1 h 40 min. Subsequently, cells were fixed with Zamboni's fixative buffer for 40min and then incubated with peroxidase-conjugated anti-rabbit antibody (1:1000, Amersham

Biosciences) for 2 h at room temperature. After three times wash with PBS for each 5 minutes, colour was developed with 250 µl of ABTS solution per well. After 10–20 min of rotation, 200 µl of the substrate solution from each well were transferred to a 96-well plate and measured at 405/492 nm with a microtiter plate reader. Compared to the cells which were same treated in agonist-free medium, agonist-induced receptor internalization was quantified as the percentage loss of surface receptors.

3.8 Preparation of cell lysates and Western blotting analysis for MAP kinase

The activation of p38 MAP kinase was determined by Western blotting with Phospho-p38 MAPK (Thr180/Tyr182) antibody which is specific for phosphorylated, activated form of p38 MAPK. Cells were seeded in 12-well plate at a density of 2.0×105 per well. After 24 hours, cells were further serum-starved for 16 h with DMEM containing 1% FCS. After stimulation with 1 µM DPDPE or 10 µM DAMGO in serum-free medium at 37°C for indicated time, cells were washed with ice-cold PBS once and lysed on ice with 100 µl of fresh-prepared ice-cold RIPA buffer containing proteinase inhibitors and phosphatase inhibitors for 1 hour. The cell lysate was centrifuged at 14,000 rpm for 20 min and the total protein in supernatant was quantified. Supernatants containing same amount of total proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis. After electroblotting, the nitrocellulose membrane were blocked with 3% non-fat milk for 30 min, and incubated with 1 µg/ml phospho-p38 MAPK (Thr180/Tyr182) rabbit antibody overnight at 4°C. Binding of primary antibody was detected with secondary anti-rabbit antibody for 1 h at room temperature, followed by detection using enhanced chemiluminescence (ECL) detection system. To detect β-actin, the same membrane was treated with 1% sodium azide in TBS for 45 min to inactive the peroxidase signal. After that the membrane was incubated with β-actin mouse antibody and further detected with secondary anti-mouse antibody and ECL system.

3.9 cAMP measurement

1.0 x 105 cells per well were seeded in poly-L-lysine-coated 24-well plate overnight. For the measurement of cAMP accumulation, cells were first washed one time with 0.5 ml serum-free Ultra MEM. Immediately, medium was removed and replaced by 0.25 ml serum-free DEEM medium containing 25 µM forskolin with or without agonist. The cells were incubated at 37°C for 15 min. After one time wash with cold PBS, the intracellular cAMP was extracted immediately with 0.5 ml of cold HCl/ethanol (1

volume of 1 N HCl/100 volumes of ethanol, stored at -20°C). The supernatant was transferred into a 1.5 ml tube, and then evaporated by vacuum at 30°C. The residue of cAMP was frozen in -20°C or for further examination. The extracted cAMP content was dissolved in TE buffer and determined using a commercially available cyclic AMP [^{3}H] assay system.

3.10 Data analysis

Statistic significant difference was analyzed by Student's t-test or ANOVA followed by bonferroni test using GraphPad Prism 4.0 software. All graphs in the following were drawed by the same software.

4. Results

4.1 δ-opioid receptor activates PLD2

Since our group has previously demonstrated that μ-opioid receptor can mediate phospholipase D2 activation (Koch et al., 2003), we speculated that this may be the case also for the δ-opioid receptor. Therefore we stably expressed PLD2 and HA-tagged δ-opioid receptor in HEK293 cells. δ-opioid receptor and PLD2 expression were monitored by ligand binding experiments, Western blot, and immunocytochemical analyses. Saturation binding experiments revealed no substantial differences between DOR and DOR-PLD2 expressing cells with respect to their affinities (K_D) to selective δ antagonist [^{3}H]naltrindol (0.142 ± 0.048 nM and 0.173 ± 0.049 nM for DOR and DOR-PLD2 cells, respectively) and their numbers of binding sites (B_{max}) (1258 ± 361 fmol/mg protein and 1247 ± 352 fmol/mg protein for DOR and DOR-PLD2, respectively). Then using a transphosphatidylation assay, we examined whether PLD2 can be activated by agonist exposure of δ-opioid receptor in PLD2-DOR stably expressing cells (Fig. 3A). After a treatment for 30 min with the δ-opioid receptors selective agonist [D-Pen^2, D-Pen^5]enkephalin (DPDPE), PLD2 activity was increased about to 1.4 times. The incubation with the opioid antagonist naloxone could block this DPDPE-induced activation of PLD2, indicating that this PLD2 activation was mediated by δ-opioid receptor. Since the alkaloid agonist morphine has been reported to activate both δ- and μ-opioid receptors but not to cause their rapid internalization (Keith et al., 1996), we further tested its effect on the receptor-mediated PLD activation. The exposure to morphine failed to induce an increase of PLD2 activity. Furthermore, inhibition of protein kinase C (PKC) by the PKC inhibitor chelerythrine chloride did not attenuate the DPDPE-induced PLD2 activation. This finding suggests that the δ-opioid receptor-induced PLD2 activation is not caused by PKC activation. In contrast, in PLD1b-HADOR coexpressing cells, DPDPE stimulation did not increase PLD1b activity (Fig. 3B) showing that δ-opioid receptor specifically activates PLD2.

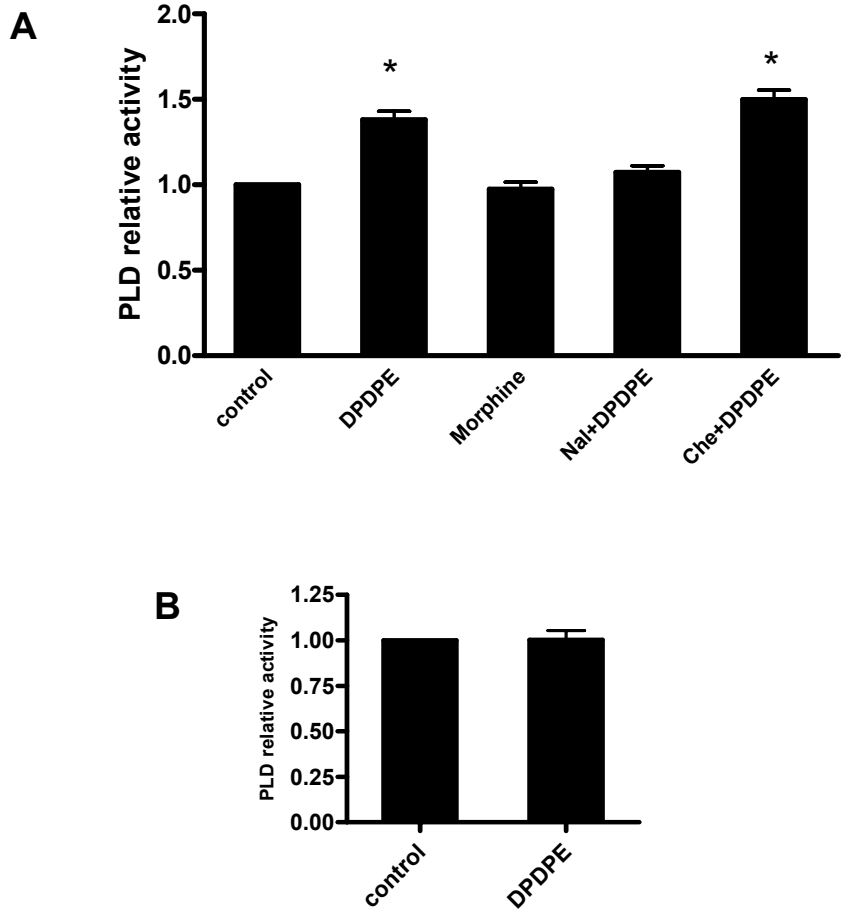


Fig. 3. δ-opioid receptor mediates PLD2 activation but not PLD1b activation. A, HEK293 cells coexpressing PLD2 and DOR were treated with 1μM DPDPE with or without 10μM naloxone (Nal) or 5μM chelerythrine chloride (Che), or treated with 1μM morphine alone for 30min, or not treated (control). B, HEK293 cells coexpressing PLD1b and DOR were treated with 1μM DPDPE for 30min or not treated (control). PLD relative activity was calculated as the ratio of PLD activity of each treatment to controls. Asterisk represents a significant difference ($P<0.05$) compared with the control cells using ANOVA followed by Bonferroni test. The values represent means ± S.E. of at least three independent experiments.

4.2 δ-opioid receptor interacts with PLD2 in HEK293 cells

To analyze the interaction between δ-opioid receptor and PLD2 in HEK293 cells, we carried out coimmunoprecipitation studies. δ-opioid receptor and PLD2 were NH_2-terminally tagged with a HA epitope tag and a Myc epitope tag, respectively. Expression of HADOR and MycPLD2 was examined by directly immunoblotting lysates from these cells with specific antibodies against HA tag and Myc tag (Fig. 4,

lysate). For coimmunoprecipitation, HADOR receptors were precipitated respectively from the lysates of HADOR expressing cells, MycPLD2 expressing cells and HADOR-MycPLD2 coexpressing cells using an anti-HA antibody. The resulting precipitates were immunoblotted with antibody directed against Myc tag. As shown in Fig. 4 (lane 2, 3), Myc-tagged PLD2 was detected in immunoprecipitates from cells coexpressing HADOR and MycPLD2, suggesting that δ-opioid receptor is physically associated with PLD2 in vivo. Even after the cells were treated with 0.1 μM DPDPE for 30 min, PLD2 could still be detected in the resulting immunoprecipitate. However, as a control, in immunoprecipitate from MycPLD2 expressing cells, no MycPLD2 was detected (Fig. 4, lane 5), indicating that PLD2 is not unspecifically immunoprecipitated by anti-HA antibody.

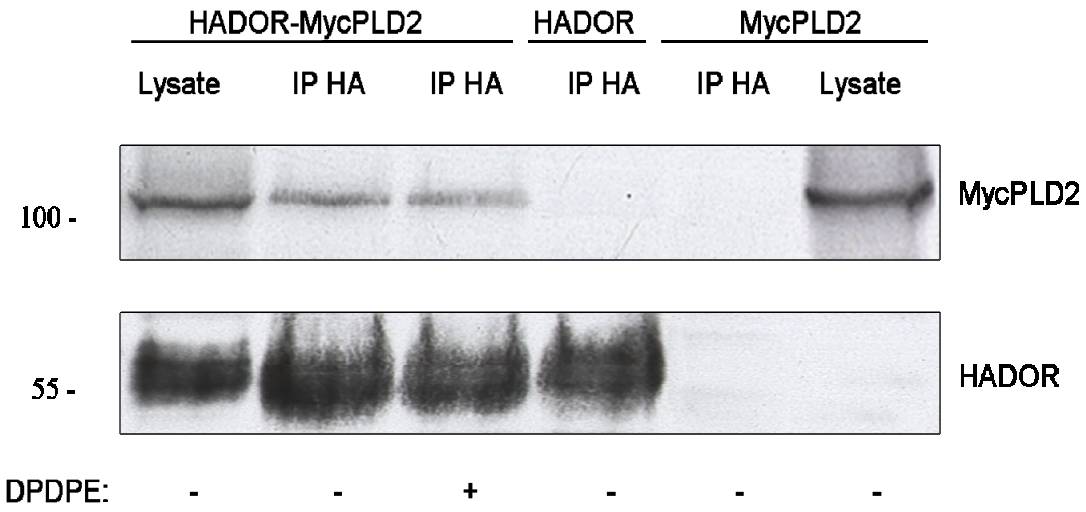


Fig. 4. DOR physically associates with PLD2. Lysates of cells or the immunoprecipitates (IP) generated with the anti-HA antibody from the cell lysates were subjected to Western blot analysis. Lane 1, lysate of HADOR-mycPLD2 coexpressing HEK293 cells; lane 2, IP of HADOR-mycPLD2 coexpressing HEK293 cells; lane 3, IP of HADOR-mycPLD2 coexpressing HEK293 cells which were preincubated with 0.1μM DPDPE for 30 min; lane 4, IP of HADOR expressing cells; lane 5, IP of mycPLD2 expressing cells; lane 6, lysate of mycPLD2 expressing cells.

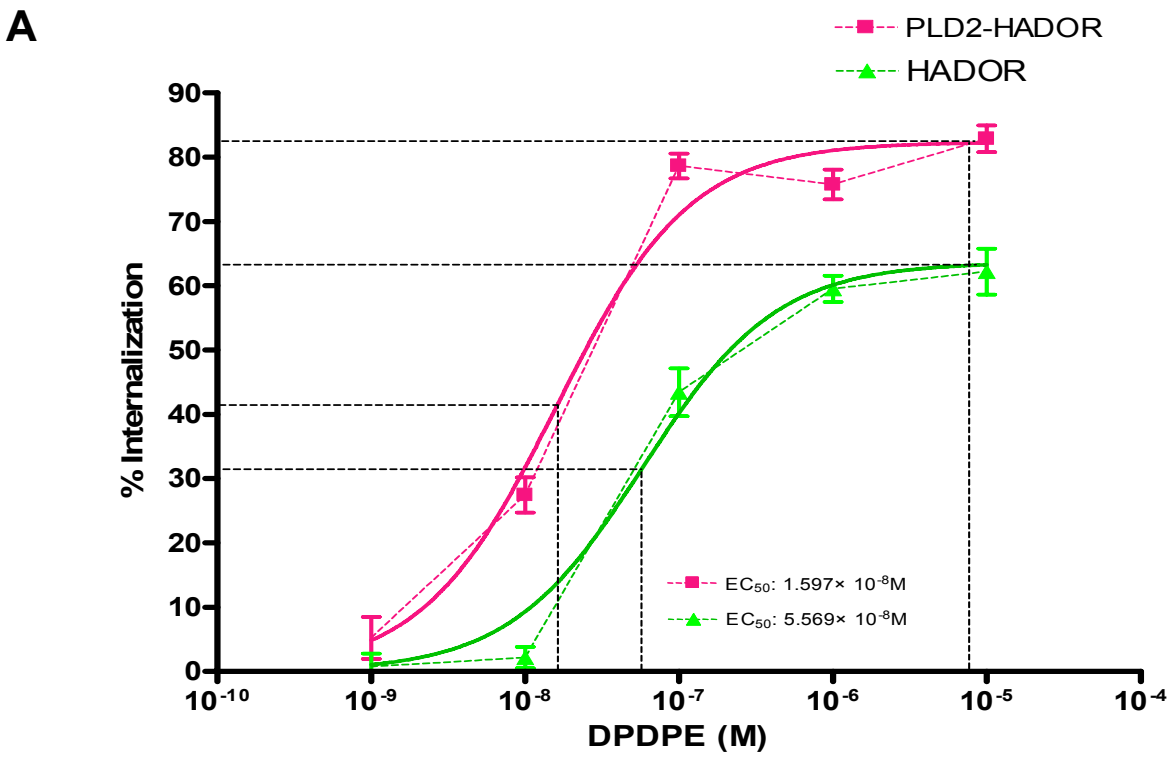
4.3 PLD2 activity is required for agonist-induced δ-opioid receptor endocytosis

