G Protein-Coupled Receptor Signaling to Phospholipase D1 Mediated by G_{12}-Type G Proteins, LIM-kinase and Cofilin

Inaugural-Dissertation

zum
Erwerb des Doktorgrades
Dr. rer. nat.
des Fachbereiches
Bio- und Geowissenschaften, Landschaftsarchitektur
an der Universität Essen

vorgelegt von
Li Han

aus Binzhou (Shan Dong, V.R. China)

November 2002

Die der vorliegenden Arbeit zugrundeliegenden Untersuchungen wurden am Institut für Pharmakologie des Universitätsklinikums Essen durchgeführt.

1. Gutachter:	Prof. Dr. K. H. Jakobs
2. Gutachter:	Prof. Dr. H. Esche
3. Gutachter:	Prof. Dr. H. Grunz
Vorsitzender des Prüfungsausschusses:	Prof. Dr. Dr. H. de Groot
Tag der mündlichen Prüfung:	12.03.2003

Index

1. Abbreviations

ARF	ADP-ribosylation factor
BCA	2,2'-Bischinoline-4,4'-dicarbonic acid
BSA	Bovine serum albumin
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNase	Deoxyribonuclease
dNTP	2'-Deoxyribonucleoside-5'-triphosphate
DTT	1,4-Dithiothreitol
EGF	Epidermal growth factor
FCS	Fetal calf serum
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
G protein	Guanine nucleotide-binding protein
GST	Glutathione *S*-transferase
GTPγS	Guanosine-5'-O-[γ-thio]-triphosphate
HA	Influenza hemagglutinin epitope tag
HBSS	Hank's balanced salt solution
HeBS	HEPES-buffered solution
HEK-293 cells	Human embryonic kidney 293 cells
HEPES	*N*-(2-Hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid
IP_3	Inositol-1,4,5-trisphosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside
LPA	Lysophosphatidic acid
mAChR	Muscarinic acetylcholine receptor
MOI	Multiplicity of infection
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PH	Pleckstrin homology
PIP 5-kinase	Phosphoinositide-4-phosphate 5-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein kinase C

PKN	Protein kinase N
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethyl sulfonylfluoride
PtdCho	Phosphatidylcholine
PtdEtOH	Phosphatidylethanol
PTX	Pertussis toxin
PX	Phox homology
RBD	Rho-binding domain of Rhotekin
RGS	Regulators of G protein signaling
RNase	Ribonuclease
SDS	Sodium dodecylsulfate
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TE	Tris-EDTA
TEMED	*N*,*N*,*N*',*N*'-Tetramethylethylenediamine
TNM-FH	*Trichoplusiani* nutrient medium-formulation hink
Tris	Tris-(hydroxymethyl)-aminomethane

2. Introduction

2.1 Phospholipase D and signal transduction

The hydrolysis of cellular phospholipids leads to the formation of various bioactive lipid mediators, acting either as extracellular signaling molecules or as intracellular second messengers. A well-known second messenger-forming system involving phospholipid hydrolysis is the stimulation of phosphoinositide-specific phospholipase C (PLC) isoforms, (Rhee, 2001). Upon activation by membrane receptors, these PLC enzymes hydrolyze the membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP_2), and thereby generate the two second messengers, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3), leading to the activation of several protein kinase C (PKC) isoforms and the release of Ca^{2+} from intracellular stores, respectively. It is now well established that PLC stimulation plays a major role in many early and late cellular responses to receptor activation, including smooth muscle contraction, secretion, neuronal signaling as well as cell growth and differentiation (Clapham, 1995; Nishizuka, 1995; Newton, 1997; Berridge *et al.*, 2000; Battaini, 2001).

In 1948, Hanah and Chaikoff were the first to discover phospholipase D (PLD) as a distinct, phospholipid-specific phosphodiesterase activity in cabbage leaves. Their pioneering work indicated that PLD hydrolyzes phosphatidylcholine to phosphatidic acid (PA) and choline (Figs. 1 and 2). However, only in the mid-1980s, the fundamental discovery that PLD is rapidly and dramatically activated in response to extracellular stimuli in cultured animal cells has brought PLD signaling to the very forefront of current biological and biomedical research (Exton, 1999; Liscovitch *et al.*, 2000; Cockcroft, 2001; Steed & Cow, 2001; Exton, 2002). Thus, PLD was classified, together with phosphoinositide-specific PLC isoforms, as a *bona fide* signal-activated phospholipase, generating biologically active products which were expected to play important roles in the regulation of cell function and cell fate. Meanwhile, phosphatidylcholine-hydrolyzing PLD has been identified in bacteria, protozoa, fungi, plants and animals, and, due to this widespread distribution, is generally assumed to be involved in the regulation of fundamental cellular functions. Indeed, activation of PLD by a vast number of membrane receptors has now been established to modulate a wide array of cellular responses, such as calcium mobilization, secretion, superoxide production, endocytosis, exocytosis, vesicle trafficking, glucose transport, rearrangements of the actin cytoskeleton, mitogenesis and apoptosis (Daniel *et al.*, 1999; Jones *et al.*, 1999a; McPhail *et al.*, 1999; Venable *et al.*, 1999; Nakashima & Nozawa, 1999; Liscovitch *et al.*, 2000; Exton, 2002; Cummings *et al.*, 2002a; Joseph *et al.*, 2002).

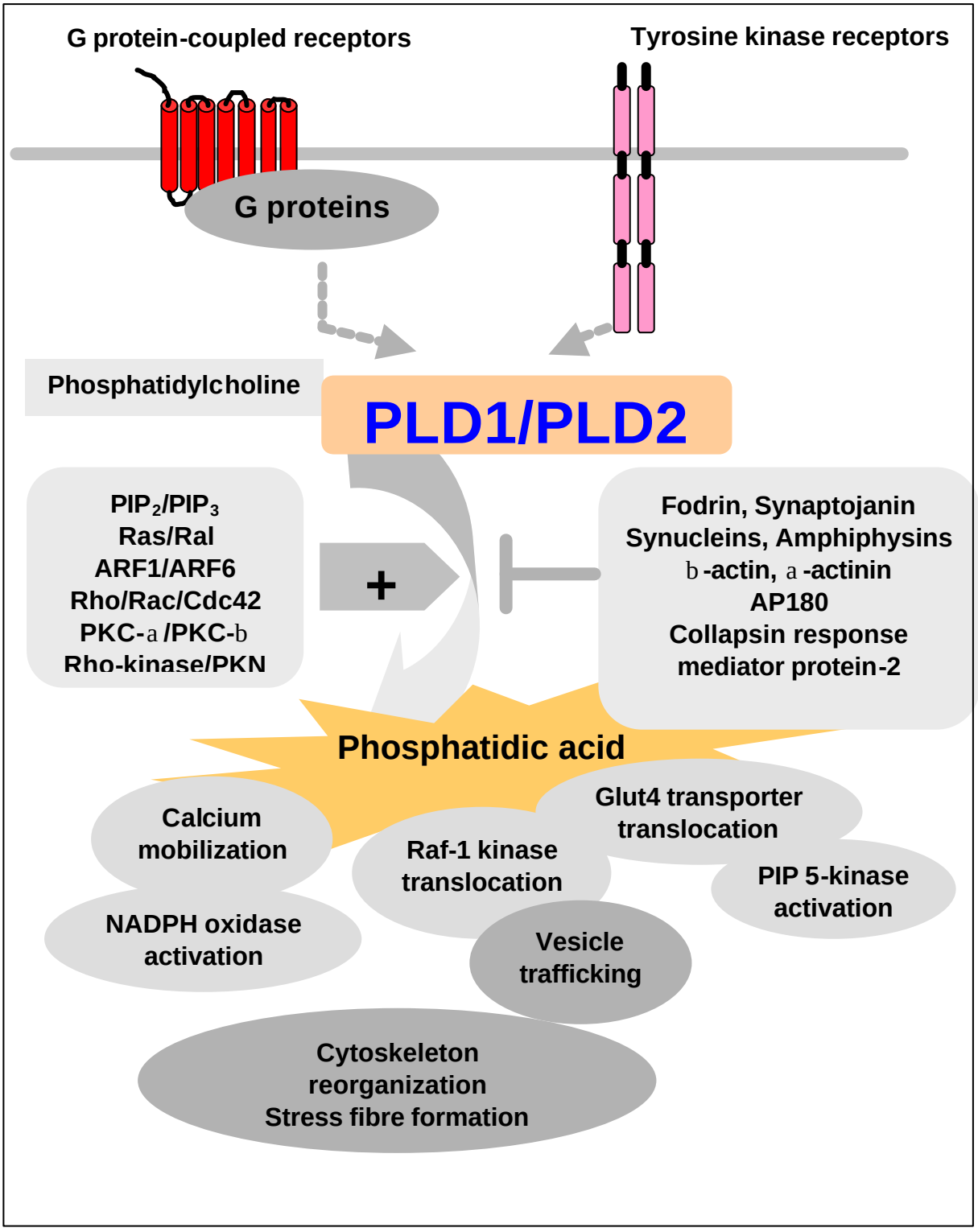


Fig. 1: Regulation of PLD enzymes and cellular roles of PA. For further explanation, see text.

2.2 Cellular roles of phosphatidic acid

The regulation of cellular responses by PLD is likely mediated by its immediate reaction product PA (English, 1996). PA has been demonstrated to bind to various cellular proteins, such as Raf-1 kinase, protein phosphatase 1 and the mammalian target of rapamycin, probably affecting both cellular localization and activity of these PA-binding proteins (Ghosh *et al.*, 1996; Kishikawa *et al.*, 1999; Rizzo *et al.*, 2000; Fang *et al.*, 2001; Manifava *et al.*, 2001; Jones & Hannun, 2002). Furthermore, PA can be metabolized to DAG by phosphatidate phosphohydrolase and to lysophosphatidic acid (LPA) by phospholipase A_2 (Dennis, 1994; Sciorra & Morris, 1999). While PA-derived DAG is believed to stimulate a specific subset of PKC isoforms (Pettitt *et al.*, 1997; Hodgkin *et al.*, 1998), LPA acts as an extracellular signaling molecule, activating specific heptahelical receptors coupled to heterotrimeric guanine nucleotide-binding proteins (G proteins) (Contos *et al.*, 2000; Fukushima *et al.*, 2001). Thus, due to the formation of various bioactive lipid mediators (PA, DAG and LPA), regulation of cellular functions by PLD can involve complex signaling networks.

A role for PLD in actin organization has initially been observed upon addition of bacterial PLD from *Streptomyces chromofuscus* and exogenous PA to IIC9 fibroblasts (Ha & Exton, 1993). As exogenous PA can be metabolized to LPA (van Dijk *et al.*, 1998), the question of whether the effects are directly mediated by PA or *via* generation of LPA is still not definitively answered. Primary alcohols have proven useful tools to test the involvement of PLD in cellular processes. These alcohols, such as ethanol and butan-1-ol, decrease PA formation by shunting phosphatidyl moieties into biologically inactive phosphatidylalcohols (Fig. 2).For example, treatment of porcine aortic endothelial cells as well as human airway epithelial cells with butan-1-ol reduced agonist-stimulated formation of actin stress fibres (Cross *et al.*, 1996; Porcelli *et al.*, 2002). Primary alcohols further inhibited agonist-induced secretion of interleukin-8 from and activation of extracellular signal-regulated kinase in human bronchial epithelial cells, calcium mobilization in U937 cells, activation of p38 mitogen-activated protein kinase in HL-60 cells, phosphorylation of $p22^{phox}$ [a subunit of flavocytochrome b_{558} of the superoxide-generating NADPH oxidase] in human neutrophils, activation of phosphoinositide 3-kinase and Akt in CHO cells, activation of type Iα phosphatidyl-4-phosphate (PIP) 5-kinase in porcine aortic endothelial cells, secretion of metalloproteinase-9 from human fibrosarcoma cells, release of noradrenaline from chromaffin cells and extracellular signal-regulated kinase-driven mitogenesis in NIH 3T3 cells (Caumont *et al.*, 1998; Melendez *et al.*, 1998; Williger *et al.*, 1999a; Caumont *et al.*, 2000; Jones *et al.*, 2000; Regier *et*

al., 2000; Banno *et al.*, 2001; Bechoua & Daniel, 2001; Hong *et al.*, 2001; Cummings *et al.*, 2002b; Wang *et al.*, 2002). Inhibition of PA formation also interfered with the recruitment of the coatomer complex during formation of transport vesicles (Ktistakis *et al.*, 1996; Bi *et al.*, 1997; Chen *et al.*, 1997; Jones *et al.*, 1999b), but the importance of PLD in the budding of COP1-coated vesicles has been questioned by more recent findings (Kuai *et al.*, 2000). Taken together, these findings strongly support an essential role for PLD in a variety of cellular functions.

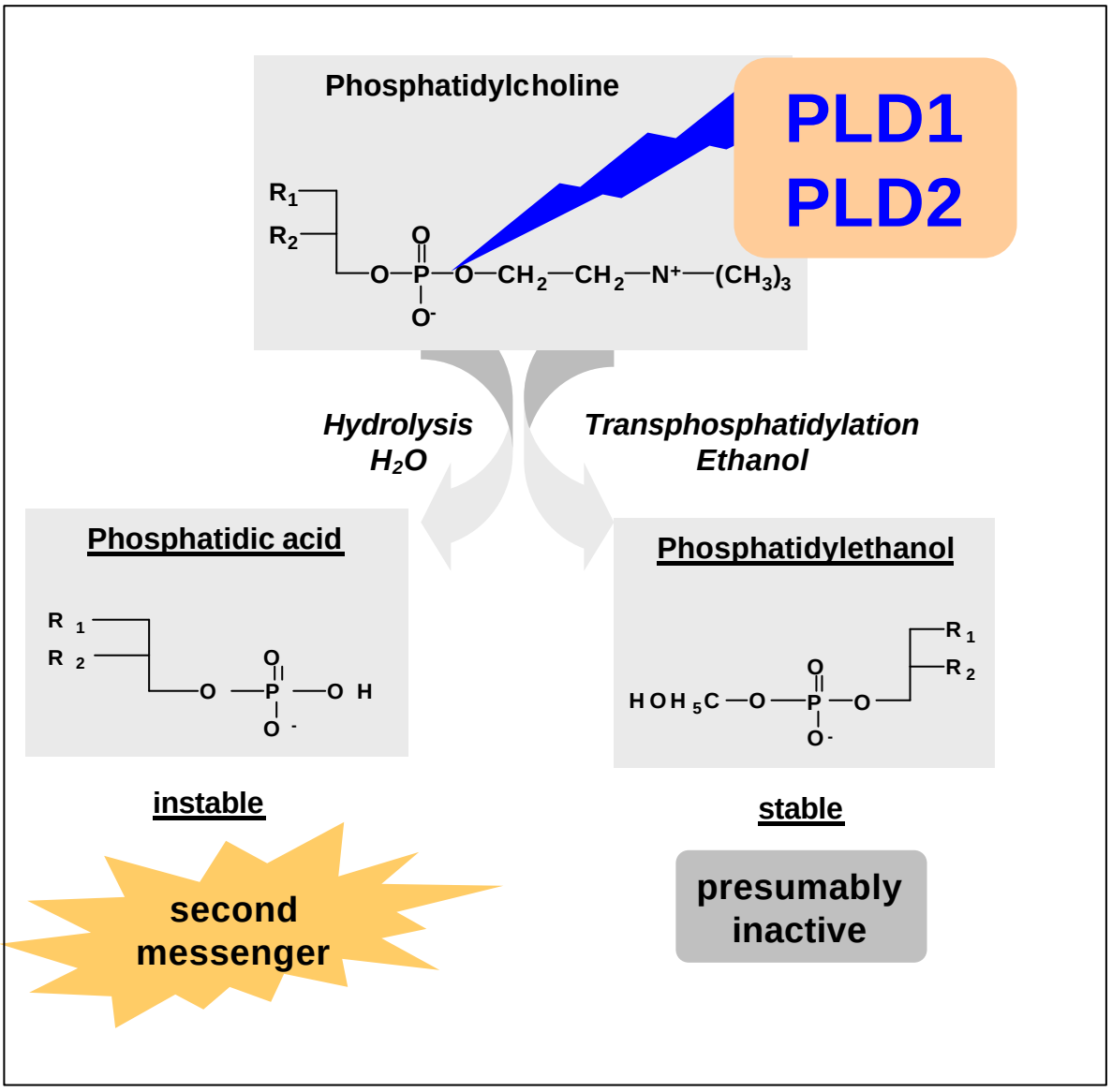


Fig. 2: Transphosphatidylation reaction catalyzed by PLD enzymes. For further explan-ation, see text.

2.3 PLD isoforms

Several PLD genes have recently been cloned, and a PLD gene superfamily, characterized by a number of structural domains and sequence motifs, has emerged. The PLD superfamily includes bacterial phosphatidylserine and cardiolipin synthases, bacterial endonucleases, pox virus envelope proteins and a murine toxin from *Yersenia pestis* (Koonin, 1996; Ponting & Kerr, 1996; Stuckey & Dixon, 1999; Frohman *et al.*, 1999; Exton, 1999; Leiros *et al.*, 2000; Liscovitch *et al.*, 2000; Exton, 2002). In mammalian cells, two PLD isoforms, PLD1 and PLD2, have been identified as mediators of cellular signaling. Additional PLD activities have been described, such as an oleate-activated PLD and phosphoinositide-specific PLD enzymes, but their contribution to the regulation of cellular signaling is not well understood (Ching *et al.*, 1999; Liscovitch *et al.*, 2000; Cockcroft, 2001; Exton, 2002; Cummings *et al.*, 2002a).

Mammalian PLD1 cDNA, encoding a 1074-amino acid protein, was initially cloned from a HeLa cell cDNA libary, whereas the splice variant PLD1b, encoding a 1036-amino acid protein, was identified by quantitative reverse transcriptase PCR in HL-60 cells (Hammond *et al.*, 1995; Morris *et al.*, 1996; Hammond *et al.*, 1997; Colley *et al.*, 1997a; Sung *et al.*, 1999a). The identification of related sequences in expressed sequence databases resulted in the cloning of PLD2, encoding a 933-amino acid protein and sharing 55 % identity to PLD1 (Colley *et al.*, 1997b; Redina & Frohman, 1998; Sung *et al.*, 1999b). PLD1 and PLD2 contain the four highly conserved domains I-IV and other motifs (see Fig. 14). The domains I and IV bear NH_2- and COOH-terminally located *HKD* motifs, which have been shown to be essential for catalytic activity (Sung *et al.*, 1997), whereas conserved regions in domain III and the COOH-terminus are required to gain full activation of PLD enzymes (Sung *et al.*, 1999a,b; Xie *et al.*, 2000). At the NH_2-terminus, PLD1 and PLD2 comprise a pleckstrin homology (PH) domain as well as a phox homology (PX) domain. PH and PX domains modulate the intracellular localization and activity state of a large number of proteins involved in signal transduction, apparently due to interaction with phosphoinositides, especially PIP_2 and phosphatidylinositol-3,4,5-trisphosphate (PIP_3) (Kavran *et al.*, 1998; Hirata *et al.*, 1998; Teruel & Meyer, 2000; Cullen *et al.*, 2001; Sato *et al.*, 2001; Xu *et al.*, 2001). These phosphoinositide-binding motifs also modulate the membrane association and activity of PLD enzymes (Hodgkin *et al.*, 2000; Sciorra *et al.*, 1999). PLD1, but not PLD2, contains a 116-amino acid loop, and it has been proposed that this sequence may mediate interaction of PLD1 with negative regulatory elements (Sung *et al.*, 1999a,b). In addition, specific direct interaction sites for several signaling components have been identified on PLD enzymes, which may contribute to enzyme

regulation (see below; Exton, 1999; Cockcroft, 2001; Liscovitch *et al.*, 2001; Exton, 2002; Cummings *et al.*, 2002a). Based on this molecular analysis, catalytically inactive mutants have very recently been generated, K898R PLD1 and K758R PLD2, respectively, which have been successfully used to further reveal the physiological role of PLD enzymes in mammalian cells. Using this approach, both PLD1 and PLD2 were shown to be required for endocytosis of the epidermal growth factor (EGF) receptor in 3Y1 rat fibroblasts (Shen *et al.*, 2001). Furthermore, PLD2 was found to be essential for apical constitutive protein transit, whereas PLD1 was implicated in agonist-triggered apical secretion in HT29-cl19A cells (Denmat-Ouisse *et al.*, 2001). Microinjection of catalytically inactive PLD1 in *Aplysia* neurons reduced the release of acetylcholine (Humeau *et al.*, 2001), and expression of catalytically inactive PLD1 largely reduced catecholamine secretion from stimulated chromaffin and PC12 cells (Vitale *et al.*, 2001). Finally, expression of catalytically inactive PLD1 reduced agonist-induced formation of actin stress fibers in Rat-2 fibroblasts and COS-7 cells (Kam & Exton, 2001). Thus, the use of catalytically inactive PLD mutants represents an attractive tool to study the physiological importance of PLD enzymes in various cell types. However, in many mammalian cells, specifically in primary cultured cells, e.g. cardiomyocytes, this approach is hampered due to low transfection efficiencies.

2.4 Subcellular localization of PLD isoforms

The subcellular localization of PLD isoforms was studied by enzyme activity analysis and Western blotting of subcellular fractions, immunochemistry and expression of epitope-tagged PLD enzymes in several cell types. PLD was found at the plasma membrane as well as in intracellular compartments, including the endoplasmic reticulum, the Golgi compartment, endosomes and lysosomes (Cockcroft, 2001; Liscovitch *et al.*, 2001; Exton, 2002). Using immunofluorescence, immunogold electron microscopy and subcellular fractionation, endogenous PLD1 was found to primarily localize to the Golgi apparatus in GH_3 pituitary and NRK kidney cells (Freyberg *et al.*, 2001). Other studies have localized PLD1 to vesicular structures in the perinuclear region, to the plasma membrane, Golgi or endosomes (Colley *et al.*, 1997a; Brown *et al.*, 1998; Kim *et al.*, 1999; Emoto *et al.*, 2000; Hughes & Parker, 2001; Kam & Exton, 2001; Kristiansen *et al.*, 2001; Lee *et al.*, 2001; Vitale *et al.*, 2001; Hughes *et al.*, 2002). Thus, while PLD1 seems to be associated with various intracellular compartments in mammalian cells, PLD2 has been primarily localized to the plasma membrane (Colley *et al.*, 1997b; Emoto *et al.*, 2000; Park *et al.*, 2000; Lee *et al.*, 2001). Both PLD enzymes have also been shown to constitutively associate with membrane

receptors, and it is speculated that this binding might relocate PLD to the plasma membrane (Min *et al.*, 1998; Slaaby *et al.*, 1998). In line with this, PLD1 and PLD2 have been reported to be present in caveolae (Czarny *et al.*, 1999; Sciorra & Morris, 1999), which represent, together with lipid rafts, compartimentalized membrane signaling microdomains, believed to confer specificity into the complex mechanisms of signal transduction (Okamoto *et al.*, 1998; Kurzchalia & Parton, 1999; Anderson & Jacobson, 2002; Zajchowski & Robbins, 2002). Finally, the association of PLD1 with the detergent-insoluble cytoskeleton has been reported (Iyer & Kusner, 1999; Hodgkin *et al.*, 1999). Lee *et al.* (2001) reported recently colocalization of PLD1 and PLD2 with the actin cytoskeleton in the periphery of COS-7 cells and PC12 cells. In addition, posttranslational modification of PLD enzymes apparently altered its subcellular localization and may thus affect PLD-dependent signaling as well (Rudge *et al.*, 1998; Exton, 2002).

2.5 Regulation of PLD by membrane receptors

As mentioned above, in the presence of primary alcohols, PLD enzymes catalyze a transphosphatidylation reaction, generating specific phosphatidylalcohols. Due to their unique origin and their relative metabolic stability, the formation of phosphatidylalcohols has served as a convenient and sensitive marker for PLD activation in cultured cells (Morris *et al.*, 1997). Signal-dependent PLD activation has been demonstrated in numerous cell types stimulated with a variety of hormones, neurotransmitters, growth factors, cytokines, adhesion molecules, drugs and physical stimuli (Exton, 1999; Cockcroft, 2001; Liscovitch *et al.*, 2000; Exton, 2002). As nearly every membrane receptor known to stimulate PIP_2-specific PLC isoforms also caused PLD stimulation, it was assumed that stimulation of PLD might be secondary to PLC activation and actually initiated by the increase in cytosolic Ca^{2+} concentration and activation of PKC isoforms. The 12 PKC isoforms currently known are classified according to their differential Ca^{2+} and DAG requirements: Ca^{2+}/DAG-dependent conventional PKC enzymes (e.g. PKC-α and PKC-β), DAG-dependent novel PKC enzymes and Ca^{2+}/DAG-independent atypical PKC enzymes. Isoform-specific PKC inhibitors and down-regulation (inactivation) of DAG-dependent PKC isoforms by long-term cell treatment with phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), were used to study the involvement of PKC isoforms in PLD activation (Newton, 1997; Battaini, 2001). Indeed, PLD responses to EGF and platelet-derived growth factor (PDGF), activating on tyrosine kinase receptors, as well as LPA and carbachol, acting on G protein-coupled receptors, turned out to be PKC-dependent in some cell types (Exton, 1999; Caulfield & Birdsall, 1998; Contos *et al.*, 2000;

Schlessinger, 2000; Cockcroft, 2001; Liscovitch *et al.*, 2001; Exton, 2002; Fukushima *et al.*, 2001). Likewise, in mouse embryo fibroblasts lacking PLC-γ1, known to be primarily activated by tyrosine kinase receptors, stimulation of PLD by PDGF was largely reduced (Hess *et al.*, 1998; Hess *et al.*, 2000; Schlessinger, 2000; Rhee, 2001). Furthermore, a physical association of PLD with PKC-α and PKC-β has been reported, resulting in strong activation of *in vitro* PLD1, but not PLD2, activity. Mutagenesis and binding studies identified the major interaction site between PLD1 and PKC-α within the NH_2-terminus of PLD1 (Park *et al.*, 1998; Slaaby *et al.*, 2000). Based on the molecular characterization of the PLD1/PKC-α interaction site, PLD1 mutants unresponsive to PKC have been generated which, upon expression in HEK-293 cells, did not respond to activation of coexpressed bombesin and M_1 or M_3 muscarinic acetylcholine receptors (mAChRs) (Zhang *et al.*, 1999; Du *et al.*, 2000). PKC activation by bombesin and M_1/M_3 muscarinic receptors is primarily caused by the stimulation of PLC-β isoforms by pertussis toxin (PTX)-insensitive $G\alpha_q$ proteins (Neer, 1995; Gudermann *et al.*, 1996; Caulfield & Birdsall, 1998; Rhee, 2001; Watling, 2001). In line with this, expression of the PKC-unresponsive PLD1 mutant in COS-7 cells resulted in PLD activity which was no longer responsive to constitutively active $G\alpha_q$ (Xie *et al.*, 2002).

The M_3 mAChR, stably expressed in HEK-293 cells, was shown to couple to both PLC and PLD *via* a PTX-insensitive mechanism (Peralta *et al.*, 1988; Offermanns *et al.*, 1994; Schmidt *et al.*, 1994), however, stimulation of PLD by the agonist carbachol was found to be not affected by PKC inhibition (Schmidt *et al.*, 1994; Rümenapp *et al.*, 1997). Likewise, PLD stimulation by the α_1-adrenoceptor in Mardin-Darby canine kidney cells and in rabbit aortic vascular smooth muscle cells was shown to be PKC-independent (Kiss, 1996; Balboa & Insel; 1998; Muthalif *et al.*, 2000; Parmentier *et al.*, 2001), strongly indicating that PLD stimulation by membrane receptors must not necessarily be secondary to PLC stimulation.

G protein-coupled receptors constitute the largest group of membrane receptors known to regulate a wide range of biological responses, including cell growth, differentiation, migration and inflammatory processes (Morris & Malbon, 1999; Lefkowitz, 2000; Marinissen & Gutkind, 2001; Pierce *et al.*, 2002). These receptors induce cellular signaling via GTP-bound α-subunits, classified into the four families, G_s, G_i, G_q and G_{12}, as well as free βγ-dimers (Neer, 1995; Gudermann *et al.*, 1996; Wieland *et al.*, 1997; Lefkowitz, 2000; Marinissen & Gutkind, 2001; Pierce *et al.*, 2002). α-Subunits of G_s and G_i proteins, but not members of the G_q and G_{12} families, are targets for the bacterial toxins, cholera toxin from *Vibrio cholerae*, which specifically ADP-ribosylates $G\alpha_s$ proteins, leading to their persistent activation, and PTX from *Bordetella pertussis*, which ADP-ribosylates $G\alpha_i$ proteins, causing their inactivation (Neer, 1995). These toxins have been used to

characterize G protein-coupled receptor signaling to PLD. PLD stimulation by chemoattractants in neutrophils and by the LPA-like lysophospholipid sphingosine-1-phosphate in human bronchial epithelial cells was PTX-sensitive (Fensome *et al.*, 1998; Watling, 2001; Cummings *et al.*, 2002b; Wang *et al.*, 2002). As mentioned above, M_3 mAChR signaling to PLD in HEK-293 cells as well as PLD stimulation by endothelin-1, thrombin and noradrenaline in neonatal rat cardiomyocytes was mediated via PTX-insensitive G proteins (Offermanns *et al.*, 1994; Schmidt *et al.*, 1994; Fahimi-Vahid *et al*., 2002; Gosau *et al*., 2002). PLD activation by angiotensin II in vascular smooth muscle cells was PTX-insensitive too, but also $\beta\gamma$-subunits were found to modulate the receptor response (Ushio-Fukai *et al.*, 1999). Likewise, PLD stimulation by endothelin-1 in rat myometrium was controlled by both α-subunits of PTX-insensitive G proteins as well as by $\beta\gamma$-subunits (Le Stunff *et al.*, 2000a). Treatment with PTX does not discriminate between members of the G_q and G_{12} family of heterotrimeric G proteins, as both are PTX-insensitive (Neer, 1995; Dhanasekaran & Dermott, 1996; Gudermann *et al.*, 1996; Fields & Casey, 1997). G_{12} proteins have also been suggested to be involved in PLD regulation (Plonk *et al.*, 1998). Recently, regulators of G protein signaling (RGS) have been identified as GTPase-activating proteins (GAPs), promoting GTP hydrolysis and deactivation of α-subunits, resulting in the inhibition of agonist responses (Druey *et al.*, 1996; Dohlman & Thorner, 1997; Wieland & Chen, 1999; de Vries *et al*., 2000; Ross & Wilkie, 2000). While inhibition of receptor signaling to PLC by RGS proteins has been reported (Huang *et al.*, 1997; Neill *et al.*, 1997), RGS proteins have not yet been used to study G protein-coupled receptor signaling to PLD.

The question of whether PLD1 and PLD2 are differentially activated by receptors has been addressed only in a few reports. For example, Zhang *et al.* (1999) reported PLD1 stimulation by the G_q-coupled bombesin and M_1/M_3 receptors, upon coexpression in HEK-293 cells. Furthermore, coexpression of PLD1 together with the AT_1 receptor as well as the Edg4 receptor resulted in PLD stimulation by angiotensin II and LPA, respectively (Du *et al.*, 2000). These studies suggested that G protein-coupled receptors may couple primarily to PLD1. However, Parmentier *et al.* (2001) have reported recently that the α_1-adrenoceptor signals to PLD2 in rabbit aortic smooth muscle cells. In addition, it has been shown recently that sphingosine-1-phosphate signaling in human bronchial epithelial cells may involve both, PLD1 and PLD2 (Wang *et al.*, 2002). Thus, G protein-coupled receptor signaling to the two PLD isoforms needs to be further analyzed.

Earlier reports employing tyrosine kinase inhibitors, such as genistein and tyrphostins, implicated that tyrosine kinase activity may affect some PLD responses (Schmidt *et al.*, 1994; Rümenapp *et al.*, 1998; Houle & Bourgoin, 1999; Le Stunff *et al.*, 2000b; Fahimi-Vahid *et al.*,

2002; Gosau *et al.*, 2002). Indeed, Marcil *et al.* (1997) reported tyrosine phosphorylation of PLD1 in HL-60 cells, but the posttranslational modification did not change enzyme activity. More recent studies have supplied some insights in PLD stimulation by tyrosine kinase receptors. In Rat-1 fibroblasts expressing the human insulin receptor, the PLD response to insulin was reported to be mediated by PLD2, but not PLD1 (Rizzo *et al.*, 1999), whereas in HEK-293 cells the insulin response was mediated by both PLD1 and PLD2 (Slaaby *et al.*, 2000). Neither study analyzed whether activation of PLD by the insulin receptor was accompanied by tyrosine phosphorylation of the enzymes. Cotransfection of HEK-293 cells with PLD1 or PLD2 together with the EGF receptor resulted in activation of both PLD enzymes by EGF, but only PLD2 became tyrosine phosporylated upon receptor activation and associated with the EGF receptor in a ligand-independent manner. However, mutation of the tyrosine phosphorylation site in PLD2 did not affect the ability of EGF to activate the enzyme (Slaaby *et al*., 1998). Interestingly, it has been recently shown in HeLa cells that PLD1b is not simply localized in endosomes, but is relocated in these endosomal compartments after EGF stimulation (Hughes *et al.*, 2002). Finally, stimulation of PLD1 by PDGF has been reported in Swiss 3T3 fibroblasts, which was accompanied by tyrosine phosphorylation of PLD1 and its physical association with the PDGF receptor (Min *et al.*, 1998). Taken together, whereas the stimulation of PLC-γ isoforms by tyrosine kinase receptors has been characterized in detail, our understanding of receptor coupling to PLD is still incomplete.

2.6 Interaction of PLD with phosphoinositide metabolism

In contrast to PLC-γ isoforms, where direct interaction with tyrosine kinase receptors is needed and sufficient for activation (Schlessinger, 2000; Rhee, 2001), association of PLD enzymes with tyrosine kinase receptors did not trigger enzyme activity (Min *et al.*, 1998; Slaaby *et al.*, 1998). Thus, it has been assumed that receptor regulation of PLD may involve additional signaling molecules (Liscovitch *et al.*, 2000; Cockcroft, 2001; Exton, 2002). Attractive candidates are phosphoinositides, especially PIP_2 and PIP_3, which profoundly affect the enzyme activity and cellular localization of PLD1 and PLD2 (Brown *et al.*, 1993; Liscovitch *et al.*, 1994; Pertile *et al.*, 1995; Ohguchi *et al.*, 1996a; Schmidt *et al.*, 1996a; Schmidt *et al.*, 1996b; Hodgkin *et al.*, 1999; Hodgkin *et al.*, 2000). Thus, reduction of the cellular phosphoinositide content by fodrin (Lukowski *et al*., 1996) or synaptojanin (Chung *et al.*, 1997) inhibited PLD activities. The actin-binding protein fodrin most likely reduced the cellular content of PIP_2 and PIP_3 by inhibition of phosphoinositide kinases (Lukowski *et al.*, 1998), which generate PIP_2 and PIP_3 by sequential phosphorylation of PI

at the 3, 4 or 5 position of the inositol ring (Fruman *et al.*, 1998; Anderson *et al.*, 1999; Chan *et al.*, 1999; Rameh & Cantley, 1999; Vanhaesebroeck *et al.*, 2001), whereas synaptojanin acts as a phosphoinositide phosphatase (Chung *et al.*, 1997; Lee *et al.*, 1997; Majerus *et al.*, 1999; Lee *et al.*, 2000; Toker, 2002). Phosphoinositide kinases and PLD might directly cooperate in the control of cellular processes. So was the coupling of phosphoinositide phosphorylation and PLD activation found to be necessary to initiate clathrin-coat assembly in rat brain lysosomes (Arneson *et al.*, 1999). Divecha *et al.* (2000) demonstrated that both PLD1 and PLD2 interact with type Iα PIP 5-kinase, but only PLD2 activity was regulated by coexpression with the lipid kinase. By binding to proteins with PH/PX domains, PIP_2 and PIP_3 modulate a remarkable number of cellular processes, such as clathrin-coated endocytosis, vesicular trafficking, membrane movement and actin cytoskeleton assembly (Martin, 1998; Toker, 1998; Janmey *et al.*, 1999; Czech, 2000; Martin, 2001; Simonsen *et al.*, 2001; Lemmon *et al.*, 2002). Intriguingly, PLD enzyme activity, primarily PLD2, is modulated by β-actin, the actin- binding protein, α-actinin, as well as proteins involved in synaptic vesicle trafficking, such as AP180 [previously named clathrin assembly protein 3], amphiphysins, synucleins and collapsin response mediator protein-2 (Lee *et al.*, 1997; Jenco *et al.*, 1998; Lee *et al.*, 2000; Park *et al.*, 2000; Lee *et al.*, 2001; Ahn *et al.*, 2002; Lee *et al.*, 2002). Thus, these findings suggest that PLD enzymes may be implicated in various phosphoinositide-driven cellular transport processes.