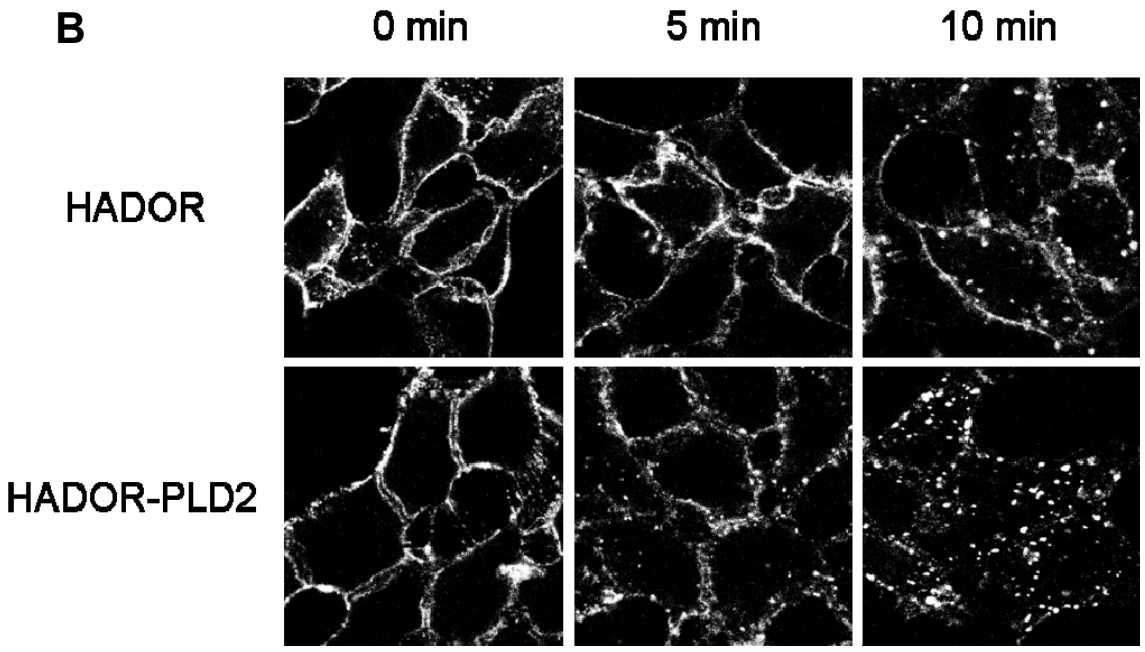
4.3.1 Coexpression of PLD2 enhances agonist-induced δ-opioid receptor endocytosis

Because PLD2 is physically associated with the δ-opioid receptor and can be activated through the δ-opioid receptor, we further investigated the effects of PLD2 on δ-opioid receptor endocytosis in PLD2-HADOR coexpressing cells and in HADOR expressing cells. We first studied δ-opioid receptor internalization induced by δ-opioid receptor selective agonist by a quantitative internalization assay. Cell surface δ-opioid receptors in both PLD2-HADOR coexpressing cells and HADOR expressing cells were labeled with anti HA antibody at 4°C. The cells were treated with the DOR agonist DPDPE in a series of concentrations for 30 minutes at 37°C, and the remaining cell surface δ-opioid receptors were measured by ELISA. Based on the control cells which were exposed in agonist-free medium in the same conditions, agonist-induced receptor internalization was calculated as the loss of the surface δ-opioid receptor. As shown in Fig. 5A, in the presence of 1 nM DPDPE, very little δ-opioid receptor endocytosis was observed in cells coexpressing PLD2-HADOR or cells expressing HADOR, and there was no distinct difference between these two cell lines. As the concentration of DPDPE was raised, the δ-opioid receptor internalization increased in both cells lines in a dose-dependent manner. However, in PLD2-HADOR coexpressing cells, the DPDPE-induced δ-opioid receptor internalization increased more promptly than that in DOR alone expressing cells, and the difference between them became gradually larger and significant. Compared to the cells expressing δ-opioid receptors and only endogenous PLD2, the coexpression of PLD2 led to a left shift of the DPDPE dose-response curve of receptor endocytosis with a 3-fold increase in the ability of DPDPE, indicating a role of PLD2 in promoting δ-opioid receptor endocytosis. When the concentration of DPDPE reached 10 μM, the δ-opioid receptor internalization was maximal for both cells, and almost 20% of the cellular δ-opioid receptors were more endocytosed in PLD2-HADOR coexpressing cells than in HADOR expressing cells. These data reveal that the PLD2 enhances agonist-induced δ-opioid receptor endocytosis.

Next we studied the time-dependency of DPDPE-induced δ-opioid receptor endocytosis in HADOR expressing cells and PLD2-HADOR coexpressing cells by confocal microscopy analysis. After labeling of cell surface δ-opioid receptors, cells were treated with DPDPE for different time periods. Then cells were fixed and subjected to immunofluorescence staining. As shown in Fig. 5B, compared to untreated cells, no distinct alteration in the cellular localization of δ-opioid receptors was

observed in the cells expressing HADOR after 5 minutes stimulation with DPDPE, in contrast, there were already small vesicles with receptors forming in the cells coexpressing PLD2 and HADOR. After 20 minutes of DPDPE stimulation, the most vesicles in PLD2 coexpressing cells were already inside of the cells, whereas a lot of vesicles in the cells expressing only DOR were still on or near the plasma membrane. A





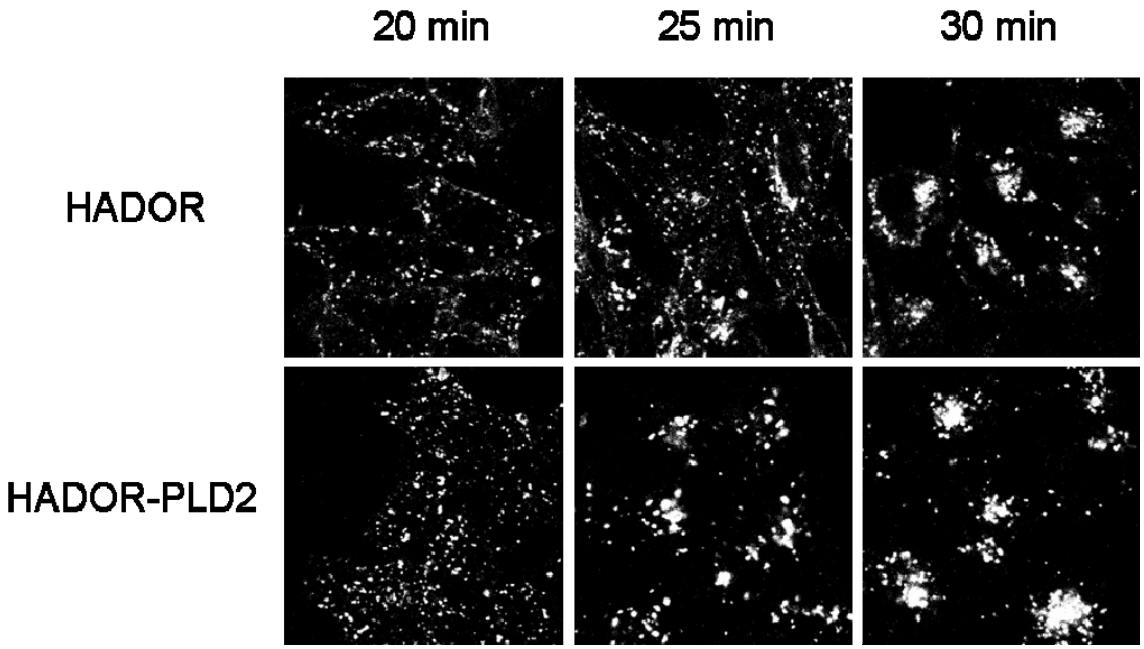


Fig. 5. Coexpression of PLD2 enhances agonist-induced δ-opioid receptor endocytosis. A, Quantitative analysis of DOR internalization in HEK293 cells expressing HADOR or coexpressing PLD2 and HADOR, which were exposed to DPDPE in a series of concentrations for 30 min. Agonist-induced DOR endocytosis, quantified as the percentage loss of surface receptors in agonist-treated cells, was measured by ELISA. Data are presented as means ± S.E. from at least three independent experiments performed in triplicate. B, Confocal microscopy analysis of time-dependent DOR endocytosis induced by DPDPE in HADOR-PLD2 coexpressing cells and HADOR expressing cells. After the labeling of cell surface δ-opioid receptors, cells were treated with 0.1 μM DPDPE for different times as indicated.

striking difference of δ-opioid receptor endocytosis between the two cell lines was observed after DPDPE treatment for 25 min, when most of the vesicles in PLD2 and HADOR coexpressing cells fused and accumulated in the perinuclear region. Taking together, these observations indicate the role of PLD2 in enhancing of δ-opioid receptor endocytosis.

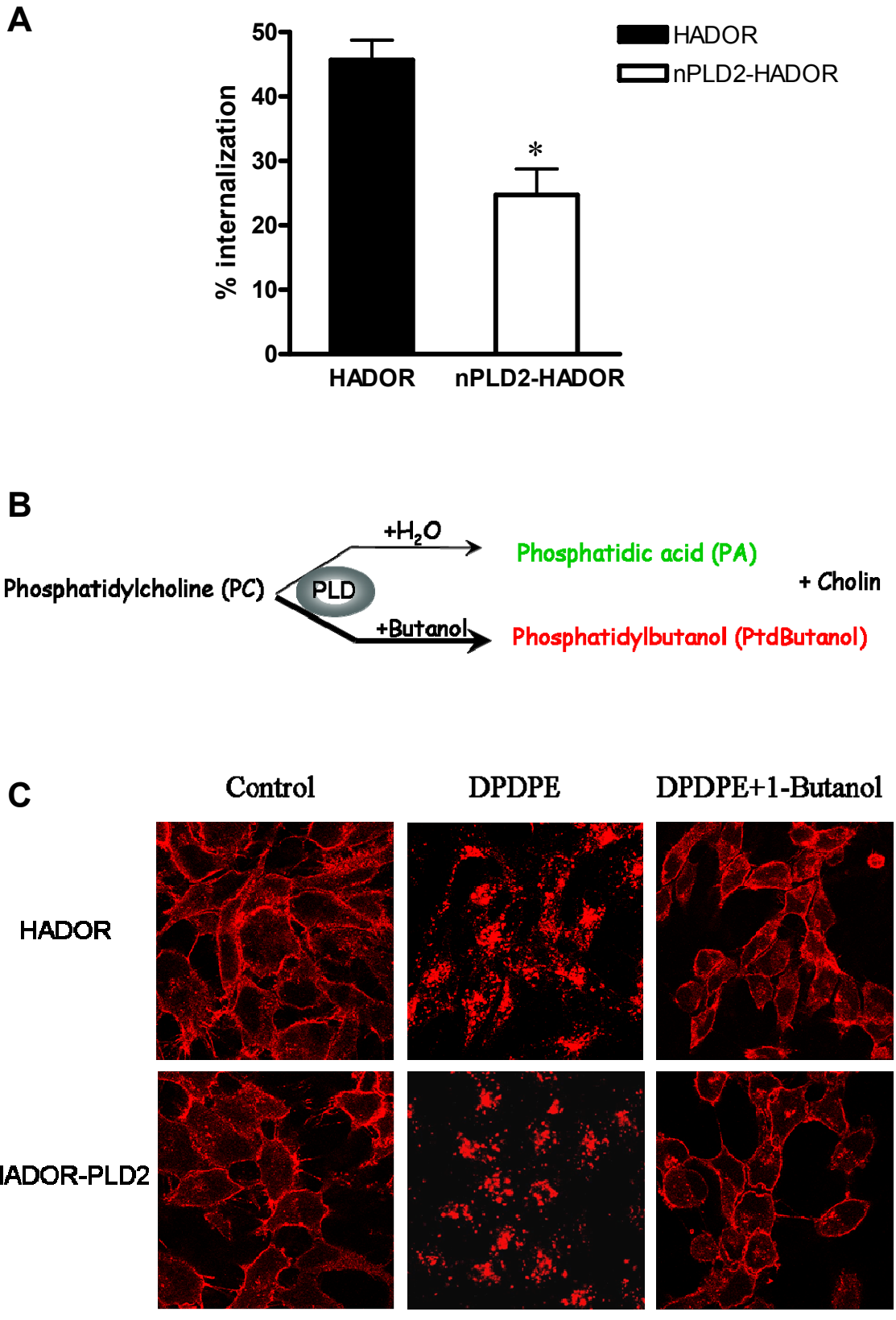
4.3.2 Coexpression of a PLD2 negative mutant (K758R) decreases agonist-induced δ-opioid receptor endocytosis

To further confirm the function of PLD2 in δ-opioid receptor endocytosis, we made a point mutation in the catalyzing motif of PLD2, in which the amino acid Lysine758 was substituted by Arginine. The mutation deletes the catalytic activity of

PLD2 and the negative mutant construct was generally used for silencing endogenous PLD2 activity (Denmat-Ouisse et al., 2001; Sung et al., 1997; Wang et al., 2003). Then this negative PLD2 mutant (nPLD2) was used to transfect the cells expressing HADOR. The coexpression of this K758R PLD2-catalytically inactive mutant significantly attenuated the DPDPE-induced DOR endocytosis in HADOR expressing cells (nPLD2-HADOR) (Fig. 6A). This suggests that the catalytically inactive PLD2 competes with the endogenous PLD2 for the interaction with DOR, and its coexpression impairs the DOR-mediated PLD2 activation, so that the DOR internalization was impaired. Thus PLD2 activity is essential for agonist-induced δ-opioid receptor endocytosis.

4.3.3 Inhibition of agonist-induced δ-opioid receptor endocytosis by primary alcohol 1-butanol

Using H_2O as an electron donor, PLD2 plays a function as a phospholipid degrading enzyme, cleaving phosphatidylcholine (PC) to yield phosphatidic acid (PA) and choline. By the same mechanism, PLD2 can generate phosphatidylbutanol instead of PA from 1-butanol. In the presence of 1-butanol, PLD2 will predominantly utilize 1-butanol instead of H_2O (Fig. 6B). Therefore 1-butanol is generally used as a PLD inhibitor to inhibit PA production. Using 1-butanol, we examined the role of PLD2-mediated PA production in δ-opioid receptor endocytosis in HEK293 cells. Applying confocal microscopy, we found that after DPDPE treatment for 30 min, δ-opioid receptors were distinctly endocytosed in both HADOR and PLD2-HADOR expressing cells, and this DPDPE-induced δ-opioid receptor endocytosis was strongly blocked by 1-butanol in both cell lines (Fig. 6C). Consistent with the results of the confocal analysis, the quantitative internalization assay further confirmed the effect of 1-butanol on the inhibition of DPDPE-mediated DOR endocytosis (Fig. 6D). These observations strongly suggest that the production PA by PLD2 is required for agonist-induced δ-opioid receptor endocytosis.