2.7 Regulation of PLD by monomeric GTPases

The superfamily of monomeric GTPases with molecular masses of approximately 20 kDa consists of more than 100 members, which are divided into five groups: Ras, Ran, Rho, Rab and ADP-ribosylation factor (ARF) GTPases (Hall, 2000; Takai *et al.*, 2001). These GTPases control distinct fundamental processes. Ras proteins primarily regulate gene expression (Feig *et al.*, 1996; Vjtek & Der, 1998; Reuther & Der, 2000), Rho proteins control actin cytoskeleton organization (Hall, 1998; Schmidt & Hall, 1998; Kjøller & Hall, 1999; Schmitz *et al.*, 2000), Rab and ARF proteins are involved in the regulation of intracellular transport and membrane trafficking (Moss & Vaughan, 1998; Chavrier & Goud, 1999; Donaldson & Jackson, 2000), and Ran proteins in the regulation of nucleocytoplasmic transport and microtubule organization (Hall, 2000; Takai *et al.*, 2001). The cycling of monomeric GTPases from their GDP-bound inactive state to their GTP-bound active state is catalyzed by specific guanine nucleotide exchange factors (GEFs), whereas the recycling to the GDP-bound inactive state is promoted by GAPs (Hall, 2000; Jackson & Casanova,

2000; Reuther, & Der, 2000; Takai *et al.*, 2001; Zheng, 2001).

Members of the monomeric GTPases, specifically ARF, Rho and Ras proteins, play an important role in PLD signaling. ARF proteins, particularly ARF1 and ARF6, initially identified as *in vitro* regulators of PLD activity (Brown *et al.*, 1993; Cockcroft *et al.*, 1994), are now well established activators of both PLD enzymes, but PLD1 is much more responsive to ARF than PLD2 (Hammond *et al.*, 1997; Redina & Frohman, 1998; Sung *et al.*, 1999a; Sung *et al.*, 1999b). However, the evidence for a participation of ARF proteins in receptor-mediated PLD stimulation is largely indirect. It is mainly based on *in vitro* reconstitution studies with membranes and ARF-depleted permeabilized cells (Houle *et al.*, 1995; Rümenapp *et al.*, 1995; Shome *et al.*, 1997; Caumont *et al*, 1998; Shome *et al.*, 1998; Caumont *et al*, 2000) and on studies with the ARF-binding protein, Arfaptin (Kanoh *et al.*, 1997; Tsai *et al.*, 1998; Williger *et al.*, 1999b). Furthermore, PLD stimulation by various receptors in intact cells was found to be reduced by brefeldin A, an inhibitor of some ARF-specific GEFs (Rümenapp *et al.*, 1995; Karnam *et al.*, 1997; Shome *et al.*, 1997; Bacon *et al.*, 1998; Fensome *et al.*, 1998; Mitchell *et al.*, 1998; Shome *et al.*, 1998; Rizzo *et al.*, 1999; Andresen *et al.*, 2000; Fahimi-Vahid *et al*., 2002). Similarly, the ARF-related protein ARP, which can sequester ARF-specific GEFs, was shown to inhibit M_3 mAChR signaling to PLD in HEK-293 cells (Schürmann *et al.*, 1999). It should also be noted that ARF1 and ARF6 may indirectly modulate PLD signaling by activation of PIP 5-kinase, thus by the supply of the PLD cofactor PIP_2 (Martin *et al.*, 1996; Godi *et al.*, 1999; Honda *et al.*, 1999; Jones *et al.*, 2000; Skippen *et al.*, 2002). Likewise, it has recently been shown that ARF1 reversed the inhibition of PLD, particularly of PLD2, by β-actin and α-actinin which bind PIP_2 (Park *et al.*, 2000; Lee *et al.*, 2001).

Rho GTPases, in particular Rho, Rac and Cdc42, exclusively activate PLD1 by direct interaction with its COOH-terminus, and this activation can be triggered by PKC-α and ARF1 (Hammond *et al.*, 1997; Colley *et al*., 1997a; Sung *et al*., 1999a,b; Frohman, *et al.*, 2000; Cai & Exton, 2001). C3 transferase from *Clostridium botulinum*, known to specifically inactivate Rho by ADP-ribosylation (Aktories & Just, 1993), and the expression of inactive Rho mutants have been used to study the involvement of Rho in receptor signaling to PLD. Using these approaches, Rho was found to be involved in PLD stimulation by bradykinin and sphingosine-1-phosphate in human adenocarcinoma A459 cells, by PDGF and LPA in Rat-1 fibroblasts and by RANTES in Jurkat T cells (Malcolm *et al.*, 1996; Hess *et al*., 1997; Meacci *et al*., 1999; Bacon *et al.*, 1998), whereas apparently both, Rho and Rac, mediated tyrosine kinase receptor (PDGF and EGF) signaling to PLD in Rat-1 fibroblasts (Hess *et al.*, 1997). In HEK-293 cells, C3 transferase specifically

suppressed the PLD response to the M_3 mAChR, without changing PLD stimulation by tyrosine kinase receptors (Schmidt *et al.*, 1999; Voß *et al.*, 1999). The selective involvement of Rho in mAChR signaling to PLD in HEK-293 cells was confirmed by studies with *Clostridium difficile* toxin B, known to inactivate Rho proteins by monoglucosylation (Just *et al.*, 1995), and further work identified RhoA as the essential GTPase (Schmidt *et al.*, 1996a; Rümenapp *et al.*, 1998; Schmidt *et al.*, 1998; Schmidt *et al.*, 1999). Since then, toxin B has been widely used to analyze the role of Rho proteins in receptor signaling to PLD (Ojio *et al.*, 1996; Ohguchi *et al.*, 1996b; Ohguchi *et al.*, 1997; Fahimi-Vahid *et al.*, 2002; Gosau *et al.*, 2002).

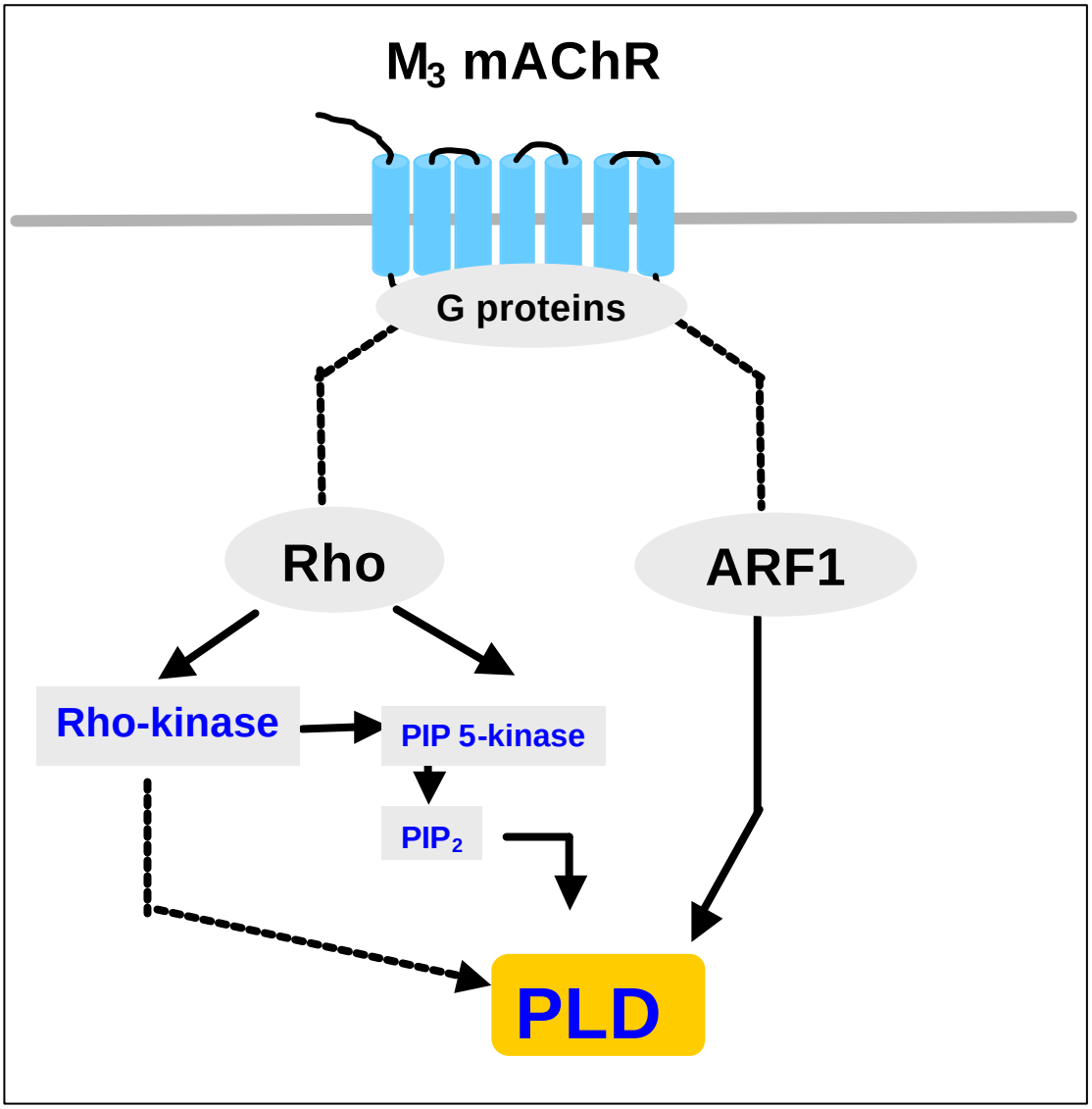


Fig. 3: Regulation of PLD stimulation by the M_3 mAChR in HEK-293 cells. For further explanation, see text.

A few studies reported that both, ARF and Rho proteins, contribute to receptor signaling to PLD. However, the interaction between these GTPases has either not been evaluated or a dominant role for ARF proteins has been proposed (Karnam *et al.*, 1997; Bacon *et al.*, 1998; Fensome *et al.*, 1998; Fahimi-Vahid *et al.*, 2002; Gosau *et al.*, 2002). In HEK-293 cells, the PLD response to the M_3 mAChR, which can activate both, ARF1 and RhoA, was shown to be regulated in a concerted action by independent ARF and Rho pathways (Rümenapp *et al.*, 1995; Keller *et al.*, 1997; Bayer *et al.*, 1999) (Fig. 3). In line with such a dual regulation, ARF and Rho have been found to localize in PLD-containing caveolae (Michaely *et al.*, 1999; Teruel & Meyer, 2000). The recent identification of ARAP proteins, which can link ARF and Rho signaling pathways, may be helpful to further understand the mechanism of PLD regulation by these GTPases (Bar-Sagi & Hall, 2000; Krugmann *et al.*, 2002; Miura *et al.*, 2002).

Regulation of PLD by Ras GTPases (Ras and Ral) was first reported in *v*-Src-transformed Balb/c and NIH-3T3 fibroblasts (Jiang *et al.*, 1995; Feig *et al.*, 1996). Further work suggested that Ral, which can directly interact with PLD1, cooperates with ARF (Luo *et al.*, 1997; Kim *et al.*, 1998; Luo *et al.*, 1998) and Rho proteins (Frankel *et al.*, 1999; Wilde *et al.*, 2002) to achieve full PLD activation. Treatment of HEK-293 cells with *Clostridium sordellii* lethal toxin and *Clostridium difficile* toxin B-1470, known to inactivate Ras and Ral by monoglucosylation, reduced tyrosine kinase receptor, but not M_3 mAChR, signaling to PLD; the toxin effects were fully mimicked by expression of inactive Ras and RalA. Additional work demonstrated that the Ras/Ral signaling cascade to PLD was dependent on a Ral-specific GEF and triggered by PKC-α (Just *et al.*, 1996; Chaves-Olarte *et al.*, 1999; Schmidt *et al.*, 1998; Voß *et al.*, 1999). Likewise, it was reported that Ras GTPases are involved in PLD responses to PDGF in NIH-3T3 and Rat-2 cells (Lucas *et al.*, 2000) and RalA in EGF receptor signaling to PLD in 3Y1 rat fibroblasts (Lu *et al.*, 2000). Ben El Hadj *et al.* (1999) reported that *Clostridium sordellii* lethal toxin, apparently by inactivating endogenous Ras and Ral proteins, also interfered with G protein activation of PLD in HL-60 cells. Finally, extracellular signal-regulated kinase, an effector system of Ras proteins, was found to modulate PLD signaling (Muthalif *et al.*, 2000; Parmentier *et al.*, 2001; Wang *et al.*, 2002; Chen *et al.*, 2002), suggesting that Ras proteins may regulate the PLD response by this pathway.

2.8 Involvement of Rho-dependent kinases in PLD stimulation

As mentioned above, PLD stimulation by RhoA can be caused by a direct PLD1-RhoA interaction. However, there is also evidence for indirect, Rho-dependent mechanisms. For example, inhibition of PLD by toxin B was accompanied by a reduction of the cellular PIP_2 content, and addition of PIP_2 to cell membranes could rescue this inhibition (Schmidt *et al.*, 1996b; Schmidt *et al.*, 1996c; Zhang *et al.*, 1996; Oude Weernink *et al.*, 2000a; Fahimi-Vahid *et al.*, 2002; Gosau *et al.*, 2002). RhoA was actually shown to stimulate the PIP_2 synthesis by PIP 5-kinase in HEK-293 cells (Oude Weernink *et al.*, 2000b). Intriguingly, Rho-kinase, a serine/threonine kinase, which activity depends on GTP-loaded Rho (Leung *et al.*, 1995; Kimura *et al.*, 1996; Leung *et al.*, 1996; Lim *et al.*, 1996; Matsui *et al.*, 1996; Nakagawa *et al.* 1996; Maekawa *et al.*, 1999), was found to mediate the RhoA-dependent PLD response to the M_3 mAChR (Schmidt *et al.*, 1999) (Fig. 3), and to stimulate PIP 5-kinase activity as well (Oude Weernink *et al.*, 2000b). Based on these initial reports, Rho-kinase was confirmed to regulate PIP 5-kinase in N1E-115 neuroblastoma cells and PLD-driven cell responses in Rat-2 fibroblasts and human bronchial epithelial cells (Kam & Exton, 2001; Cummings *et al.*, 2002b; Yamazaki *et al.*, 2002). Rho-kinase belongs to a family of Rho effectors, including the PKC-related protein kinase N (PKN), rhophilin, rhotekin, p21-activated protein kinase (PAK) and the Wiskott-Aldrich syndrome protein (WASP), which are differentially regulated by Rho proteins. Whereas Rho regulates Rho-kinase, PKN, rhophilin and rhotekin, Rac/Cdc42 control PAK and WASP functions (Aspenström, 1999; Kaibuchi *et al.*, 1999; Amano *et al.*, 2000; Bishop & Hall, 2000). Also these Rho effectors are candidates to be involved in the regulation of PLD activity. Indeed, Oishi *et al.* (2001) recently reported that PKN regulates PLD1 through direct interaction. As PKN also interacts with α-actinin, which by itself inhibits PLD enzymes, PKN may modulate PLD signaling by reversing the inhibitory effect of α-actinin on PLD1, as well as by direct interaction with PLD1 (Mukai *et al.*, 1997; Lee *et al.*, 2001).

2.9 LIM-kinase

PLD enzymes were not found to directly interact with, or to be phosphorylated by Rho-kinase (Schmidt *et al.*, 1999), indicating that regulation of PLD by Rho/Rho-kinase obviously involves additional, yet undefined, components. As Rho-kinase profoundly affects the reorganization of the actin cytoskeleton by interacting with various signaling molecules (Aspenström, 1999; Kaibuchi *et al.*, 1999; Amano *et al.*, 2000; Bishop & Hall, 2000), mechanisms involved in this

process may be linked to the regulation of PLD as well. Upon activation by Rho, Rho-kinase most likely directs the actin architecture *via* two mechanisms: 1) Rho-kinase phosphorylates, and thereby inhibits, myosin light chain phosphatase and consequently increases actomyosin-based contractility (Kimura *et al*., 1996; Amano *et al.*, 1997; Oshiro *et al.*, 1998; Maekawa *et al*., 1999), and 2) Rho-kinase phosphorylates the serine/threonine kinase LIM-kinase, which in turn phosphorylates and inactivates the actin depolymerization factor cofilin, leading to enhanced actin polymerization (Maekawa *et al.*, 1999; Ohashi *et al.*, 2000; Lou *et al.*, 2001; Sumi *et al*., 2001a,b; Matsui *et al.*, 2002). Two LIM-kinases (*LIM* domain containing kinase; approximately 65 kDa) have been identified, which are located in the cytosol and nucleus of mammalian cells; in addition, LIM-kinase was found to be abundantly expressed in the peripheral and central nervous system (Mizuno *et al.*, 1994; Pröschel *et al.*, 1995; Edwards *et al*., 1999; Meng *et al*., 2002). LIM-kinase bears two NH_2-terminal LIM domains and a PDZ domain; while the LIM domains seem to modulate enzyme activity, the PDZ domain probably is responsible for membrane association (Edwards & Gill, 1999; Edwards *et al*., 1999). Phosphorylation of LIM-kinase at threonine 508 by Rho-kinase or PAK activates IIM-kinase, which in turn phosphorylates its mere substrate cofilin, leading to profound changes in the dynamics of the actin cytoskeleton (Arber *et al*., 1998; Rosenblatt & Mitchison, 1998; Yang *et al*., 1998; Edwards *et al.*, 1999; Lawler, 1999; Maekawa *et al.*, 1999; Minamide *et al.*, 2000; Sumi *et al*., 2001a,b; see also Fig. 4). Meanwhile, LIM-kinase has been implicated in several processes, including the regulation of gene transcription by the serum response factor, chemotaxis of T lymphocytes, mitosis of HeLa cells, *Listeria*-induced phagocytosis and regulation of the actin growth cone in neuronal cells, and is associated with the manifestation of the William-Beuren Syndrome [a neurological condition characterized by mild mental retardation and defects in visu-spatial cognition] (Tassabehji *et al.*, 1996, Wu *et al.*, 1998; Sotiropoulos *et al.*, 1999; Wang *et al.*, 1999; Donnai & Karmiloff-Smith, 2000; Aizawa *et al.*, 2001a; Bierne *et al.*, 2001; Amano *et al.*, 2002; Geneste *et al*., 2002; Nishita *et al.*, 2002).

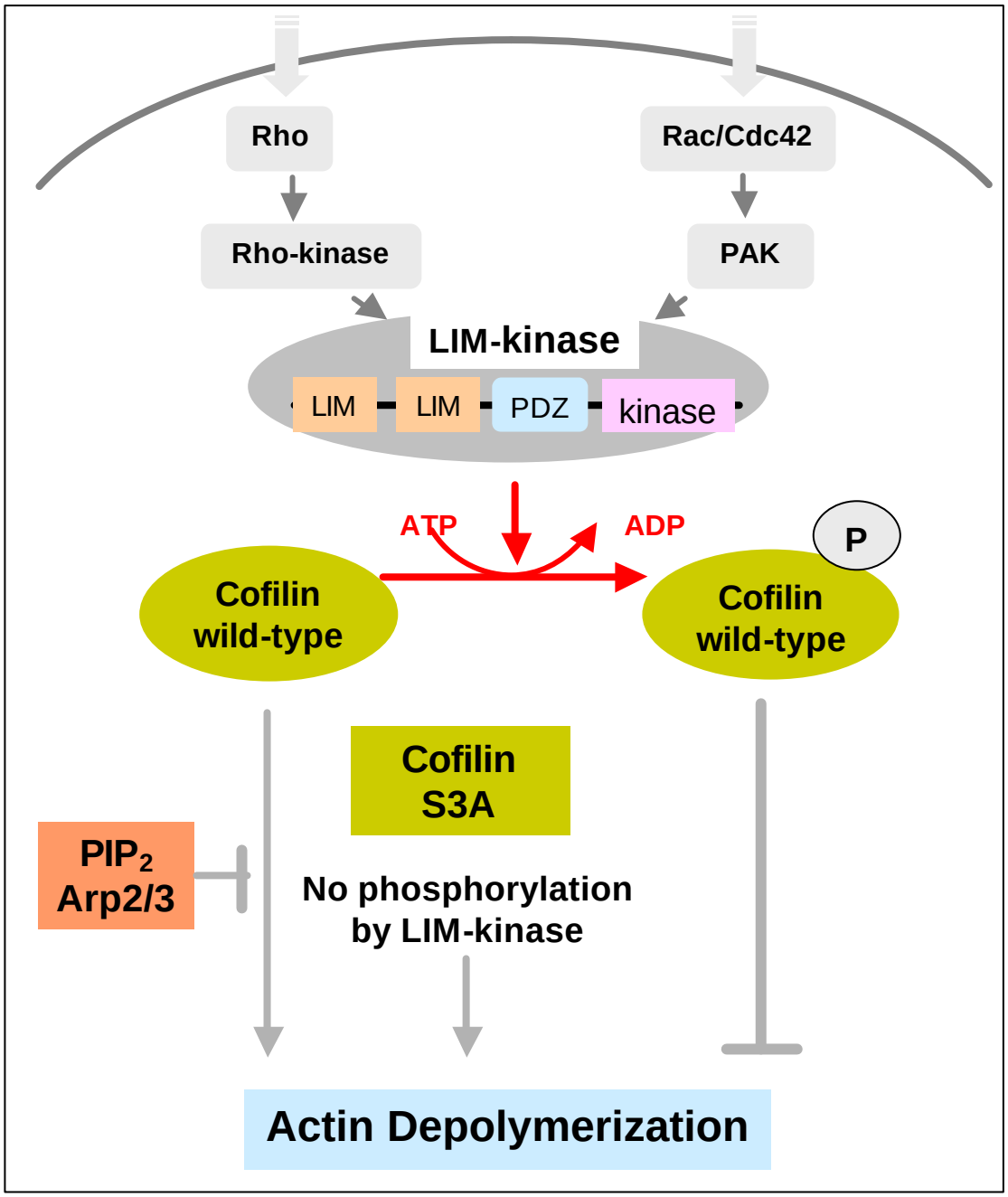


Fig. 4: LIM-kinase and cofilin signaling. For further explanation, see text.

Cofilin (approximately 20 kDa), the only LIM-kinase substrate identified so far, was named for its ability to form cofilamentous structures with actin; it is widely expressed, in particular in hematopoietic cells, osteoclasts and fibroblasts. Cofilin bears an actin- and a phosphoinositide-binding domain and a sequence confering nuclear localization (Nishida *et al.*, 1984; Ott *et al.*, 1996; Lappalainen *et al.*, 1998; Aizawa *et al.*, 2001b). Cofilin is implicated in the regulation of actin

cytoskeleton dynamics during cell division, exo- and endocytosis as well as motility. It binds to actin monomers as well as actin filaments (F-actin), thereby promoting actin filament turnover (Lappalainen & Drubin, 1997; Bamburg, 1999; Higgs & Pollard, 2001; Chen *et al.*, 2000; Birkenfeld *et al.*, 2001). The cofilin-actin interaction is tightly controlled by phosphocycling. Cofilin preferentially binds to ADP-actin monomers and thereby inhibits the nucleotide exchange on them. Consequently, the actin monomer pool increases and F-actin rapidly depolymerizes. Phosphorylation of cofilin by LIM-kinase at serine 3, located in the actin-binding domain, results in its inactivation. Mutation of serine to alanine (S3A) generates a nonphosphorylatable cofilin, which is used to study the role of cofilin in cellular responses (Agnew *et al.*, 1995; Bamburg, 1999; Higgs & Pollard, 2001; Toshima *et al.*; 2001; see also Fig. 4). The transient phosphorylation (inactivation) of cofilin permits free nucleotide exchange on actin monomers and F-actin reassembly; dephosphorylation by a cofilin phosphatase mobilizes cofilin for another round of actin monomer sequestering, allowing actin structure reorganization (Meberg *et al*. 1998; Bamburg, 1999; Chen *et al.*, 2000; Higgs & Pollard, 2001). In *Drosophila*, the slingshot phosphatase, a family of phosphatases that have the property of F-actin binding, was found to commit the reactivation of cofilin (Niwa *et al*., 2002). In addition, it has been demonstrated that phosphoinositides, the actin-related protein complex Arp2/3 and the modulation of intracellular pH affected the functions of cofilin (Yonezawa *et al*., 1985; Nagaoka *et al.*, 1995; Sakisaka *et al.*, 1997; Mullins *et al.*, 1998; Kusano *et al.*,1999; Bernstein *et al.*, 2000; Ono & Ono, 2002). A role for LIM-kinase and cofilin in the reorganization of the actin cytoskeleton by Rho/Rho-kinase is now well accepted, but it is not known whether LIM-kinase and cofilin affect the PLD response as well.

2.10 Aims of the study

Aim of the present study was to gain further insights into the regulation of PLD by the G protein-coupled M_3 mAChR in HEK-293 cells. For comparison, the PKC-dependent tyrosine kinase receptor signaling to PLD in these cells was studied. In particular, it was intended 1) to define the type of PTX-insensitive heterotrimeric G protein mediating the M_3 mAChR-induced PLD stimulation, in comparison to the PLC response by the same receptor, 2) to identify the PLD isoform(s) activated by the M_3 mAChR, 3) to generate adenoviruses encoding wild-type and catalytically inactive PLD1 and PLD2 mutants for future work on the cellular role of these PLD enzymes in receptor action, and 4) to identify the components and mechanisms by which Rho/Rho-kinase controls PLD stimulation by the M_3 mAChR.

3. Materials and Methods

3.1 Materials

3.1.1 Chemical reagents

Name	Source (supplier / company)
Acetic acid	Baker
Acrylamide	Serva
Agarose	Invitrogen
Ammonia	Merck
Ammonium formiate	Fluka
Ammonium peroxodisulfate	Serva
Ampicillin	Grünenthal
Aprotinin	Sigma
ATP	Roche Molecular Biochemicals
Benzamidine	Sigma
Bisacrylamide	Serva
Boric acid	Merck
Bradykinin	Sigma
BSA	Biomol
BSA, fatty acid free	Sigma
1-Butanol	Baker
Calcium acetate	Merck
Calcium chloride	Merck
Calcium dihydrogenphosphate	Merck
Carbachol	Sigma
Chloroform	Baker
Chloropan	Maniatis
Chloroquin diphosphate	Sigma
Coomassie-blue	Serva
Diethylether	Merck
DMEM/Nutrient Mix F-12	Invitrogen
DMSO	Sigma

DTT	Serva
EDTA	Merck
EGF	Biomol
EGTA	Merck
Ethanol	Baker
Ethidium bromide	Sigma
Ethylacetate	Merck
FCS	Invitrogen
Geneticin (G418)	Invitrogen
GDP	Roche Molecular Biochemicals
γ-Globulin (calf)	BIO-RAD
D(+)-Glucose	Merck
Glutathione	Amersham
Glycerol	Fluka
Glycine	Sigma
GTPγS	Roche Molecular Biochemicals
HEPES	Serva
Imidazole	Sigma
Insulin	Sigma
Iodine	Merck
IP_3	Sigma
IPTG	Stratagene
Isooctane	Riedel de Haen
Isopropanol	Baker
Kanamycin	Sigma
LB agar	Invitrogen
LB medium	Invitrogen
Leupeptin	Sigma
Lithium chloride	Merck
LPA	Sigma
Magnesium chloride, hexahydrate	Merck
Magnesium sulfate, heptahydrate	Merck
2-Mercaptoethanol	Merck
Methanol	Baker

Nonidet P 40	Fluka
PA	Sigma
Penicillin G	Seromed
Phenol	Fluka
Phosphoric acid	Merck
PMA	Sigma
PMSF	Roche Molecular Biochemicals
Poly-D-lysine	Sigma
Poly-L-lysine	Seromed
Ponceau S	Sigma
PtdCho	Sigma
PtdEtOH	Avanti
PIP_2	Roche Molecular Biochemicals
Hydrochloric acid (37 %)	Merck
SDS	Serva
Serva-blue (G/R)	Serva
Skim milk powder	Merck
SOC medium	Invitrogen
Sodium acetate	Merck
Sodium dihydrogenphosphate	Merck
Sodium carbonate	Merck
Sodium chloride	Merck
Sodium deoxycholate	Fluka
Sodium cholate	Sigma
Sodium bicarbonate	Merck
Sodium hydroxide	Merck
Soybean trypsin inhibitor	Sigma
Streptomycin	Seromed
Sucrose	Merck
TCA	Merck
TEMED	Serva
Thrombin	Pharmingen
TNM-FH	Sigma
Trichlorotrifluoroethane	Merck

Triethanolamine	Sigma
Tris	Merck
TRITC-phalloidin	Sigma
Triton X-100	Roth
Trypsin	Invitrogen
Tween 20	Sigma

3.1.2 Radioactive reagents

Name (specific activity)	**Source (supplier / company)**
[γ-^{32}P]ATP (3,000 Ci/mmol)	PerkinElmer Life Sciences
myo-[^{3}H]Inositol (18 Ci/mmol)	PerkinElmer Life Sciences
[^{3}H]IP_3 (21 Ci/mmol)	PerkinElmer Life Sciences
Levo-[2,5,6-^{3}H]Noradrenaline (56.4 Ci/mmol)	PerkinElmer Life Sciences
[9,10-^{3}H]Oleic acid (5 Ci/mmol)	PerkinElmer Life Sciences
L-α-Dipalmitoyl [2-palmitoyl-9,10-^{3}H] PtdCho (89 Ci/mmol)	PerkinElmer Life Sciences

3.1.3 Antibodies / Enzymes / Miscellaneous

Name	**Source (supplier / company)**
Alexa-488 goat anti-mouse IgG (H+L) conjugate	Molecular Probes, MoBiTec
Alexa-633 goat anti-rabbit IgG (H+L) conjugate	Molecular Probes, MoBiTec
Alkaline phosphatase, shrimp	Roche Molecular Biochemicals
anti-ARF1 antibody (rabbit)	Santa Cruz Biotechnology
anti-Cdc42 antibody (rabbit)	Santa Cruz Biotechnology
anti-*flag* antibody (mouse)	Strategene
anti-Gα_{12} (rabbit)	Santa Cruz Biotechnology
anti-Gα_{13} (rabbit)	Santa Cruz Biotechnology
anti-Gα_{q} (rabbit)	Santa Cruz Biotechnology
anti-HA antibody (mouse)	Dr. J. Bos
anti-His antibody (mouse)	Santa Cruz Biotechnology
anti-myc antibody (mouse)	Calbiochem

anti-PLD1 antibody (rabbit)	Dr. S. Bourgoin
anti-PLD2 antibody (rabbit)	Dr. S. Bourgoin
anti-Rac1 antibody (rabbit)	Santa Cruz Biotechnology
anti-RGS4 antibody (rabbit)	Dr. T. Wieland
anti-RhoA antibody (mouse)	Santa Cruz Biotechnology
anti-Rho-kinase antibody (goat)	Santa Cruz Biotechnology
DNase I	Roche Molecular Biochemicals
goat anti-mouse IgG/Peroxidase-conjugate	Dianova
goat anti-rabbit IgG/Peroxidase-conjugate	Sigma
Proteinase K	Promega
Restriction endonucleases	New England Biolabs
RNase A	Qiagen
T4 DNA ligase kit	Promega
Glutathione sepharose beads	Amersham Biosciences
Ni-NTA superflow suspension beads	Qiagen
Molecular weight standards	Sigma
1 Kb DNA ladder	Invitrogen
Lambda DNA/ EcoRI + HindIII marker	Promega

3.1.4 Plasmids

Name	**Supplier**
pAcGHLT	
RhoA	Dr. K. Kaibuchi
Rho-kinase CAT	Dr. K. Kaibuchi
ARF1	Dr. B. Lohmann
PLD1	Drs. A. Morris and M.A. Frohman
PLD2	Drs. A. Morris and M.A. Frohman
pAdEasy-1	
LacZ	Dr. T. Wieland
Lsc-RGS	Dr. T. Wieland
RGS4	Dr. T. Wieland

pAdTrack-CMV	Dr. T. Wieland
pcDL-SRa	
HA-tagged cofilin	Dr. K. Mizuno
HA-tagged cofilin (S3A)	Dr. K. Mizuno
pCGN	
PLD1	Dr. M.A. Frohman
PLD1 (K898R)	Dr. M.A. Frohman
PLD2	Dr. M.A. Frohman
PLD2 (K758R)	Dr. M.A. Frohman
pCIS	
$G\alpha_q$	Dr. T. Wieland
$G\alpha_{12}$	Dr. T. Wieland
$G\alpha_{13}$	Dr. T. Wieland
$G\alpha_q$ (R183C)	Dr. T. Wieland
$G\alpha_{12}$ (Q229L)	Dr. T. Wieland
$G\alpha_{13}$ (Q226L)	Dr. T. Wieland
$G\alpha_{12}$ (G228A)	Dr. S. Offermanns
$G\alpha_{13}$ (G225A)	Dr. S. Offermanns
pEF	
myc-tagged Rho-kinase	Dr. K. Kaibuchi
myc-tagged Rho-kinase (amino acids 6-553)	Dr. K. Kaibuchi
myc-tagged Rho-kinase (amino acids 941-1388)	Dr. K. Kaibuchi
myc-tagged C3 transferase	Dr. A. Hall
pEXV	
myc-tagged Cdc42 (T17N)	Dr. A. Hall
myc-tagged Rac1 (T17N)	Dr. A. Hall
pQE60	

His_6-tagged cofilin	Dr. K. Mizuno
His_6-tagged cofilin (S3A)	Dr. K. Mizuno

pRK5

ARF1 (T31N)	Dr. B. Lohmann
ARF1 (Q71L)	Dr. B. Lohmann

pUCD2

HA-tagged LIM-kinase	Dr. K. Mizuno
HA-tagged LIM-kinase (T508EE)	Dr. K. Mizuno
HA-tagged LIM-kinase (D460A)	Dr. K. Mizuno

pUCD2 SRa

GST/flag-tagged LIM-kinase	Dr. K. Mizuno

pGEX

GST-tagged Rhotekin-RBD	Dr. S. Offermanns

3.1.5 Bacteria

Strains	**Remarks**
DH5α/DH10B	Large-scale production of plasmids
BJ5183	Homologous recombination of adenovirus

3.1.6 Other materials

Name	**Source (supplier / company)**
Agefix Neutol Liquid NE	Agfa Gevaert
Agefix Fixer	Agfa Gevaert
Anion exchange AG 1-X8 resin	BIO-RAD
BCA protein assay reagent	Pierce
Chromatography paper	Whatman
Cryo vials	Greiner

Electroporation cuvette	BIO-RAD
Emulsifier scintillator Plus/299	Canberra Packard
Insulin syringe	Falcon
Kodak X-OMAT AR-films	Kodak
Lipofectamine reagent	Promega
Nitrocellulose membrane	Advanced Microdevices
Plasmid purification kit	Qiagen
QIAquick Gel Extraction kit	Qiagen
Slide-A-Lyzer cassette	Pierce
Sterile filter (0,20 µm)	Sartorius
Scintillation vials	Packard
Silica gel 60C	Merck
Western Lightning™	PerkinElmer Life Sciences

3.2 Methods

3.2.1 Cell culture

3.2.1.1 HEK-293 cells, N1E-115 neuroblastoma cells and PC12 cells

Cell culture was performed in a sterile laminar airflow (Laminar Air HA 2472 GS, Heraeus). Medium and solutions were either autoclaved (121°C, 30 min) or sterile filtered (0.2 µm, Sartorius). The cells were routinely grown as monolayer in 20 ml DMEM/F12 medium, containing 10 % (v/v) FCS, 100 U/ml penicillin G and 100 µg/ml streptomycin, on 145-mm culture dishes at 37°C in an incubator (B5060 EK/CO_2, Heraeus) with a humidified, 5 % CO_2 atmosphere. For experiments, cells were subcultured on 35- or 60-mm culture dishes unil near confluency. For storage in liquid nitrogen, cells were centrifuged for 5 min at 1,800 rpm (Megafuge 1.0 R, Heraeus) and resuspended in preserving medium (45 % (v/v) DMEM/F12 medium, 45 % (v/v) FCS, 10 % (v/v) DMSO). This cell suspension was slowly frozen at −80°C for 24 h before final storage in liquid nitrogen. For cell recovery, the cells were quickly thawed at 37°C, centrifuged to remove the DMSO-containing medium and seeded in 20 ml DMEM/F12 medium on 145-mm culture dishes.

HEK-293 wild-type cells transformed by E1 of human adenovirus type 5 (Ad 5) DNA were used to generate recombinant PLD adenoviruses. HEK-293 cells, stably expressing the M_3

mAChR at a density of about 200,000 receptors per cell (Peralta *et al*., 1988), were maintained in the presence of 0.5 mg/ml G418 (neomycin analogue). Experiments with HEK-293 cells were performed on 35- or 60-mm culture dishes pretreated with 0.02 mg/ml poly-L-lysine for 10 min to improve attachment of the cells. N1E-115 neuroblastoma cells, established by cloning the C-1300 spontaneous mouse neuroblastoma tumor (Amano *et al.*, 1972), were detached by resuspension in Ca^{2+}-free versene (137 mM NaCl, 2.6 mM KCl, 0.54 mM EDTA, 8.4 mM KH_2PO_4). PC12 cells, derived from a transplantable rat pheochromocytoma, were detached by incubation with 0.25 % (w/v) trypsin for 1 min (Alemany *et al*., 2001).

3.2.1.2 *Sf9* cells

Sf9 cells were cultured in TNM-FH medium supplemented with 10 % (v/v) FCS, 4.2 mM $NaHCO_3$, 100 U/ml penicillin G and 100 µg/ml streptomycin at 27°C (HERAcool 40, Heraeus, CO_2 supply was not required). For storage in liquid nitrogen, *Sf9* cells were centrifuged for 5 min at 1,800 rpm (Megafuge 1.0 R, Heraeus) and resuspended in preserving medium (45 % (v/v) TNM-FH medium, 45 % (v/v) FCS, 10 % (v/v) DMSO). This cell suspension was frozen at -80°C for 24 h and transferred for storage in liquid nitrogen. For cell recovery, the frozen cells were thawed at 37°C and incubated with 4 ml TNM-FH medium for 60 min in 25-cm^2 culture flasks. After attachment of the cells, the DMSO-containing medium was removed and the cells were cultured in 20 ml TNM-FH medium.

3.2.2 Transformation of bacteria

3.2.2.1 Calcium chloride method

A DH5α/DH10B *E. coli* culture in 20 ml LB medium was transferred into 500 ml LB medium and cultured at 37°C with constant shaking (Certomat, Braun) until the OD_{600} reached ~0.4. The bacteria were harvested by centrifugation for 10 min at 5,000 rpm at 4°C (Sorvall RC-5B, DuPont), resuspended in ice-cold 150 ml 50 mM $CaCl_2$ and incubated for 5 min on ice. After recentrifugation, the bacteria were incubated with ice-cold 8 ml 50 mM $CaCl_2$ for 1 h, before 8 ml 50 mM $CaCl_2$ containing 40 % (v/v) glycerol were added. The mixture was aliquoted, snap-frozen in liquid nitrogen and stored at -80°C. For transformation, 100 µl $CaCl_2$-treated bacteria were mixed with an appropriate amount of DNA in a pre-chilled polypropylene tube for 15 min on ice. The mixture was incubated for 90 s at 42°C, followed by another 5 min incubation on ice. 300 µl SOC

medium was added and the mixture was incubated at 37°C for 30 min. Thereafter, the bacteria were plated onto the LB agar plates containing the appropriate antibiotics and incubated at 37°C overnight (GTR 0241, Memmert).

LB-ampicillin plates	LB agar	32 mg/ml
	Ampicillin	100 mg/ml
LB-kanamycin plates	LB agar	32 mg/ml
	Kanamycin	30 mg/ml
LB-streptomycin plates	LB agar	32 mg/ml
	Streptomycin	30 mg/ml

3.2.2.2 Electroporation method

A DH10B/BJ5183 *E. coli* culture in 100 ml LB medium was diluted in 1000 ml LB medium and cultivated at 37°C, until OD_{600} reached ~0.8. The bacteria were harvested by centrifugation (see above) and incubated on ice for 1 h. They were washed twice with 100 ml ice-cold 10 % (v/v) glycerol. Finally, the bacteria were resuspended in 5 ml ice-cold 10 % (v/v) glycerol, aliquoted, snap-frozen in liquid nitrogen and stored at -80°C.

40 µl electrocompetent DH10B/BJ5183 *E. coli* were mixed with 50 - 300 ng DNA in a pre-chilled polypropylene tube and transferred into an ice-prechilled cuvette, which was pretreated for 15 min under UV. Electroporation was performed in a Gene Pulser electroporator (200 Ohm / 25 µF / 2.5 KV; BIO-RAD). Afterwards, 1 ml SOC medium was added, and the bacteria were incubated at 37°C for 1 h. Finally, transformed bacteria were plated onto agarose plates containing appropriate antibiotics and incubated at 37°C overnight (GTR 0241, Memmert).

3.2.3 Transfection of cells

3.2.3.1 Calcium phosphate method

Subconfluent cell monolayers grown on 145-mm culture dishes were fed with fresh DMEM/F12 medium one day before transfection. Appropriate amounts of cDNAs were transferred into reaction tubes (Eppendorf 1,5 ml) and adjusted with H_2O to 80 µl. The cDNAs were

precipitated by addition of 20 µl 3 M sodium acetate (pH 5.2) and 250 µl ethanol. The mixture was centrifuged for 10 min at 13,000 rpm (Biofuge 13, Heraeus). The DNA pellets were air-dried at RT and resuspended in 700 µl H_2O. Thereafter, DNA solutions were thoroughly mixed with 300 µl 2 M $CaCl_2$ and 1 ml 2 × HeBS solution and incubated for 10 min. 200 µl 10 mM chloroquine were added to the cell monolayers to reduce the degradation of cDNA by lysosomal hydrolases. The cDNA/$CaCl_2$/HeBS-mixture was carefully transferred onto the chloroquine-pretreated cell monolayer, and the cells were placed for 4 - 5 h in an incubator (B5060 EK/CO_2, Heraeus). Afterwards, the cell monolayers were washed once with FCS-free DMEM/F12 medium and then cultured in fresh DMEM-F12 medium. After 24 h, the cells were splitted on appropriate dishes and used for experiments 48 – 72 h after transfection. The transfection efficiency was 50 - 70 %.

To examine protein expression, cells on 35- or 60-mm culture dishes were washed twice with HBSS and scraped into ~0.3 ml lysis buffer (10 mM Tris/HCl, 1 % (m/v) SDS, pH 7.4). The lysates were incubated for 10 min at 95°C (Thermoblock, Eppendorf). After protein determination by the Pierce method, 75 - 100 µg protein of the samples were loaded onto polyacrylamide gels, and protein expression was analyzed by immunoblotting with appropriate antibodies.

2 × HeBS, pH 7.05	NaCl	280 mM
	HEPES	50 mM
	Na_2HPO_4	1.48 mM

3.2.3.2 Lipofectamine method

cDNA was incubated with 20 µl lipofectamine reagent in 1 ml FCS-free DMEM/F12 medium for 15 min at RT. Cell monolayers (70 - 80 % confluent) on 145-mm culture dishes were washed twice with FCS-free DMEM/F12 medium. Thereafter, the DNA-lipid mixture was transferred to the cell monolayers with 4 ml DMEM/F12 medium, and the cells were incubated for 4 h (B5060 EK/CO_2, Heraeus). Finally, the cells were washed two times with FCS-free DMEM/F12 medium and cultivated in DMEM-F12 medium.

3.2.4 Membrane preparation (Schmidt *et al*., 1996c)

All steps were performed at 4°C. Cells were harvested, washed with 50 ml HP buffer and resuspended in 60 ml buffer B. The cell suspension (~2 x 10^7 cells/ml) was subjected to nitrogen

cavitation at 500 p.s.i. for 30 min on ice. Thereafter, the EGTA concentration of the cavitate was adjusted to 1.25 mM, followed by centrifugation for 10 min at 2,400 rpm (Megafuge 1.0R, Heraeus) to pellet nuclei and unbroken cells. The supernatant was filtered through a two-layer gauze filter to remove light lipid debris and centrifuged for 20 min at 20,000 rpm (Sorvall RC-5B, DuPont). The precipitated membrane fraction was washed in buffer E and stored in aliquots (~20 mg/ml in buffer E) in liquid nitrogen.

HP buffer, pH 7.4	Triethanolamine/HCl	10 mM
	NaCl	140 mM
Buffer B, pH 7.5	Tris/HCl	20 mM
	Sucrose	250 mM
	$MgCl_2$	1.5 mM
	ATP	1 mM
	Benzamidine	3 mM
	Leupeptin	1 µM
	PMSF	1 mM
	Soybean trypsin inhibitor	2 µg/ml
Buffer E, pH 7.5	Tris/HCl	20 mM
	EDTA	1 mM
	DTT	1 mM
	Benzamidine	3 mM
	Leupeptin	1 µM
	PMSF	1 mM
	Soybean trypsin inhibitor	2 µg/ml

3.2.5 Purification of recombinant proteins

3.2.5.1 *Sf9* cells

To obtain GST-tagged fusion proteins of RhoA, ARF1, Rho-kinase CAT, PLD1 and PLD2, *Sf9* cells were infected with the appropriate recombinant baculoviruses. For that, 1 ml virus stock and 4 ml TNM-FH medium were added to the cell monolayer (~5 MOI) and incubated at 27°C for ~3 h. Afterwards, 20 ml TNM-FH medium were added, and the cells were cultured for at least 3 days.

The cells were harvested, centrifuged for 15 min at 1,800 rpm at RT (Sorvall RC-5B, DuPont), washed once with ice-cold Ca^{2+}/Mg^{2+}-free PBS and resuspended in TegD buffer I. The cells were disrupted by sonification (4 × 30 s, 4°C, Labsonic U, Braun) and centrifuged for 1 h at 20,000 rpm at 4°C (Sorvall RC-5B, DuPont). The supernatant containing the GST-tagged proteins was mixed with glutathione Sepharose beads, which were equilibrated with 2 × 5 ml TegD buffer II. The mixture was incubated for 2 h at 4°C under gentle rocking (REAX 2, Heidolph). Finally, the beads were washed twice with appropriate buffer and stored at 4°C for experiments.

Alternatively, for purification of Rho-kinase CAT, RhoA, and ARF1, glutathione Sepharose beads were filled into a chromatography column (BIO-RAD) and equilibrated with TegD buffer II. The supernatant was loaded on the column at 4°C. Thereafter, the column was washed with 30 ml TegD buffer II, and the bound recombinant protein was eluted with 1 ml TegD buffer III. The protein concentration (either bound on beads or soluble) was determined by the Bradford method. Preparations were analyzed for homogeneity by SDS-PAGE and Coomassie-blue staining. The GST-tagged proteins were identified by immunoblotting with appropriate antibodies.

TEgD buffer I, pH 7.5	Tris/HCl	50 mM
	EGTA	2 mM
	DTT	1 mM
	Sucrose	250 mM
	PMSF	10 µM
	Leupeptin	1 µg/ml
TEgD buffer II, pH 7.5	Tris/HCl	50 mM
	EGTA	2 mM
	DTT	1 mM
TEgD buffer III, pH 7.5	Tris/HCl	50 mM
	EGTA	2 mM

	DTT	1 mM
	Reduced glutathione	10 mM
PBS, pH 7.2	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO4	6.5 mM
	KH_2PO_4	1.5 mM
	$CaCl_2$	0.9 mM
	$MgCl_2$	0.5 mM

3.2.5.2 *E. coli*

His_6-tagged cofilin and GST-tagged Rho-binding domain (RBD) of Rhotekin (amino acids 7-89, Ren & Schwartz, 2000) were expressed in *E. coli*. The selected colony grown on a LB-agar plate was inoculated in 100 ml LB medium containing 100 µg/ml ampicillin and incubated overnight at 37°C. The culture was diluted in 1000 ml LB medium containing 100 µg/ml ampicillin and further incubated at 37°C until its OD_{600} reached ~0.4. Then, IPTG was added up to a final concentration of 0.5 mM to induce the expression of recombinant proteins. After additional incubation for 3 h at 37°C, the bacteria were centrifuged for 15 min at 6,500 rpm at 4°C (Sorvall RC-5B, DuPont) and resuspended in 35 ml buffer A for His_6-tagged cofilin or 40 ml PBS supplemented with proteinase inhibitors for GST-tagged RBD. The bacteria were disrupted by sonification (4 × 30 s, 4°C, Labsonic U, Braun) and Triton X-100 was added up to a final concentration of 1 % (v/v). The mixture was incubated for 30 min at 4°C with gentle shaking (REAX 2, Heidolph) and centrifuged for 15 min at 18,000 rpm at 4°C (Sorvall RC-5B, DuPont). The supernatant was incubated overnight with either Ni-NTA superflow suspension beads for His_6-tagged cofilin or glutathione Sepharose beads for GST-tagged RBD at 4°C while gently rocking (REAX 2, Heidolph).

The glutathione Sepharose beads with GST-tagged RBD were washed with appropriate buffer and stored at 4°C. The Ni-NTA beads with His_6-tagged cofilin were packed in a chromatography column (BIO-RAD) and washed three times with 20 ml buffer B to remove nonspecifically bound proteins. Cofilin was eluted by addition of 5 × 1 ml buffer C. The eluate was consecutively dialyzed overnight and 2 × 2 h against 500 ml buffer A at 4°C. Finally, cofilin was concentrated (microconcentrator, Centricon 10) by centrifugation for 30 min at 6,000 rpm at 4°C

(Sorvall RC-5B, DuPont). The protein concentration was determined by the Bradford method. The concentrated eluate was analyzed by SDS-PAGE and cofilin visualized by Coomassie-blue staining.

Buffer A, pH 8.0	Tris/HCl	50	mM
	NaCl	100	mM
	$MgCl_2$	2	mM
	2-Mercaptoethanol	6	mM
	Glycerol	5 %	v/v
Buffer B, pH 8.0	Buffer A plus		
	Imidazole	25	mM
Buffer C, pH 8.0	Buffer A plus		
	Imidazole	400	mM
PBS (supplemented)	PBS (without Ca^{2+}, Mg^{2+})		
	DTT	0.5	mM
	PMSF	1	mM
	Leupeptin	1	µg/ml
	Benzamidine	1	µg/ml
	Aprotinin	0.1	µM
	Soybean trypsin inhibitor	2	µg/ml

3.2.5.3 TSA cells

TSA cells are HEK-293 cells transformed with temperature-sensitive tsA SV-40 virus and stably expressing the large SV40 T antigen. To obtain recombinant LIM-kinase, subconfluent TSA cells were transfected by the calcium phosphate method with pUCD2 SRα GST/flag-tagged LIM-kinase. The transfected cells were harvested by centrifugation for 15 min at 1,800 rpm at RT (Sorvall RC-5B, DuPont), washed once with ice-cold Ca^{2+}/Mg^{2+}-free PBS and resuspended in TegD buffer I. Purification of LIM-kinase was performed as described in 3.2.5.1.

3.2.6 Protein determination

3.2.6.1 **Bradford method** (Bradford, 1976)

Protein samples were appropriately diluted, adjusted to 800 µl with H_2O (800 µl H_2O was used as reference value) and thoroughly mixed with 200 µl Bradford reagent in a disposable Polystyrol cuvette. The samples were measured at OD_{595} nm (LKB Biochrom Ultraspec II spectrometer, Pharmacia) after they had reacted for 10 min. Protein concentrations were calculated against a standard curve ranging from 0.4 - 2.0 µg/ml calf γ-globulin.

Bradford reagent	Serva-blue G	350 µg/ml
	Ethanol	25 % (v/v)
	Phosphoric acid (85 %)	25 % (v/v)

3.2.6.2 **Pierce method** (Smith *et al.*, 1985)

To determine the protein concentration of samples in SDS-containing lysis buffer, the standard BCA Protein Assay kit (Pierce) was used as indicated. Protein concentration was measured at OD_{562} nm (LKB Biochrom Ultraspec II spectrometer, Pharmacia).

3.2.7 SDS-PAGE and immunoblotting (Laemmli, 1970; Towbin *et al.*, 1979)

For production of polyacrylamide gels, acrylamide and bis-acrylamide were mixed in a proportion of 30:0.8, and the polymerization was initiated by addition of TEMED and ammonium peroxodisulfate. The stacking gel contained 6 % (m/v) acrylamide, while acrylamide concentration (10 %, 12.5 %, 15 % (m/v)) of the separating gel was dependent on molecular size of the proteins of interest. Samples were incubated in Laemmli sample buffer for 10 min at 95°C before loading onto the gel. Electrophoresis was performed at 36 mA for 4 h in electrophoresis buffer.

For immunoblotting, the separated proteins on the gel were transferred onto nitrocellulose membranes by electrophoresis (100 mA) overnight at 4°C in blot buffer. The membranes were stained with Ponceau S. To eliminate unspecific binding, the membranes were blocked for 2 h with TBS containing 5 % (w/v) skim milk powder. After short rinse with TBS, the membranes were

incubated for at least 1 h with appropriate primary antibodies, which were diluted in TBS containing 0.1 % (m/v) BSA. The membranes were then rinsed three times with TBS containing 0.2 % (m/v) Tween 20, followed by 10 min blot in TBS containing 5 % (w/v) skim milk powder. Afterwards, the membranes were incubated for 1 h with the suitable peroxidase-conjugated secondary antibodies (dilution 1:5,000 to 1:10,000). The membranes were rinsed three times with TBS containing 0.2 % (m/v) Tween 20, then incubated for 1 min with ECL reagent. The chemiluminescent reaction was detected on Kodak X-ray films by exposure for 30 s - 20 min.

Alternatively, the separated proteins on the gel were directly stained with Coomassie-blue staining solution. The gels were vacuum dried at 80°C (Gel Dryer 543, BIO-RAD).

2 × Separating buffer, pH 8.8	Tris/HCl	750 mM
	SDS	0.2 % (m/v)
2 × Stacking buffer, pH 6.8	Tris/HCl	250 mM
	SDS	0.2 % (m/v)
Laemmli sample buffer, pH 7.5	Tris/HCl	20 mM
(3 × concentrated)	SDS	6.6 % (m/v)
	2-Mercaptoethanol	20 % (v/v)
	Glycerol	33 % (v/v)
	Bromphenol blue dye	0.1 % (m/v)
Electrophoresis buffer, pH 8.3	Tris	25 mM
	Glycine	200 mM
	SDS	1 % (m/v)
Blot buffer, pH 8.3	Tris/HCl	40 mM
	Glycine	300 mM
	SDS	0.01 % (m/v)
	Methanol	20 % (v/v)
TBS, pH 7.4	Tris/HCl	10 mM
	NaCl	150 mM

Coomassie-blue staining solution	Serva blue R	1.4	mg/ml
	Methanol	50 %	(v/v)
	Acetic acid	10 %	(v/v)
Ponceau S	Ponceau S	0.1 %	(m/v)
	Acetic acid	5 %	(v/v)
High molecular weight standards	Myosin	205	kDa
	β-Galactosidase	116	kDa
	Phosphorylase b	97.4	kDa
	Albumin, bovine	66	kDa
	Albumin, egg	45	kDa
	Carbonic anhydrase	29	kDa
Low molecular weight standards	Albumin, bovine	66	kDa
	Albumin, egg	45	kDa
	Glyceraldehyde-3-phosphate-dehydrogenase	36	kDa
	Carbonic anhydrase	29	kDa
	Trypsinogen	24	kDa
	Soybean trypsin inhibitor	20	kDa
	α-Lactalbumin	14.2	kDa

3.2.8 Interaction of PLD and cofilin

Glutathione Sepharose beads loaded with GST-PLD1 or GST-PLD2 were rinsed with TEgD buffer II (see 3.2.5.1) and equally divided in 1.5 ml Eppendorf tubes (10 - 30 µg protein per tube). The beads were incubated overnight with 30 µg recombinant cofilin (wild type or S3A) or with buffer A (see 3.2.5.2) at 4°C with gentle shaking (REAX 2, Heidolph). The beads were collected by centrifugation (2 min, 4°C, 1800 rpm) and washed three times with 700 µl ice-cold buffer A to remove unspecifically bound proteins. Finally, the beads were resuspended in Laemmli

sample buffer. PLD and cofilin were separated by SDS-PAGE and detected by immunoblotting (see 3.2.7.) with anti-PLD and anti-His antibodies, respectively.

3.2.9 Phosphorylation of recombinant proteins

To study whether LIM-kinase phosphorylates PLD1 *in vitro*, GST-LIM-kinase-bound glutathione Sepharose beads (15 - 25 µg protein) were equilibrated with P-buffer and mixed with 20 - 25 µg recombinant PLD1 or membranes of PLD1-overexpressing *Sf9* cells in the presence and absence of recombinant ARF1, Rho-kinase CAT or cofilin. Alternatively, 10 - 30 µl eluted LIM-kinase in TEgD buffer III was used (see 3.2.5.1). The mixtures were preincubated for 5 min at 37°C and ATP-mix was added at a dilution of 1:5 to start the phosphorylation reaction. After 15 min, the reaction was stopped by addition of Laemmli sample buffer and heating for 5 min at 95°C. The proteins were separated by SDS-PAGE, and phosphorylated proteins detected by autoradiography using Kodak X-Omat AR films. Phosphorylation of cofilin by LIM-kinase was typically performed for 45 min at 25°C.

P-buffer, pH 7.4	$MgCl_2$	5 mM
	EDTA	1 mM
	EGTA	0.1 mM
	DTT	1 mM
	Tris/HCl	25 mM
ATP-mix, pH 7.5	$MgCl_2$	25 mM
	$CaCl_2$	1 mM
	MgATP	250 µM
	[γ-^{32}P]ATP	5 µCi/sample
	Tris/HCl	100 mM

3.2.10 Immunofluorescence laser confocal microscopy

HEK-293 cells, stably expressing the M_3 mAChR, were transiently transfected with PLD and/or cofilin and grown on coverslips (Falcon) pretreated with 0.1 mg/ml poly-D-lysine. The cells

were rinsed with Moscona and incubated with agonists dissolved in Moscona for 15 min at 37°C. Thereafter, the cells were fixed and permeabilized with ethanol/acetone (1/1, v/v) for 10 min at RT, followed by two washes with Moscona. The unspecific binding sites of the cells were blocked for 15 min by incubation with Moscona/BSA (Moscona supplemented with 0.5 % BSA). The cells were rinsed, incubated with the primary antibodies (anti-PLD or anti-His) diluted in Moscona/BSA. Afterwards, the cells were rinsed three times with Moscona/BSA, incubated for 45 min in darkness with the corresponding fluorescence-conjugated secondary antibodies (Alexa-633 red for PLD, Alexa-488 green for cofilin) at RT. The secondary antibodies were washed out in darkness with Moscona/BSA, and the cell specimens were mounted in Moscona supplemented with 90 % (v/v) glycerol and 1.0 % (v/v) p-phenylenediamine. Confocal immunofluorescence imaging was performed using Zeiss LSM 510 Axiovert 100M confocal laser scanning microscopy system (Plan-Neofluor 40×/1.3 oil objective, 488 nm / 633 nm excitation).

Moscona buffer, pH 7.4	NaCl	13.6 mM
	D(+)-Glucose	10 mM
	$NaHCO_3$	12 mM
	KCl	4 mM
	NaH_2PO_4	0.36 mM
	KH_2PO_4	0.18 mM

3.2.11 Phalloidin staining and fluorescence microscopy

Cell monolayers on coverslips (as described above) were rinsed with Moscona and fixed with 3 % (v/v) para-formaldehyde for 15 min, followed by two washes with Moscona. Cells were permeabilized by incubation with 0.05 % (v/v) Triton X-100. The cells were washed three times with Moscona and then incubated for 15 min with TRITC-phalloidin (25 µg/ml) to stain the actin filaments. After washout of TRITC-phalloidin, the cells were mounted with Moviol (Calbiochem) and examined by fluorescence microscopy (Zeiss Axiovert S100, 546 nm excitation).

3.2.12 Subcellular translocation of ARF1 and RhoA (Heneweer *et al.*, 2002)

To determine the subcellular distribution of ARF1 and RhoA, cell monolayers were detached, the cells were washed with PBS and resuspended in PBS at a concentration of ~4×10^7 cells/ml. 40 µl aliquots were incubated with receptor agonists for 10 min at 37°C. 950 µl lysis buffer A were added, and the cells were lysed by three freeze/thaw cycles (snap-freezing in liquid nitrogen followed by thawing at 37°C). The lysates were centrifuged for 10 min at 10,000 rpm at 4°C (Biofuge 13, Heraeus). The pellets were resuspended in 500 µl lysis buffer B, sonified for 10 s at 4°C (Labsonic U, Braun) and again centrifuged for 10 min at 10,000 rpm at 4°C (Biofuge 13, Heraeus). The content of ARF1/RhoA in the supernatant was determined by SDS-PAGE and immunoblotting with appropriate antibodies.

Lysis buffer A, pH 7.4	EDTA	2 mM
	EGTA	2 mM
	DTT	1 mM
	Tris/HCl	20 mM
	PMSF	1 mM
	Leupeptin	10 µM
	Aprotinin	2 µg/ml
	Soybean trypsin inhibitor	50 µg/ml
Lysis buffer B, pH 7.4	Lysis buffer A plus	
	Triton X-100	1 % (v/v)

3.2.13 "Pull-down" assay of RhoA (Ren & Schwartz, 2000)

Alternatively, RhoA activation was assessed by measuring the binding of RhoA to GST-tagged RBD of Rhotekin. Subconfluent cell monolayers were rinsed with HBSS and incubated with agonists for 2 - 5 min at 37°C. The stimulation was stopped by addition of ice-cold 700 µl GST-fish buffer. The cells were scraped, incubated for 10 min on ice in 1.5 ml Eppendorf tubes and then centrifuged for 10 min at 10,000 rpm at 4°C (Biofuge 13, Heraeus). The RhoA-containing supernatant was incubated for 2 h with the GST-tagged RBD-bound glutathione Sepharose beads (30 µg protein pro sample) at 4°C with gentle shaking (REAX 2, Heidolph). Thereafter, the beads were spun down, washed twice with 1 ml GST-fish buffer and resuspended in Laemmli sample

buffer for SDS-PAGE. The presence of RhoA was detected by immunoblotting with the anti-RhoA antibody.

HBSS, pH 7.4	NaCl	118	mM
	D(+)-Glucose	5	mM
	KCl	5	mM
	$CaCl_2$	1	mM
	$MgCl_2$	1	mM
	HEPES	15	mM
GST-fish buffer	Tris/HCl	50	mM
	NaCl	100	mM
	$MgCl_2$	2	mM
	PMSF	1	mM
	Glycerol	10 %	(v/v)
	Nonidet P-40	1 %	(v/v)
	Leupeptin	1	µg/ml
	Aprotinin	100	nM
	Soybean trypsin inhibitor	10	µg/ml

3.2.14 Assay of PLD activity in intact cells (Schmidt *et al*. 1999)

In the presence of ethanol, PLD catalyzes the transphosphatidylation of its substrate phosphatidylcholine (PtdCho) to produce the metabolically stable phosphatidylethanol (PtdEtOH) (Exton, 2002). Cells were replated 24 h after transfection on 35-mm culture dishes, and cellular phospholipids were labeled by incubating monolayers for 20-24 h with [^{3}H]oleic acid (2 µCi/ml) in culture medium. The cell monolayers were rinsed with HBSS and incubated with agonists/inhibitors in HBSS containing 2 % (v/v) ethanol at 37°C. The reactions were stopped by addition of 500 µl ice-cold methanol. Cell monolayers were scraped and washed with another 500 µl methanol and transferred into 6.5 ml reaction tubes. Cell suspensions were vigorously mixed with 1 ml chloroform and 0.5 ml H_2O, and the mixtures were centrifuged for 10 min at 3,400 rpm at 4°C (Megafuge 1.0R, Heraeus). Thereafter, the lower organic phase was transferred into 3.5 ml reaction tubes and vacuum dried (Speed Vac plus SC 110A, Savant). Dried lipids were resuspended in 25 µl

chloroform/methanol (1/1, v/v), and 10 µl samples were spotted onto thin layer plates (Silica gel 60C) preloaded with lipid standards (PA and PtdEtOH). Chromatography was performed in ethylacetate/iso-octane/acetic acid/H_2O (13/2/3/10, v/v/v/v). Afterwards, the separated lipids on the plates were stained in an iodine chamber, and the positions were marked according to the standards. Radioactive fractions were scraped out and mixed with 3 ml scintillation solution for liquid scintillation counting (Beckman LS6000 SC). Measurements in the absence of ethanol were used to determine unspecific radioactivity. The formation of [^{3}H]PtdEtOH was expressed as percentage of the total amount of labeled phospholipids.

Alternatively, cells were replated on 145-mm culture dishes and incubated for 20 - 24 h with [^{3}H]oleic acid (2 µCi/ml) in culture medium. Thereafter, the cells were detached from the dishes, washed twice in HBSS with centrifugation at 1,700 rpm for 5 min at 4°C and resuspended at 1×10^7 cells/ml. Reaction mixtures (200 µl) containing 100 µl cell suspension, 2 % (v/v) ethanol and the indicated agonists or inhibitors were incubated for 45 min at 37°C. The reaction was stopped by addition of 1 ml ice-cold chloroform/methanol (1/1, v/v) and 0.5 ml H_2O. After vortexing, the samples were centrifuged for 10 min at 3,400 rpm at 4°C. The lower organic phase was transferred into 3.5 ml reaction tubes, and the samples were further processed as described above.

3.2.15 PLD activity measurement *in vitro*

[^{3}H]PtdCho and PIP_2 in a molar ratio of 10:1 were vacuum dried (Speed Vac plus SC 110A, Savant) and emulsified in vesicle solution by sonication (3 × 20 s; Labsonic U, Braun). The lipids (200 µM [^{3}H]PtdCho, 500,000 cpm per assay; 25 µM PIP_2) were mixed with membrane fractions (200 µg protein), 2 % (v/v) ethanol, agonists and regulatory factors in 100 µl assay buffer. The mixture was incubated for 15 min at 30°C for PLD measurement. The reaction was stopped by addition of 1 ml ice-cold chloroform/methanol (1/1, v/v) and 0.5 ml H_2O. The further procedure was as decribed in 3.2.14. The PLD activity was expressed as the formation of PtdEtOH in nmol × min^{-1} × mg^{-1} protein.

Alternatively, to study the role of phosphorylated cofilin on PLD activity, GST-tagged LIM-kinase-bound glutathione Sepharose beads and His_6-tagged cofilin (wild type or S3A) were incubated together at an appropriate ratio of protein content in the presence or absence of 1 mM MgATP for 45 min at 25°C. The GST-tagged LIM-kinase-bound glutathione Sepharose beads were removed by centrifugation for 3 min at 2,400 rpm at 4 °C. The supernatant containing phosphorylated or non-phosphorylated cofilin was incubated with GST-tagged PLD1/PLD2-bound

glutathione Sepharose beads (20 - 30 µg / reaction tube), [^{3}H]PtdCho/PIP_2 vesicles, 2 % (v/v) ethanol and stimulatory agents for 30 min at 30 °C. The reactions were stopped, and further procedures were as described above.

Vesicle solution, pH 7.5	EGTA	3 mM
	KCl	80 mM
	DTT	1 mM
	HEPES	50 mM
Assay buffer, pH 7.5	EGTA	3 mM
	KCl	80 mM
	$MgCl_2$	3 mM
	$CaCl_2$	2 mM
	DTT	1 mM
	HEPES	50 mM

3.2.16 Inositol phosphate formation assay (Schmidt *et al.*, 2000)

Cells were labeled with *myo*-[^{3}H]inositol (1 µCi/ml) in inositol-free DMEM/F12 medium for 16 - 24 h and equilibrated with HBSS/LiCl (HBSS supplemented with 10 mM LiCl) for 10 min at 37°C in 35-mm dishes before challenge with agonists/inhibitors in HBSS/LiCl for 30 min. LiCl was included to inhibit the dephosphorylation of *myo*-[^{3}H]inositol monophosphate (Berridge *et al.*, 1993). The reaction was stopped by addition of 1 ml ice-cold methanol to the cell monolayers. The cells were scraped on ice and transferred into 3.5 ml reaction tubes. Thereafter, 1 ml chloroform and 0.5 ml H_2O were added, and the mixture was vortexed and centrifuged at 3,500 rpm for 10 min at 4°C (Megafuge 1.0R, Heraeus). The aqueous upper phase was loaded onto an anion exchange resin column (AG 1-X8, 200-400 mesh, chloride form) to determine the formation of inositol phosphates. The columns were washed with 6 ml H_2O and 5 ml 50 mM ammonium formiate containing 0.1 M formic acid. Finally, *myo*-[^{3}H]IP_x (= [^{3}H]IP + [^{3}H]IP_2 + [^{3}H]IP_3) were eluted by addition of 6 ml 1 M ammonium formiate containing 0.1 M formic acid. Alternatively, measurement of PLC activity was performed in cell suspension (see 3.2.14). The upper hydrophilic phase was transferred into 3.5 ml reaction tubes, and the samples were further handled as described above. The radioactivity was

measured in 1 ml aliquots by liquid scintillation spectrometry (Beckman LS6000 SC). Unlabeled cells were scraped in 500 µl 0.1 M NaOH for protein determination by the Braford method. [^{3}H]IP_x formation was expressed as cpm $\times 10^{-3} \times mg^{-1}$ protein or as cpm $\times 10^{-3} \times 10^{-6}$ cells.

3.2.17 IP_3 and PIP_2 mass assays (Schmidt *et al.*, 2000)

For direct measurement of the cellular mass of IP_3 and PIP_2, transfected cell monolayers on 35-mm culture dishes (~80 % confluence) were incubated for 1 min with agonists in HBSS. After aspiration of the HBSS, 0.5 ml 0.5 M cold TCA was added and allowed to extract the cellular IP_3 for 20 min on ice. The TCA solution was transferred into 6.5 ml reaction tubes and extracted 3 times with 3 vol. H_2O/diethylether (1/2, v/v) to remove the TCA. 200 µl of the IP_3-containing aqueous lower phase was transferred to 1.5 ml Eppendorf tubes and mixed with 50 µl 30 mM EDTA (pH 7.0) and 50 µl 60 mM $NaHCO_3$.