A
HADOR
nPLD2-HADOR
% internalization
50
40
30
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10
0
*
HADOR
nPLD2-HADOR
B
+H2O
Phosphatidic acid (PA)
+ Cholin
Phosphatidylcholine (PC)
PLD
+Butanol
Phosphatidylbutanol (PtdButanol)
C
Control
DPDPE
DPDPE+1-Butanol
HADOR
HADOR-PLD2

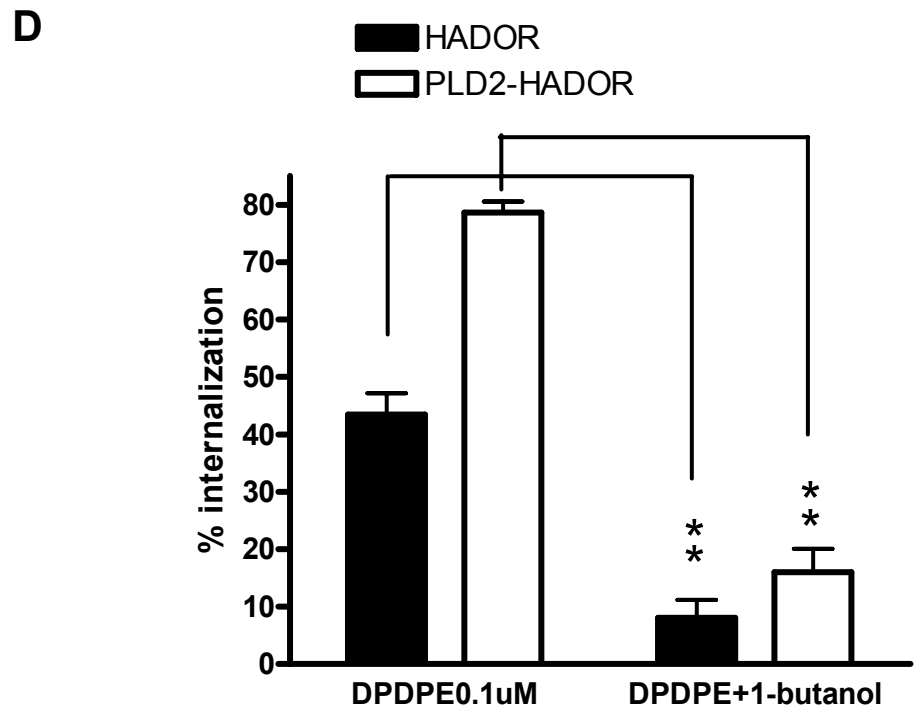


Fig.6. Inhibition of agonist-induced DOR endocytosis by expression of a negative mutant PLD2 (K758R) or by the primary alcohol 1-butanol. A, HEK293 cells coexpressing HADOR and the negative PLD2 mutant or expressing HADOR alone were treated with 0.1 μM DPDPE for 30 min, and then subjected to a quantitative internalization assay. B, The mechanism of 1-butanol to inhibit PLD. C, Confocal microscopy analysis of DOR internalization and its inhibition by the primary alcohol 1-butanol in HADOR expressing and PLD2-HADOR coexpressing HEK293 cells. Cells were left untreated or treated with 0.1 μM DPDPE for 30 min in the presence or absence of 1-butanol in serum-free medium. D, Quantitative analysis of the inhibition of δ-opioid receptor endocytosis by 1-butanol. HEK293 cells coexpressing PLD2 and HADOR or expressing HADOR alone were treated with 0.1 μM DPDPE for 30 min in the presence or absence of 0.5% 1-butanol. Cell surface opioid receptors were determined by ELISA. Agonist-induced DOR endocytosis was quantified as the percentage loss of surface receptors compared to the cells which were same treated in agonist-free medium. Data are presented as means ± S.E. from at least three independent experiments performed in triplicate. Double asterisks indicate a significant difference ($P<0.001$) using Student's t-test.

4.4 Role of PA-derived DAG in agonist-induced DOR and MOR endocytosis

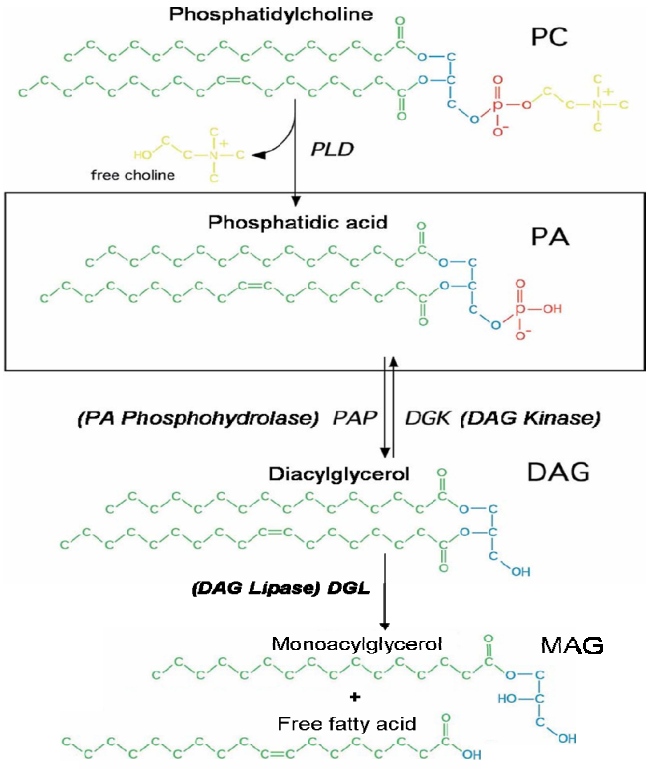
PLD hydrolyzes PC to form PA. In the lipid metabolism related to PLD (Fig. 7A), PA can be further dephosphorylated to diacylglycerol (DAG) by PA phosphohydrolase (PAP or PPH) (Brindley and Waggoner, 1996; Sciorra and Morris, 1999). The resulted DAG can also be turned back to PA by DAG kinase (DGK) (Topham and Prescott, 1999). DAG is an important intracellular lipid signaling molecule which is under

intensive investigation. Therefore we investigated what role the conversion from PA to DAG plays for the function of PLD2 in δ-opioid receptor endocytosis.

4.4.1 Inhibition of PA-derived DAG synthesis attenuates agonist-induced opioid receptors internalization

First we inhibited the conversion from PA to DAG by using propranolol, an effective PAP inhibitor used since 1980's (Albert et al., 2005; Billah et al., 1989; Deretic et al., 2004; Grkovich et al., 2006; Koul and Hauser, 1987). Fig. 7B shows the results of the quantitative internalization analysis of DPDPE-induced DOR endocytosis during the inhibition of PAP activity. We found, when the conversion from PA to DAG was inhibited, δ-opioid receptor endocytosis was not enhanced because of the increase of PA, but rather was distinctly decreased, revealing a role of PA-derived DAG in agonist-induced DOR endocytosis. Because our group has previously demonstrated that PLD2 activity is also required for agonist-induced μ-opioid receptor (MOR) endocytosis, we further tested this effect on [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin (DAMGO)-induced μ-opioid receptor endocytosis in HEK293 cells expressing HAMOR. Similar to δ-opioid receptor endocytosis, the inhibition of PAP also remarkably attenuated μ-opioid receptor endocytosis (Fig. 7B). These data suggest that the conversion from PA to DAG is important for the function of PLD2 in opioid receptors endocytosis.

A



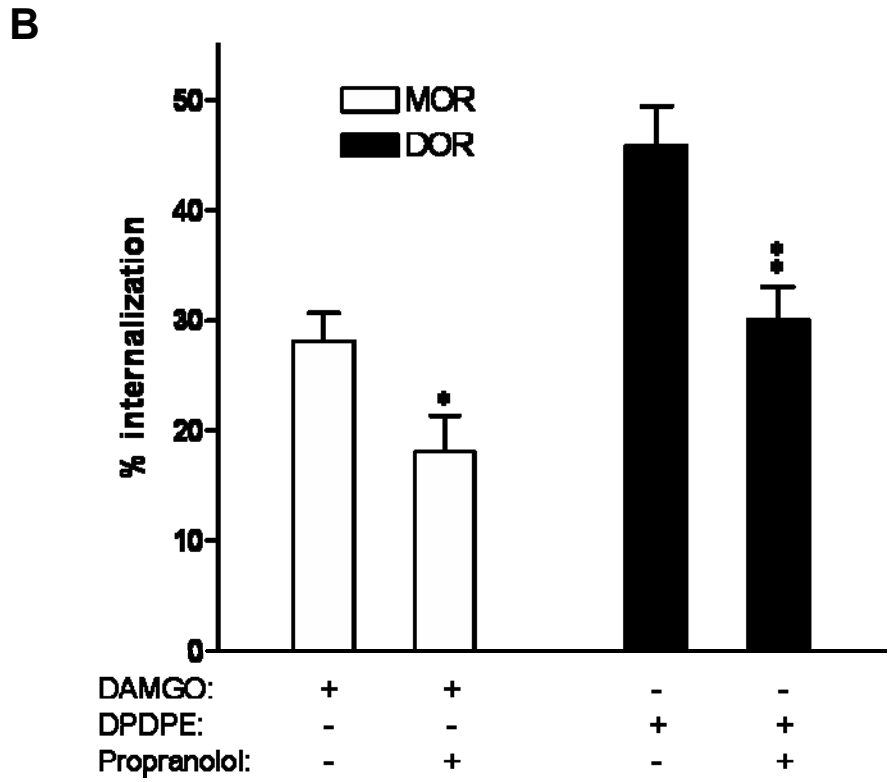
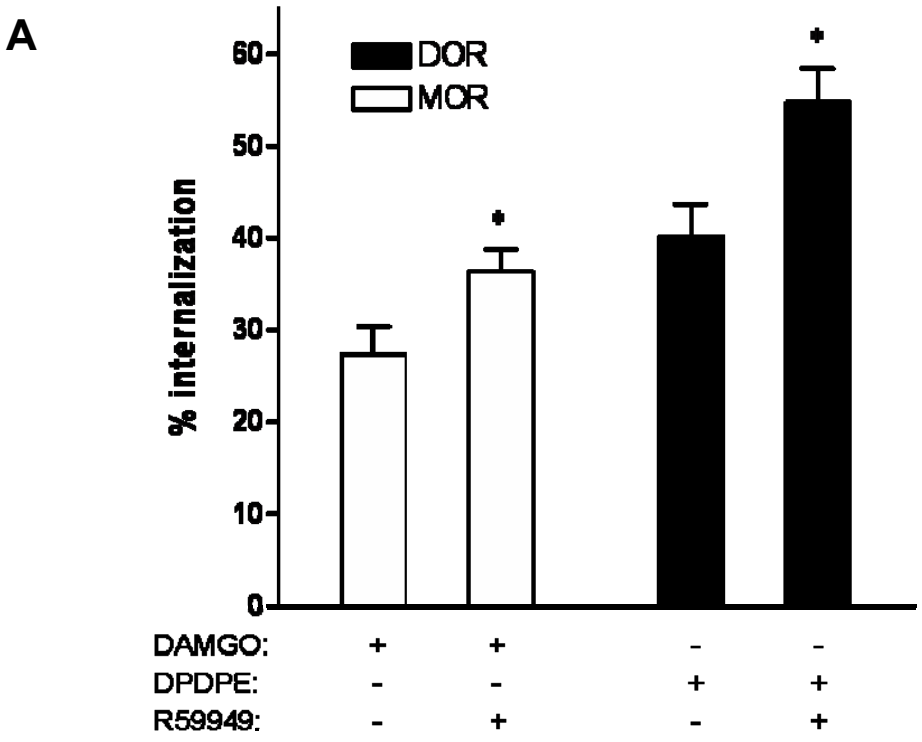


Fig. 7. Inhibition of the conversion from PA to DAG attenuates agonist-induced DOR and MOR endocytosis. A, The lipid metabolism related to PLD. B, HEK293 cells expressing HADOR or HAMOR were stimulated with 0.1 μM DPDPE or 1 μM DAMGO respectively for 30 min in the presence or absence of 250 μM propranolol. Cell surface opioid receptors were determined by ELISA and agonist-induced receptor internalization was quantified as the percentage loss of surface receptors compared to the cells which were same treated in agonist-free medium. Single asterisk indicates a significant difference ($P<0.05$) and double asterisks indicate a significant difference ($P<0.001$) between in the presence and the in the absence of propranolol using Student's t-test. Data are presented as means ± S.E. from three independent experiments performed in triplicate.

4.4.2 Increasing the level of PA-derived DAG augments agonist-stimulated opioid receptors endocytosis

To further verify the effect of the conversion between PA and DAG on opioid receptor endocytosis, we inhibited the reversed conversion from DAG to PA. To increase DAG accumulation, we applied the most commonly used DGK inhibitor, R59949. R59949 specifically acts on the catalytic domain of DGK and inhibits its catalytic activity (Jiang et al., 2000). As shown in Fig. 8A, the inhibition of conversion from DAG to PA augmented the DPDPE-induced δ-opioid receptor endocytosis significantly. The same effect of the DGK inhibition was also detected on the

DAMGO-induced μ-opioid receptor internalization. These data confirm the above results and further demonstrate that PA-derived DAG is involved in opioid receptor endocytosis.