After TCA extraction, the remaining cell monolayers were analyzed for PIP_2 mass. For this, 0.94 ml cold acidified chloroform/methanol (chloroform/methanol/12 N HCl, 40/80/1, v/v/v) were added to the cells, and the cells were immediately scraped, transferred into 6.5 ml reaction tubes and mixed with 0.31 ml chloroform and 0.56 ml 0.1 M HCl. After centrifugation for 10 min at 2,400 rpm at 4°C (Megafuge 1.0R, Heraeus), 400 µl of the lower phase was transferred to 1.5 ml Eppendorf tubes and vacuum dried (Speed Vac, plus SC 110A, Savant). PIP_2 was chemically hydrolyzed into IP_3 by incubation with 0.25 ml 1 M KOH for 15 min at 95°C (Thermoshaker, Schutron). For neutralization, KOH-treated samples were loaded onto ion exchange columns (AG 50-X8, 200-400 mesh, hydrogen form) and eluted with 5 × 0.25 ml H_2O. The eluate was adjusted to ~pH 7 with 1 M $NaHCO_3$ and thoroughly mixed with 2 ml 1-butanol/light petroleum ether (5/1, v/v). The mixture was centrifuged for 10 min at 2,400 rpm at 4°C (Megafuge 1.0R, Heraeus), and 1 ml of the aqueous lower phase was used for the IP_3 receptor binding assay. The columns were regenerated by 5 ml 1 M HCl and 5 × 5 ml H_2O.

IP_3-binding protein was obtained from bovine adrenal cortex. For this, adrenal cortex tissue was thoroughly homogenized (Ultra-Turrax, setting 5-6, Janke & Kunkel) in buffer (20 mM $NaHCO_3$, 1 mM dithiothreitol, pH 8.0) and centrifuged at 5,000 rpm for 10 min at 4°C (Megafuge 1.0R, Heraeus). The supernatant was collected, and the pellet was resuspended in buffer for renewed homogenization and centrifugation. The resulting supernatants were combined and centrifuged at 15,000 rpm for 20 min at 4°C (Sorvall RC-5B, DuPont). The supernatant was discarded, and the pellet was resuspended in 50 ml homogenization buffer and recentrifuged for 20

min at 15,000 rpm at 4°C. The pellet was washed two times, adjusted to 15 mg/ml and stored at -80°C.

The IP_3 receptor binding assay included 30 µl standard IP_3 (0.036 - 36 pmol) or the unknown samples, 30 µl assay buffer (100 mM Tris/HCl, 4 mM EDTA, pH 8.0), 30 µl [^{3}H]IP_3 (5,000 cpm) and 30 µl IP_3-binding protein. Steady-state receptor occupation was allowed to be established for 30 min on ice. Thereafter, the mixtures were filtrated on GF/C-filter, and the filters were washed 3 times with 3 ml buffer (25 mM Tris/HCl, 1 mM EDTA, 5 mM $NaHCO_3$, pH 8.0). Bound [^{3}H]IP_3 was determined by liquid scintillation counting. The cellular content of IP_3 and PIP_2 was calculated and expressed as pmol × mg^{-1} protein.

3.2.18 Noradrenaline release

PC12 cells in 35-mm dishes were loaded for 40 min at 37°C with 0.1 µCi/ml [^{3}H]noradrenaline in HBSS containing 0.2 % fatty acid-free BSA. After washing two times with Ca^{2+}/Mg^{2+}-free PBS, the cell monolayers were challenged with 1 ml 30 mM KCl/HBSS or HBSS containing 10 µM bradykinin for 10 min at 37°C. The supernatant containing released [^{3}H]noradrenaline was directly transferred in a scintillation vial for radioactivity measurement. To determine the total cellular content of [^{3}H]noradrenaline, cells were lysed in 1 % (m/v) SDS, followed by liquid scintillation counting.

30 mM KCl/ HBSS, pH 7.4	NaCl	93 mM
	D(+)-Glucose	5 mM
	KCl	30 mM
	$CaCl_2$	1 mM
	$MgCl_2$	1 mM
	HEPES	15 mM

3.2.19 Generation of recombinant PLD adenoviruses (He *et al.*, 1998)

3.2.19.1 Cloning of PLD into pAdTrack-CMV

To produce recombinant PLD adenovirus, the PLD genes were first excised from the eukaryotic expression vector pCGN and cloned into the pAdTrack-CMV shuttle vector, encoding green fluorescent protein (GFP; see also Fig. 5). 7.5 - 10 µg pCGN plasmid encoding hPLD1 or

mPLD2 were digested by XbaI and SmaI restriction endonucleases (3 U pro µg plasmid) in a 20 µl reaction system (Sambook *et al*, 1989). The PLD gene fragments were directly separated by electrophoresis on 0.8 % (m/v) agarose and extracted by water-saturated phenol and chloroform/isoamylethanol (24/1, v/v). Afterwards, PLD gene fragments were precipitated by addition of 1/10 volume 3 M sodium acetate (pH 5.2) and the same volume of isopropanol at -20°C for 30 min. After centrifugation at 13,000 rpm for 30 min at 4°C, the DNA pellet was washed twice with 70 % (v/v) ethanol, air dried and finally dissolved in 10 µl H_2O. The DNA concentration was determined at 260 nm (LKB Biochrom Ultraspec II Spectrophotometer, Pharmacia). In parallel, 10 µg pAdTrack-CMV vector was digested with XbaI and EcoRV restriction endonucleases, and the linearized vector was extracted, precipitated and washed as described above. The 5'-ends of linearized vector DNAs were dephosphorylated with alkaline phosphatase (1 U pro pMol 5'-end of linearized vector) for 30 min at 37°C. Thereafter, the dephosphorylated vectors were extracted using the "QIAquick" column (Qiagen) and eluted in 30 µl H_2O. The DNA concentration was determined at 260 nm. 300 ng dephosphorylated pAdTrack-CMV vector were mixed with PLD inserts at a molar ratio of 1:3 (vector:insert) and adjusted with H_2O to 8.5 µl. The mixture was incubated at 45°C for 5 min, followed by cooling down on ice. 1 µl 10 × ligation buffer (Promega) and 5 U T4-DNA-ligase were added and incubated at 4°C overnight or, alternatively, at 16°C for 4 h (Thermoblock, Eppendorf). 50 - 300 ng of the ligation mixture DNA was transformed in DH10B *E. coli* by the electroporation method (see 3.2.2.2).

To screen for the pAdTrack-PLD clones, 3 ml *E. coli* culture in LB medium from each selected colony were centrifuged for 2 min at 6,000 rpm at RT (Megafuge 1.0R, Heraeus). The bacteria were resuspended in 250 µl TELT lysis buffer and incubated on ice for 5 min. Digestion was performed with 5 µl 50 mg/ml lysozyme for 5 min. Afterwards, the mixture was heated for 1 min at 95°C (Thermoblock, Eppendorf), followed by incubation for 5 min on ice. After centrifugation for 10 min at 12,000 rpm (Megafuge 1.0R, Heraeus), the plasmid-containing supernatant was mixed with 500 µl 100 % ethanol and incubated for 15 min at RT. The precipitated plasmids were rinsed with 80 % ethanol, air dried and dissolved in 10 µl H_2O. The plasmids were digested by the appropriate restriction endonucleases. Once the right clone (pAdTrack-PLD) was confirmed, its corresponding culture was amplified in 500 - 1000 ml LB medium with appropriate antibiotics for large-scale purification (Qiagen, maxi-prep kits).

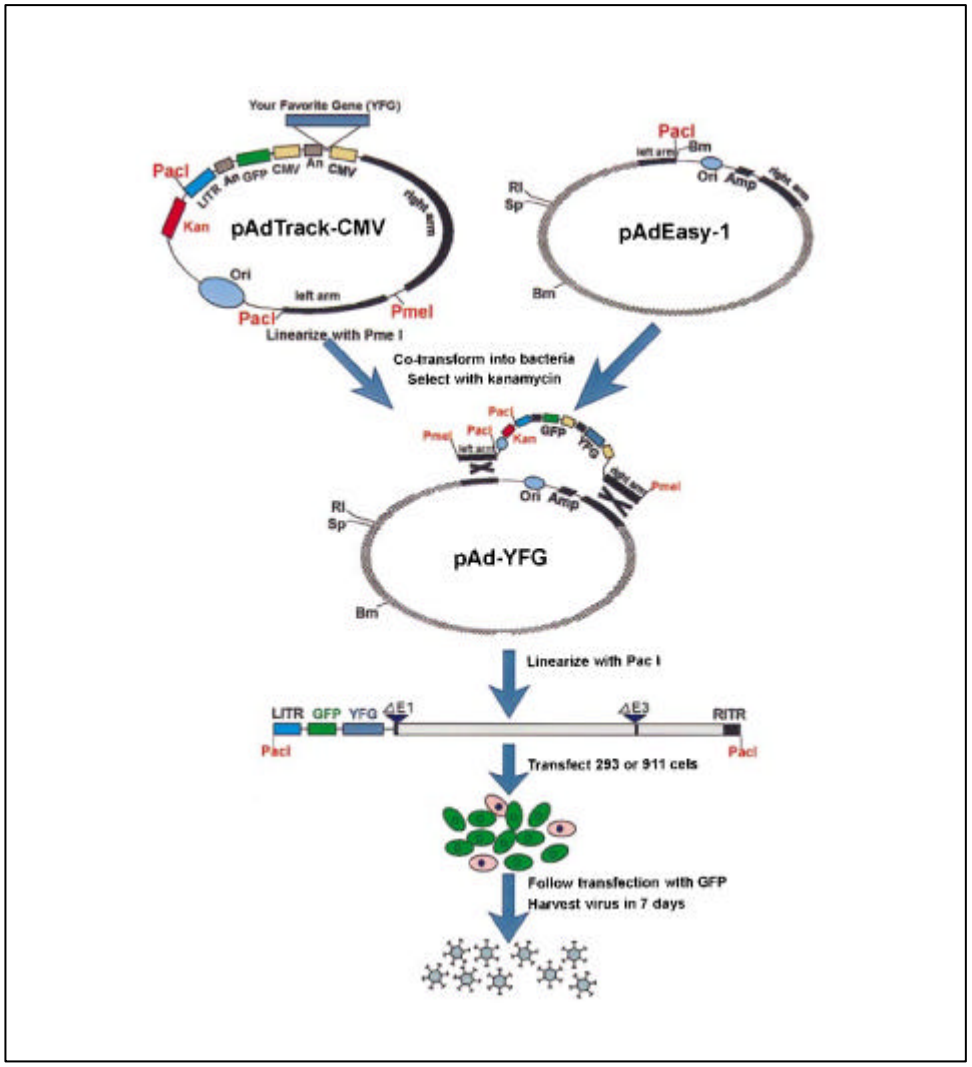


Fig. 5: Schematic outline of the AdEasy System. The gene of interest is first cloned into a shuttle vector, e.g. pAdTrack-CMV. The resultant plasmid is linearized by restriction endonuclease PmeI and subsequently cotransformed into *E. coli* BJ5183 cells with an adenoviral backbone plasmid, e.g. pAdEasy-1. Recombinants are selected for kanamycin resistance and confirmed by restriction endonuclease analyses. Finally, the PacI-linearized recombinant plasmid is transfected into adenovirus-packaging cell lines, e.g. HEK-293 cells. Recombinant adenoviruses are typically generated within 7-12 days. The "left arm" and "right arm" represent the regions mediating homologous recombination between the shuttle vector and the adenoviral backbone vector. An, polyadenylation site; Bm, BamHI; RI, EcoRI; LITR, left-hand inverted terminal repeat and packaging signal; RITR, right-hand inverted terminal repeat; Sp, SpeI (He *et al.*, 1998).

TBE buffer, pH 8.0	Tris-borate	89 mM
	Boric acid	89 mM
	EDTA	2 mM
TELT lysis buffer, pH 7.4	Tris/HCl	50 mM
	LiCl	2.5 mM
	EDTA	62.5 mM
	Triton X-100	0.4 % (v/v)

3.2.19.2 Homologous recombination

1 mg pAdTrack-PLD was linearized with PmeI. After extraction by phenol-chloroform-isoamylethanol and precipitation by ethanol (see 3.2.19.1), the linearized pAdTrack-PLD in 10 µl H_2O was co-transformed with 500 ng supercoiled adenoviral backbone pAdEasy-1 vector in 40 µl electrocompetent BJ5183 *E. coli* by the electroporation method (see 3.2.2.2; West, 1994). After incubation for 45 min in 1 ml SOC medium at 37°C, the BJ5183 *E. coli* was plated on LB kanamycin agar plates and incubated at 37°C overnight. Small clones were picked up for plasmid mini-preparation as described in 3.2.19.1. To determine the right recombinants (pAdEasy-PLD), the plasmids were screened by NdeI digestion. Finally, 1 µl of the correct recombinant plasmid was transformed into DH10B *E. coli* for large-scale purification (Qiagen, maxi-prep kits).

3.2.19.3 Preparation of high titer virus stock

30 µg pAdEasy-PLD were digested with PacI to expose its inverted terminal repeats for virus particle formation (Chartier *et al*., 1996). The linearized pAdEasy-PLD was extracted by phenol-chloroform-isoamylethanol, precipitated by ethanol and dissolved in 10 µl H_2O (see 3.2.19.1). HEK-293 wild-type cells on 145-mm culture dishes (~70 % confluence) were transfected with the linearized pAdEasy-PLD by the lipofectamine method (see 3.2.4.2). GFP expression was daily monitored by fluorescence microscopy (Zeiss Axiovert S100, ACHROPLAN 10 × / 0.25 Ph1). After 7 - 10 days, the cells were harvested by centrifugation at 1,700 rpm for 5

min at 4°C and resuspended in 10 ml DMEM/F12 medium. To release the viral particles, the cells were three times repetitively snap-frozen in liquid nitrogen and thawed at 37°C. The lysates were centrifuged at 2,200 rpm for 10 min at 4°C (Megafuge 1.0R, Heraeus), and the viruses (in the supernatant) were directly amplified or stored at -20°C. For virus amplication, HEK-293 cells on 145-mm culture dishes were washed with FCS-free DMEM/F12 medium, incubated with 0.4 ml virus-containing supernatant and 4 ml DMEM/F12 medium (2 % FCS) at 37°C for 30 min. Thereafter, 16 ml DMEM/F12 (10 % FCS) were added. Cell lysis and green fluorescence became evident at 2 - 3 days post infection. The cells were harvested in 10 ml DMEM/F12 medium and lysed by three freeze/thaw cycles. Typically, the amplification procedure resulted in at least a 10-fold increase in viral particles in the supernatant.

To produce high-titer virus stock, HEK-293 cells (30 x 145-mm culture dishes) were infected with virus supernatant (~5 MOI). The infected cells were harvested as described above, and the lysates incubated for 30 min with 175 µl 2 M $MgCl_2$, 100 µl 10 mg/ml RNase A and 60.2 µl 1 mg/ml DNase I at 37°C. After centrifugation at 3,000 rpm for 30 min at 4°C (Megafuge 1.0R, Heraeus), the virus-containing supernatant was extracted two times with an equal volume of trichlorotrifluoroethane (with centrifugation at 3,400 rpm for 5 min at 4°C) and transferred into a sterile Falcon tube. The remaining pellet was washed with DMEM/F12 medium, resuspended in Murakami buffer and stored at -80°C. A CsCl gradient was carefully built up in a centrifuge tube (Beckman No. 326814) with equal volumes of 1.46 g/ml and 1.2 g/ml CsCl, respectively, and the virus-containing supernatant was loaded on top of the gradient. The gradient was centrifuged at 35,000 rpm for 16 h at 10°C (L7-55 Ultracentrifuge, Beckman). The interphase was transferred with a syringe into a Slide-a-Lyzer for dialysis (2 × 2 h against 2 L Ca^{2+}/Mg^{2+}-free PBS, 1 × overnight against 2 L virus storage buffer). The virus solution was mixed with an equal volume of filter-sterilized virus storage buffer containing 50 % glycerol and stored at -20°C.

3.2.19.4 Virus titration and infection of cells

100 µl virus suspension or stock were mixed with 5 µl 10 mg/ml proteinase K (cleaving the polypeptide of virus particles) and 2.7 ml 20 % (m/v) SDS (dissolving the virus capsule) and incubated for 2 h at 55°C. Thereafter, the proteins were three times extracted with 300 µl chloropan and the virus DNA-containing aqueous phase was concentrated in a microconcentrator (Centricon 10) by centrifugation at 6,000 rpm for 30 min at 4°C (Sorvall RC-5B, DuPont). The concentrated DNA was separated by electrophoresis on 0.8 % (m/v) agarose, together with different amounts (10

- 30 ng) of purified pAdEasy-PLD plasmids. The titer of the virus stock was roughly estimated by comparing the thickness of the DNA bands of the virus DNA with the pAdEasy-PLD plasmids (21 ng DNA ≅ 6.2×10^8 adenovirus particles; 30 virus particles ≅ 1 biological active virus).

Cells on 145-mm culture dishes (~70 % confluence) were washed with FCS-free DMEM/F12 medium and infected with the appropriate amount of virus stock (~5 MOI). Viral infection was monitored by visualization of GFP reporter, that is incorporated into the viral backbone, by fluorescence microscopy (see 3.2.19.3).

Virus storage buffer, pH 7.4	Tris/HCl	10 mM
	NaCl	137 mM
	KCl	5 mM
	$MgCl_2$	1 mM
Murakami buffer, pH 7.4	Tris/HCl	50 mM
	$MgCl_2$	50 mM
	EDTA	5 mM
	EGTA	1 mM
	Aprotinin	5 µg/ml

3.2.20 Data analysis

Graphs were generated with GraphPad Prism (version 3.02, 2000). All experiments (if not indicated otherwise) were performed in triplicate. Data shown in figures are mean ± S.D. of representative experiments or mean ± S.E. of *n* independent experiments. Comparisons between means were performed with either the Student's paired *t* test or one-way analysis of variance test. Confocal laser microscopy images were processed by the Image Pro Plus software (version 4.5; Media cybernatics, Inc., Crofton, MD, USA).

4. Results

4.1 Specific requirement of G_{12}-type G proteins for M_3 mAChR signaling to PLD

As nearly every G protein-coupled receptor that stimulates PIP_2-specific PLC also causes PLD stimulation, it has been supposed that stimulation of PLD ensues from PLC activation with subsequent increase in cytoplasmic Ca^{2+} concentration and activation of PKC isoforms (Exton, 199; Cockcroft, 2001; Liscovitch *et al*., 2001; Exton, 2002). In HEK-293 cells, the G protein-coupled M_3 mAChR couples to both PLC and PLD *via* a PTX-insensitive mechanism (Peralta *et al.*, 1988; Offermanns *et al.*, 1994; Schmidt *et al.*, 1994), but stimulation of PLD by the M_3 mAChR agonist, carbachol, was not affected by PKC inhibition (Schmidt *et al.*, 1994; Rümenapp *et al.*, 1997). Additional work demonstrated that regulation of PLD by PMA-responsive PKC lies downstream of tyrosine kinase receptors (Schmidt *et al.*, 1998; Voß *et al.*, 1999). Thus, as the M_3 mAChR couples to PLC most likely *via* G_q-type G proteins (Offermanns *et al*., 1994), it was considered that the receptor couples to PLD *via* G_q proteins as well, or that two distinct PTX-insensitive G proteins mediate receptor coupling to the two phospholipases.

To determine the type of PTX-insensitive G protein mediating M_3 mAChR-PLD coupling in comparison to M_3 mAChR-PLC coupling, we transiently transfected HEK-293 cells with expression vectors encoding for mutants (wild-type and constitutively active) of the α-subunits of G_{12}, G_{13} and G_q. Expression of the individual Gα subunits was examined by immunoblotting with specific antibodies (Figs. 6A and 7A; Insets). In cells overexpressing wild-type $G\alpha_q$, both basal and carbachol-stimulated PLC activities were enhanced by 3- to 5-fold (Fig. 6B). Expression of constitutively active R183C $G\alpha_q$ caused an even larger increase in basal PLC activity, which was not further enhanced by carbachol (Fig. 7B). Despite this marked PLC stimulation, neither basal nor carbachol-stimulated PLD activities were altered by the expression of R183C $G\alpha_q$ (Fig. 7A).

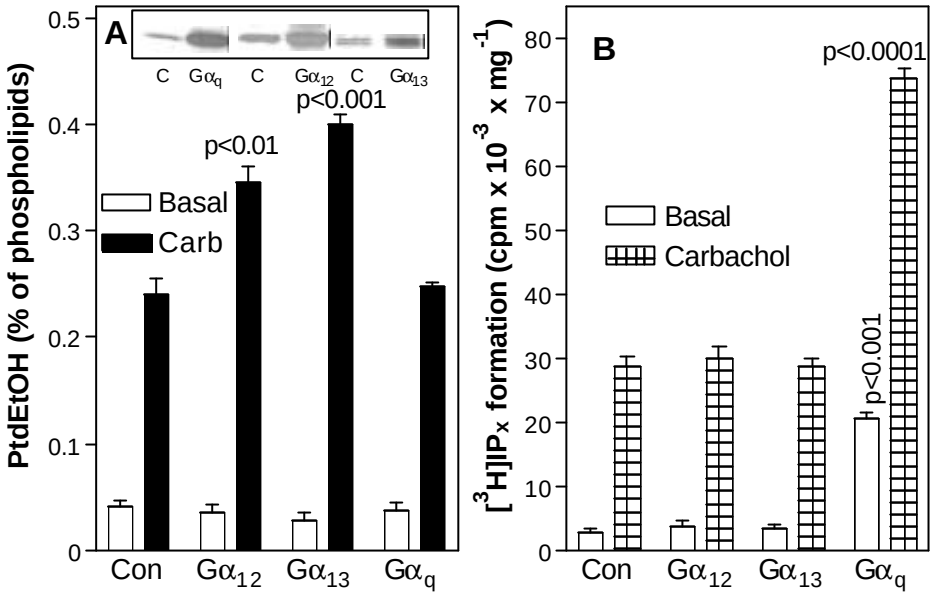


Fig. 6: Effects of overexpression of wild-type Gα proteins on PLD and PLC responses to M_3 mAChR activation. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Con*, *C*) and expression plasmids for $G\alpha_{12}$, $G\alpha_{13}$ or $G\alpha_q$ (100 µg each). After 48 h, PLD (**A**) and PLC (**B**) activities were determined by measuring accumulation of [^{3}H]PtdEtOH and [^{3}H]IP_x, respectively, in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*) (n = 4). **Inset:** Immunoblots of $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ with specific antibodies.

On the other hand, in cells overexpressing either (wild-type and constitutively active) $G\alpha_{12}$ or $G\alpha_{13}$, the PLD response to carbachol (1 mM) was increased by 2-fold compared to control cells, leaving basal PLD activity unaffected (Figs. 6A and 7A). Coexpression of constitutively active $G\alpha_{12}$ and $G\alpha_{13}$ led to a strong, receptor-independent increase in basal PLD activity (Fig. 8). In contrast, overexpression of either (wild-type and constitutively active) $G\alpha_{12}$ or $G\alpha_{13}$ did not modify M_3 mAChR signaling to PLC (Figs. 6B and 7B). Furthermore, PLD stimulation by the phorbol ester PMA (100 nM) was not affected by the overexpression of constitutively active $G\alpha_{12}$, $G\alpha_{13}$ or $G\alpha_q$ (Fig. 9), which is line with the finding that regulation of PLD by PMA-responsive PKC is triggered by tyrosine kinase receptors, but not by the M_3 mAChR, in HEK-293 cells (Schmidt *et al.*, 1994; Voß *et al.*, 1999). These data suggested that the M_3 mAChR couples specifically to PLD *via* G_{12}-type G proteins.

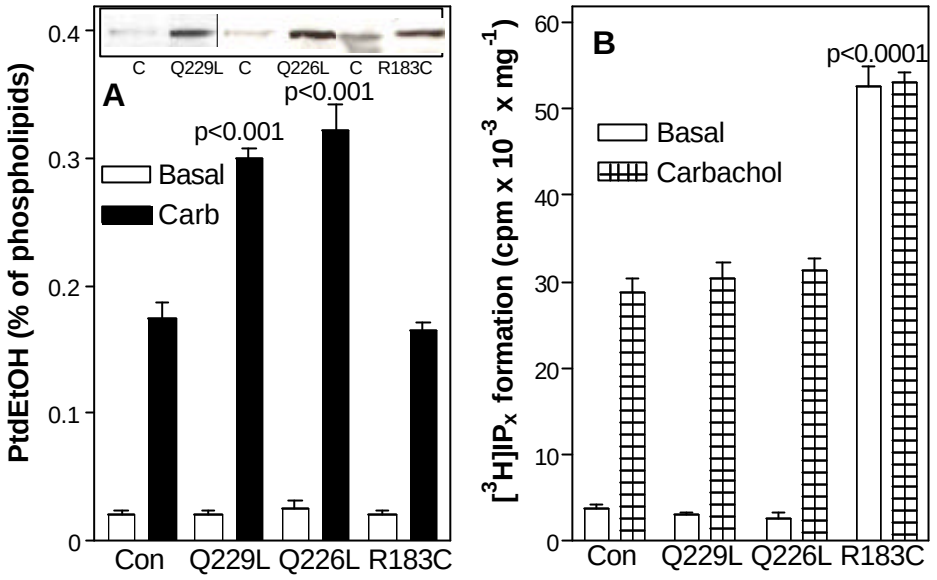


Fig. 7: Effects of expression of constitutively active Gα proteins on PLD and PLC responses to M_3 mAChR activation. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Con*, *C*) and expression plasmids for Q229L $G\alpha_{12}$, Q226L $G\alpha_{13}$ or R183C $G\alpha_q$ (50 µg each). After 48 h, PLD (**A**) and PLC (**B**) activities were determined by measuring accumulation of [^{3}H]PtdEtOH and [^{3}H]IP_x, respectively, in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*) (n = 4). **Inset:** Immunoblots of Q229L $G\alpha_{12}$, Q226L $G\alpha_{13}$ and R183C $G\alpha_q$ with specific antibodies.

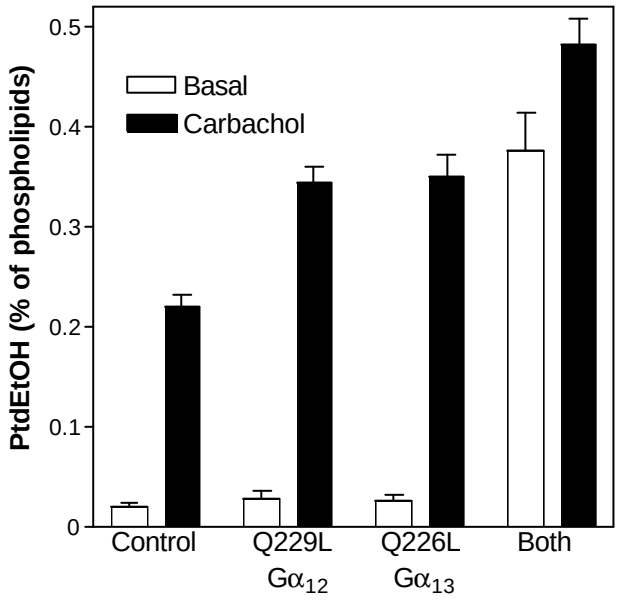


Fig. 8: Receptor-independent PLD stimulation by coexpression of constitutively active $G\alpha_{12}$ *plus* $G\alpha_{13}$. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Control*) and expression plasmids for Q229L $G\alpha_{12}$, Q226L $G\alpha_{13}$ or Q229L $G\alpha_{12}$ *plus* Q226L $G\alpha_{13}$ (*Both*) (10 µg each). After 48 h, [^{3}H]PtdEtOH accumulation was determined in the absence (*Basal*) and presence of 1 mM carbachol (n = 3).

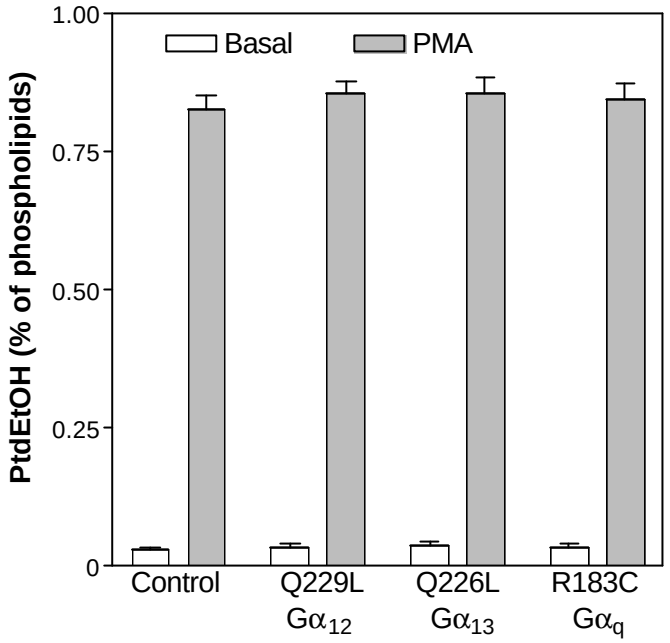


Fig. 9: Lack of effect of Gα proteins on PLD stimulation by PMA. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Control*) and expression plasmids for Q229L $G\alpha_{12}$, Q226L $G\alpha_{13}$ or R183C $G\alpha_q$ (50 µg each). After 48 h, [^{3}H]PtdEtOH accumulation was determined in the absence (*Basal*) and presence of 100 nM PMA (n = 3).

To determine the endogenous G protein subtype mediating the coupling of the M_3 mAChR to PLD and PLC, we made use of the two RGS proteins, RGS4 and the RGS homology domain of Lsc (Lsc-RGS; a murine homolog of a Rho-specific GEF), which act as GAPs for $G\alpha_q$ and $G\alpha_{12}$ family members, respectively (Druey *et al.*, 1996; Dohlman & Thorner, 1997; Wieland & Chen, 1999; de Vries *et al.*, 2000; Ross & Wilkie, 2000). Expression of the RGS proteins was proven by immunoblotting with specific antibodies (Fig. 10A; Inset). The transient expression of Lsc-RGS reduced by about 50 % the PLD response to carbachol, whereas expression of RGS4 was without effect (Fig. 10A). In contrast, expression of RGS4 attenuated the PLC response to carbachol by about 50 %, whereas the expression of Lsc-RGS did not alter PLC signaling (Fig. 10B). As shown in Fig. 11, the reduction of PLD stimulation induced by Lsc-RGS was mimicked by transient expression of G228A $G\alpha_{12}$ or G225A $G\alpha_{13}$, which act as dominant-negative mutants of $G\alpha_{12}$ and $G\alpha_{13}$, respectively (Gohla *et al.*, 1999).

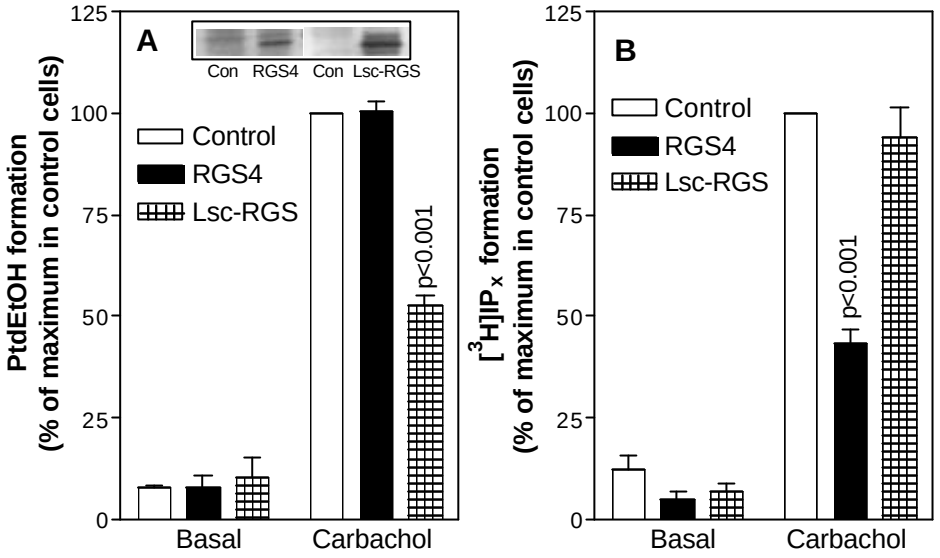


Fig. 10: Effects of transient expression of RGS4 and Lsc-RGS on PLD and PLC stimulation by the M_3 mAChR. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Control, Con*) and expression plasmids for flag-tagged RGS4 or myc-tagged Lsc-RGS (100 µg each). After 48 h, PLD **(A)** and PLC **(B)** activities were determined in the absence (*Basal*) and presence of 1 mM carbachol. Data are representative of four experiments. **Inset:** Immunoblot of flag-tagged RGS4 and myc-tagged Lsc-RGS with specific antibodies.

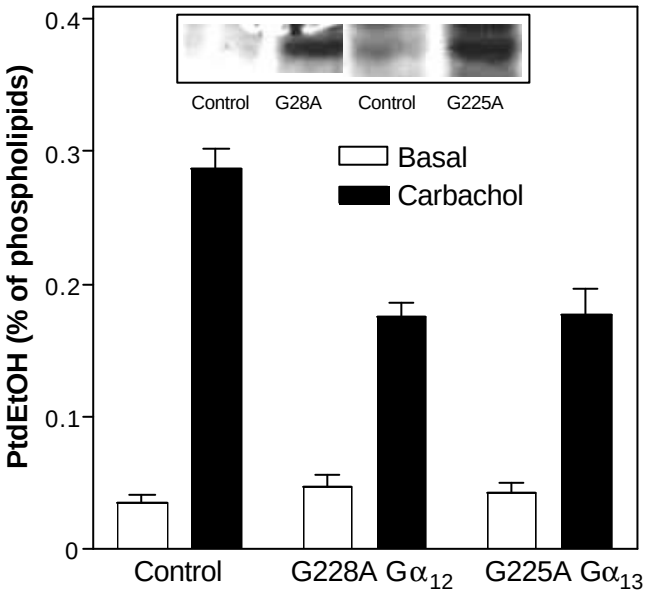


Fig. 11: Inhibition of the PLD response to the M_3 mAChR by dominant-negative $G\alpha_{12}$ and $G\alpha_{13}$. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Control*) and expression plasmids for G228A $G\alpha_{12}$ or G225A $G\alpha_{13}$ (100 µg each). After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 1 mM carbachol (n = 3). **Inset:** Immunoblots of G228A $G\alpha_{12}$ and G225A $G\alpha_{13}$ with specific antibodies.

In addition, the cells were infected with adenoviruses encoding RGS4 and Lsc-RGS, as this approach results in high efficient gene transfer (He *et al.*, 1998; Young & Mautner, 2001; Armstrong *et al.*, 2002). Expression of the individual adenoviruses was examined by expression of GFP (data not shown; see also Fig. 17) or immunoblotting with specific antibodies (Fig. 12A; Inset). The expression of RGS4 by adenoviral infection was without any effect on PLD stimulation by the M_3 mAChR (Fig. 12A). Neither the maximal extent nor the concentration dependence of PLD stimulation by carbachol was altered in RGS4-expressing cells, compared with control cells infected with an adenovirus encoding LacZ. In contrast, the carbachol-induced PLD stimulation was blunted (by about 80 %) by the expression of Lsc-RGS (Fig. 12A). On the other hand, adenoviral expression of Lsc-RGS did not affect the stimulation of PLC activity at any carbachol concentration, whereas PLC stimulation was strongly reduced (by about 70 %) by the expression of RGS4 (Fig. 12B). Taken together, expression (transient or by infection with recombinant adenoviruses) of RGS4 or Lsc-RGS in HEK-293 cells strongly indicates that G_q- and G_{12}-type G proteins specifically mediate the coupling of the M_3 mAChR to PLC and to PLD, respectively. Likewise, expression of dominant-negative mutants of $G\alpha_{12}$ and $G\alpha_{13}$ demonstrated that both heterotrimeric G proteins are indispensable to achieve full activation of PLD by the M_3 mAChR.

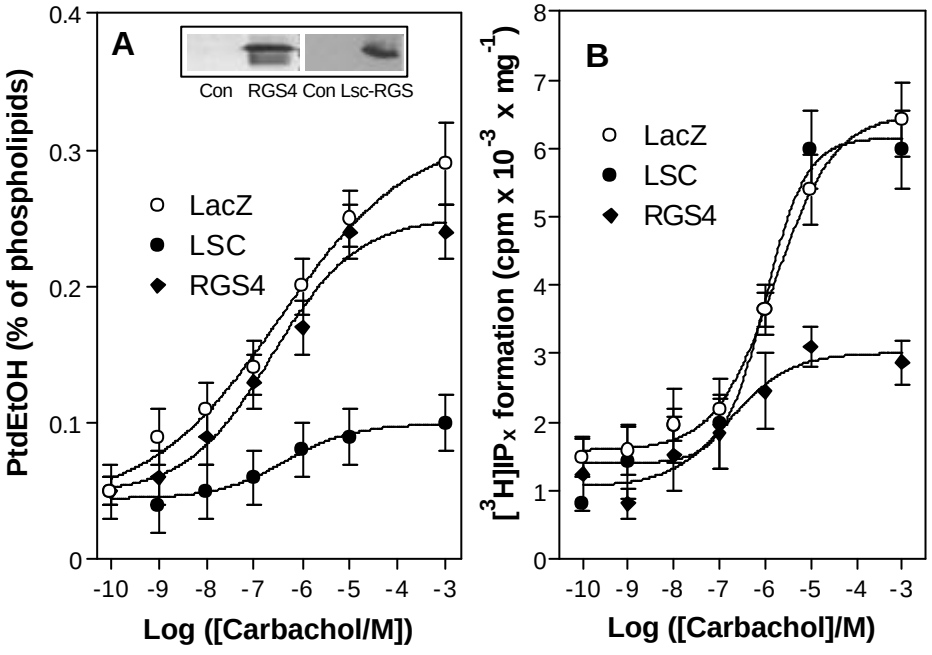


Fig. 12: Effects of adenoviral expression of RGS4 and Lsc-RGS on PLD and PLC stimulation by the M_3 mAChR. M_3 mAChR-expressing HEK-293 cells were infected with recombinant adenoviruses encoding LacZ, His_6-tagged Lsc-RGS and RGS4 at a MOI = 30. At 48 h later, PLD **(A)** and PLC **(B)** activities were determined at the indicated concentrations of carbachol. Data are representative of two experiments. **Inset:** Immunoblot of RGS4 and His_6-tagged Lsc-RGS with specific antibodies.

4.2 Involvement of distinct PLD isoforms in receptor signaling to PLD

Regulation of PLD by membrane receptors in HEK-293 cells involves apparently distinct signaling cascades (Schmidt *et al.*, 1994; Rümenapp *et al.*, 1995; Schmidt *et al.*, 1996b; Schmidt *et al.*, 1999; Voß *et al.*, 1999). In particular, as shown above, stimulation of PLD by the M_3 mAChR is mediated by the G_{12}-type G proteins, $G\alpha_{12}$ and $G\alpha_{13}$. Additional work demonstrated that the PLD response to the G protein-coupled M_3 mAChR depends on ARF and Rho GTPases, but not PKC. In contrast, stimulation of PLD by tyrosine kinase receptors is triggered by PKC-α and a Ras/Ral-GEF/Ral-dependent signaling cascade (see Introduction). As HEK-293 cells endogenously express both, PLD1 and PLD2 (Schmidt *et al.*, 1998; Schmidt *et al.*, 1999), it was considered that the distinct membrane receptors may signal to both PLD isoforms, or that PLD1 and PLD2 are differentially activated by these receptors.

As a first approach to gain insights into the regulation of PLD1 and PLD2 by membrane receptors in HEK-293 cells, we transiently transfected the cells with expression vectors encoding for catalytically inactive PLD mutants. Expression of the PLD mutants was probed by immunoblotting with specific antibodies (Fig. 13A; Inset). As shown in Fig. 13, expression of either mutant had only small effects on basal PLD activity. However, expression of the catalytically inactive K898R PLD1 mutant reduced by about 50 % the M_3 mAChR action (Fig. 13A). In contrast, expression of the catalytically inactive K758R PLD2 mutant only sightly affected PLD stimulation by carbachol. However, this mutant was not inactive. Expression of catalytically inactive PLD2 suppressed by about 60 % stimulation of PLD by PMA-responsive PKC (Fig. 13B). In contrast, expression of K898R PLD1 failed to inhibit PLD stimulation by PMA. As regulation of PLD by PMA-responsive PKC is triggered by tyrosine kinase receptors (Voß *et al.*, 1999), the effects of the catalytically inactive PLD mutants on EGF receptor signaling was analyzed as well. As shown in Fig. 13C, expression of catalytically inactive PLD2 reduced specifically PLD stimulation by EGF. Thus, in HEK-293 cells the M_3 mAChR signals most likely primarily to PLD1, whereas tyrosine kinase receptors signal primarily to PLD2.

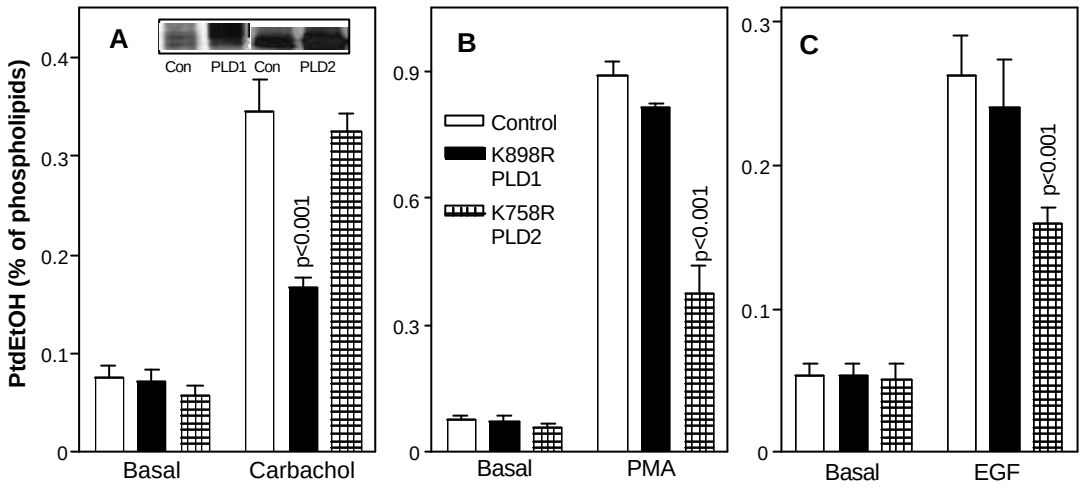


Fig. 13: Effects of catalytically inactive PLD isoform mutants on PLD stimulation by carbachol, PMA and EGF. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Control, Con*) and expression plasmids for K898R PLD1 or K758R PLD2 (150 µg each). After 48 h, [^{3}H]PtdEtOH formation was determined in the absence (*Basal*) and presence of 1 mM carbachol **(A)**, 100 nM PMA **(B)** or 100 ng/ml EGF **(C)** (*n* = 3-5). **Inset:** Immunoblot of PLD1 and PLD2 with specific antibodies.

4.3 Generation of recombinant PLD adenoviruses

Based on the promising findings with recombinant adenoviruses encoding RGS4 and Lsc-RGS (see also 4.1), adenoviruses encoding wild-type as well as catalytically inactive PLD1 and PLD2 mutants were generated for future work on the cellular role of these PLD enzymes in receptor action. To insert the PLD mutants into the shuttle vector pAdTrack-CMV (see also Fig. 5), wild-type and catalytically inactive PLD1 and PLD2 were excised from the pCGN vector using the XbaI + SmaI endonucleases. The size of PLD1 is about 3.5 kb and can be cut out by the restriction endonuclease BglII at position 685 and by the restriction endonuclease Nde I at position 1026. The size of PLD2 is about 3.0 kb and can be cut by Bgl II at position 2576, however PLD2 exhibits no NdeI cutting site. In addition, PLD1 and PLD2 did not contain cutting sites for the restriction endonucleases PmeI and PacI (Fig. 14).

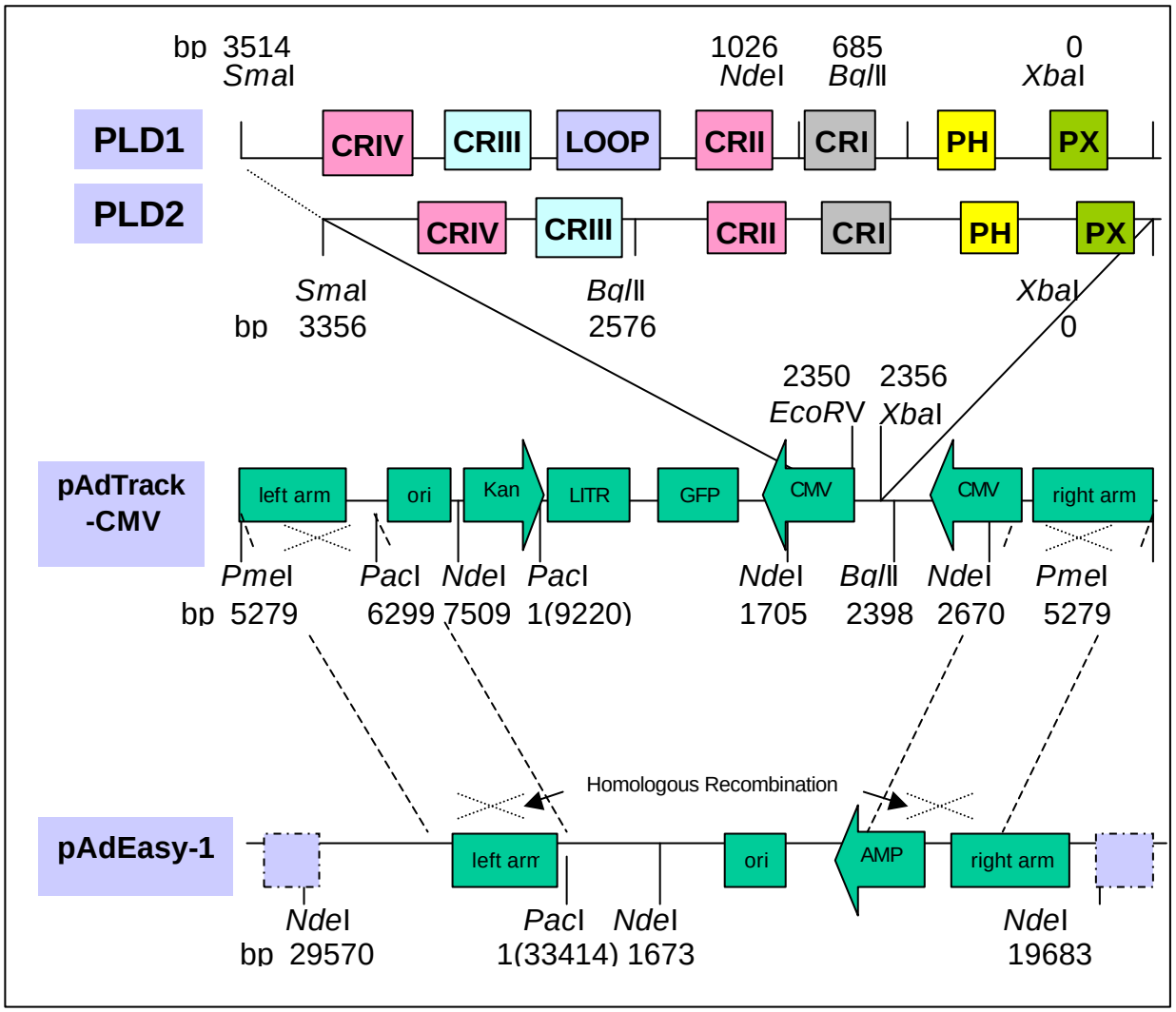


Fig. 14: Scheme of PLD1 and PLD2 subcloning into pAdTrack-CMV and pAdEasy-1. CRI-CRIV, conserved regions I to IV; PH, pleckstrin homology; PX, phox homology; ori, origin; Kan, kanamycin; LITR, left-hand inverted terminal repeats; GFP, green fluorescent protein; CMV, cytomegalovirus; AMP, ampicillin. For further explanation, see text and also Fig. 5.

The pAdTrack-CMV shuttle vector (9.2 kb; see also Fig. 5) was linearized with Xba I + EcoR V in its multicloning site and dephosphorylated to minimize the false-positive ligations. In these experiments, ligation was quite difficult due to the generation of blunt ends from EcoR V cutting of pAdTRack-CMV and Sma I cutting of PLD inserts. The plasmid DNA from candidate clones were screened with Bgl II digestion. As shown in Fig. 15, the right pAdTrack-PLD1 constructs were excised with a characteristic band of around 700 bp between the position 685 in PLD1 and the position 2398 in pAdTrack-CMV by BglII. For pAdTrack-PLD2, the characteristic band was around 2600 bp from the position 2576 in PLD2 to position 2398 in pAdTrack-CMV (Figs. 14 and 15).

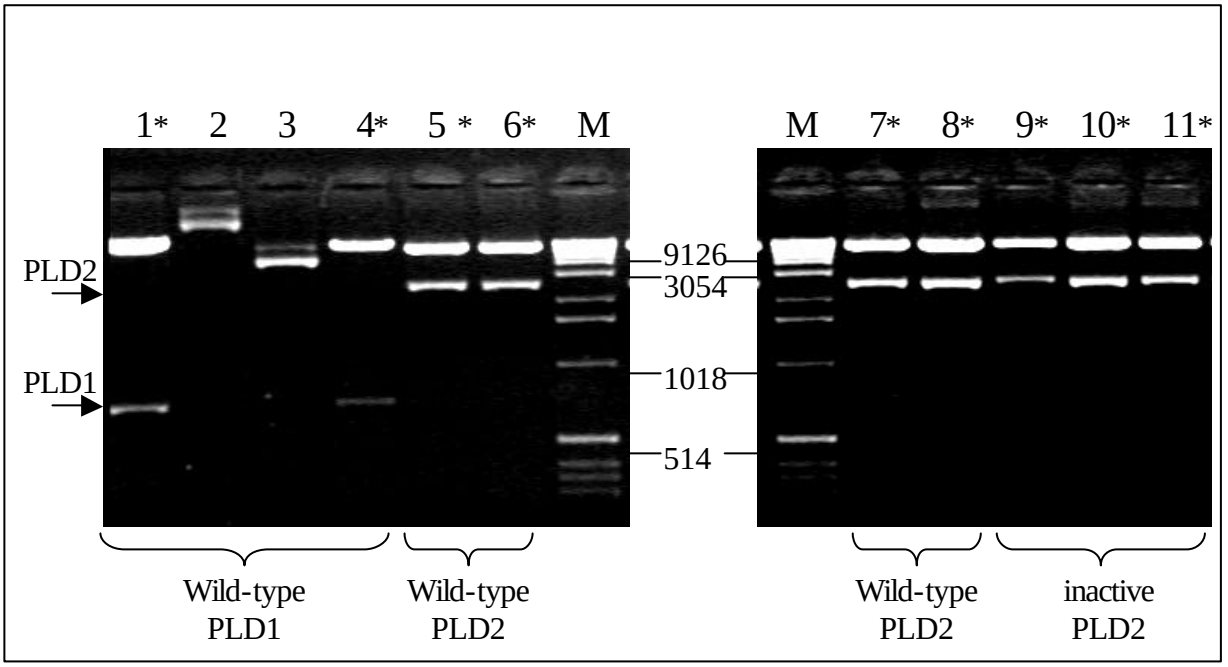


Fig. 15: Subcloning of PLD inserts into pAdTrack-CMV. PLD inserts derived from the pCGN vectors were ligated into pAdTrack-CMV. pAdTrack-PLD constructs were purified, digested with the restriction endonuclease BglII and screened by electrophoresis on 0.8 % (m/v) agarose. **(M)**, 1Kb DNA ladder marker; Lanes 1-4, wild-type PLD1 in pAdTrack-CMV; Lanes 5-8, wild-type PLD2 in pAdTrack-CMV; Lanes 9-11, catalytically inactive PLD2 in pAdTrack-CMV. Positive pAdTrack-PLD clones were marked with "*".

To achieve homologous recombination and generation of the recombinant adenoviruses, we used the restriction endonuclease NdeI to select pAdEasy-PLD recombinants. The restriction endonuclease NdeI has 3 cutting sites on the backbone adenoviral vector pAdEasy-1, 4 cutting sites on pAdTrack-PLD1 and 3 cutting sites on pAdTrack-PLD2 (Fig. 14). In case of correct homologous recombination between PmeI-linearized pAdTrack-PLD1 and supercoiled pAdEasy-1 (Fig. 14), the 4 cutting sites of NdeI on pAdTrack-PLD1 took place at the cutting site 1673 of Nde I on pAdEasy-1. Correct pAdEasy-PLD1 constructs were excised by NdeI into 6 bands. PLD1 inserts were distributed

into two bands. The characteristic smallest band was about 1.3 kb between position 1026 in PLD1 and position 2670 in pAdTrack-CMV. The second band was about 3.1 kb between position 1705 in pAdTrack-CMV and position 1026 in PLD1 (Fig. 16). As PLD2 has no NdeI cutting site, digestion by NdeI of correct pAdEasy-PLD2 constructs produced 5 bands. The PLD2-containing bands were of about 4.3 kb between position 1705 and position 2670 in pAdTrack-CMV. The other four bands were identical to the pattern obtained by NdeI cut of pAdEasy-PLD1. The biggest band was by about 14 kb between position 2670 in pAdTrack-CMV and position 19,683 in pAdEasy-1 (Figs. 14 and 16). Thus, employing such cloning strategy, recombinant adenoviruses encoding for various PLD mutants were obtained.

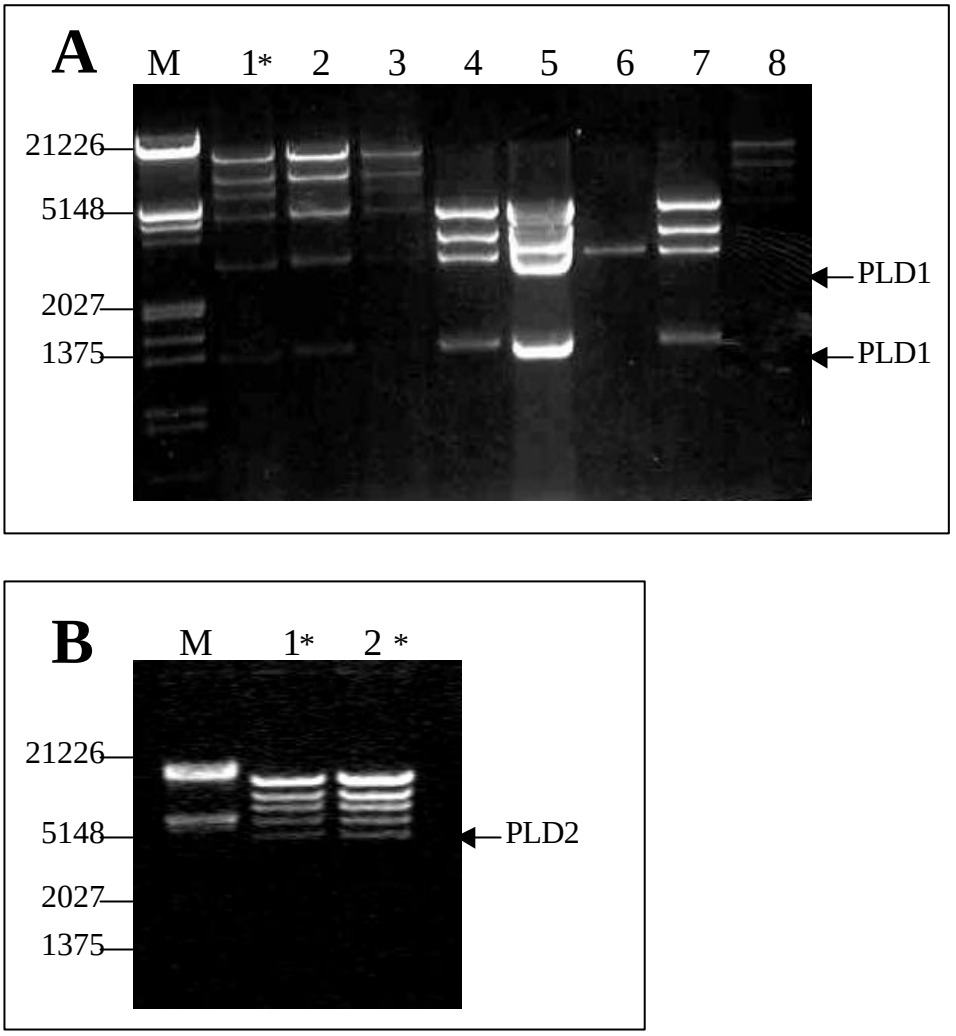
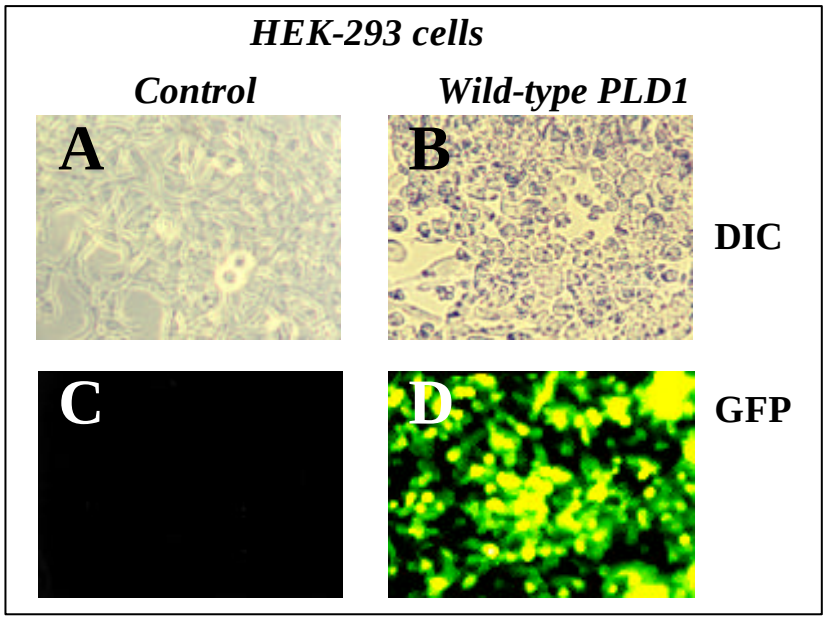
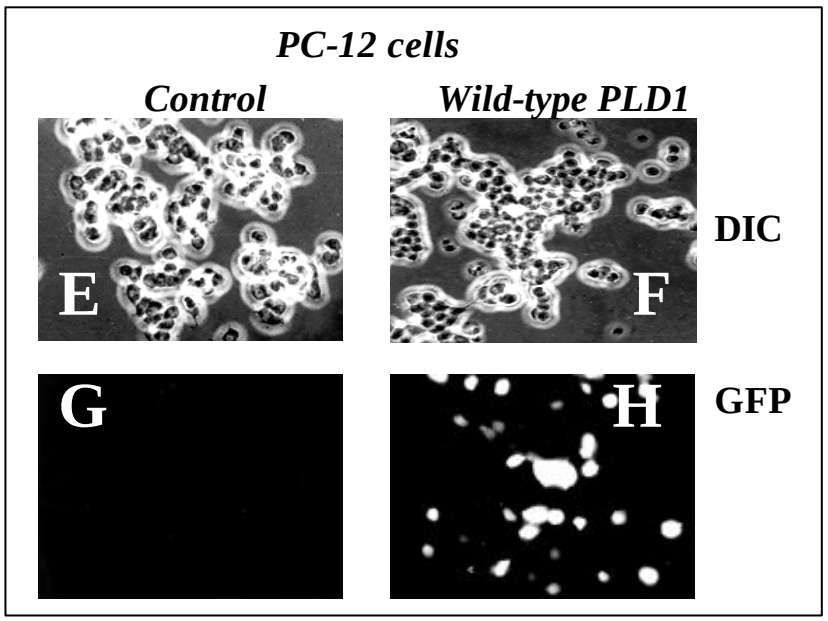


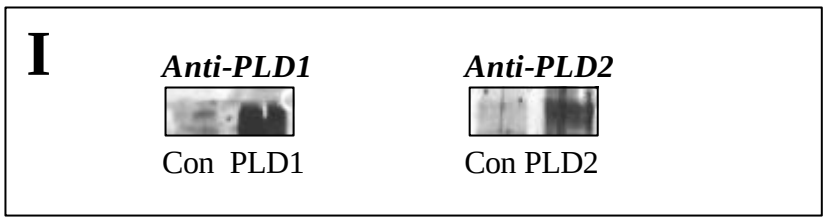
Fig. 16: Generation of homologous recombinants in BJ5183 *E. coli* cultures. pAdTrack-PLD constructs were linearized by PmeI, purified and cotransformed with the pAdEasy-1 vector into BJ5138 *E. coli* cultures for homologous recombination. Possible pAdEasy-PLD recombinants were purified, digested with the restriction endonuclease NdeI and screened by electrophoresis on 0.8 % (m/v) agarose. Various catalytically inactive pAdEasy-PLD1 recombinants **(A)**, wild-type PLD2 recombinant (lane 1) and catalytically inactive PLD2 recombinant (lane 2) **(B). (M)**, Lamda DNA / EcoRI and HindIII marker. Positive pAdEasy-PLD constructs were marked with "*".

The recombinant adenoviral plasmids encoding wild-type PLD1 and catalytically inactive PLD2 were re-transformed into DH10B *E. coli*. As illustrated in Fig. 17, the high titer viral stock infected the HEK-293 cells (Fig. 17 A-D) and PC12 cells (Fig. 17 E-H) efficiently and the comet-like signals of recombinant PLD adenovirus foci became apparent at 3 - 4 days post infection. In addition, as verified by immunoblotting with specific antibodies, adenoviral infection of HEK-293 cells with wild-type PLD1 and catalytically inactive PLD2 resulted in strong overexpression of the PLD isoforms in the cells (Fig. 17I). Similar results were obtained by infection of HEK-293 and PC12 cells with the recombinant adenoviruses encoding for wild-type PLD2 and catalytically inactive PLD1 (data not shown). Thus, recombinant adenoviruses encoding wild-type and catalytically inactive PLD mutants were generated which are obviously capable to successfully infect various cell types resulting in expression of PLD1 and PLD2, as shown by GFP expression using the fluorescence microscope and by immunoblotting with specific antibodies.

Fig. 17: Adenoviral expression of wild-type PLD1 and catalytically inactive PLD2 in HEK-293 and PC12 cells. Linearized pAdEasy-GFP-PLDs were infected into HEK-293 wild-type cells. Viruses were subsequently produced and purified until high titer viral stock was successfully harvested. Thereafter, 1 µl of virus storage buffer (*Control*; **A**, **C**, **E**, **G**) or recombinant adenovirus encoding wild-type PLD1 (*Wild-type PLD1*; **B, D, F, H)** was used to infect HEK-293 cells (upper panel) and PC12 cells (lower panel) cultured on 145-mm dishes. Viral infection was monitored by GFP expression using the fluorescence microscope. **(A**, **B**, **E**, **F):** DIC, Differential interference contrast; **(C**, **D**, **G**, **H):** GFP signal. Shown are representative images. **I**: Immunoblots of PLD1 and PLD2 with specific antibodies of lysates of control (*Con*) HEK-293 cells and HEK-293 cells infected with adenoviruses encoding wild-type PLD1 and catalytically inactive PLD2.


HEK-293 cells
Control
Wild-type PLD1
A
B
DIC
C
D
GFP


PC-12 cells
Control
Wild-type PLD1
E
F
DIC
G
H
GFP


I
Anti-PLD1
Anti-PLD2
Con PLD1
Con PLD2

4.4 LIM-kinase mediates PLD stimulation by G protein-coupled receptors

In HEK-293 cells, PLD stimulation by RhoA is apparently not caused by direct RhoA-PLD interaction, but rather involves indirect Rho-dependent mechanisms. For example, stimulation of PIP 5-kinase by RhoA elevates the cellular level of PIP_2, which profoundly affects PLD enzyme activity in HEK-293 cells (Schmidt *et al.*, 1996; Schmidt *et al.*, 1996c; Schmidt *et al.*, 1999; Oude Weernink *et al.*, 2000a). Additional work demonstrated that Rho-kinase, a member of the Rho effector family, mediates stimulation of PLD by the M_3 mAChR (Schmidt *et al.*, 1999) and stimulates PIP 5-kinase activity as well (Oude Weernink *et al.*, 2000b). As PLD enzymes do not directly interact with, or are phosphorylated by Rho-kinase (Schmidt *et al.*, 1999), regulation of PLD by Rho/Rho-kinase obviously involves additional, yet undefined, signaling components. As Rho-kinase modulates, at least in part, the reorganization of the actin cytoskeleton by activating LIM-kinase (Aspenström, 1999; Kaibuchi *et al.*, 1999; Amano *et al.*, 2000; Bishop & Hall, 2000), it was considered that stimulation of PLD by Rho/Rho-kinase may involve LIM-kinase as well.

To test this hypothesis, HEK-293 cells were transiently transfected with expression vectors encoding for mutants (wild-type, constitutively active and kinase-deficient) of LIM-kinase. Substitution of threonine by two glutamic acid residues at position 508 (T508EE) generates a constitutively active LIM-kinase mutant (CAT LIM-kinase), which is unresponsive to Rho/Rho-kinase-dependent phosphorylation at position 508, whereas a mutation at position 460 (Asp→Ala) (D460A) abolishes LIM-kinase activity (kinase-deficient LIM-kinase, KD LIM-kinase; Pröschel *et al*, 1995; Edwards & Gill, 1999). Expression of the HA-tagged LIM-kinase mutants was examined by immunoblotting with a specific antibody (Fig. 18A; Inset). As illustrated in Fig. 18A, overexpression of wild-type LIM-kinase markedly increased PLD stimulation by the M_3 mAChR, by about 2.5-fold. Expression of constitutively active LIM-kinase (T508EE LIM-kinase) potentiated PLD stimulation by carbachol even more pronounced, by about 4-fold (Fig. 18B), whereas overexpression of kinase-deficient LIM-kinase (D460A LIM-kinase) reduced PLD stimulation by the M_3 mAChR by about 60 % (Fig. 18C). None of these LIM-kinase mutants altered basal PLD activity and stimulation of PLD by PMA-responsive PKC (Figs. 18 and 19). Thus, LIM-kinase triggers specifically regulation of PLD by the G protein-coupled M_3 mAChR.

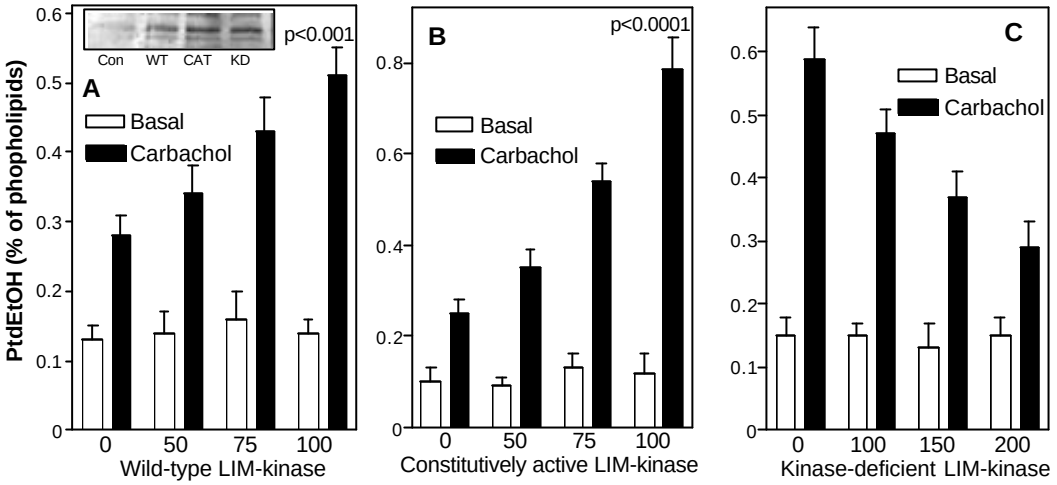


Fig. 18: Effect of LIM-kinase on PLD stimulation by the M_3 mAChR. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vector (200 µg pUCD2, *0*, *Con*) and the indicated concentrations of HA-tagged wild-type LIM-kinase **(A)**, HA-tagged constitutively active LIM-kinase (T508EE LIM-kinase) **(B)** or HA-tagged kinase-deficient LIM-kinase (D460A LIM-kinase) **(C)**. After 48 h, [^{3}H]PtdEtOH accumulation was determined in the absence (*Basal*) and presence of 1 mM carbachol (n = 2-4). **Inset:** Immunoblot of HA-tagged wild-type LIM-kinase, HA-tagged T508EE LIM-kinase and HA-tagged D460A LIM-kinase with a specific antibody.

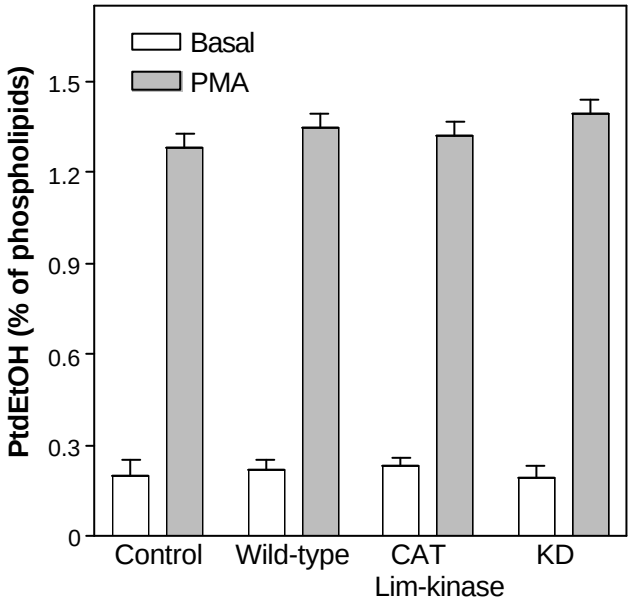


Fig. 19: Lack of effect of LIM-kinase on PLD stimulation by PMA. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vector (*Control*, 200 µg pUCD2) and expression plasmids for HA-tagged wild-type LIM-kinase, HA-tagged T508EE LIM-kinase (100 µg each) or HA-tagged D460A LIM-kinase (200 µg). After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 100 nM PMA (n = 3).

To gain insights into the mechanisms of PLD regulation by LIM-kinase, we studied the effect of purified recombinant LIM-kinase on PLD activity in HEK-293 cell membranes. Addition of recombinant LIM-kinase (2 µM) in the presence of 1 mM MgATP largely increased PLD activity, similar as, but not additive with, GTPγS-activated RhoA or Rho-kinase (Fig. 20). These data suggest that regulation of PLD by LIM-kinase is enabled by Rho/Rho-kinase signaling.