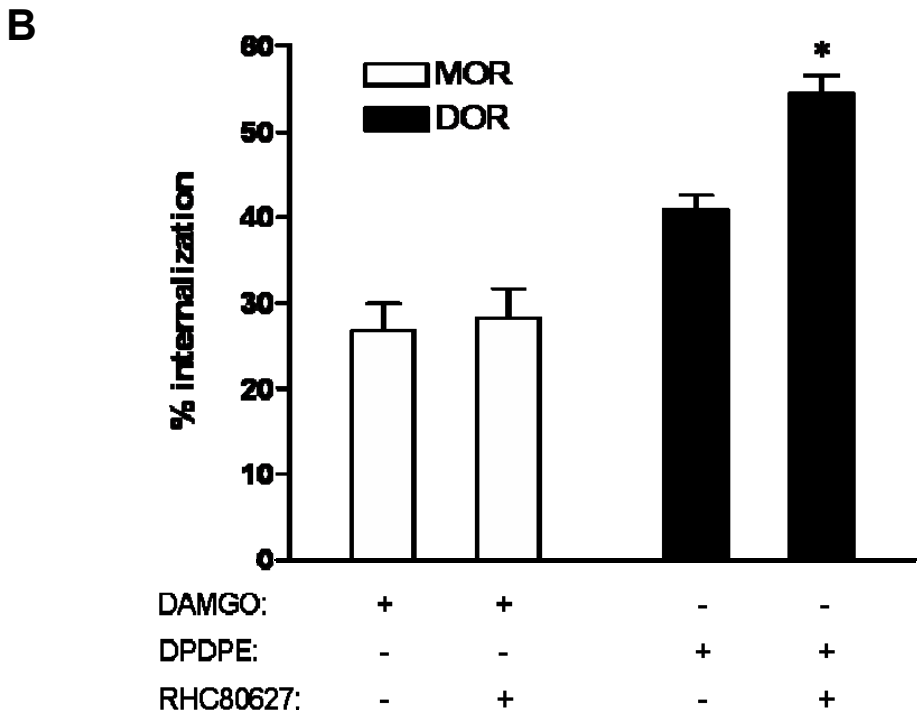


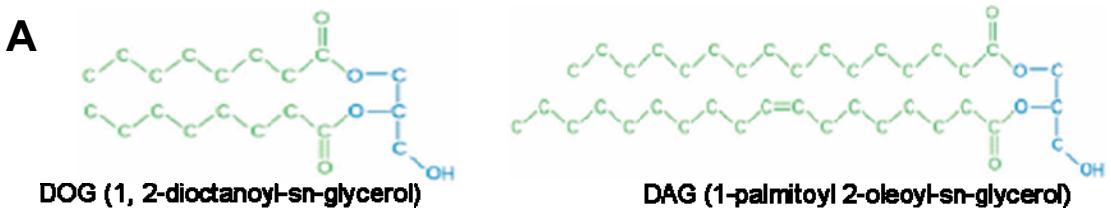
Fig. 8. Increasing the level of PA-derived DAG augments agonist-stimulated opioid receptor endocytosis. A, Inhibition of DAG kinase. HEK293 cells expressing HADOR or HAMOR were stimulated with 0.1μM DPDPE or 1μM DAMGO for 30 min after the preincubation with or without 40μM R59949. B, Inhibition of DAG lipase. HEK293 cells expressing HADOR or HAMOR were stimulated with 0.1μM DPDPE or 1μM DAMGO respectively for 30 min after the preincubation with or without 100μM RHC80627. Cell surface opioid receptors were determined by ELISA and agonist-induced opioid receptor internalization was quantified as the percentage loss of surface receptors compared to the cells which were same treated in agonist-free medium. Single asterisk indicates a significant difference ($P<0.05$) between with and without R59949 or RHC80627 incubation using Student's t-test. Data are presented as means ± S.E. from three independent experiments performed in triplicate.

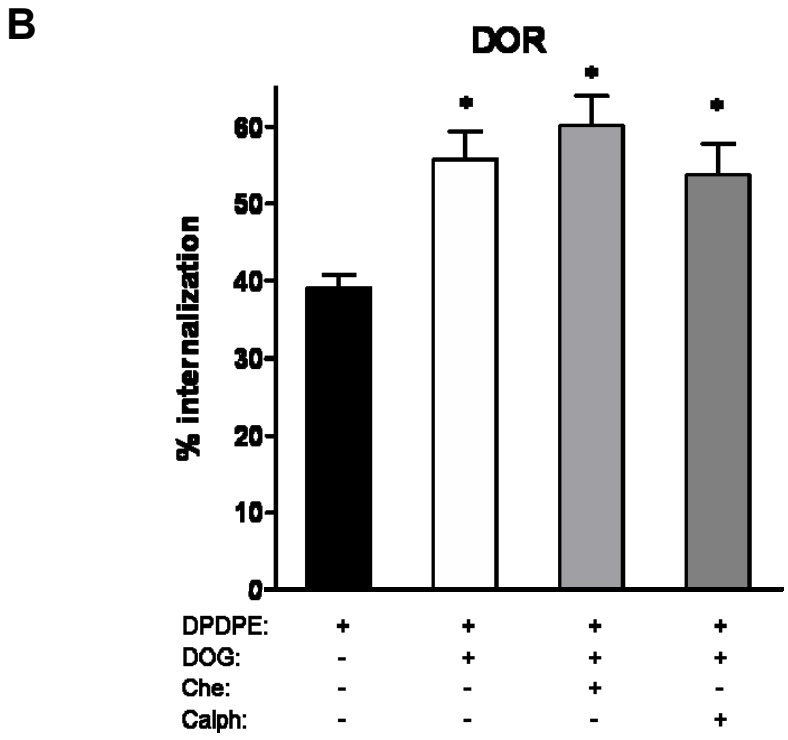
DAG may also be metabolized to monoacylglycerol (MAG) and free fatty acids by DAG lipase (DGL) (Amin et al., 1986; Migas and Severson, 1996; Pasquare et al., 2004). We further examined whether this metabolism might have effect on opioid receptor endocytosis. Fig. 8B shows that, when DAG lipase was inhibited by its specific inhibitor RHC80627 (Amin et al., 1986; Konrad et al., 1994), DPDPE-induced δ-opioid receptor internalization was significantly increased, which can be due to more DAG accumulated after the inhibition of DGL. For μ-opioid receptor internalization, no significant increase was observed. It might be because that less DAG needs to be converted through this DGL pathway and more DAG is need in other physiological events of μ-opioid receptor. On the other hand, because neither μ-opioid receptor internalization nor δ-opioid receptor internalization was reduced by the inhibition of DGL, it can be deduced that the downstream of DGL, for example MAG, does not contribute to agonist-induced opioid receptors endocytosis.

4.4.3 DOG, a synthetic cell-permeable DAG analogue increases opioid receptor endocytosis

Diacylglycerol is a lipid composed of two fatty acid chains linked to glycerol. In order to obtain more direct evidence that the resulted DAG contributes to opioid receptor endocytosis, we applied a cell-permeable DAG analog, 1,2-dioctanoyl-sn-glycerol (DOG). DOG is a synthetic DAG of short chain fatty acid, which is a powerful cell biology research tool for DAG function (Fig. 9A) (Lucas et al., 2003; Ma et al., 2000). We examined whether the presence of DOG influenced the agonist-induced opioid receptor endocytosis. Compared to the treatment with DPDPE

alone, the presence of DOG remarkably increased the DPDPE-stimulated δ-opioid receptor endocytosis in HADOR expressing cells (Fig. 9B). Confocal microscopy analysis again confirmed the effect of DOG on enhancing the rate of DPDPE-induced δ-opioid receptor endocytosis in HADOR expressing HEK293 cells. After 30 min of incubation with a very low concentration of DPDPE, 1nM, hardly could the receptor endocytosis be observed. If DOG was present, the δ-opioid receptor exhibited some obvious receptor endocytosis. When the concentration of DPDPE was raised to 10 nM, very distinct difference could be observed between in the presence and absence of DOG (Fig. 9D). We further examined whether DOG treatment also promotes agonist-induced μ-opioid receptor endocytosis by quantitative internalization assay. As shown in Fig. 9C, similar to δ-opioid receptor endocytosis, in the presence DOG, DAMGO induced distinctly more μ-opioid receptor endocytosis in HAMOR expressing cells. These data strongly suggest a role of resulted DAG in opioid receptor endocytosis and give further evidence that the conversion from PA to DAG is involved in the function of PLD2 in opioid receptor endocytosis.





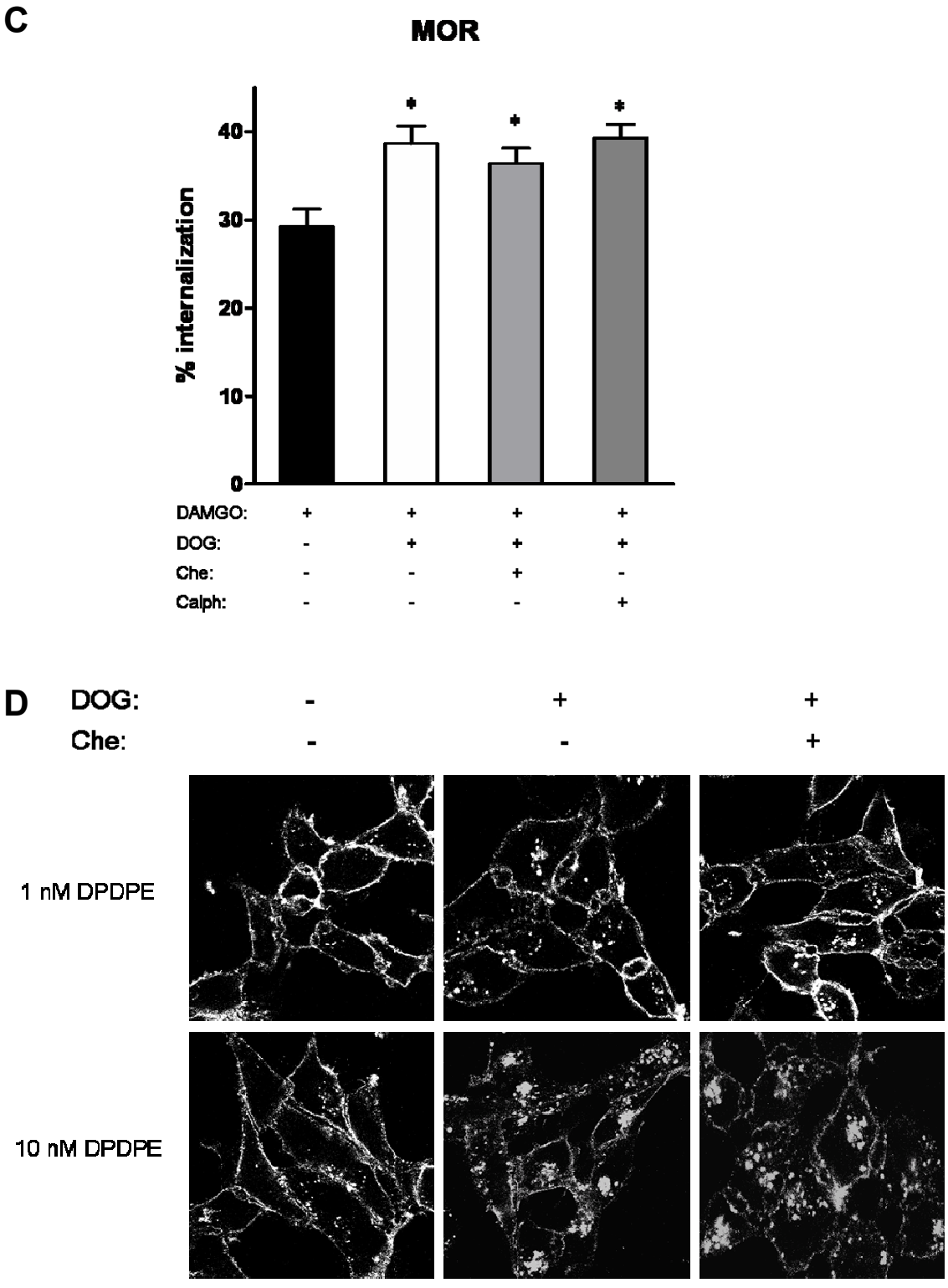


Fig. 9. The DAG analog, DOG, enhances agonist-induced opioid receptor endocytosis in a PKC-independent manner. A, Structure of DOG (1,2-dioctanoyl-sn-glycerol, a synthetic cell-permeable DAG with short chain fatty acids) and DAG. B, Quantitative analysis of delta-opioid receptor internalization in HEK293 cells expressing HADOR, which were treated with 0.1 μM DPDPE for 30 min in the presence or absence of 150 μM DOG with or without 5 μM chelerythrine chloride (Che) or 1μM

calphostin C (Calph). C, Quantitative analysis of μ-opioid receptor internalization in HAMOR expressing HEK293 cells, which were stimulated with 1 μM DAMGO in the presence or absence of 150 μM DOG with or without either 5 μM chelerythrine chloride or 1μM calphostin C for 30 min. Cell surface opioid receptors were determined by ELISA and agonist-induced opioid receptor internalization was quantified as the percentage loss of surface receptors compared to the cells which were same treated in agonist-free medium. D, Confocal microscopy analysis of delta-opioid receptor subcellular distribution in HADOR expressing cells which were treated 1 or 10 nM DPDPE in the presence or absence of 150 μM DOG with or without 5 μM chelerythrine chloride. Single asterisk indicates a significant difference ($P<0.05$) between with and without DOG treatment using ANOVA followed by Bonferroni test. Data are presented as means ± S.E. from three independent experiments performed in triplicate.

4.4.4 Role of PKC in the Agonist-induced DOR and MOR endocytosis

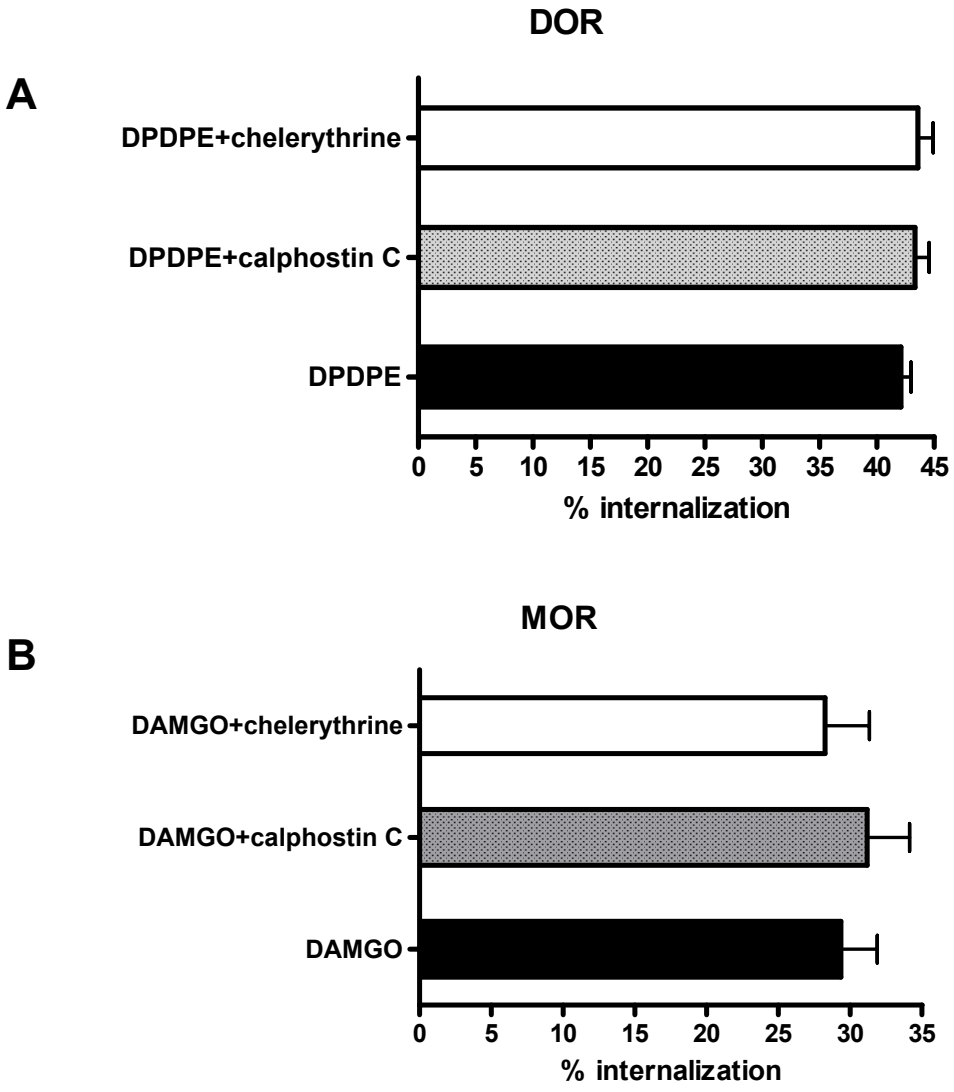


Fig. 10. Inhibition of PKC does not impair agonist-induced DOR and MOR endocytosis. A, HADOR expressing HEK293 cells were stimulated with 0.1μM DPDPE for 30 min in the presence or

absence of 1 μM calphostin C or 5 μM chelerythrine chloride. B, HAMOR expressing HEK293 cells were stimulated with 1μM DAMGO for 30 min in the presence or absence of 1 μM calphostin C or 5 μM chelerythrine chloride. Cell surface opioid receptors were determined by ELISA and agonist-induced opioid receptor internalization was calculated as the percentage loss of surface receptors compared to the cells which were same treated in agonist-free medium. Data are presented as means ± S.E. from three independent experiments performed in triplicate.

DAG or DOG is usually thought that it can activate protein kinase C (PKC). So we investigated whether the activation of PKC was involved in the process of opioid receptor endocytosis by application of two specific PKC inhibitors, chelerythrine chloride and calphostin C. We found, the inhibition of PKC by either chelerythrine chloride or calphostin C did not impair DPDPE-induced δ-opioid receptor endocytosis in HADOR expressing cells, nor impaired DAMGO-induced μ-opioid receptor endocytosis in HAMOR expressing cells (Fig. 10A and 10B), suggesting that PKC is not involved in agonist-induced δ- and μ-opioid receptor endocytosis. Consistent with these findings, in the investigation of the DOG's effect which is showed above, we also made PKC inhibition to assess the influence of PKC. In quantitative receptor internalization analysis the inhibition of PKC by chelerythrine chloride or calphostin C could not block the effect of DOG on increasing agonist-induced both δ-opioid receptor and μ-opioid receptor endocytosis (Fig. 9B and 9C). Confocal analysis also showed that after inhibition of PKC by chelerythrine chloride, the enhancement of δ-opioid receptor endocytosis by DOG was not attenuated for both concentrations of DPDPE treatment (Fig. 9D).

4.4.5 DAG enhances the clathrin-dependent agonist-induced opioid receptor internalization

Eukaryotic cells exhibit at least two endocytic pathways: the clathrin-dependent pathway and the clathrin-independent pathway. Clathrin-independent endocytosis is mainly mediated by glycolipid rafts, in which caveolae are a well-characterized subdomain. The sensitivity of endocytosis via caveolae to cholesterol depletion by filipin distinguishes the pathway from the clathrin-dependent endocytic pathway which is sensitive to hypertonic sucrose (Anderson, 1998; Heuser and Anderson, 1989). It is generally believed that the internalization of opioid receptors occurs through clathrin-coated vesicles (von Zastrow, 2003). We investigated whether the

enhancement of opioid receptor internalization by either the endogenous PA-derived DAG or the heterologous DAG analog DOG, is via clathrin-dependent endocytosis or via caveolae-dependent endocytosis. As shown in Fig. 11, in the absence of DOG, the inhibition of caveolae-dependent endocytic pathway by filipin did not change the endocytic rate of DPDPE-stimulated δ-opioid receptor, and hypertonic sucrose blocked the receptor endocytosis stimulated by DPDPE in HADOR expressing cells, showing that DPDPE induces δ-opioid receptor via clathrin-dependent pathway in line with previous reports (Chu et al., 1997; Gaudriault et al., 1997; Hasbi et al., 2000). In the presence of DOG, the DPDPE-induced endocytosis of δ-opioid receptor was significantly increased. Filipin had no distinct effect on this DOG enhancement of agonist-induced receptor endocytosis. Whereas hypertonic sucrose blocked the DPDPE-induced δ-opioid receptor endocytosis also in the presence of DOG, and there was no significant difference of DPDPE-stimulated δ-opioid receptor endocytosis between in the presence and in the absence of DOG. These results suggest that the enhancement of agonist-induced opioid receptor endocytosis by the resulted DAG occurs via the clathrin-dependent pathway but not via the caveolae-dependent pathway.

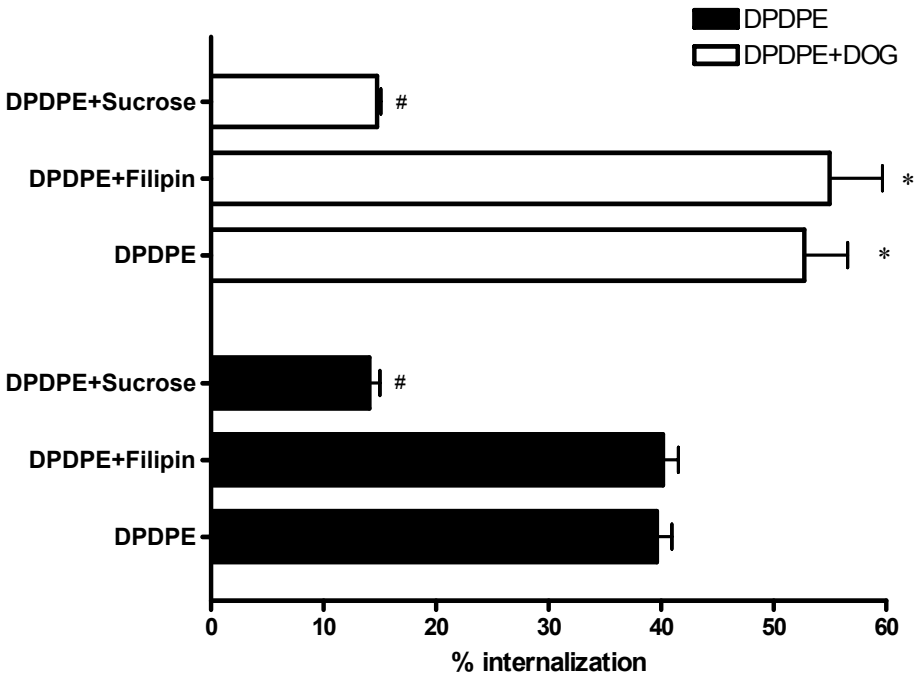


Fig. 11. The enhancement of agonist-induced δ-opioid receptor endocytosis by DAG is via the clathrin-dependent pathway. In the presence or absence 150 μM DOG, HADOR expressing HEK293 cells were treated with 0.1 μM DPDPE for 30 min followed a preincubation with or without either 10

ng/ml filipin or 400 μM sucrose. The cells in the same treatment without the agonist were set as zero-controls. Cell DOR endocytosis was determined by quantitative receptor internalization assay. "*" indicates a significant difference ($P<0.05$) of the same treatments between in the presence and in the absence of DOG, and "#" indicates a significant difference ($P<0.05$) of the same treatments between in the presence and in the absence of sucrose, using Student's t-test. Data are presented as means ± S.E. from three independent experiments performed in triplicate.

4.5 The role of PLD2 in opioid receptor endocytosis involves p38 kinase activation

Recently p38 MAP kinase was shown to regulate endocytic trafficking (Cavalli et al., 2001; Huang et al., 2004; Mace et al., 2005; McLaughlin et al., 2006; Vergarajauregui et al., 2006). It was described that the activation of p38 MAPK promotes endocytosis by stimulating the formation of guanyl nucleotide dissociation inhibitor (GDI):Rab5 complex (Cavalli et al., 2001; Huang et al., 2004), or by phosphorylation of the Rab5 effectors EEA1 (early endosome antigen 1) and Rabenosyn-5 to regulate their recruitment to membranes (Mace et al., 2005). These events facilitate the functions of the small GTPase Rab5, a key regulator of clathrin-dependent endocytosis. Rab5 coordinates multiple processes, such as the formation of clathrin-coated vesicles, their fusion with early endosomes, homotypic early endosome fusion, as well as motility of endosomes (reviewed in Zerial and McBride, 2001). It has been shown that p38 MAPK activation is required for μ-opioid receptor endocytosis (Mace et al., 2005). The inhibition of p38 MAPK by the inhibitor SB203580 impaired DAMGO-induced μ-opioid receptor endocytosis, whereas the activation of p38 MAPK by coexpressing a constitutively active form of MKK6 enhanced μ-opioid receptor endocytosis. Consistent with this, we also found that the DPDPE-induced δ-opioid receptor endocytosis was attenuated by the inhibition of p38 kinase with the inhibitor SB203580 but not by the inhibition of other MAPKs with ERK (extracellular signal-regulated kinase) inhibitor PD98059 and JNK (c-jun N-terminal kinase) inhibitor SP600125 (Fig. 12). Furthermore, DAMGO rapidly stimulated an activation of p38 MAPK in μ-opioid receptor expressing cells as examined by Western blotting using phospho-specific antibodies. In contrast, short-term morphine, which is not able to trigger μ-opioid receptor endocytosis, was not able to induce p38 activation (Mace et al., 2005). We hypothesized that, there might be a relation between the activation of p38 MAPK and opioid receptor-mediated PLD2

activation so that the opioid receptor-mediated PLD2 activity is involved in the activation of p38 MAPK.

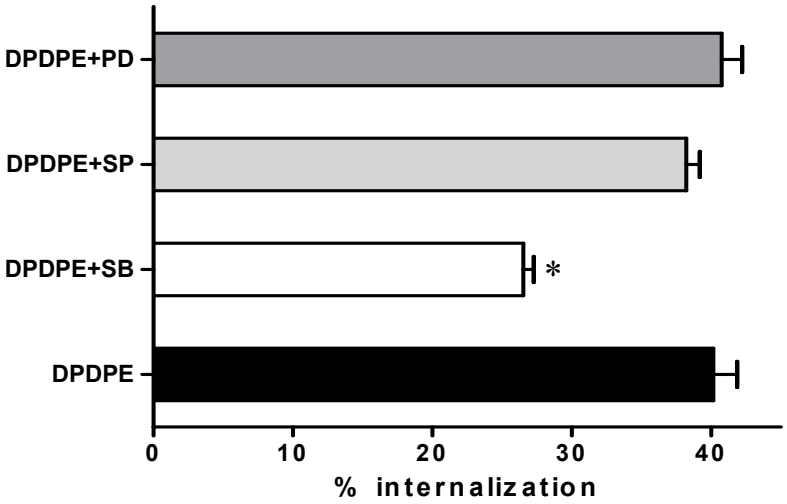


Fig. 12. DPDPE-induced δ-opioid receptor endocytosis was attenuated by inhibition of p38 kinase. HEK293 cells expressing HADOR were treated with 0.1 μM DPDPE for 30 min in the presence or absence of 10 μM SB203580 (SB) (P38 inhibitor), 50 μM PD98059 (PD) (ERK inhibitor) or 10 μM SP600125 (SP) (JNK inhibitor). Agonist-induced DOR endocytosis was determined by quantitative receptor internalization assay as described in Material and Methods. . *Significant difference ($p < 0.05$) compared to the treatment with DPDPE alone (ANOVA followed by Bonferroni test). Data are presented as means ± S.E. from three independent experiments performed in triplicate.

4.5.1 PLD activity is required for opioid receptor-mediated p38 activation

Using a specific antibody against phosphorylated p38 MAPK (Thr180/Tyr182), we first tested the activation of p38 MAPK by the agonist DPDPE in HADOR expressing HKE293 cells as well as by DAMGO in HAMOR expressing HEK293 cells (Fig. 13A). The increase of p38 MAPK phosphorylation was readily detected with the peaking after 5 min of stimulation with DPDPE in HADOR-expressing cells. Same finding also occurred in HAMOR expressing cells after DAMGO stimulation, which is in agreement with previous report (Mace et al., 2005). However, in the presence of the PLD inhibitor 1-butanol, which inhibits PA production by PLD, the p38 phosphorylation, induced by both DPDPE and DAMGO, was strongly blocked, indicating that PLD activity and PA production are necessary for opioid receptors-mediated p38 MAPK activation. To ensure that the inhibiting effect of 1-butanol for p38 MAPK signaling is not because of an unspecific toxic inhibition, we

also examined the activation of protein kinase Akt as a control. As shown in Fig. 13A, the Akt activation was not affected in the presence of 1-butanol.

Then we further examined the influence of PLD2 coexpression on δ-opioid receptor-mediated p38 MAPK activation (Fig. 13B). It was found that coexpression of PLD2 caused stronger and more sustained DPDPE-induced activation of p38 MAPK as compared to cells expressing only endogenous PLD2. In both cells, p38 MAPK was rapidly phosphorylated after 5 min of DPDPE treatment, then in HADOR expressing cells the p38 MAPK phosphorylation began to decrease quickly, whereas the phosphorylation of p38 MAPK in HADOR-PLD2 coexpressing cells was still distinct even after 20 min of DPDPE stimulation, indicating a potential role of PLD2 in opioid receptor-mediated p38 MAPK activation. These data suggest that PLD2 activity is required for opioid receptor-mediated p38 activation.