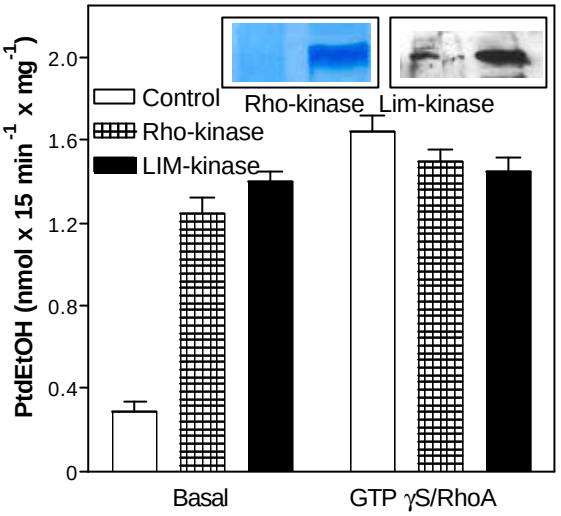


Fig. 20: Stimulation of PLD activity in HEK-293 cell membranes by recombinant Rho-kinase and LIM-kinase. PLD activity was measured in HEK-293 cell membranes for 15 min at 30°C in the presence of 1 mM MgATP without (*Control*) and with recombinant constitutively active Rho-kinase or LIM-kinase (2 µM each) alone (*Basal*) or in the presence of recombinant GTPγS-activated RhoA (10 µg RhoA + 100 µM GTPγS; *GTPgS/RhoA*). Data are representative of two experiments. **Inset:** Coomassie blue staining of GST-tagged Rho-kinase and immunoblot of flag-tagged LIM-kinase with a specific antibody.

To define whether PLD potentiation by LIM-kinase is indeed Rho- and Rho-kinase-dependent, cotransfection experiments were performed with the Rho-inactivating C3 transferase (Aktories & Just, 1993) and a dominant-negative Rho-kinase mutant, which lacks the Rho-binding and PH domains of Rho-kinase (RB/PH(TT); Oshiro *et al.*, 1998). Expression of C3 transferase and RB/PH(TT) reduced PLD stimulation by the M_3 mAChR and fully prevented the potentiating effect of wild-type LIM-kinase (Figs. 21A and 21B). In contrast, cotransfection of C3 transferase or RB/PH(TT) did not inhibit PLD potentiation by the Rho/Rho-kinase-independent constitutively active LIM-kinase (Figs. 21C and 21D).

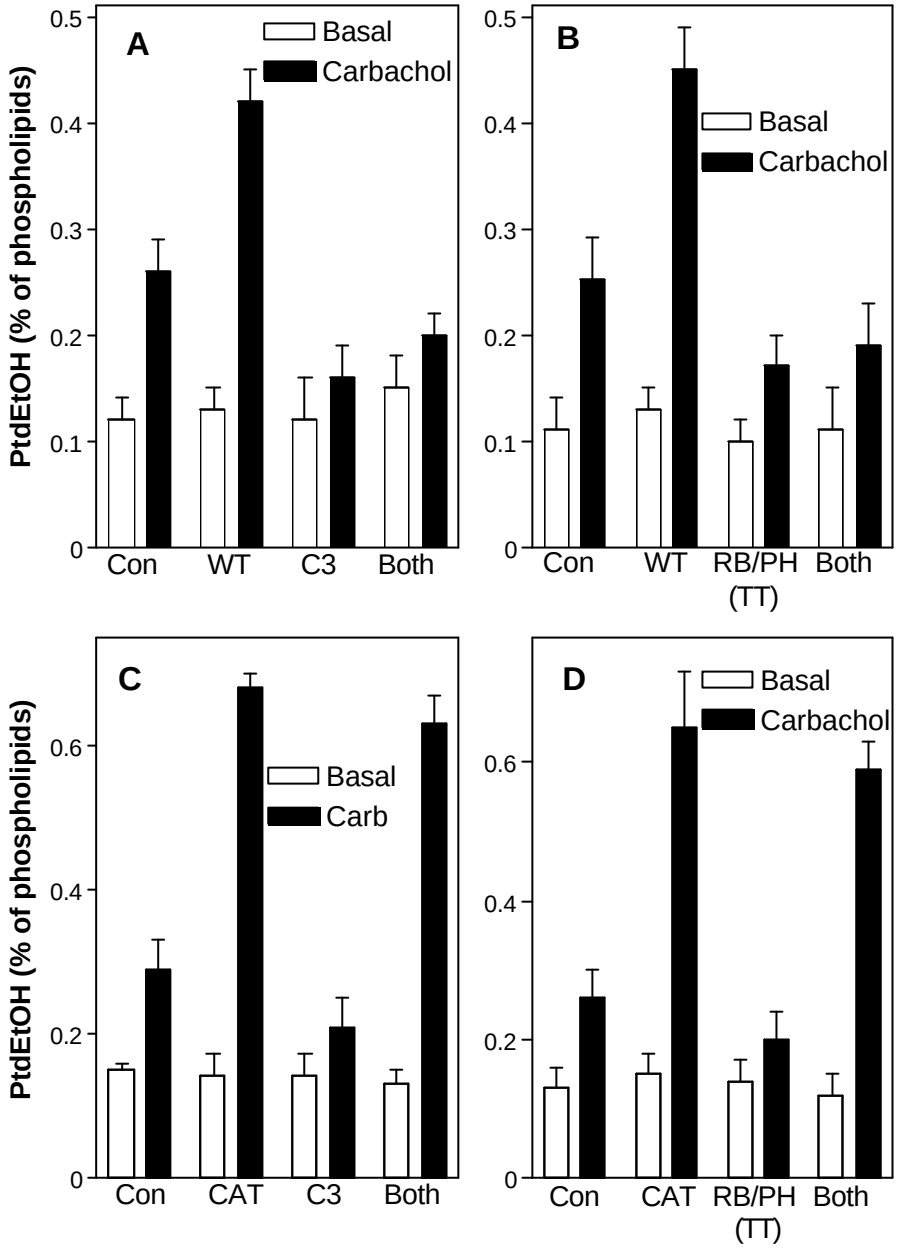


Fig. 21: PLD potentiation by LIM-kinase is Rho- and Rho-kinase-dependent. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Con*), 100 µg wild-type LIM-kinase **(A, B)**, 100 µg C3 transferase **(A, C)**, 100 µg dominant-negative Rho-kinase (RB/PH(TT) **(B, D)**, 100 µg constitutively active LIM-kinase (*CAT*) **(C, D)**, wild-type LIM-kinase *plus* either C3 transferase (*Both*) **(A)** or RB/PH(TT) (*Both*) **(B)**, and constitutively active LIM-kinase *plus* either C3 transferase (*Both*) **(C)** or RB/PH(TT) (*Both*) **(D)**. After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*) (n = 2-5).

As LIM-kinase can also be activated by Rac and Cdc42 signals (Yang *et al.*, 1998; Edwards *et al.*, 1999), we transiently transfected the cells with dominant-negative mutants of Rac1 (T17N Rac1) and Cdc42 (T17N Cdc42). However, expression of these two Rho-like GTPases did neither affect PLD stimulation by the M_3 mAChR nor PLD potentiation caused

by overexpression of LIM-kinase (Fig. 22). Thus, PLD potentiation by LIM-kinase depends on Rho/Rho-kinase, but not Rac1 and Cdc42, signals.

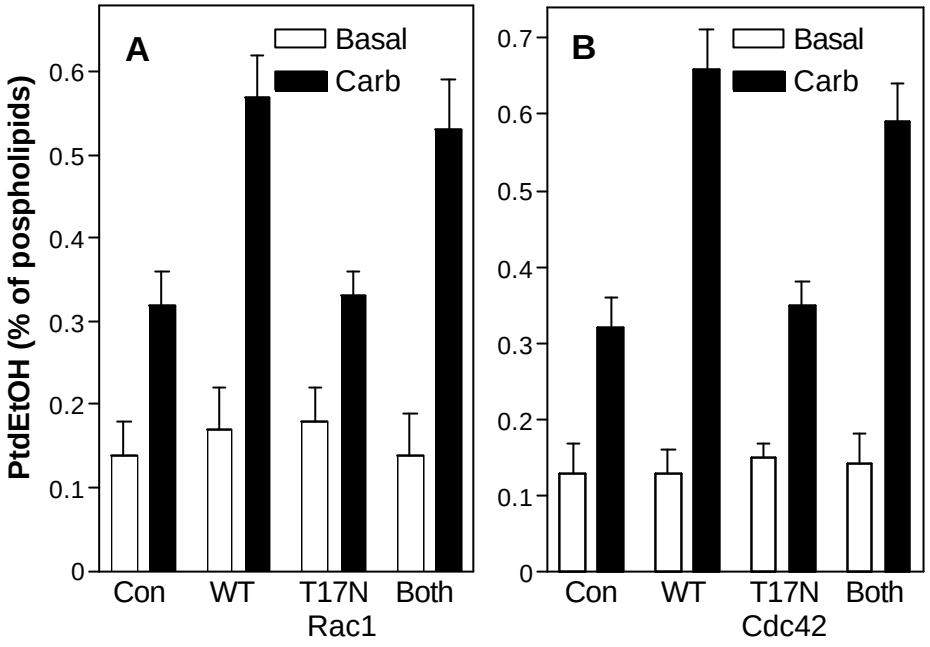


Fig. 22: PLD potentiation by LIM-kinase does not depend on Rac1 and Cdc42. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Con*), 100 µg wild-type LIM-kinase (*WT*) **(A, B)**, 100 µg dominant-negative Rac1 (T17N Rac1) **(A)**, 100 µg dominant-negative Cdc42 (T17N Cdc42) **(B)**, wild-type LIM-kinase *plus* either T17N Rac1 (*Both*) **(A)** or T17N Cdc42 (*Both*) **(B)**. After 48 h, [^{3}H]PtdEtOH accumulation was determined in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*). Overexpression of T17N Rac1 and T17N Cdc42 was examined by immunoblotting with specific antibodies (data not shown). Data are representative of three similar experiments.

To study whether regulation of PLD by Rho-kinase is indeed mediated by LIM-kinase, we transiently transfected the cells with wild-type Rho-kinase or constitutively active Rho-kinase (CAT Rho-kinase), which upon deletion of the Rho-binding domain of Rho-kinase is unresponsive to Rho (Schmidt *et al.*, 1999), together with kinase-deficient LIM-kinase. Coexpression of kinase-deficient LIM-kinase with either wild-type Rho-kinase or CAT Rho-kinase completely abolished PLD potentiation by both, wild-type Rho-kinase and CAT-Rho-kinase mutant (Fig. 23), strongly suggesting that LIM-kinase is in fact a downstream effector of Rho-kinase in M_3 mAChR signaling to PLD.

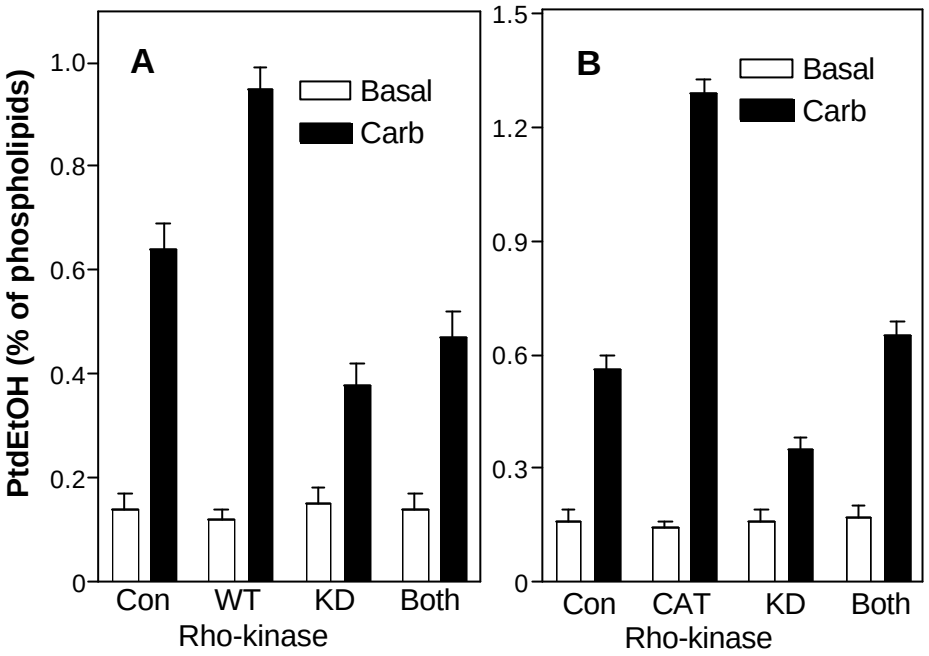


Fig. 23: PLD potentiation by Rho-kinase is LIM-kinase dependent. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Con*), wild-type Rho-kinase (*WT*) **(A)**, constitutively active Rho-kinase (*CAT*) **(B)**, 150 µg kinase-deficient LIM-kinase (*KD*), wild-type Rho-kinase *plus* kinase-deficient LIM-kinase (*Both*) **(A)** or constitutively active LIM-kinase *plus* kinase-deficient LIM-kinase (*Both*) **(B)**. After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*) (n = 3-4).

Stimulation of PLD by the M_3 mAChR in HEK-293 cells is obviously the result of ARF and Rho signals (Rümenapp *et al.*, 1995; Schmidt *et al.*, 1996b; Schmidt *et al.*, 1999; Schürmann *et al.*, 1999; Voß *et al.*, 1999). In line with such a dual regulation, expression of constitutively active LIM-kinase largely enhanced PLD stimulation by the M_3 mAChR but did not cause an increase in basal PLD activity (see Figs. 18 and 21), possibly because active ARF is missing. To examine whether PLD potentiation by LIM-kinase requires activated ARF and *vice versa*, we transiently transfected the cells with various mutants of LIM-kinase and ARF1. Expression of dominant-negative ARF1, T31N ARF1, which exhibits its inhibitory effect most likely by the sequestration of endogenous ARF-specific GEFs (Dascher & Balch, 1994), reduced by about 70 % PLD stimulation by carbachol and fully abolished PLD potentiation by constitutively active LIM-kinase (Fig. 24A). On the other hand, expression of constitutively active ARF1 (Q71L ARF1; Teal *et al.*, 1994) enhanced by about 2.5-fold PLD stimulation by carbachol leaving basal PLD activity unaffected (Fig. 24B). However, coexpression of kinase-deficient LIM-kinase fully prevented PLD potentiation by

constitutively active ARF1 (Fig. 24B). Thus, regulation of PLD by the M_3 mAChR in HEK-293 cells is a concerted action of Rho/Rho-kinase/LIM-kinase and ARF signaling cascades.

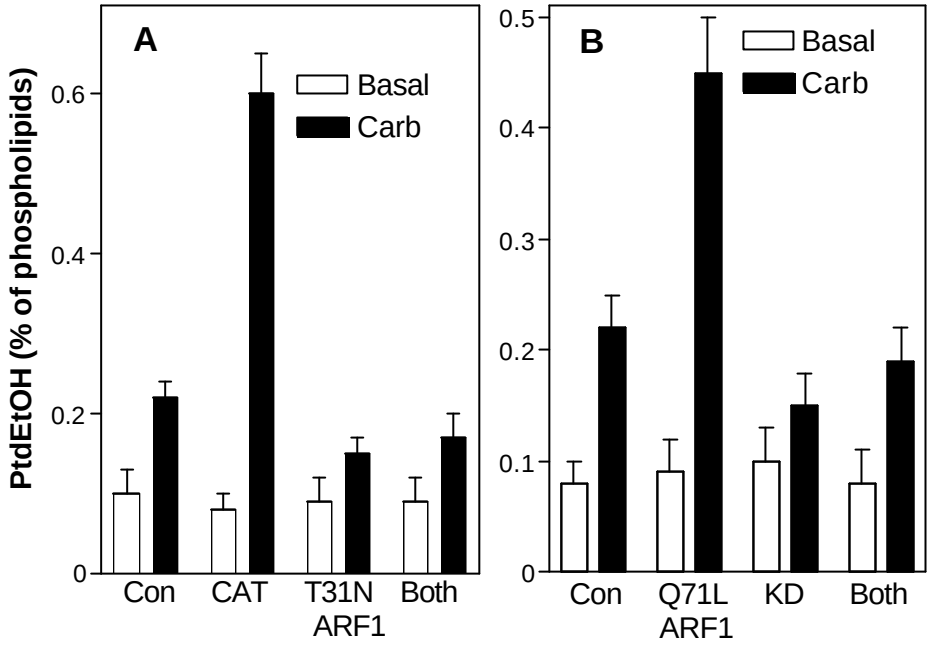


Fig. 24: PLD stimulation by the M_3 mAChR is a concerted action of ARF and Rho signaling. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Con*), constitutively active LIM-kinase (*CAT*), dominant-negative ARF1 (T31N ARF1), constitutively active LIM-Kinase *plus* T31N ARF1 (*Both*) **(A)**, constitutively active ARF1 (Q71L ARF1) (100 µg each), 150 µg kinase-deficient LIM-kinase (*KD*) and Q71L ARF1 *plus* kinase-deficient LIM-kinase (*Both*) **(B)**. After 48 h, [^{3}H]PtdEtOH accumulation was determined in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*) (n = 3-4).

The results obtained by expression of catalytically inactive PLD mutants (see 4.2) suggested that the M_3 mAChR signals in HEK-293 cells primarily to PLD1, but not to PLD2. Therefore, it was assumed that LIM-kinase may preferentially signal to PLD1 as well. To study this, the cells were cotransfected with wild-type LIM-kinase together with either catalytically inactive PLD1 (K898R PLD1) or catalytically inactive PLD2 (K758R PLD2). As illustrated in Fig. 25, coexpression of K898R PLD1, but not K758R PLD2, fully prevented PLD potentiation by LIM-kinase. Thus, regulation of PLD acctivity by LIM-kinase signals primarily to PLD1, but not to PLD2.

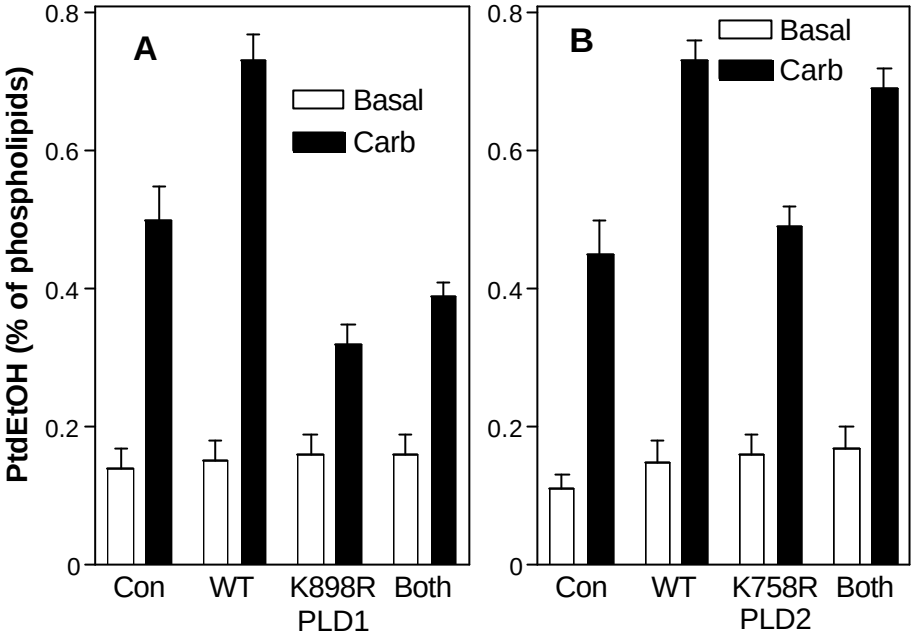


Fig. 25: LIM-kinase signals to PLD1. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Con*), wild-type LIM-kinase (*WT*), 150 µg catalytically inactive PLD1 (K898R PLD1) **(A)**, 150 µg catalytically inactive PLD2 (K758R PLD2) **(B)** and wild-type LIM-kinase *plus* either K898R PLD1 (*Both*) **(A)** or K758R PLD2 (*Both*) **(B)**. After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*). Data are representative of three similar experiments.

To study whether regulation of receptor signaling to PLD by LIM-kinase is restricted to the M_3 mAChR, we stimulated PLD activity in kinase-deficient LIM-kinase-expressing HEK-293 cells by various agonists of receptors endogenously expressed in HEK-293 cells (Schmidt *et al.*, 2000). As expected, expression of kinase-deficient LIM-kinase largely reduced PLD stimulation by the M_3 mAChR (Fig. 26). Most important, LPA receptor signaling to PLD was similarly reduced by kinase-deficient LIM-kinase, by about 50 %. In contrast, tyrosine kinase receptor (EGF and insulin) signaling to PLD was not changed by expression of kinase-deficient LIM-kinase (Fig. 26). Taken together, LIM-kinase seems to be indispensable for G protein-coupled receptor signaling to PLD, but not for PLD stimulation by tyrosine kinase receptors.

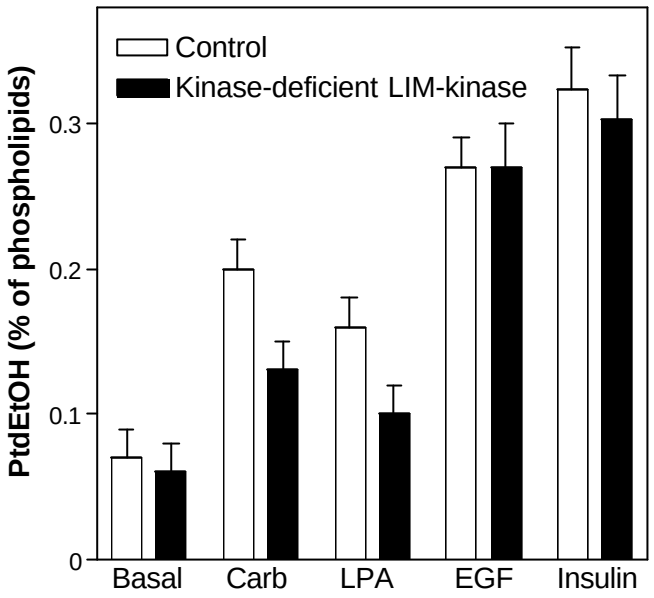


Fig. 26: G protein-coupled receptor signaling to PLD depends on LIM-kinase. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Control*) or with 150 µg kinase-deficient LIM-kinase. After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*), 10 µM LPA, 100 ng/ml EGF or 10 µg/ml insulin (n = 3).

As it has been hypothesized that LIM-kinase signaling is critically involved in brain function *via* regulation of actin dynamics (Meng *et al.*, 2002), and as it has been recently demonstrated that Rho-kinase/LIM-kinase signaling regulates neurite remodeling in N1E-115 neuroblastoma cells (Yamazaki *et al.*, 2002), we transiently transfected the LIM-kinase mutants in these neuronal cells and studied their effects on mAChR signaling to PLD. As shown in Fig. 27, overexpression of wild-type LIM-kinase increased PLD stimulation by the agonist carbachol by about 30 %. Expression of the constitutively active LIM-kinase potentiated PLD stimulation by carbachol by even about 250 %. In contrast, expression of kinase-deficient LIM-kinase inhibited carbachol-induced PLD stimulation by about 60 %. None of the LIM-kinase mutants changed basal PLD activity in N1E-115 neuroblastoma cells (Fig. 27). Thus, LIM-kinase signaling is not only essential for the regulation of actin dynamics in neuronal cells, but apparently also for PLD signaling.

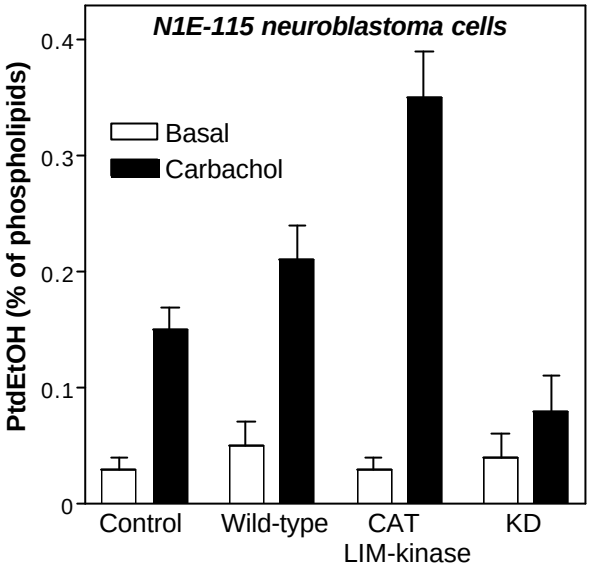


Fig. 27: Regulation of PLD by LIM-kinase in N1E-115 neuroblastoma cells. N1E-115 neuroblastoma cells were transfected with empty expression vectors (*Control*), wild-type LIM-kinase, constitutively active LIM-kinase (*CAT*) or kinase-deficient LIM-kinase (*KD*) (100 µg each). After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 100 µM carbachol (n = 3).

4.5 LIM-kinase signals *via* cofilin to PLD

As LIM-kinase was now identified as a novel player in receptor signaling to PLD, it was first assumed that LIM-kinase may directly interact with PLD enzymes, resulting in their phosphorylation. However, as illustrated in Fig. 28, purified recombinant LIM-kinase (Fig. 28A) did neither phosphorylate PLD1 or PLD2 nor bind to the PLD enzymes (Fig. 28B, *right panel*; data not shown). The recombinant LIM-kinase was, however, not inactive. Under identical experimental conditions, LIM-kinase phosphorylated wild-type cofilin, but not the nonphosphorylatable cofilin mutant, S3A cofilin (Fig. 28B, *left panel*). As cofilin, a member of the actin depolymerization family, is the only LIM-kinase substrate identified so far (Bamburg, 1999; Maekawa *et al.*, 1999; Higgs & Pollard, 2001), it was then studied whether cofilin is involved in PLD regulation by LIM-kinase.

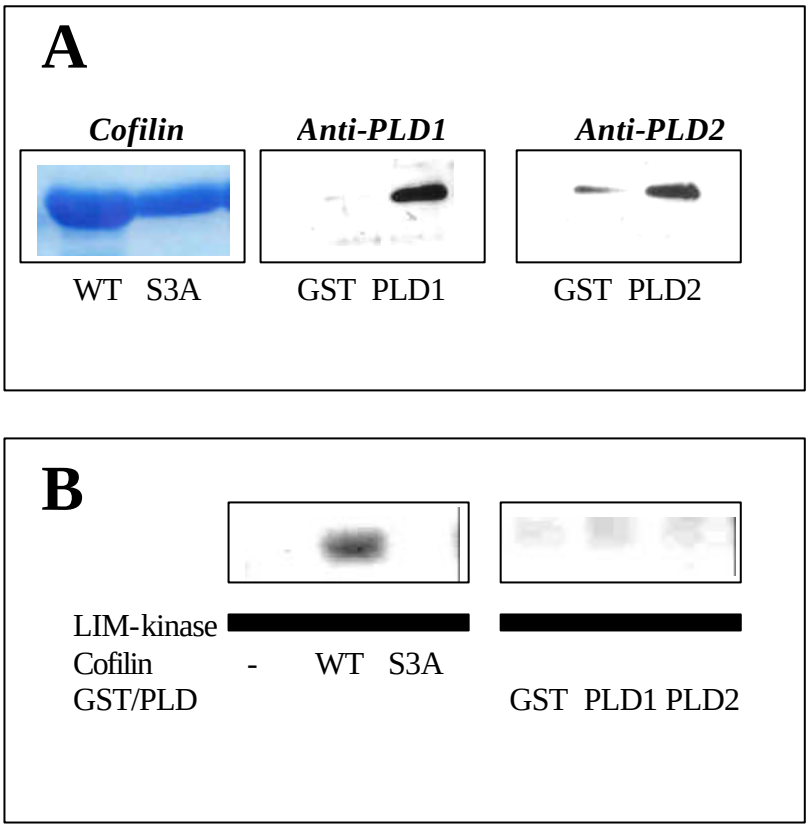


Fig. 28: LIM-kinase does not phosphorylate recombinant PLD1 and PLD2. (A) SDS-PAGE (15 % (m/v) of purified His_6-tagged wild-type cofilin (*WT*) and nonphosphorylatable cofilin (*S3A*) (10 µg each) stained with Coomassie blue (*left panel*). *Sf9* cells were infected with baculoviruses encoding GST, GST-tagged PLD1 or GST-tagged PLD2. After purification, the proteins were separated on SDS-PAGE (12.5 % (m/v) (10 µg each), transferred onto nitrocellulose membranes and probed with specific antibodies. **(B)** Recombinant LIM-kinase (1.5 µM) was incubated with wild-type cofilin, S3A cofilin (10 µg each), GST, GST-tagged PLD1 or GST-tagged PLD2 (50 µg each) in the presence of [γ-^{32}P]ATP for 45 min at 25°C. Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. Data are representative of three to four similar experiments.

First, HEK-293 cells were transiently transfected with wild-type cofilin and the nonphosphorylatable cofilin mutant, S3A cofilin. Expression of the HA-tagged cofilin mutants was verified by immunblotting with a specific antibody (Fig. 29A; Inset). Expression of wild-type cofilin largely enhanced, by about 2.5-fold, carbachol-induced PLD stimulation (Fig. 29A). In contrast, expression of S3A cofilin suppressed the M_3 mAChR response by about 50 % (Fig. 29B). Wild-type cofilin and S3A cofilin did not alter basal PLD activity and regulation of PLD by PMA-responsive PKC (Fig. 30). Thus, cofilin seems to be specifically involved in PLD stimulation by the G protein-coupled M_3 mAChR.

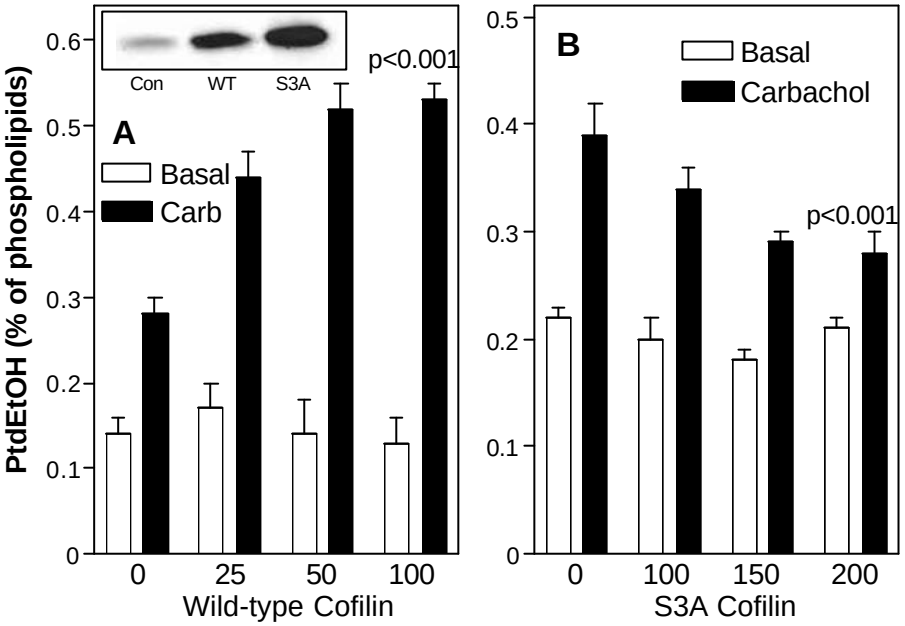


Fig. 29: Effect of cofilin on PLD stimulation by the M_3 mAChR. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vector (200 µg pCDL-SRα, *0*, *Con*) and the indicated concentrations of HA-tagged wild-type cofilin **(A)** or HA-tagged nonphosphorylatable cofilin (*S3A Cofilin*) **(B)**. After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*) (n = 3-4). **Inset:** Immunoblot of HA-tagged wild-type (*WT*) and S3A cofilin (*S3A*) with a specific antibody.

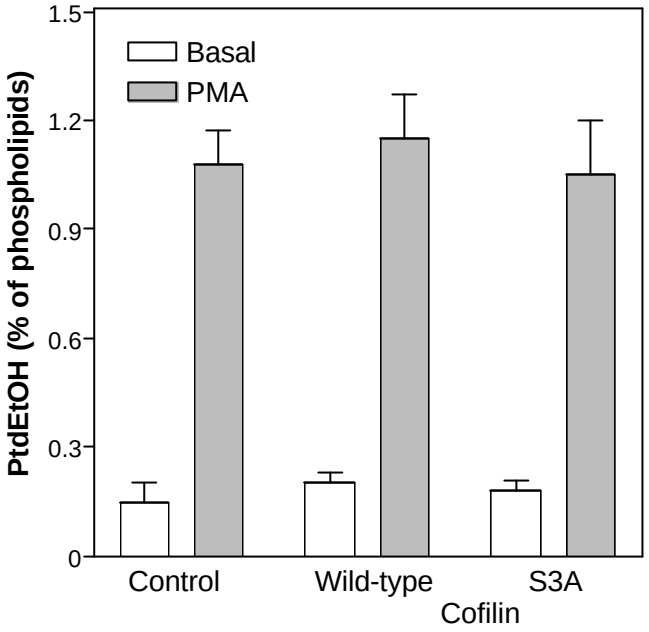


Fig. 30: Lack of effect of cofilin on PLD stimulation by PMA. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Control*, 150 µg pCDL-SRα), 100 µg wild-type cofilin or 150 µg S3A cofilin. After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 100 nM PMA (n = 4).

In the following, it was studied whether regulation of PLD activity by cofilin requires Rho/Rho-kinase/LIM-kinase signals. As shown in Fig. 31, expression of either the Rho-inactivating C3 transferase (Fig. 31A), the dominant-negative Rho-kinase (RB/PH(TT) (Fig. 31B) or the kinase-deficient LIM-kinase (Fig. 31C) fully abrogated the stimulatory effect of wild-type cofilin on PLD stimulation by carbachol. These data, thus, suggested that cofilin is part of the M_3 mAChR-initiated Rho/Rho-kinase/LIM-kinase signaling cascade to PLD.

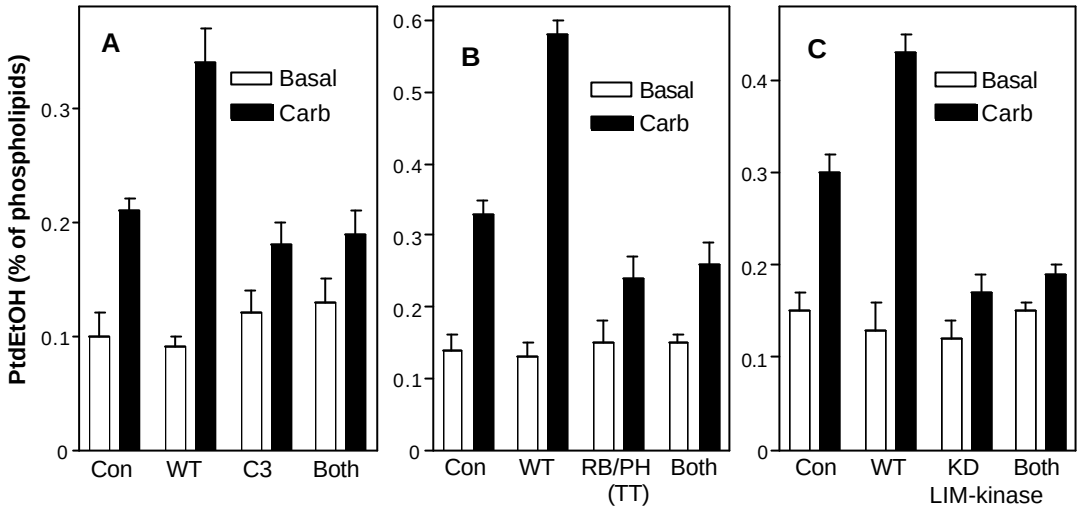


Fig. 31: PLD potentiation by cofilin requires Rho, Rho-kinase and LIM-kinase. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Con*), wild-type cofilin (*WT*) **(A-C)**, C3 transferase **(A)**, dominant-negative Rho-kinase (RB/PH(TT) (100 µg each) **(B)**, 150 µg kinase-deficient LIM-kinase (*KD LIM kinase*) **(C)**, wild-type cofilin (*WT*) *plus* either C3 transferase (*Both*) **(A),** RB/PH(TT) (*Both*) **(B)** or kinase-deficient LIM-kinase (*Both*) **(C)**. After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*) (n = 2-4).