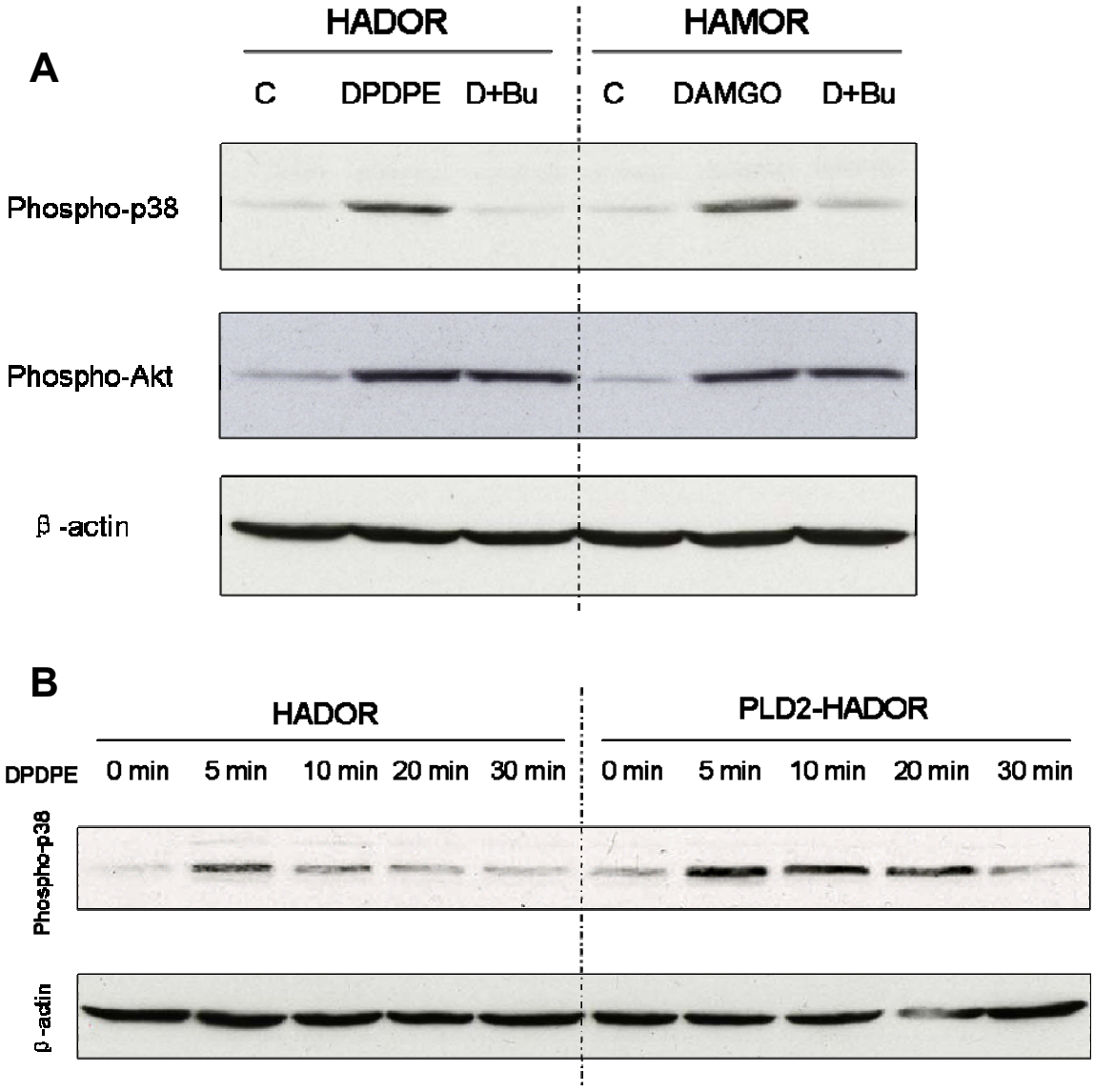


Fig. 13. PLD2 is required for opioid receptor-mediated p38 MAPK activation. A, Blocking of opioid receptor-mediated p38 MAPK activation by the PLD inhibitor 1-butanol. As indicated, δ- or μ-opioid receptors expressing HEK293 cells were unstimulated (C) or stimulated with 0.1 μM DPDPE or 1 μM DAMGO with (D+Bu) or without 0.5% 1-butanol for 5 min. B, Comparison of DPDPE-induced p38 MAPK activation in HADOR expressing cells with that in PLD2-HADOR coexpressing cells. Cells were treated with 0.1μM DPDPE for different time courses as indicated. Cell lysates were subjected to Western blotting to analyze phosphorylated p38 kinase or phosphorylated Akt.

4.5.2 DAG generated from PA is involved in opioid receptor-mediated p38 activation

Because the PLD2 product PA can be further converted to DAG, which is important for opioid receptors endocytosis as shown above, it is possible that PA-derived DAG is also involved in the function of PLD2 on opioid receptors-mediated p38 MAPK activation. To address this issue, we inhibited the conversion of PA to DAG by propranolol. P38 MAPK phosphorylation induced by either DPDPE in HADOR expressing cells or DAMGO in HAMOR expressing cells was remarkably impaired after inhibition of the conversion (Fig. 14A), indicating the important role of PA-derived DAG in opioid receptors-mediated p38 MAPK activation.

In addition, we analyzed the effect of DOG, an analog of endogenous DAG, on p38 MAPK activation. As mentioned before, DOG is a synthetic cell-permeable DAG with short chains of fatty acids. As shown in Fig. 14B, in HEK293 cells expressing HADOR, treatment with DOG alone was sufficient to induce p38 MAPK phosphorylation, which could not be blocked by the inhibition of PKC with chelerythrine chloride. When the cells were stimulated with the DOR agonist DPDPE, p38 MAPK phosphorylation was triggered rapidly. The presence of DOG augmented the DPDPE-induced phosphorylation of p38 MAPK. Inhibition of PKC with chelerythrine chloride could not impair this augmented phosphorylation of p38 MAPK, too. The same results were also obtained in HAMOR expressing cells. Fig. 14C shows the activating effect of DOG on p38 MAPK phosphorylation in HAMOR expressing cells which were treated or not treated with the MOR agonist DAMGO. Taken together, these data indicate the PA-derived DAG can activate p38 MAPK in a PKC-independent manner.

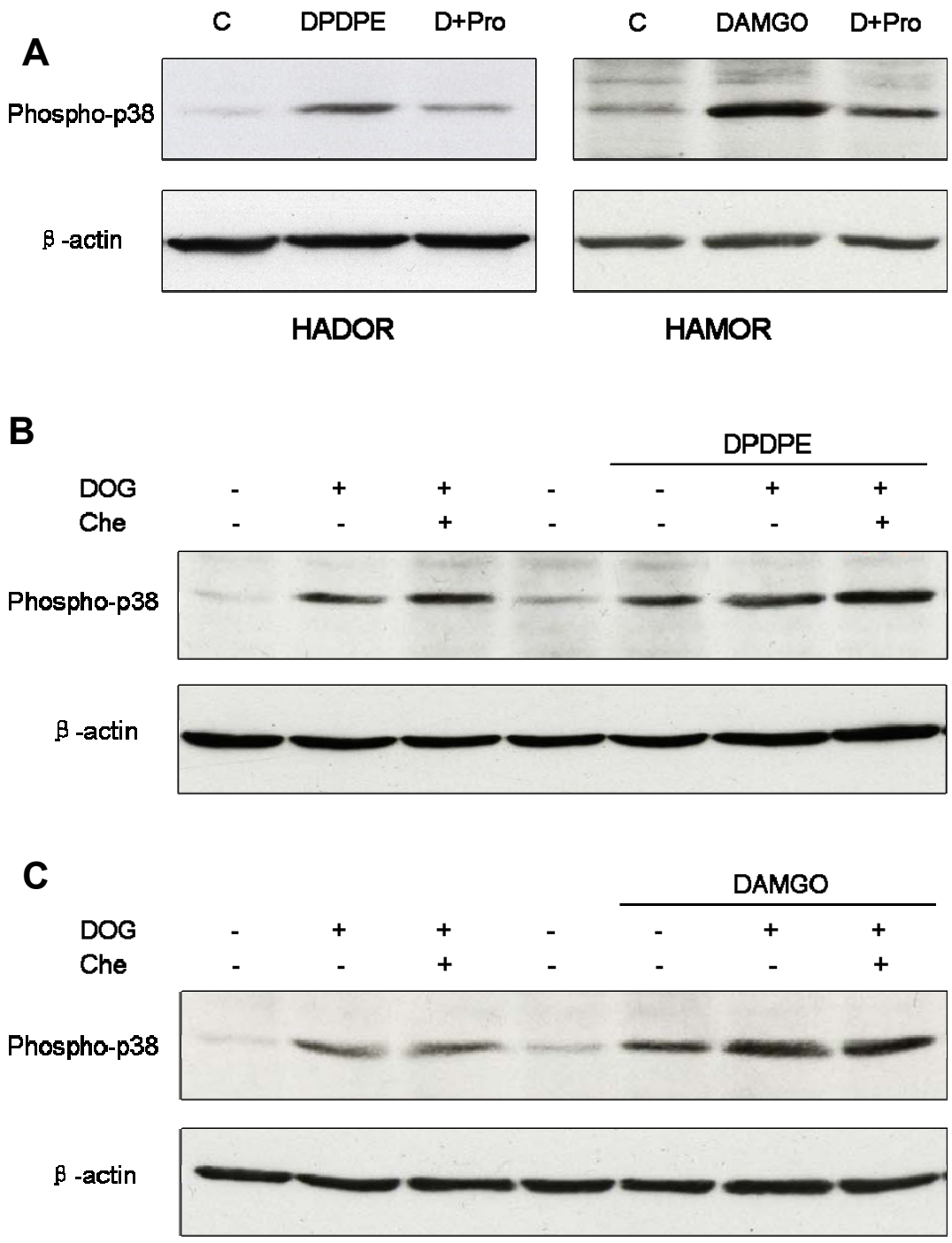


Fig. 14. PA-derived DAG is involved in opioid receptor-mediated p38 activation. A, Attenuation of opioid receptor-mediated p38 MAPK activation by inhibition of the conversion from PA to DAG with propranolol. As indicated, HEK293 cells expressing δ- or μ-opioid receptor were unstimulated (C) or stimulated with 0.1 μM DPDPE or 1 μM DAMGO for 5 min in the absence or presence (D+Pro) of 250 μM propranolol. B, Effect of DOG, a DAG analog, on the activation of p38 MAPK in HADOR-expressing cells. HADOR expressing cells were treated as indicated with or without 150 μM DOG, 0.1 μM DPDPE, and 5 μM chelerythrine chloride. C, Effect of DOG on the activation of p38

MAPK in HAMOR-expressing cells. HAMOR expressing cells were treated as indicated with or without 150 μM DOG, 1 μM DAMGO, and 5 μM chelerythrine chloride. Cell lysates were subject to Western blotting and phosphorylated p38 kinase was analyzed.

5. Discussion

PLD widely distributes in organisms ranging from bacteria, plants to animals. It hydrolyzes phosphatidylcholine to generate choline and the bioactive lipid phosphatidic acid, which is believed to have many different functions in signal transduction, membrane trafficking, transformation and cytoskeletal dynamics (Liscovitch et al., 1999; Liscovitch et al., 2000). In addition, PLD activity has been shown to be regulated by a number of GPCRs as reviewed in the introduction. Our group has reported previously that the μ-opioid receptor can mediate PLD2 activation (Koch et al., 2003). In mammalian cells, PLD2 is mainly associated with the plasma membrane (Jenkins and Frohman, 2005; Liscovitch et al., 1999). In the present study, it was found that PLD2 but not PLD1b can also be activated by stimulating δ-opioid receptor with the agonist DPDPE. The opioid agonist morphine which is not able to induce opioid receptor internalization failed to induce an increase of PLD2 activity. The opioid receptor antagonist naloxone blocked the DPDPE-stimulated PLD2 activation in HADOR-PLD2 coexpressing cells, suggesting that it is the δ-opioid receptor which specifically mediates this PLD2 activation. Both δ-opioid receptor and μ-opioid receptor-mediated PLD2 activation are PKC-independent, which is consistent with the reported mechanism of PLD activation in other receptor systems, such as M3 muscarinic and α1-adrenergic receptors (Balboa and Insel, 1998; Muthalif et al., 2000; Rumenapp et al., 1997; Schmidt et al., 1994). On the other hand, an association of PKC with PLD resulting in strong activation of PLD1 in vitro has been reported, in which the major interaction site of PLD1 was identified within its N-terminus (Park et al., 1998). A physiological association between δ-opioid receptor and PLD2 was identified by coimmunoprecipitation experiments in HEK293 cells stably expressing δ-opioid receptor and PLD2. In the NH_2 terminus of PLD2 there is a phox homologous (PX) domain (Liscovitch et al., 2000). This PX domain, which is found in a number of signaling and adaptor proteins, is thought to mediate protein-protein interaction or to bind phosphatidylinositol phosphates (Xu et al., 2001). PX domains of proteins mediating protein trafficking were shown to be required not only for the association with cellular membranes but also for the association with various receptors, such as epidermal growth factor receptor and platelet-derived growth factor receptor (Haft et al., 1998; Phillips et al., 2001). The PX domain in PLD2 is the site for the interaction with the COOH terminus of μ-opioid receptor as revealed by a yeast two-hybrid screen

(Koch et al., 2003). Therefore we speculate that this PX domain in PLD2 may also be the site which binds to the δ-opioid receptor.

Chronic opioid application is well known to produce tolerance, limiting the therapeutic use of these drugs. To uncover and eventually control the adaptive mechanisms, several cellular functions with regard to tolerance development have been intensively investigated. Special interest focused on opioid receptors internalization. In general, opioid receptor endocytosis is accomplished by complicated interactions of various determinants. Our previous work has shown that PLD2 activation is required for agonist-induced MOR endocytosis (Koch et al., 2003). The present study provides evidence for an essential role of PLD2 in the agonist-induced DOR endocytosis. Coexpression of PLD2 in DOR expressing cells remarkably enhanced DPDPE-induced DOR endocytosis. The effect of PLD2 on agonist-induced DOR endocytosis was further proved by inhibition of endogenous PLD2 activity with a catalytically inactive PLD2 mutant (K758R). PLD2 enzymatic action produces PA, a bioactive lipid. The inhibition of PLD2 activity results in the reduction of PA production. So we further tested the effect of blocking PA production with 1-butanol, which causes the replacement of PA by phosphatidylbutanol, on agonist-induced DOR endocytosis. In agreement with the data acquired by coexpressing the catalytically inactive PLD2 mutant, the results further confirmed the role of PLD2-mediated PA formation in agonist-induced DOR endocytosis, which is similar to MOR, providing further support that PLD2 activity is required for agonist-induced endocytosis of opioid receptors via its product PA. Consistent with our results, in recent reports PLD activity has been shown to regulate angiotensin II receptor endocytosis (Du et al., 2004), B-cell antigen receptor trafficking (Snyder and Pierce, 2006) and class 1 metabotropic glutamate receptor endocytosis (Bhattacharya et al., 2004) by coexpression of wild type PLD, catalytically inactive PLD mutant as well as applying PLD inhibitor 1-butanol. A recent report showed that PLD increases epidermal growth factor (EGF) receptor endocytosis through its PX domain, which is described to have GTPase-activating protein (GAP) activity and can directly activate the GTPase domain of dynamin to facilitate EGF receptor internalization (Lee et al., 2006). But demonstrably this mechanism is not sufficient for the role of PLD2 in agonist-induced opioid receptor endocytosis, because coexpression of the negative mutant PLD2, which has intact wild-type PLD2-PX domain, decreased opioid receptor endocytosis, moreover, the inhibition of PA

production from PLD with 1-butanol does not affect function of the PX domain of PLD, but inhibited the agonist-induced opioid receptor endocytosis. In fact the results in present study reveal that the lipase activity of PLD2 and its product PA are crucial for agonist-induced opioid receptor endocytosis.

Having identified PA is required for opioid receptor endocytosis, we asked whether the metabolism of PA could contribute to the function of PLD2 in this process. The PLD-produced PA can be converted into DAG by the family of enzymes known as phosphatidic acid phosphohydrolases (PAPs), which are highly active in vivo (Brindley and Waggoner, 1996; Sciorra and Morris, 1999). Thus, activation of PLD results in the increase of both PA and DAG. The produced DAG can be reconverted to PA via phosphorylation by diacylglycerol kinases (DGKs). The regulation of PA and DAG levels appears to be tightly controlled via the activities of PAP and DGK. Thus inhibition of PAP and DGK can be used to analyze the influence of the conversion from PA to DAG on opioid receptor endocytosis. We found when the PAP activity was inhibited, DPDPE-induced δ-opioid receptor endocytosis was remarkably reduced in HADOR expressing cells. Similarly, inhibition of PAP activity also resulted in strong reduction of DAMGO-induced μ-opioid receptor endocytosis in the cells expressing HAMOR. These experiments indicate that the PA-derived DAG is involved in the function of PLD2 to facilitate agonist-induced opioid receptor endocytosis. If so, the converse inhibition of DGK should increase agonist-induced opioid receptor endocytosis. Indeed the DGK inhibition significantly augmented both DPDPE-induced δ-opioid receptor endocytosis and DAMGO-induced μ-opioid receptor endocytosis. This finding was further supported by addition of the cell-permeable analog of endogenous DAG, DOG (Lucas et al., 2003; Ma et al., 2000). The presence of DOG strongly enhanced both DPDPE-induced δ-opioid receptor endocytosis and DAMGO-induced μ-opioid receptor endocytosis. Collectively, our results reveal that the metabolism from PA to DAG is involved in the role of PLD2 in agonist-induced opioid receptor endocytosis.

But how can the PA-derived DAG regulate agonist-induced opioid receptor endocytosis? DAG is an extensively studied lipid second messenger, and has been shown to activate lipid-dependent kinases such as the classical and most novel PKC families. It may also be further hydrolyzed to monoacylglycerol and related fatty acid by DAG lipase (Amin et al., 1986; Migas and Severson, 1996; Pasquare et al., 2004).

Using two broad-spectrum PKC inhibitors, chelerythrine chloride and calphostin C, we exclude the mediation by PKC for the function of PA-derived DAG in agonist-induced opioid receptor internalization. Inhibition of PKC impeded neither DPDPE-induced δ-opioid receptor endocytosis nor DAMGO-induced μ-opioid receptor endocytosis. Furthermore, inhibition of PKC failed to reduce the effect of DOG in promoting both DPDPE-induced δ-opioid receptor endocytosis and DAMGO-induced μ-opioid receptor endocytosis. Therefore PKC activity is not required for agonist-induced opioid receptor endocytosis. It should be noted that the studies on the specificity of the lipids hydrolyzed and produced in vivo by PLD defined the fatty acid composition of these lipids, indicating that PLD seems to hydrolyze PC with either monounsaturated and/or saturated acyl chains and to form PA of the same diacyl composition (Pettitt et al., 1997; Pettitt et al., 2001). The diacyl composition of the DAG species resulted from PAP dephosphorylation of PA is likewise primarily of the monounsaturated and/or saturated species (Pettitt et al., 1997). The signaling function of PA and DAG are strongly dependent on their fatty acid contents. In the studies of compositions and functions of diacylglycerol and phosphatidate, the more saturated forms of DAG produced from PA have been suggested to have less potency or not to stimulate PKC activity *in vivo* (Hodgkin et al., 1998; Pettitt et al., 1997) and *in vitro* (Marignani et al., 1996). Also PA is only active when it contains predominantly saturated fatty acids (Hodgkin et al., 1998). Though a report also showed that a metabotropic glutamate receptor associated to phospholipase D might influence translocation of protein kinase C subtype ε in a calcium-independent manner (Pastorino et al., 2000). Thus the following discussed effect of DAG may be one of the important functions of the more saturated DAG species derived from PA other than activating PKC.

On the other hand, the hydrolytic products of DAG are not responsible for the role of the produced DAG in agonist-stimulated opioid receptor endocytosis, since the inhibition of DAG lipase by RHC80627 was unable to decrease either δ-opioid receptor endocytosis stimulated by DPDPE or μ-opioid receptor endocytosis stimulate by DAMGO. Moreover, both in the absence and in the presence of DOG, hypertonic sucrose, the inhibitor of clathrin-dependent endocytosis, but not filipin, the inhibitor of caveolae-dependent endocytosis, blocked agonist-induced opioid receptor endocytosis, showing that the enhancement of agonist-induced opioid receptor endocytosis by the PA-derived DAG is mediated via clathrin-dependent pathway but not caveolae-

dependent pathway.

In this study, evidence for a functional link between PLD2 activity and p38 MAP kinase activation was provided. P38 MAPK is a member of the MAP kinase family, which can regulate numerous cellular responses, for example, stress related transcription and cell cycle regulation (Ono and Han, 2000; Takeda and Ichijo, 2002). The activation of p38 MAPK requires its phosphorylation. Recently p38 MAP kinase has been identified to have the ability to regulate receptor endocytic trafficking (Cavalli et al., 2001; Huang et al., 2004; Mace et al., 2005; McLaughlin et al., 2006; Samuvel et al., 2005; Vergarajauregui et al., 2006). The function of P38 in endocytosis is tightly related to the small GTPase Rab5, one of the key regulators of clathrin-dependent endocytosis. Rab5 coordinates multiple processes, such as the formation of clathrin-coated vesicles, their fusion with early endosomes and homotypic early endosome fusion, as well as motility of endosomes (Seachrist and Ferguson, 2003; Zerial and McBride, 2001). Rab5 cycles between GTP- and GDP-bound states (Martinez and Goud, 1998). In addition, it also cycles between membrane-bound and cytosolic states, and this cycling requires guanyl-nucleotide dissociation inhibitor (GDI) (Wu et al., 1996). GDI functions as a vehicle. It extracts the GDP-bound Rab proteins from membranes and forms a cytosolic GDI:Rab complex, and it delivers Rab proteins to the appropriate target membrane where Rab proteins are reloaded by a GDI displacement factor (Dirac-Svejstrup et al., 1997; Pfeffer and Aivazian, 2004). The activation of p38 MAPK has been identified to accelerate endocytosis by stimulating the activity of GDI to form the GDI:Rab5 complex, enhancing its activity in retrieving Rab5 from the endosomal membranes (Cavalli et al., 2001; Huang et al., 2004). For the μ-opioid receptor it has been found that p38 MAPK modulates endocytosis by phosphorylating the Rab5 effectors EEA1 (early endosome antigen 1) and Rabenosyn-5 regulating the recruitment of them to membranes (Mace et al., 2005) (Fig. 15B). Stimulation of the μ-opioid receptor with DAMGO rapidly induced an activation of p38 MAPK, in contrast, morphine, which is not able to trigger μ-opioid receptor endocytosis, was not able to induce P38 MAPK activation. Moreover, P38 MAPK activation was shown to be required for μ-opioid receptor endocytosis. The inhibition of p38 MAPK by inhibitor SB203580 impaired DAMGO-induced μ-opioid receptor endocytosis (Mace et al., 2005). Consistent with this, we also found that DPDPE-induced δ-opioid receptor endocytosis involves p38 MAPK activation. We

further uncovered a functional relation between p38 MAPK activation and PLD2 activity in both μ-opioid receptor and δ-opioid receptor signaling (Fig. 15A). Firstly, by inhibition of PLD2 activity or overexpression of PLD2, PLD2 activity was found to be required for opioid receptor-mediated p38 activation. Secondly, by inhibition of the enzyme PAP and heterologous administration of DAG analog, it was identified that conversion from PA to DAG is involved in opioid receptor-mediated p38 MAPK activation. Combining together, these data indicate that the role of PLD2 in regulating agonist-induced opioid receptor endocytosis involves p38 MAPK. Like all MAP kinases, p38 MPK is activated by the MAP kinase kinases (MKKs). MKK6, MKK3, MKK4 and MKK7 have been reported to activate all or part of the p38 MAPK isoforms (Hu et al., 1999; Jiang et al., 1997; Keesler et al., 1998). The further upstream activators of MKK/p38 pathway are further diversified. Several MKK kinases (MAP3K) including MTK1, MLK2, MLK3, DLK, ASK1 and TAK1, as well as p21 activated kinases (PAKs) have been demonstrated to cause p38 activation (Ono and Han, 2000). The further upstream activators are not very clear so far. Some small GTP-binding proteins, like Rac and Cdc42, were identified as potential regulators of the p38 pathway (Bagrodia et al., 1995; Zhang et al., 1995). Others like Rap1 are also reported to be able to activate p38 MAPK (Huang et al., 2004). Concerning the mediators between DAG and p38 signaling, some reports described that activation of PKC by DAG is involved in p38 MAPK activation in respective cell response research (Shimizu et al., 1999; Tanaka et al., 2003). In contrast, in the present study the PA-derived DAG activated p38 MAPK in a PKC independent manner, since inhibition of PKC did not diminish p38 MAPK activation. We speculate that there might be some other mediators which contain C-1 domain linking DAG to upstream of p38 signaling. For example, one potential molecule is protein kinase D (PKD). PKD is a novel DAG-dependent kinase (Baron and Malhotra, 2002; Yang and Kazanietz, 2003). In fact PKC-independent PKD activation has been reported to be involved in p38 MAPK activation induced by bone morphogenetic protein 2 (Lemonnier et al., 2004). One of the other possible candidates is RasGRP, a Ras guanyl nucleotide-releasing protein with DAG-binding motif. Ras can be activated by DAG through RasGRP directly (Ebinu et al., 1998). On the other hand, Ras has been reported to be able to activate p38 MAPK signaling (Norman et al., 2004; Shin et al., 2005; Zhu et al., 2001), though it is generally believed as an upstream activator of ERK MAPK signaling.

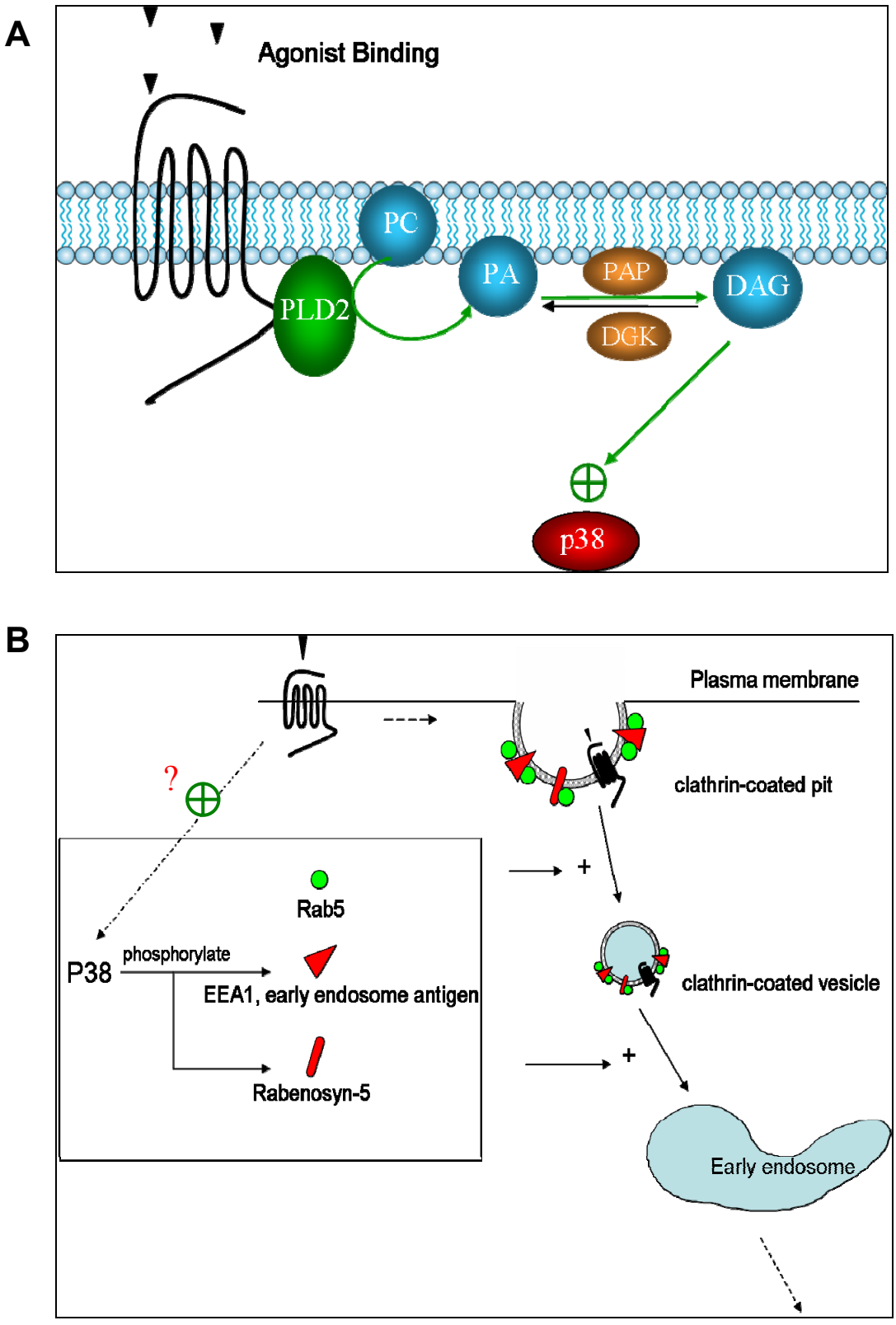


Fig.15. A, Opioid receptors mediate p38 MAPK activation through PLD2 in a PKC-independent way (drawn based on the present study). B, Phosphorylation of EEA1 by p38 MAP kinase regulates opioid receptor endocytosis (drawn according to Mace et al., 2005).