If cofilin functions as downstream effector of LIM-kinase in the regulation of PLD by the M_3 mAChR, expression of S3A cofilin, which by itself reduced receptor signaling to PLD (see Fig. 29), should prevent the stimulatory effect of LIM-kinase on PLD activity. Indeed, coexpression of S3A cofilin largely abrogated the stimulatory effect of wild-type LIM-kinase (Fig. 32A) and, most important, also of constitutively active LIM-kinase on M_3 mAChR-induced PLD stimulation (Fig. 32B). In line with a specific role of cofilin in M_3 mAChR signaling to PLD, coexpression of K898R PLD1, but not K758R PLD2, fully prevented PLD potentiation by cofilin (Fig. 33). Thus, LIM-kinase seems to signal *via* cofilin specifically to PLD1.

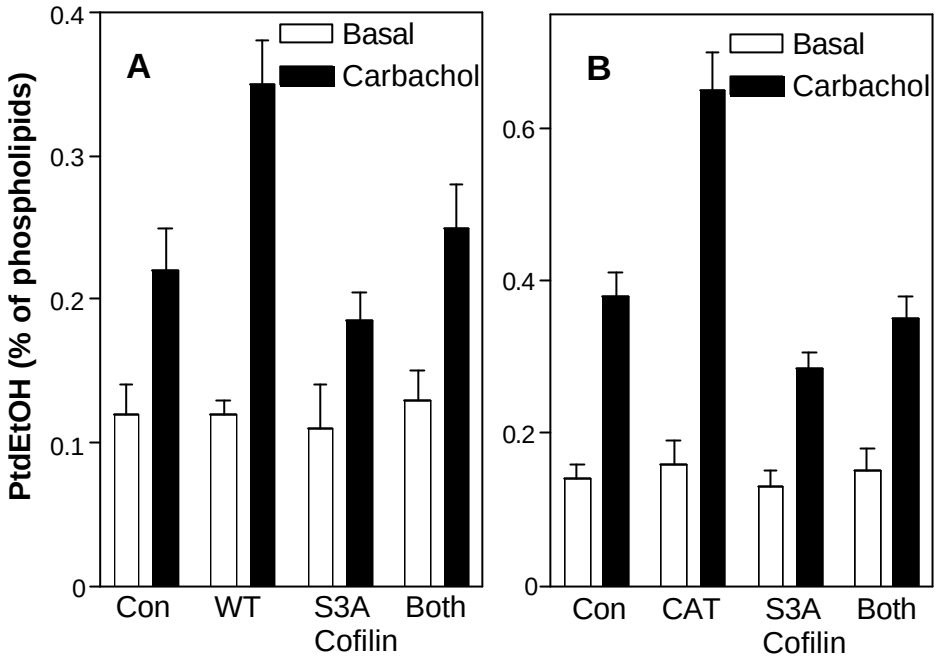


Fig. 32: Inhibition of LIM-kinase-induced PLD potentiation by S3A cofilin. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Con*), wild-type LIM-kinase (*WT*) **(A)**, constitutively active LIM-kinase (*CAT*) (100 µg each) **(B)**, 150 µg S3A cofilin, wild-type LIM-kinase *plus* S3A cofilin (*Both*) **(A)** or constitutively active LIM-kinase *plus* S3A cofilin (*Both*) **(B)**. After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 1 mM carbachol (n = 4-6).

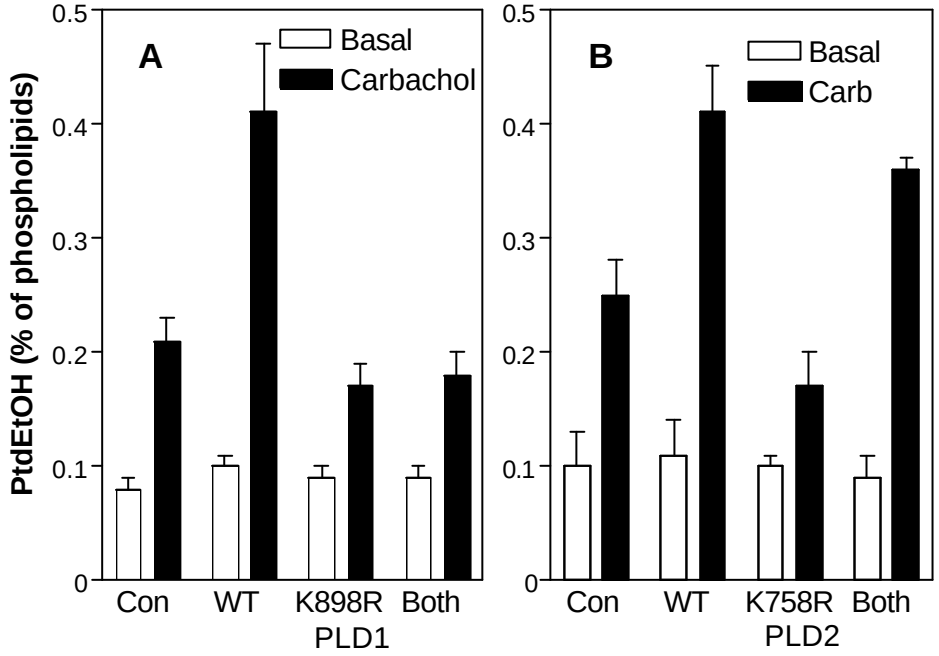


Fig. 33: Cofilin signaling to PLD1. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Con*), 100 µg wild-type cofilin (*WT*), 150 µg catalytically inactive PLD1 (K898R PLD1) **(A)**, 150 µg catalytically inactive PLD2 (K758R PLD2) **(B)** and wild-type cofilin *plus* either K898R PLD1 (*Both*) **(A)** or K758R PLD2 (*Both*) **(B)**. After 48 h, PLD activity was determined in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*). Data are representative of three similar experiments.

The following experiments were set up to gain insights into the mechanisms of PLD regulation by cofilin. As regulation of the phosphoinositide metabolism can profoundly affect PLD signaling (Liscovitch *et al.*, 2000; Cockcroft, 2001; Exton, 2002) and as Rho-kinase regulates PIP 5-kinase activity (Oude Weernink *et al.*, 2000a; Yamazaki *et al.*, 2002), it was considered that LIM-kinase and cofilin may affect PLD stimulation *via* regulation of the cellular PIP_2 content. To study this, HEK-293 cells were transiently transfected with the various LIM-kinase and cofilin mutants, followed by measurements of the cellular PIP_2 level. As illustrated in Fig. 34, none of the LIM-kinase and cofilin mutants altered the cellular level of PIP_2 (Fig. 34A). Likewise, the LIM-kinase and cofilin mutants did not change basal and carbachol-stimulated IP_3 formation (Fig. 34B). Thus, LIM-kinase/cofilin triggers PLD stimulation most likely not *via* modulation of the cellular phosphoinositide metabolism.

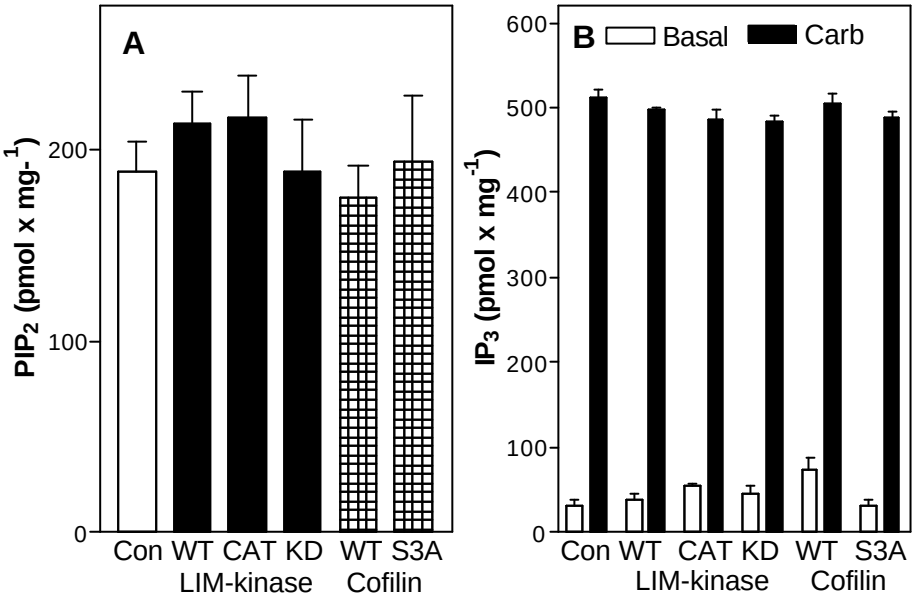
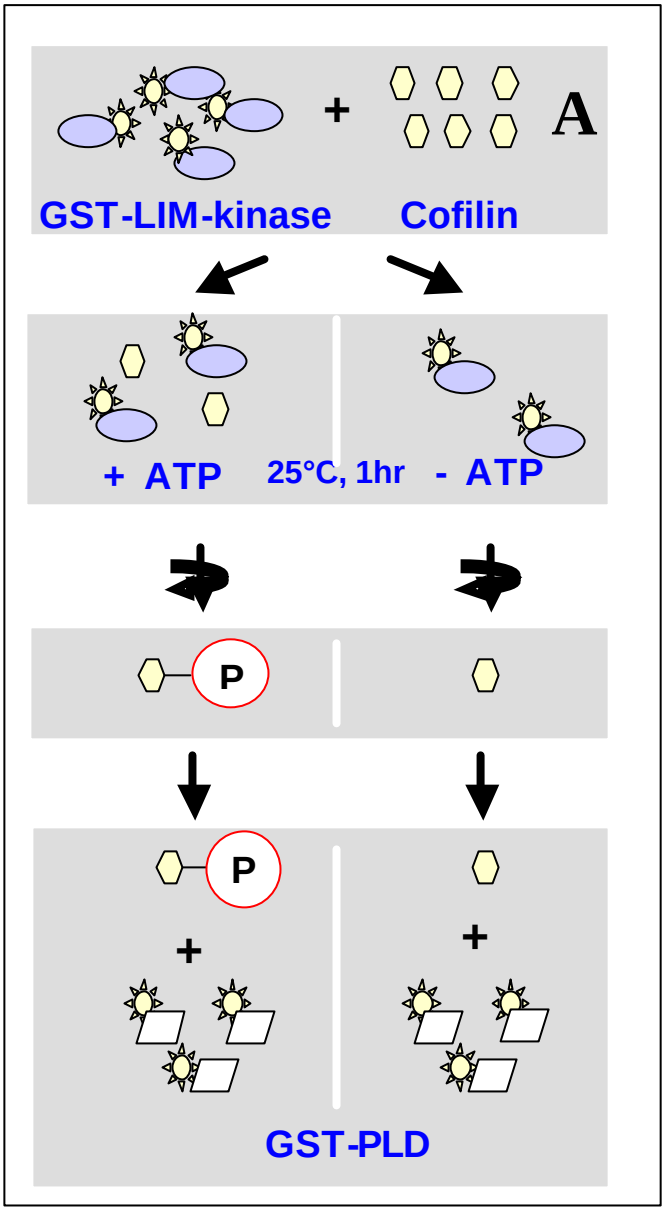


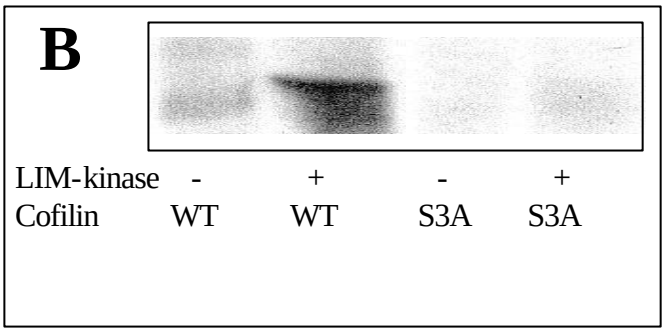
Fig. 34: LIM-kinase and cofilin do not change cellular PIP_2 and PLC signaling. M_3 mAChR-expressing HEK-293 cells were transfected with empty expression vectors (*Con*), wild-type LIM-kinase (*WT*), constitutively active LIM-kinase (*CAT*, 100 µg each), 150 µg kinase-deficient LIM-kinase (*KD*), 100 µg wild-type cofilin (*WT*) or 150 µg S3A cofilin. After 48 h, either cellular PIP_2 level was determined in unstimulated cells **(A)** or IP_3 formation was determined in the absence (*Basal*) and presence of 1 mM carbachol (*Carb*) **(B)** (n = 3).

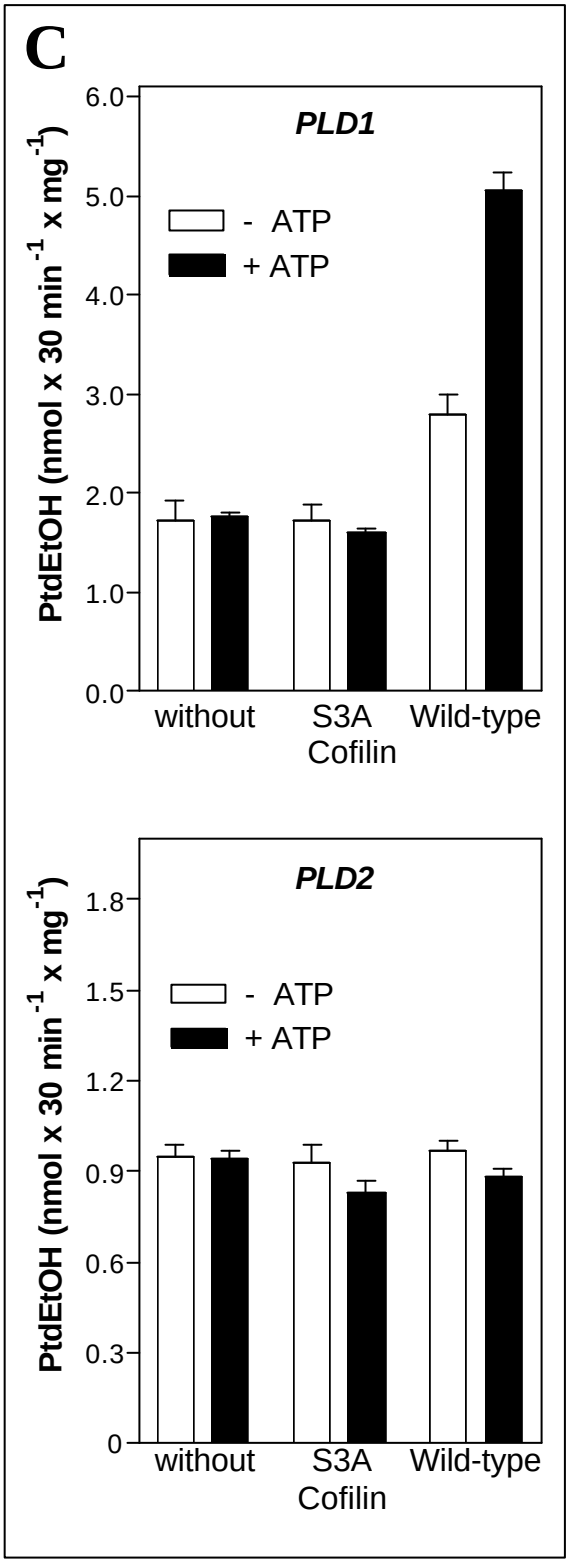
The interaction of cofilin with actin is tightly controlled by a phosphocycling process. Specifically, binding of cofilin to actin, inducing F-actin depolymerization, is lost upon phosphorylation of cofilin at serine 3 by LIM-kinase, resulting in F-actin reassembly

(Bamburg, 1999; Chen *et al.*, 2000; Higgs & Pollard, 2001; see also Fig. 4). Therefore, it was studied whether PLD stimulation by cofilin is controlled its phosphorylation state. For this, cofilin was phosphorylated by LIM-kinase, and the effects of unphosphorylated and phosphorylated cofilin on the activity of purified recombinant PLD enzymes were studied. As shown in Fig. 35B, LIM-kinase phosporylated wild-type cofilin, but not the S3A mutant. Addition of purified recombinant wild-type cofilin pretreated with LIM-kinase in the absence of MgATP increased the activity of PLD1 by about 50 % (Fig. 35C, upper panel). Most important, wild-type cofilin phosphorylated by LIM-kinase (in the presence of MgATP) enhanced PLD1 activity by about 300 %. Wild-type cofilin pretreated without LIM-kinase was without effect on PLD1 activity (data not shown). Furthermore, neither unphosphorylated nor phosphorylated wild-type cofilin altered PLD2 activity (Fig. 35C, lower panel). Finally, S3A cofilin, pretreated or not with LIM-kinase in the absence or presence of MgATP, had no effect on the activity of either PLD enzyme. Thus, upon phosphorylation by LIM-kinase cofilin specifically enhances PLD1 activity.

Fig. 35: Phosphorlyated cofilin stimulates PLD1, but not PLD2, *in vitro.* Schematic illustration of cofilin phosphorylation by LIM-kinase and subsequent PLD assay **(A)**. In brief, GST-tagged LIM-kinase (30 µg,) was incubated without and with wild-type cofilin or S3A cofilin (1 mg each,) in the absence or presence of 1 mM MgATP at 25°C for 45 min. Then, GST-tagged LIM-kinase was pelleted, and the supernatant (10 µg protein / sample) was incubated with GST-PLD1 ( )(*upper panel*) or GST-PLD2 (*lower panel*) (15 µg each) to measure PLD activity **(C)**. GST-tagged LIM-kinase (30 µg) was incubated with 1 mg wild-type or S3A cofilin in the presence of [γ-^{32}P]ATP for 45 min at 25°C. Phosphorylated cofilin was detected by autoradiography with Kodak X-Omat AR-films **(B)**. Data are representative of three experiments.


A
GST-LIM-kinase
Cofilin
+ ATP
25°C, 1hr
- ATP
P
P
GST-PLD


B
LIM-kinase - + - +
Cofilin WT WT S3A S3A


C
PLD1
- ATP
+ ATP
PtdEtOH (nmol x 30 min-1 x mg-1)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
without
S3A
Wild-type
Cofilin
PLD2
- ATP
+ ATP
PtdEtOH (nmol x 30 min-1 x mg-1)
1.8
1.5
1.2
0.9
0.6
0.3
0
without
S3A
Wild-type
Cofilin

PLD, primarily PLD2, enzyme activity is known to be regulated by actin, actin-binding proteins as well as proteins involved in synaptic vesicle trafficking, probably due to direct interactions (Lee *et al.*, 1997; Jenco *et al.*, 1998; Lee *et al.*, 2000; Park *et al.*, 2000; Lee *et al.*, 2001; Ahn *et al.*, 2002; Lee *et al.*, 2002). However, it has to be emphasized that all such molecules do inhibit PLD enzyme activity. In contrast, here for the first time a stimulatory effect of an actin-binding protein (cofilin) on PLD, specifically PLD1 activity is presented. It was, therefore, of interest to study whether cofilin directly interacts with PLD1. As illustrated in Fig. 36, wild-type cofilin, but not S3A mutant, specifically bound to PLD1, but not PLD2. These data reflect the differential stimulatory effect of wild-type cofilin on PLD1, but not PLD2 activity.

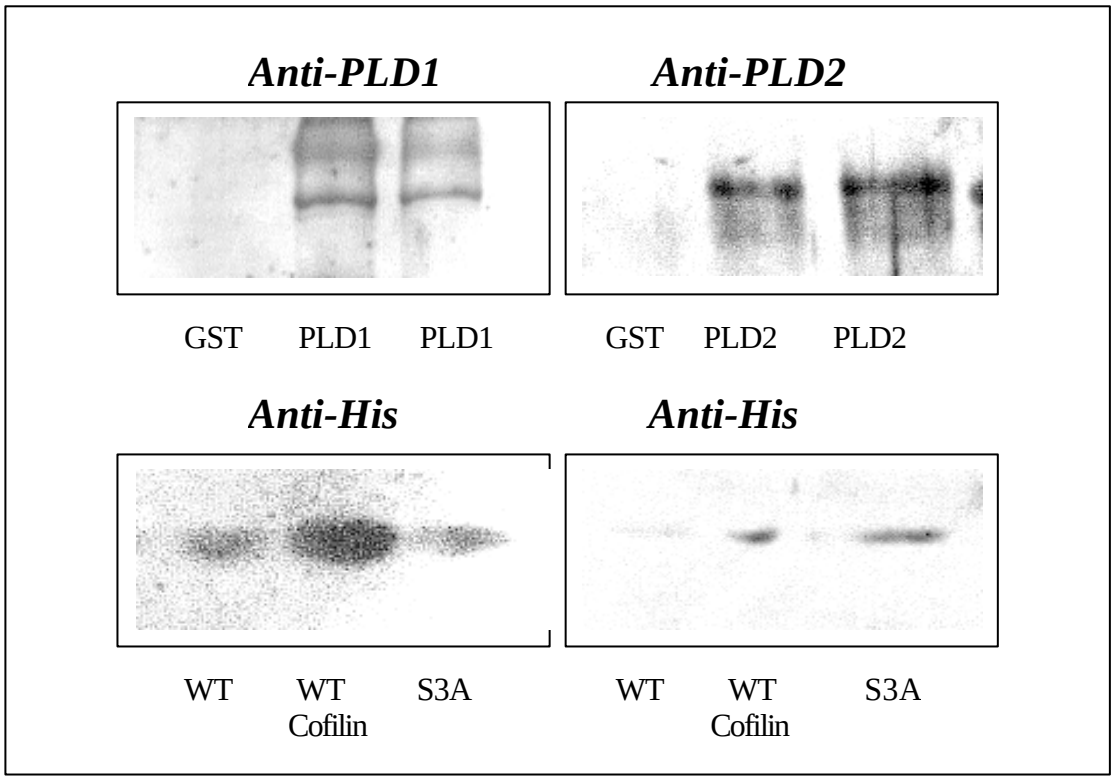


Fig. 36: Cofilin binds to PLD1, but not PLD2, *in vitro.* Glutathione Sepharose beads loaded with GST, GST-PLD1 and GST-PLD2 (20 µg each) were incubated with recombinant His_6-tagged wild-type or S3A cofilin (30 µg each) overnight at 4°C. Specifically bound proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane and detected by immunoblotting with anti-PLD and anti-His antibodies. Data are representative of three experiments.

As wild-type cofilin is able to directly and specifically interact with PLD1 *in vitro*, we transiently transfected HEK-293 cells with PLD and cofilin mutants to analyze by immunofluorescence laser confocal microscopy whether cofilin affects subcellular localization of PLD enzymes. As reported before in other cell types (reviewed in: Cockcroft, 2001; Liscovitch *et al.*, 2001; Exton, 2002), in HEK-293 cells PLD1 localized to intracellular compartments and the plasma membrane, whereas PLD2 was found exclusively at the plasma membrane (Fig. 37; compare A and D). Coexpression of wild-type cofilin altered subcellular localization of PLD1 (Fig. 37B). In cells coexpressing PLD1 and wild-type cofilin PLD1 was found primarily at the plasma membrane (Fig. 37B). Coexpression of S3A cofilin and PLD1 did not cause such drastic subcellular redistribution of PLD1 (Fig. 37C). In contrast, coexpression of either wild-type or S3A cofilin did not alter the subcellular localization of PLD2 (Fig. 37E-F).

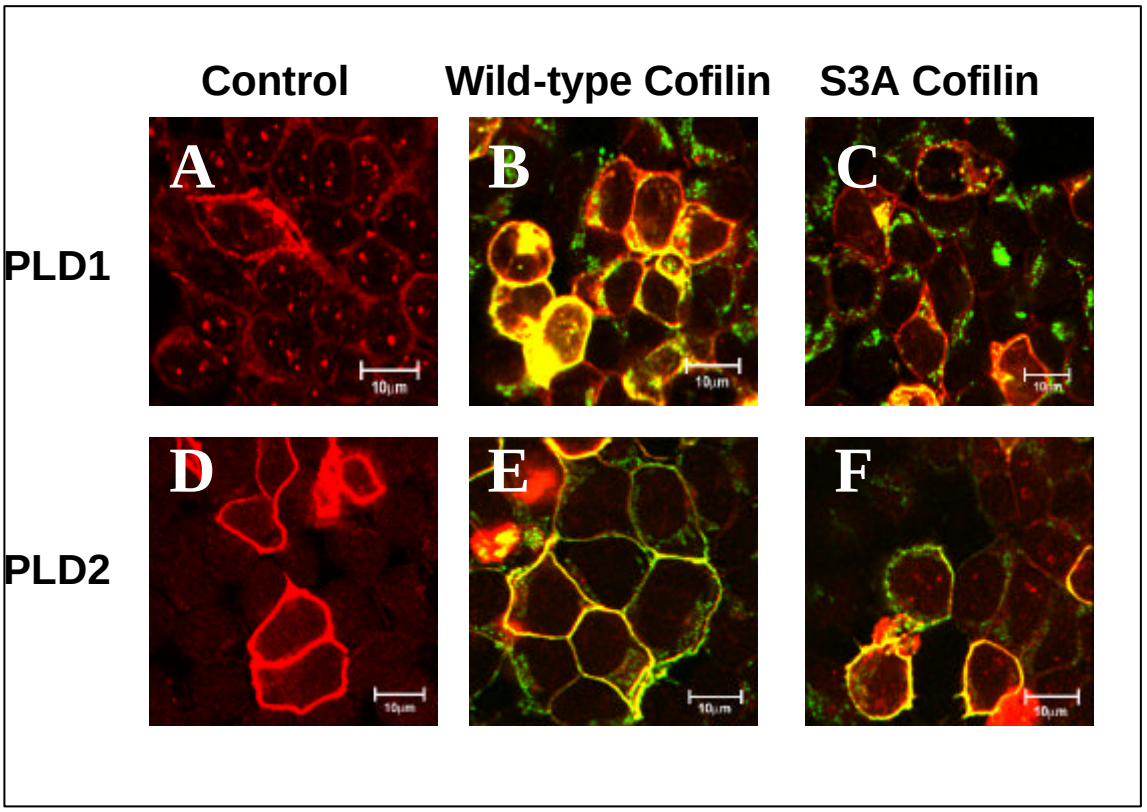


Fig. 37: Subcellular redistribution of PLD1, but not PLD2, by cofilin. M_3 mAChR-expressing HEK-293 cells were transfected with wild-type PLD1 **(A-C)** or wild-type PLD2 **(D-F)** (50 µg each) either alone **(A, D)**, with HA-tagged wild-type cofilin (50 µg) **(B, E)** or HA-tagged S3A cofilin (150 µg) **(C, F)**. After 48 h, immunofluorescence laser confocal microscopy was performed as described in the "Materials and Methods" section. After incubation with the primary antibodies (anti-PLD or anti-HA), PLD enzymes were detected with Alexa-633 goat anti-rabbit IgG (excitation: 633; emission: red colour) and cofilin with Alexa-488 goat anti-mouse IgG (excitation: 488; emission: green colour). Yellow colour: merge of red and yellow colours. Data are characteristic of three similar experiments.

5. Discussion

Phosphatidylcholine-specific phospholipase D (PLD) enzymes represent, together with the phosphatidylinositol-4,5-bisphosphate (PIP_2)-hydrolyzing phospholipase C (PLC) isoforms, an extracellular signal-activated phospholipase superfamily, which generates biologically active products (Exton, 1999; Liscovitch *et al.*, 2000; Cockcroft, 2001; Rhee, 2001; Steed & Cow, 2001; Exton, 2002). The hydrolysis of phosphatidylcholine by PLD enzymes generates the second messenger phosphatidic acid (PA), which is assumed to trigger various early and late cellular responses, such as calcium mobilization, secretion, superoxide production, endocytosis, exocytosis, vesicle trafficking, glucose transport, rearrangements of the actin cytoskeleton, mitogenesis and apoptosis (see Fig. 1; Daniel *et al.*, 1999; Jones *et al.*, 1999a; McPhail *et al.*, 1999; Venable *et al.*, 1999; Nakashima & Nozawa, 1999; Liscovitch *et al.*, 2000; Exton, 2002; Cummings *et al.*, 2002a; Joseph *et al.*, 2002). These conclusions on the cellular role of PLD are mainly based on findings with primary alcohols, which are used by PLD enzymes in a PLD-specific transphosphatidylation reaction to produce biologically inactive phosphatidylalcohols (see Fig. 2; Morris *et al.*, 1997; Liscovitch *et al.*, 2000; Cockcroft, 2001; Exton, 2002). In addition, transfection of catalytically inactive mutants of the two PLD enzymes identified in mammalian cells, PLD1 and PLD2, has been successfully used to further unravel the physiological role of PLD enzymes (Shen *et al.*, 2001; Denmat-Ouisse *et al.*, 2001; Humeau *et al.*, 2001; Vitale *et al.*, 2001; Kam & Exton, 2001). However, in many mammalian cell types, specifically in primary cultured cells, this approach is often hampered due to low transfection efficiencies. Thus, more powerful tools are needed to unravel the physiological role of PLD enzymes.

Infection of mammalian cells by recombinant adenoviruses is a powerful tool to characterize the cellular function of proteins encoded by such adenoviruses, as also shown here for adenoviruses encoding RGS4 and Lsc-RGS. Using adenoviruses encoding these proteins, we even gained insights into the signaling mechanisms of G protein-coupled receptors to PLD in cardiomyocytes, a cell type known to be resistant to transfection by classical means (Fahimi-Vahid *et al.*, 2002; Gosau *et al.*, 2002). Therefore, for future work on the cellular role of PLD enzymes in receptor action, adenoviruses encoding wild-type and catalytically inactive PLD1 and PLD2 mutants were generated, using the pAdEasy system (He *et al.*, 1998). The PLD adenoviruses successfully infected HEK-293 and PC12 cells, as shown by fluoresence microscopy and by immunoblotting with specific antibodies. In preliminary studies, it was observed that infection of PC12 cells with the adenoviruses encoding the PLD enzymes differentially affects agonist-triggered noradrenaline release (data not shown). While this work was in progress, Wang *et al.* (2002) reported the generation of

the same PLD adenoviruses and showed that infection of human bronchial epithelial cells with adenoviruses encoding PLD1 and PLD2 differentially alters receptor-mediated ERK activation and interleukin-8 secretion. Thus, the generated adenoviruses encoding wild-type and catalytically inactive PLD1 and PLD2 mutants will most likely provide a powerful tool for future work on the physiological role of PLD enzymes, specifically in primary cultured cells.

Activation of PLD enzymes by membrane receptors has been demonstrated in numerous cell types with a wide variety of hormones, neurotransmitters and growth factors (Exton, 1999; Liscovitch *et al.*, 2000; Cockcroft, 2001; Exton, 2002). However, in contrast to PLC isoforms, where direct interaction with membrane receptors and/or their associated heterotrimeric G proteins is needed and sufficient for activation (Schlessinger, 2000; Rhee, 2001), PLD enzymes are not directly activated by receptors or heterotrimeric G proteins (Min *et al.*, 1998; Slaaby *et al.*, 1998). Consequently, additional signaling molecules have been searched and analyzed for their potential involvement in receptor signaling to PLD. Among the signaling molecules meanwhile identified to be involved in receptor signaling to PLD enzymes are phosphoinositides, particularly PIP_2, members of various monomeric GTPase families, specifically ARF, Rho and Ras GTPases, and protein kinases, specifically protein kinase C (PKC) isoforms and the Rho-activated Rho-kinase (reviewed in: Exton, 1999; Liscovitch *et al.*, 2000; Cockcroft, 2001; Exton, 2002). Despite the large body of information accumulated so far, our knowledge about receptor coupling mechanisms to PLD enzymes is still far from being complete.

As nearly every membrane receptor, either G protein-coupled or tyrosine kinase receptor, known to stimulate PIP_2-specific PLC isoforms also causes PLD stimulation, it was assumed that stimulation of PLD ensues from PLC activation, with subsequent increase in cytosolic Ca^{2+} concentration and activation of PKC isoforms. In line with this assumption, it was shown that coexpression of bombesin and M_1 or M_3 muscarinic acetylcholine receptors (mAChRs), known to stimulate PLC *via* the pertussis toxin (PTX)-insensitive G_q family of heterotrimeric G proteins (Neer, 1995; Gudermann *et al.*, 1996; Caulfield & Birdsall, 1998; Watling, 2001), with PKC-unresponsive PLD1 mutants fully blunts the receptor-mediated PLD stimulation (Zhang *et al.*, 1999; Du *et al.*, 2000). Similarly, expression of a PKC-unresponsive PLD1 mutant in COS-7 cells suppressed PLD stimulation by a coexpressed constitutively active $G\alpha_q$ mutant (Xie *et al.*, 2002). However, the PLD response to α_1-adrenoceptor activation in Mardin-Darby canine kidney cells was PKC-independent (Kiss, 1996; Balboa & Insel, 1998), similarly as PLD stimulation by noradrenaline in rabbit aortic vascular smooth muscle cells (Muthalif *et al.*, 2000; Parmentier *et al.*, 2001). Thus, PLD stimulation by membrane receptors must not necessarily be secondary to PLC stimulation.

The G protein-coupled M_3 mAChR, stably expressed in HEK-293 cells, efficiently stimulates both, PLC and PLD, in a PTX-insensitive manner (Peralta *et al.*, 1988; Offermanns *et al.*, 1994; Schmidt *et al.*, 1994). However, stimulation of PLD by the agonist carbachol was found to be independent of concomitant increases in cytosolic Ca^{2+} concentration and activation of PKC isoforms, thus apparently not secondary to PLC stimulation (Schmidt *et al.*, 1994; Rümenapp *et al.*, 1997). The M_3 mAChR couples to PLC apparently *via* G_q-type G proteins (Offermanns *et al.*, 1994; Rhee, 2001). As it has been recently demonstrated in cotransfection experiments that both G_q- and G_{12}-type G proteins can cause PLD stimulation (Plonk *et al.*, 1998; Ushio-Fukai *et al.*, 1999; Xie *et al.*, 2002), it was considered that the receptor couples to PLC and PLD *via* the same type of G protein, i.e. G_q, or that two distinct G proteins, G_q and G_{12}, mediate receptor coupling to the two phospholipases. The G_q- and G_{12}-type G proteins are both not PTX substrates (Neer, 1995; Dhanasekaran & Dermott, 1996; Fields & Casey, 1997). Thus, PTX treatment of cells cannot be used to discriminate between these two types of G proteins. Regulators of G protein signaling (RGS) act as GTPase-activating proteins (GAPs) for heterotrimeric G proteins and thereby terminate the response responses, such as PLC stimulation. As different members of the RGS proteins exhibit Gα-subunit specificity (Druey *et al.*, 1996; Dohlman & Thorner, 1997; Huang *et al.*, 1997; Neill *et al.*, 1997; Wieland & Chen, 1999; de Vries *et al.*, 2000; Ross & Wilkie, 2000), expression of these proteins should help to define the G protein subtype involved in M_3 mAChR signaling to PLD.