Early evidences showed that agonist-induced opioid receptor internalization is mediated by clathrin-coated vesicles (Chu et al., 1997; Gaudriault et al., 1997; Hasbi et al., 2000; Keith et al., 1996; Law and Loh, 1999). This process is regulated by a highly conserved mechanism, involving phosphorylation of the agonist-bound receptors by G protein-coupled receptor kinases and association of the receptors with β-arrestins. Binding of β-arrestin leads to physical separation of the receptors from the G proteins. The further interaction of β-arrestin with AP-2, the clathrin adaptor protein that seems to be involved in nearly all stages of clathrin-coated vesicle formation, recruits the receptors to clathrin-coated pits. Meanwhile, based on this study and our recent report (Koch et al., 2003), the stimulation of opioid receptors by opioid agonists leads to the activation of associated PLD2, regulating the receptor internalization. PLD2 hydrolyzes PC in plasma membrane to generate the lipid PA. Then the further conversion from PA to the DAG results in the activation of p38 MAPK, which could modulate opioid receptor endocytosis by promoting the function of Rab5 (Cavalli et al., 2001; Huang et al., 2004; Mace et al., 2005), regulating the subsequent dynamin-dependent fission and formation of clathrin-coated vesicles from the plasma membrane as well as their fusion with early endosomes. In addition, some other PLD2 functions which have been demonstrated may also related to opioid receptor endocytosis to some extents. PA is known to extensively activate type I phosphoinositides-4-phosphate 5-kinase in mammalian cells, leading to an increased phopsphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P2] level of the membrane (Jones et al., 2000; Ling et al., 2002). Many proteins related to clathrin-dependent endocytosis such as clathrin, dynamin, and proteins of the AP-2 adapter complex contain domains which mediate their binding to PtdIns(4,5)P2-containing membranes (Mousavi et al., 2004). Therefore opioid receptor mediated PLD2 activation might also favor the recruitment of these endocytosis-related proteins to the local membranes. In addition, an increase of in the level of PA after PLD activation might cause a change of physical properties, e.g. charge and pH, of cellular membranes, thereby facilitating vesicle formation.

Taking all these data in this study together, we can conclude that PLD2 activity regulates agonist-induced μ- and δ-opioid receptor endocytosis and the conversion from PA to DAG is involved in this process, which may further be mediated by P38 MAPK.

6. Summary

Opioids are classically associated with effects such as analgesia, respiratory depression, and addiction, which are mediated via interaction with specific G protein-coupled opioid receptors. Opioid receptor endocytosis which occurs after agonist exposure is one important regulation process of opioid signaling. Our group has previously demonstrated that μ-opioid receptor (MOR) interacts with phospholipase D2 (PLD2) and that MOR-mediated activation of PLD2 is essential for the induction of MOR endocytosis. Here we found that delta-opioid receptor (DOR) also physiologically interacts with and agonist-dependently activates PLD2 in a PKC-independent manner in transfected HEK293 cells. As revealed by quantitative internalization assays and confocal microscopy studies, coexpression of PLD2 strongly enhanced the extent and rate of agonist-induced DOR endocytosis, whereas inhibition of PLD2 by expression of a catalytically inactive mutant PLD2 (K758R) significantly attenuated DPDPE-induced DOR endocytosis. Similarly, the inhibition of PLD2-mediated phosphatidic acid (PA) synthesis with 1-butanol blocked DOR endocytosis. These observations suggest that PLD2 activity is required for agonist-induced DOR endocytosis and that PA plays a crucial role. PA and diacylglycerol (DAG) can be converted to each other by PA phosphohydrolase and DAG kinase. Inhibition of PA phosphohydrolase, which inhibits the dephosphorylation of PA to DAG, attenuated both DPDPE-induced DOR endocytosis and DAMGO-induced MOR endocytosis. Conversely, inhibition of DAG kinase increased agonist-induced endocytosis of both receptors. Furthermore, addition of a DAG analog DOG which is a synthetic cell-permeable DAG with short chain fatty acids remarkably augmented agonist-stimulated DOR and MOR endocytosis, whereas inhibition of protein kinase C (PKC) did not influence agonist-induced opioid receptor endocytosis. These findings indicate that PA-derived DAG is involved in agonist-induced opioid-receptor endocytosis in a PKC-independent way. We also revealed that PLD2 activity and the subsequent PA-derived DAG are required for opioid receptor-mediated p38 MAPK activation which is involved in DOR and MOR endocytosis.

Taken together, PLD2 activity regulates agonist-induced δ- and μ-opioid receptor endocytosis, which involves the conversion of its product PA to DAG, and the following activation of P38 kinase.

7. Reference

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8. Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AC	Adenylate cyclase
ANOVA	Analysis of variance
ATCC	American Type Culture Collection (Rockville, MD)
Bmax	the number of maximal [3H] Naltrindol binding site
CaM	CaM Ca2+/calmodulin
cAMP	Adenosine 3',5'-cylic-monophosphate
cDNA	Complemetary deoxyribonucleic acid
CHO	Chinese hamster ovary
CNS	Central nervous system
DAG	diacylglycerol
DADLE	[D-Ala2, D-Leu5] enkephalin
DAMGO	[D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin,
DGK	DAG kinase
DGL	DAG lipase
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DOG	1, 2-dioctanoyl-sn-glycerol, a synthetic cell permeanle DAG of short chain fatty acid
DOR	The delta-opioid receptor
DPDPE	[D-Pen2, D-Pen5] enkephalin, Tyr-D-Pen-Gly-Phe-D-PenOH
DPX	Distrene, dibutyl Phthalate, Xylene
DSLET	D-Ser2, Leu5] enkephalin-Thr6
DSP	Dithiobis-(succinimi-dylpropionate)
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EEA	early endosome antigen
ELISA	Enzyme-linked immunoabsorbent assay
ERK	extracellular-signal-regulated kinases
FCS	Fetal calf serum

GAP	GTPase-activating protein
GDI	GDP dissociation inhibitor
G-protein	guanine nucletide binding protein
G418	sulfate Geneticin, a 2-deoxystreptamine antibiotic produced by Microspora rhodorangea
GASP	G protein-coupled receptor-associated sorting protein
GDP	Guanosine 5'-diphosphate
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	Guanosine 5'-triphosphate
HA	Human influenza virus hemagglutinin (YPYDVPDYA)
HA-DOR	Amino-terminally HA-tagged delta-opioid receptor
HA-MOR	Amino-terminally HA-tagged mu-opioid receptor
HEK293 cells	Human embryonic kidney 293 cells
HEPES	N-2-hydroxyethylpiperazine-N¢-2-ethanesulfonic acid
IP	Immunoprecipitation
JNK	the Jun N-terminal kinases
kb	Kilobase
K_D	Dissociation constant
kD	kiloDalton
MAG	monoacylglycerol
MAPK	Mitogen activated protein kinase
MOR1	The μ-opioid receptor isoform 1
Myc	Epitope tag (sequence: MASMQKLISEEDL)
NG108-15 cell	mouse/rat, neuroblastoma x glioma hybrid cell
nPLD2	Negative mutant of phospholipase D subtype 2
OD	optical density
ORL	orphan opioid-like receptor
PA	phosphatidic acid
PAP	PA (or phosphatidate) Phosphohydrolase
PBS	Phosphate-buffered saline

PC	phosphatidylcholine
PCR	Polymerase chain reaction
PEt	phosphatidylethanol
PLC	Phospholipase C
PLD	Phospholipase D
PKA	Protein kinase A
PKC	Protein kinae C
PKD	Protein kinase D
PMA	Phorbol-12- myristate-13- acetate
PPH	Same as PAP
Ptdbutanol	phosphatidylbutanol
PtdIns(4,5)P2	phopsphatidylinositol-4,5-bisphosphate
PX domain	phox homologous domain
RIPA buffer	Radioimmune precipitation buffer
rpm	rounds per minte
RT	Room temperature
RT-PCR	Reverse transcription-PCR
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEM	Stand error of the mean
TPBS	Tris/phosphate-buffered saline

9 Appendix

9.1 Curriculum vitae

Name:	Liquan Yang
Gender:	Male
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Education & Experience:

Oct. 2003—now	Ph.D. student of biology Department of Pharmacology and Toxicology, Medical Faculty, Otto-von-Guericke-University, Magdeburg, Germany. Supported by DAAD
Jun. 2003—Sep. 2003	DAAD scholarship-holder training interDaF e.V. am Herder-Institut der Universität Leipzig
Aug. 2001—May 2003	Assistant researcher Department of cellular and molecular biology, Shanghai Institute of Biological Products Shanghai, China
Sep. 1998—Jul. 2001	Master student of science Shanghai Institute of Biological Products Shanghai, China
Jul. 1996—Aug. 1998	Assistant researcher Chinese Research Center for Endemic Disease Control Harbin, China
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Liquan Yang

9.2 Publications and presentation

9.2.1 Publications

Yang LQ, et al. Role of phospholipase D2-derived phospatidic acid and diacylglycerol in agonist-induced μ- and δ-opioid receptor endocytosis. 2007, in review (J Bio Chem).

Wu DF, **Yang LQ**, Goschke A, Stumm RK, Liang YJ, Höllt V, and Koch T. Internalization and desensitization of the cannabinoid type 1 receptor. 2007, in revision (J Neurochem).

Liang YJ, Wu DF, **Yang LQ**, Höllt V, Koch T. Interaction of the mu-opioid receptor with synaptophysin influences receptor trafficking and signaling. Mol Pharmacol. 2007 Jan;71(1):123-31

Koch T, Wu DF, **Yang LQ**, Brandenburg LO, Höllt V. Role of phospholipase D2 in the agonist-induced and constitutive endocytosis of G-protein coupled receptors. J Neurochem. 2006 Apr;97(2):365-72.

Yang LQ, Koch T, Wu DF, Goschke A, Höllt V. Phospholipase D2 is involved in agonist-induced delta-opioid receptor endocytosis. Acta Pharmacologica Sinca. 2006, 27 (supplement): 364

9.2.2 Conference presentation and poster

Yang LQ, Wu DF, Goschke A, Höllt V, Koch T. Phospholipase D2-phosphatidic acid-diacylglycerol pathway is involved in agonist-induced delta-opioid receptor endocytosis. Poster on the 15th World Congress of Pharmacology. Beijing, China. 2006

Wu DF, Liang Y, **Yang LQ**, Höllt V, Koch T. Interaction of the mu-opioid receptor with synaptophysin influences receptor intemalization and signaling. Poster on the 37th International Narcotic Research Conference. St. Paul, Minnesota, USA.2006

Yang LQ, Koch T, Wu DF, Goschke V, Höllt V. Role of phospholipase D2 in agonist-induced δ-opioid receptor endocytosis. Oral presentation on the 47th spring meeting, the German society of experimental and clinical pharmacology and toxicology. Mainz, Germany. 2006

Wu DF, Koch T, **Yang LQ**, and Höllt V. The phospholipase D2 is involved in the endocytosis of cannabinoid receptor CB1. Oral presentation on the 47th spring meeting, the German society of experimental and clinical pharmacology and toxicology. Mainz, Germany. 2006

Koch T, Wu D, **Yang L**, Brandenburg L.O., Höllt V. Regulation of G-protein coupled receptor endocytosis by phospholipase D2. Poster on the 36th International Narcotic Research Conference. Annapolis Maryland, USA.2005

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10. Zusammenfassung

Zu den klassischen Opiatwirkungen zählen neben der Analgesie auch die Atemdepression und die Suchtauslösung. Opiate vermitteln ihre Effekte durch die Interaktion mit spezifischen G Protein-gekoppelten Opioidrezeptoren. Die Endozytose von Opioidrezeptoren nach Agonistenbehandlung gehört zu den wichtigsten regulatorischen Prozessen der Signaltransduktion von Opioiden. Unsere Arbeitsgruppe konnte kürzlich zeigen, dass der μ-Opioidrezeptor (MOR) mit der Phospholipase D2 (PLD2) interagiert und die MOR-vermittelte Aktivierung der PLD2 für die Induktion der MOR-Endozytose essentiell ist. In der vorliegenden Arbeit fanden wir heraus, dass der δ-Opioidrezeptor (DOR) ebenfalls mit der PLD2 interagiert und der DOR-Agonist DPDPE die PLD2 in HEK293 Zellen über einen PKC-unabhängigen Weg aktivieren kann. Quantitiative Internalisierungsassays und konfokalmikroskopische Studien zeigten, dass eine PLD2-Koexpression das Ausmass und die Geschwindigkeit der agonist-induzierten DOR-Endozytose erhöhte, wohingegen eine Hemmung der PLD2, mittels Expression einer katalytisch inaktiven PLD2-Mutante (PLD2-K758R), die DPDPE-induzierte DOR-Endozytose deutlich senkte. Ebenso konnte die DOR-Endozytose durch die Hemmung der PLD2-vermittelten Phosphatidsäuresynthese mittels 1-Butanol blockiert werden. Diese Beobachtungen lassen vermuten, dass die PLD2-Aktivität für die agonist-induzierte DOR-Endozytose erforderlich ist und dass Phosphatidsäure (PA) dabei eine wichtige Rolle spielt. PA kann durch die PA-Phosphohydrolase in DAG überführt und DAG durch die DAG Kinase wieder in PA umgewandelt werden. Die Inhibition der PA-Phosphohydrolase schwächte sowohl die DPDPE-induzierte DOR Endozytose als auch die DAMGO-induzierte MOR-Endozytose ab. Demgegenüber führte die Inhibition der DAG Kinase zu einem Anstieg der DPDPE-induzierten DOR-Endozytose bzw. DAMGO-induzierten MOR-Endozytose. Darüber hinaus führte die Zugabe von DOG, einem synthetischen und aufgrund von kürzeren Fettsäuren zellpermeablen Analogon des DAG, zu einem deutlichen Anstieg der agonist-vermittelten DOR und MOR Endozytose, wohingegen eine Hemmung der Proteinkinase C (PKC) die agonist-induizierte Opioidrezeptorendozytose nicht beeinflusste. Diese Ergebnisse deuten darauf hin, dass das aus PA synthetisierte DAG an der agonist-induzierten DOR- und MOR-Endozytose über einen PKC-unabhängigen Weg beteiligt ist. Wir konnten ebenfalls zeigen, dass die PLD2-Aktivität und die nachfolgende Bildung von DAG aus PA für die

opiatrezeptor-vermittelte Aktivierung der p38 MAPK erforderlich ist, welche an der Endozytose des DOR und MOR beteiligt ist.

Zusammenfassend kann man sagen, dass die PLD2-Aktivität die agonisten-induzierte Endozytose des δ- und μ-Opioidrezeptors reguliert. An diesem Prozess ist das aus dem PA gebildete DAG und im Weiteren die Aktivierung der p38 MAPK beteiligt.