Using this approach as well as expression of the relevant Gα-subunits (wild-type and mutants), it is shown herein that the M_3 mAChR signals to PLC and PLD *via* two distinct types of G proteins, i.e. G_q and G_{12}, respectively. Specifically, it is demonstrated that expression of constitutively active $G\alpha_q$ induced a strong receptor-independent increase in PLC activity. Additional work demonstrated that the M_3 mAChR signals *via* $G\alpha_q$ to PLC-β1 (Evellin *et al.*, 2002). In contrast, expression of constitutively active $G\alpha_q$ had no effect on PLD activity, which, however, was strongly enhanced upon coexpression of constitutively active $G\alpha_{12}$ and $G\alpha_{13}$, suggesting that both G_{12}-type G proteins, G_{12} and G_{13}, are required for full PLD stimulation. This hypothesis was corroborated by expression of dominant-negative mutants of $G\alpha_{12}$ or $G\alpha_{13}$ (Gohla *et al.*, 1999), which reduced PLD stimulation by the M_3 mAChR to a similar extent. The data obtained with the expression of the Gα mutants were confirmed by expression (transient or by infection with recombinant adenoviruses) of RGS4 or Lsc-RGS, which act as specific GAPs for $G\alpha_q$- and $G\alpha_{12}$-type G proteins, respectively. In detail, expression of RGS4 specifically blunted the PLC response to the M_3 mAChR, whereas expression of Lsc-RGS specifically diminished receptor signaling to PLD. As expected, adenoviral infection induced a more efficient gene transfer (He *et al.*, 1998; Young &

Mautner, 2001; Armstrong *et al.*, 2002) and consequently a more drastic inhibition of the receptor responses. Meanwhile, the adenoviruses encoding RGS4 and Lsc-RGS have been successfully used to characterize the type of G proteins involved in receptor signaling to PLD in cardiomyocytes (Fahimi-Vahid *et al.*, 2002; Gosau *et al.*, 2002).

Expression of either Gα-subunit mutants or RGS proteins did not alter PLD stimulation by phorbol 12-myristate 13-acetate (PMA)-responsive PKC. These findings confirmed the quite remarkable, differential involvement of distinct heterotrimeric G proteins in M_3 mAChR signaling to PLC and PLD. It has been demonstrated previously that the PLD response to PMA-responsive PKC in HEK-293 cells is triggered by tyrosine kinase receptors for epidermal growth factor, platelet-derived growth factor and insulin (Schmidt *et al.*, 1998; Voß *et al.*, 1999). Taken together, the findings reported here strongly corroborate the idea that in HEK-293 cells stimulation of PLD by the M_3 mAChR is in fact not a consequence of the concomitant PLC stimulation. However, the M_3 mAChR induces PLC stimulation with subsequent PKC activation (Voß *et al.*, 1999; Rümenapp *et al.*, 2001), but stimulation of PLD is apparently independent of this reaction. Thus, it is assumed that the M_3 mAChR assembles together with G_{12}-type G proteins and PLD into a highly organized signaling complex at the plasma membrane. Indeed, PLD enzymes were recently found to be present in caveolae (Czarny *et al.*, 1999; Sciorra & Morris, 1999), which in concert with lipid rafts are believed to confer specificity into the complex mechanisms of signal transduction (Okamoto *et al.*, 1998; Kurzchalia & Parton, 1999; Anderson & Jacobson, 2002; Zajchowski & Robbins, 2002).

As PLD stimulation in HEK-293 cells by the G protein-coupled M_3 mAChR and the tyrosine kinase receptors obviously involves distinct signaling cascades (Schmidt *et al.*, 1994; Rümenapp *et al.*, 1995; Schmidt *et al.*, 1996b; Schmidt *et al.*, 1999; Voß *et al.*, 1999), these cells represent an ideal model system to study a possible differential receptor signaling to PLD1 and PLD2 enzymes. It is demonstrated here, by expression of catalytically inactive PLD1 and PLD2 mutants, that the M_3 mAChR signals to PLD1, whereas the tyrosine kinase receptors signal *via* PKC to PLD2. Previous reports have also provided evidence that PLD1 and PLD2 are differentially affected by G protein-coupled and tyrosine kinase receptors, respectively (Zhang *et al.*, 1999; Du *et al.*, 2000; Slaaby *et al.*, 1998; Slaaby *et al.*, 2000). However, it has to be emphasized that these results were obtained in cotransfection studies and thus may not mimic the native cellular setting.

In previous studies, it was shown that PLD stimulation by the M_3 mAChR requires Rho GTPases, specifically RhoA. Although Rho proteins can directly interact with and activate PLD1 (Hammond *et al.*, 1997; Sung *et al.*, 1999b; Frohman, *et al.*, 2000; Cai & Exton, 2001), PLD stimulation by RhoA in HEK-293 cells was apparently not due to such a direct interaction. Additional work demonstrated that RhoA-dependent stimulation of PIP 5-

kinase, causing an increase in the cellular content of PIP_2, which acts as an essential cofactor to maintain receptor signaling to PLD, is involved in PLD stimulation (Schmidt *et al.*, 1996; Schmidt *et al.*, 1996c; Schmidt *et al.*, 1999; Oude Weernink *et al.*, 2000a). Furthermore, Rho-kinase, a Rho effector protein, was identified as an essential element in the M_3 mAChR signaling cascade to PLD (Schmidt *et al.*, 1999). However, PLD enzymes did not directly interact with, or were phosphorylated by Rho-kinase (Schmidt *et al.*, 1999). Thus, regulation of PLD by Rho/Rho-kinase obviously involves additional, yet undefined, signaling components. Here, LIM-kinase, a Rho-kinase effector (Lim *et al.*, 1996; Maekawa *et al.*, 1999; Sumi *et al.*, 2001a), is identified as a novel player in G protein-coupled receptor signaling to PLD. Expression of wild-type and constitutively active LIM-kinase potentiated PLD stimulation by the M_3 mAChR, similar to overexpression of RhoA and Rho-kinase (Schmidt *et al.*, 1999), whereas expression of kinase-deficient LIM-kinase largely reduced PLD stimulation by the receptor. The LIM-kinase mutants did not alter basal PLD activity and PLD stimulation by PMA-responsive PKC. Furthermore, purified recombinant LIM-kinase enhanced PLD activity in membranes of HEK-293 cells, similar as but not additive with activated RhoA or Rho-kinase. These findings suggested that LIM-kinase mediates PLD stimulation by Rho and Rho-kinase. In line with this hypothesis, it is demonstrated that PLD potentiation by wild-type LIM-kinase, but not by constitutively active LIM-kinase, is suppressed by coexpression of the Rho-inactivating C3 transferase (Aktories & Just, 1993) and a dominant-negative Rho-kinase mutant (Oshiro *et al.*, 1998). In contrast, expression of dominant-negative Rac1 and Cdc42 mutants did not alter the PLD response to the M_3 mAChR and LIM-kinase. These findings indicate that even the action of the overexpressed LIM-kinase is under tight control by endogenous Rho. Furthermore, expression of kinase-deficient LIM-kinase fully prevented PLD activation by both wild-type and constitutively active Rho-kinase. Finally, expression of catalytically inactive PLD1, but not of catalytically inactive PLD2, abolished PLD potentiation by LIM-kinase. Thus, overall, LIM-kinase is identified here as a novel component acting downstream of RhoA and Rho-kinase in G protein-coupled receptor signaling to PLD1.

Expression of constitutively active LIM-kinase, which is unresponsive to Rho/Rho-kinase-dependent phosphorylation at threonine 508 (Edwards & Gill, 1999), strongly enhanced the stimulatory effect of the agonist-activated M_3 mAChR but did not cause an increase in basal PLD activity. These findings indicated that a second signal, which is apparently provided by the agonist-activated receptor, is required to gain full PLD activation. Based on previous data (Rümenapp *et al.*, 1995; Schmidt *et al.*, 1996b; Schmidt *et al.*, 1999; Schürmann *et al.*, 1999; Voß *et al.*, 1999), this second signal required for PLD activation is the ARF pathway. In fact, it is demonstrated here that coexpression of dominant-negative ARF1 fully blocks PLD potentiation by constitutively active LIM-kinase, and *vice versa*

coexpression of kinase-deficient LIM-kinase fully prevents PLD potentiation by constitutively active ARF1. Thus, stimulation of PLD by the G protein-coupled receptor M_3 mAChR is apparently a concerted action of ARF and Rho signaling pathways, the latter involving Rho-activated Rho-kinase and its downstream effector, LIM-kinase (Fig. 38). Whether and how these two pathways are interconnected is presently not known. The recently identified ARAP proteins, which are apparently involved in regulation of both ARF and Rho GTPases (Krugmann *et al.*, 2002; Miura *et al.*, 2002), are possible candidates where these two may converge.

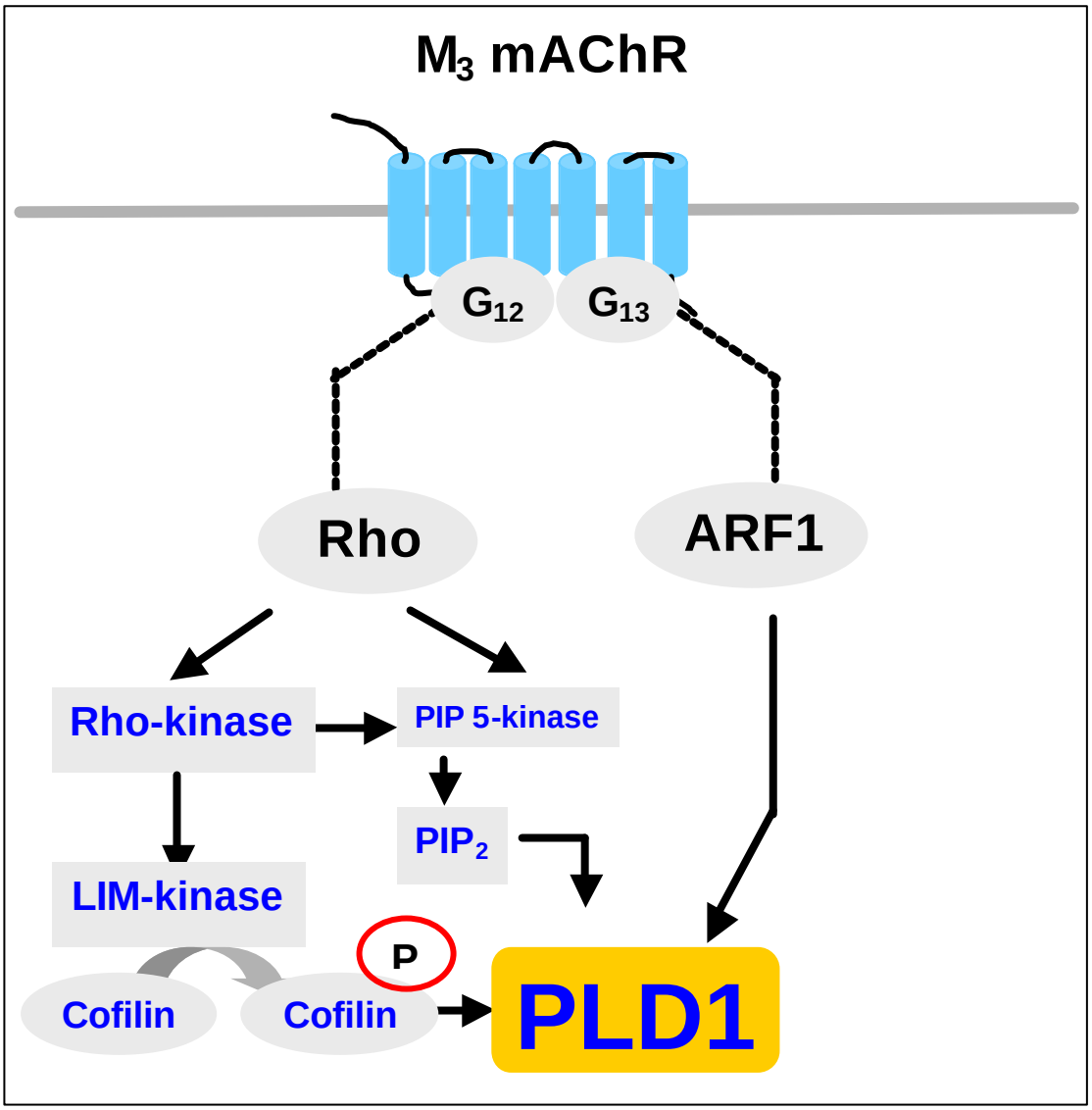


Fig. 38: M_3 mAChR signaling to PLD1 mediated by G_{12}-type G proteins, LIM-kinase and cofilin. For further explanation, see text.

Expression of kinase-deficient LIM-kinase not only suppressed PLD stimulation by the overexpressed M_3 mAChR in HEK-293 cells, but similarly reduced PLD stimulation by the endogenously expressed receptor for lysophosphatidic acid, while it had no effect on PLD stimulation by tyrosine kinase receptors. Furthermore, expression of the LIM-kinase mutants altered PLD stimulation by an endogenously expressed mAChR in N1E-115 neuroblastoma cells similarly as observed for the overexpressed M_3 mAChR in HEK-293 cells. These data strongly suggest that LIM-kinase plays a general role in PLD stimulation by G protein-coupled receptors. Very recently, the generation of a LIM-kinase knockout mice has been reported (Meng *et al.*, 2002). Studies are in progess to examine regulation of PLD signaling in cells and tissues from these LIM-kinase knockout mice.

LIM-kinase did not directly interact with either PLD1 or PLD2 and did not phosphorylate these PLD enzymes, suggesting that LIM-kinase may regulate PLD activity *via* an additional cellular component. Here it is demonstrated that cofilin, a member of the actin depolymerization family and a substrate of LIM-kinase (Bamburg, 1999; Maekawa *et al.*, 1999; Higgs & Pollard, 2001), obviously represents the missing signaling molecule. Expression of wild-type cofilin enhanced M_3 mAChR signaling to PLD1, and this stimulatory effect required a functional Rho/Rho-kinase/LIM-kinase signaling cascade. In contrast, the LIM-kinase nonphosphorylatable cofilin mutant, S3A cofilin, reduced PLD stimulation by the M_3 mAChR and its potentiation by LIM-kinase. The cofilin mutants were without effect on basal PLD activity and PLD stimulation by PMA-activated PKC. Thus, we have identified cofilin as the downstream effector of LIM-kinase in the activation of PLD1 by Rho and Rho-kinase (Fig. 38).

In contrast to Rho-kinase, which stimulates PIP 5-kinase activity (Oude Weernink *et al.*, 2000b; Yamazaki *et al.*, 2002), LIM-kinase and cofilin did not alter the cellular PIP_2 levels. These data suggested that LIM-kinase and cofilin did not trigger PLD stimulation indirectly, i.e. by providing its cofactor PIP_2. In contrast, it is demonstrated here that wild-type cofilin, but not S3A cofilin, specifically binds to PLD1 and triggers its enzyme activity, particularly upon phosphorylation by LIM-kinase. Furthermore, expression of wild-type cofilin induced redistribution of coexpressed PLD1 to the plasma membrane of HEK-293 cells. Thus, it is tempting to speculate that cofilin phosphorylated by LIM-kinase increases PLD1 activity by targeting the phospholipase to this cellular compartment, which also bears Rho and ARF proteins after stimulation of the cells by the M_3 mAChR (Rümenapp *et al.*, 1995; Keller *et al.*, 1997).

As schematically illustrated in Fig. 38, apparently many distinct proteins are involved in stimulation of PLD1 activity by the G protein-coupled M_3 mAChR. However, this may not be the end. Several additional proteins have been recently identified to be regulated by LIM-kinase and/or to modulate the function of cofilin. These include the collapsin

response mediator protein-2, wich is known to be involved in the LIM-kinase-dependent regulation of the actin growth cone in neuronal cells (Aizawa *et al.*, 2001a; Liu & Strittmatter, 2001) and which has actually been reported to inhibit PLD activity in neuronal cells (Lee *et al.*, 2002). Furthermore, the scaffold protein 14-3-3 has recently been shown to modulate LIM-kinase and cofilin signaling by stabilizing phosphorylated cofilin (Gohla & Bokoch, 2002; Birkenfeld *et al.*, 2002). Finally, phosphatases have recently been identified which may also be involved in PLD signaling, such as the slingshot phosphatase, which binds F-actin and specifically dephosphorylates cofilin (Niwa *et al.*, 2002), and protein phosphatase type 5, which is stimulated by $G\alpha_{12}$ and $G\alpha_{13}$ (Yamaguchi *et al.*, 2002), the G proteins mediating G protein-coupled receptor signaling to PLD. Studies are in progress to analyze whether these signaling molecules contribute to PLD regulation by membrane receptors.

In summary, evidence is provided that stimulation of PLD activity by G protein-coupled receptors, already known to involve ARF and Rho GTPases and the Rho-activated Rho-kinase, is mediated specifically by heterotrimeric G proteins of the G_{12}-subtype ($G\alpha_{12}$ and $G\alpha_{13}$), the Rho-kinase effector, LIM-kinase, and the LIM-kinase substrate, cofilin, which apparently in its phosphorylated form interacts with and stimulates PLD1 activity. Finally, adenoviruses encoding PLD1 and PLD2 (wild-type and mutants) have been generated, which will provide powerful tools to study the physiological function of the distinct PLD enzymes.

6. Synopsis

Stimulation of phosphatidylcholine-specific phospholipase D (PLD) enzymes, leading to the generation of the second messenger, phosphatidic acid, has been demonstrated in numerous cell types with a large number of hormones, neurotransmitters and growth factors and is implicated in a wide array of early and late cellular responses to receptor activation. In contrast to the stimulation of phosphatidylinositol-4,5-bisphosphate (PIP_2)-specific phospholipase C (PLC) isoforms, which is caused by a direct interaction with receptors and/or heterotrimeric G proteins, activation of the PLD enzymes by membrane receptors is mediated by complex signaling cascades, involving lipids and proteins of diverse families. The most important are the phosphoinositide, PIP_2, members of the monomeric GTPases superfamily, specifically ARF, Rho and Ras GTPases, and diverse protein kinases, specifically protein kinase C (PKC) isoforms and the Rho-activated Rho-kinase. However, our understanding of receptor coupling to PLD enzymes is still rather incomplete.

Aim of the present study was to gain further insights into the regulation of PLD activity by G protein-coupled receptors, using HEK-293 cells stably expressing the M_3 muscarinic acetylcholine receptor (mAChR). Previous work demonstrated that the M_3 mAChR stimulates PLD in a pertussis toxin (PTX)-insensitive manner, and that stimulation of PLD is independent of concomitant PLC and subsequent PKC activation. Additional work indicated that the PLD response to the M_3 mAChR requires ARF and Rho GTPases and the Rho-activated Rho-kinase. On the other hand, tyrosine kinase receptor signaling to PLD in HEK-293 cells is PKC-dependent and involves a Ras/Ral GTPases signaling cascade.

First, the type of PTX-insensitive heterotrimeric G protein mediating M_3 mAChR-PLD coupling *versus* M_3 mAChR-PLC coupling was determined. By transient expression of α-subunits of the PTX-resistant G proteins, G_q, G_{12} and G_{13} (wild-type, constitutively active and dominant-negative mutants), evidence is provided that the M_3 mAChR specifically couples to PLC *via* $G\alpha_q$ and to PLD *via* the G_{12}-type G proteins, $G\alpha_{12}$ and $G\alpha_{13}$, which are apparently both required for full PLD stimulation. These data were confirmed by expression (transient or by infection with recombinant adenoviruses) of RGS4 or Lsc-RGS, which act as specific GTPase-activating proteins for $G\alpha_q$- and $G\alpha_{12}$-type G proteins, respectively.

Second, the PLD isoform activated by membrane receptors in HEK-293 cells was determined. Expression of catalytically inactive PLD1 and PLD2 mutants strongly suggested that the G protein-coupled M_3 mAChR signals primarily to PLD1, whereas the tyrosine kinase receptors signal *via* PKC to PLD2. For further analysis of the cellular function of the PLD enzymes, recombinant adenoviruses encoding wild-type and catalytically inactive PLD1 and PLD2 mutants were generated, using the pAdEasy system. Infection of cells with these

adenoviruses led to strong expression of the PLD enzymes, indicating that the PLD-encoding adenoviruses can be used in future studies to define the physiological role of the distinct PLD enzymes in mammalian cells.

PLD enzymes are not phosphorylated by Rho-kinase, indicating that regulation of PLD by Rho/Rho-kinase involves additional, yet undefined, components. Here evidence is provided that LIM-kinase, a Rho-kinase effector, mediates the Rho/Rho-kinase-dependent stimulation of PLD1. Expression of wild-type and constitutively active LIM-kinase potentiated PLD stimulation by the M_3 mAChR, whereas kinase-deficient LIM-kinase had the opposite effect. Similar data were obtained with other G protein-coupled receptors in HEK-293 and N1E-115 neuroblastoma cells. On the other hand, the LIM-kinase mutants did not alter PLD stimulation by phorbol ester-activated PKC and tyrosine kinase receptors. Purified recombinant LIM-kinase stimulated PLD activity in cell membranes, similar as but not additive with activated RhoA or Rho-kinase. In line with a downstream effector role of LIM-kinase, PLD stimulation by constitutively active LIM-kinase, but not by wild-type LIM-kinase, was resistant to inactivation of Rho and Rho-kinase, whereas PLD stimulation by constitutively active Rho-kinase was fully abolished by kinase-deficient LIM-kinase. Thus, LIM-kinase is a downstream effector of Rho-kinase to gain full activation of PLD1 by Rho-dependent signals initiated by G protein-coupled receptors.

Stimulation of PLD by the M_3 mAChR is apparently the result of two signaling cascades mediated by ARF and Rho GTPases. In line with this dual signaling process, expression of dominant-negative ARF1 fully blocked PLD stimulation by constitutively active LIM-kinase, and *vice versa* coexpression of kinase-deficient LIM-kinase fully prevented PLD potentiation by constitutively active ARF1. Thus, in HEK-293 cells stimulation of PLD1 by the M_3 mAChR is a concerted action of a Rho/Rho-kinase/LIM-kinase and an ARF signaling cascade.

As reported for Rho-kinase, LIM-kinase did not directly interact with nor did LIM-kinase phosphorylate PLD enzymes, suggesting that an additional component is involved in PLD regulation by Rho-kinase/LIM-kinase. Here evidence is provided that the LIM-kinase substrate, cofilin, an actin depolymerization factor, in its phosphorylated form directly interacts with PLD1 and increases PLD1 activity. Expression of wild-type cofilin potentiated PLD stimulation by the M_3 mAChR, whereas the nonphosphorylatable cofilin mutant, S3A cofilin, reduced the receptor response. On the other hand, the cofilin mutants did not alter PLD2 stimulation by phorbol ester-activated PKC. Furthermore, PLD stimulation by cofilin was suppressed by inactivation of Rho or Rho-kinase. Finally, it is shown that cofilin, but not its S3A mutant, specifically interacts with PLD1 and, upon phosphorylation by LIM-kinase, strongly increases the activity of purified recombinant PLD1 *in vitro*. In intact cells,

expression of wild-type cofilin, but not S3A cofilin, specifically redistributed PLD1 to the plasma membrane, as demonstrated by immunofluorescence laser confocal microscopy. Taken together, evidence is provided that stimulation of PLD by G protein-coupled receptors, known to involve ARF and Rho GTPases and the Rho-activated Rho-kinase, is mediated specifically by heterotrimeric G proteins of the G_{12}-subtype ($G\alpha_{12}$ and $G\alpha_{13}$), the Rho-kinase effector, LIM-kinase, and the LIM-kinase substrate, cofilin, which apparently in its phosphorylated form interacts with and stimulates PLD1 activity.

7. References

Agnew, B.J., Minamide, L.S. & Bamburg, J.R. (1995) Reactivation of phosphorylated actin depolymerizing factor and identification of the regulatory site. *J. Biol. Chem.* 270, 17582-17587

Ahn, B.H., Rhim, H., Kim, S.Y., Sung, Y.M., Lee, M.Y., Choi, J.Y., Wolozin, B., Chang, J.S., Lee, Y.H., Kwon, T.K., Chung, K.C., Yoon, S.H., Hahn, S.J., Kim, M.S., Jo, Y.H. & Min, D.S. (2002) α-Synuclein interacts with phospholipase D isozymes and inhibits pervanadate-induced phospholipase D activation in human embryonic kidney-293 cells. *J. Biol. Chem.* 277, 12334-12342

Aizawa, H., Wakatsuki, S., Ishii, A., Moriyama, K., Sasaki, Y., Ohashi, K., Sekine-Aizawa, Y., Sehara-Fujisawa, A., Mizuno, K., Goshima, Y. & Yahara, I. (2001a) Phosphorylation of cofilin by LIM-kinase is necessary for semaphorin 3A-induced growth cone collapse. *Nat. Neurosci.* 4, 367-373

Aizawa, H., Kishi, Y., Iida, K., Sameshima, M. & Yahara, I. (2001b) Cofilin-2, a novel type of cofilin, is expressed specifically at aggregation stage of *Dictyostelium discoideum* development. *Genes Cells* 6, 913-921

Aktories, K. & Just, I. (1993) GTPases and actin as targets for bacterial toxins. In: *GTPases in Biology I*, edited by Dickey, B.F. & Birnbaumer, L. Springer Verlag, Berlin, pp.87-112

Alemany, R., Kleuser, B., Ruwisch, L., Danneberg, K., Lass, H., Hashemi, R., Spiegel, S., Jakobs, K.H., Meyer zu Heringdorf, M. (2001) Depolarisation induces rapid and transient formation of intracellular sphingosine-1-phosphate. *FEBS Lett.* 509, 239-244

Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y. & Kaibuchi, K. (1997) Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science* 275, 1308-1311

Amano, M., Fukata, Y. & Kaibuchi, K. (2000) Regulation and functions of Rho-associated kinase. *Exp. Cell Res.* 261, 44-51

Amano, T., Richelson, E., Nirenberg, M. (1972) Neurotransmitter synthesis by neuroblastoma clones. *Proc. Natl. Acad. Sci. USA* 69, 258-263

Amano, T., Kaji, N., Ohashi, K. & Mizuno, K. (2002) Mitosis-specific activation of LIM-motif containing protein kinase and roles of cofilin phosphorylation and dephosphorylation in mitosis. *J. Biol. Chem.* 277, 22093-22102

Andresen, B.T., Jackson, E.K. & Romero, G.G. (2000) Angiotensin II signaling to phospholipase D in renal microvascular smooth muscle cells in SHR. *Hypertension* 37, 635-639

Anderson, R.G.W. & Jacobson, K. (2002) A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. *Science* 296, 1821-1825

Anderson, R.A., Boronenkov, I.V., Doughman, S.D., Kunz, J. & Loijens, J.C. (1999) Phosphatidylinositol phosphate kinases, a multifaceted family of signaling enzymes. *J. Biol. Chem.* 274, 9907-9910

Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O. & Caroni, P. (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* 393, 805-809

Armstrong, A.C., Dermime, S., Allinson, C.G., Bhattacharyya, T., Mulryan, K., Gonzalez, K.R., Stern, P.L. & Hawkins, R.E. (2002) Immunization with a recombinant adenovirus encoding a lymphoma idiotype: induction of tumor-protective immunity and identification of an idiotype-specific T cell epitope. *J. Immunol.* 168, 3983-3991

Arneson, L.S., Kunz, J., Anderson, R.A. & Traub, L.M. (1999) Coupled inositide phosphorylation and phospholipase D activation initiates clathrin-coat assembly on lysosomes. *J. Biol. Chem.* 274, 17794-11805

Aspenström, P. (1999) Effectors for the Rho GTPases. *Curr. Opin. Cell Biol.* 11, 95-102

Bacon, K.B., Schall, T.J. & Dairaghi, D.J. (1998) RANTES activation of phospholipase D in Jurkat T cells: requirement of GTP-binding proteins ARF and RhoA. *J. Immunol.* 160, 1894-1900

Balboa, M.A. & Insel, P.A. (1998) Stimulation of phospholipase D via α_1-adrenergic receptors in Madin-Darby canine kidney cells is independent of PKCα and -ε activation. *Mol. Pharmacol.* 53, 221-227

Bamburg, J.R. (1999) Proteins of the ADF/cofilin family: essential regulators of actin dynamics. *Annu. Rev. Cell Dev. Biol.* 15, 185-230

Banno, Y., Takuwa, Y., Akao, Y., Okamoto, H., Osawa, Y., Naganawa, T., Nakashima, S., Suh, P.G. & Nozawa, Y. (2001) Involvement of phospholipase D in sphingosine 1-phosphate-induced activation of phosphatidylinositol 3-kinase and Akt in Chinese Hamster Ovary cells overexpressing EDG3. *J. Biol. Chem.* 276, 35622-35628

Bar-Sagi, D. & Hall, A. (2000) Ras and Rho-GTPases: A family reunion. *Cell* 103, 227-238

Battaini, F. (2001) Protein kinase C isoforms as therapeutic targets in nervous system diseases states. *Pharma. Res.* 44, 353-361

Bayer, S., Voß, M., Asmus, M., Chardin, P., Jakobs, K.H. & Schmidt, M. (1999) The ARF-specific guanine-nucleotide exchange factor ARNO potentiates phospholipase D activation by m3 muscarinic acetylcholine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 359, R59

Bechoua, S. & Daniel, L.W. (2001) Phospholipase D is required in the signaling pathway leading to p38 MAPK activation in neurophil-like HL-60 cells, stimulated by *N*-formyl-methionyl-leucyl-phenylalanine. *J. Biol. Chem.* 276, 31752-31759

Ben El Hadj, N., Popoff, M.R., Marvaud, J.C., Payrastre, B., Boquet, P. & Geny, B. (1999) G-protein-stimulated phospholipase D activity is inhibited by lethal toxin from *Clostridium sordellii* in HL-60 cells. *J. Biol. Chem.* 274, 14021-14031

Bernstein, B.W., Painter, W.B., Chen, H., Minamide, L.S., Abe, H. & Bamburg, J.R. (2000) Intracellular pH modulation of ADF/cofilin proteins. *Cell Motil. Cytoskeleton* 47, 319-336

Berridge, M.J., Lipp, P. & Bootman, M.D. (2000) The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 1, 11-21

Bi, K., Roth, M.G. & Ktistakis, N.T. (1997) Phosphatidic acid formation by phospholipase D is required for transport from the endoplasmic reticulum to the Golgi complex. *Curr. Biol.* 7, 301-307

Bierne, H., Gouin, E., Roux, P., Caroni, P., Yin, H.L. & Cossart, P. (2001) A role for cofilin and LIM kinase in *Listeria*-induced phagocytosis. *J. Cell Biol.* 155, 101-112

Birkenfeld, J., Kartmann, B., Betz, H. & Roth, D. (2001) Cofilin activation during Ca^{2+}-triggered secretion from adrenal chromaffin cells. *Biochem. Biophys. Res. Commun.* 286, 493-498

Birkenfeld, J., Betz, H. & Roth, D. (2002) Identification of cofilin and LIM-kinase 1 as novel interaction partners. *Biochem. J.*, in press.

Bishop, A.L. & Hall, A. (2000) Rho GTPases and their effector proteins. *Biochem. J.* 348, 241-255

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254

Brown, F.D., Thompson, N., Saqib, K.M., Clark, J.M., Powner, D., Thompson, N.T., Solari, R. & Wakelam, M.J.O. (1998) Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. *Curr. Biol.* 8, 835-838

Brown, H.A., Gutowski, S., Moomaw, C.R., Slaughter, C. & Sternweis, P.C. (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein stimulates phospholipase D activity. *Cell* 75, 1137-1144

Cai, S. & Exton, J.H. (2001) Determination of interaction sites of phospholipase D1 for RhoA. *Biochem. J.* 355, 779-785

Caulfield, M.P. & Birdsall, N.J.M. (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.* 50, 279-290

Caumont, A.S., Galas, M.C., Vitale, N., Aunis, D. & Bader, M.F. (1998) Regulated exocytosis in chromaffin cells. Translocation of ARF6 stimulates a plasma membrane-associated phospholipase D. *J. Biol. Chem.* 273, 1373-1379

Caumont, A.S., Vitale, N., Gensse, M., Galas, M.C., Casanova, J.E. & Bader, M.F. (2000) Identification of a plasma membrane-associated guanine nucleotide exchange factor for ARF6 in chromaffin cells. Possible role in the regulated exocytotic pathway. *J. Biol. Chem.* 275, 15637-15644

Chan, T.O., Rittenhouse, SE. & Tsichlis, P.N. (1999) AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.* 68, 965-1014

Chartier, C., Degryse, M., Gantzer, M., Dieterie, A., Pavirani, A. & Mehtali, M. (1996) Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J. Virol.* 70, 4805-4810

Chaves-Olarte, E., Löw, P., Freer, E., Norlin, T., Weidmann, M., von Eichel-Streiber, C. & Thelestam, M. (1999) A novel cytotoxin from *Clostridium difficile* serogroup F is a functional hybrid between two other large clostridial cytotoxins. *J. Biol. Chem.* 274, 11046-11052

Chavrier, P. & Goud, B. (1999) The role of ARF and Rab GTPases in membrane transport. *Curr. Opin. Cell Biol.* 11, 466-475

Chen, H., Bernstein, B.W. & Bamburg, J.R. (2000) Regulating actin-filament dynamics *in vivo*. *Trends Biochem. Sci.* 25, 19-23

Chen, H.C., Bandyopadhyay, G., Sajan, M.P., Kanoh, Y., Standaert, M., Farese Jr., R.V. & Farese, R.V. (2002) Activation of the ERK pathway and atypical protein kinase C isoforms in exercise- and aminoimidazole-4-carboxamide-1-β-D-riboside (AICAR)-stimulated glucose transport. *J. Biol. Chem.* 277, 23554-23562

Ching, T.T., Wang, D.S., Hsu, A.L., Lu, P.J. & Chen, C.S. (1999) Identification of multiple phosphoinositide-specific phospholipase D as new regulatory enzymes for phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* 274, 8611-8617

Chung, J.K., Sekiya, F., Kang, H.S., Lee, C., Han, J.S., Kim, S.R., Bae, Y.S., Morris, A.J. & Rhee, S.G. (1997) Synaptojanin inhibition of phospholipase D activity by hydrolysis of phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* 272, 15980-15985

Clapham, D.E. (1995) Calcium signaling. *Cell* 80, 259-268

Cockcroft, S. (2001) Signalling roles of mammalian phospholipase D1 and D2. *Cell. Mol. Life Sci.* 58, 1674-1687

Cockcroft, S., Thomas, G.M., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N.F., Truong, O. & Hsuan, J.J. (1994) Phospholipase D: A downstream effector of Arf in granulocytes. *Science* 263, 523-526

Colley, W.C., Altshuller, Y.M., Sue-Ling, C.K., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Branch, K.D., Tsirka, S.E., Bollag, R.J., Bollag, W.B. & Frohman, M.A. (1997a) Cloning and expression analysis of murine phospholipase D1. *Biochem. J.* 326, 745-753

Colley, W.C., Sung, T.C., Roll, R., Jenco, J., Hammond, S.M., Altshuller, Y., Bar-Sagi, D., Morris, A.J. & Frohman, M.A. (1997b) Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr. Biol.* **7**, 191-201

Contos, J.J.A., Ishii, I. & Chun, J. (2000) Lysophosphatidic acid receptors. *Mol. Pharmacol.* 58, 1188-1196

Cross, M.J., Roberts, S., Ridley, A.J., Hodgkin, M.N., Stewart, A., Claesson-Welsh, L. & Wakelam,

M.J.O. (1996) Stimulation of actin stress fibre formation mediated by activation of phospholipase D. *Curr. Biol.* 6, 588-597

Cullen, P.J., Cozier, G.E., Banting, G. & Mellor, H. (2001) Modular phosphoinositide-binding domains – their role in signalling and membrane trafficking. *Curr. Biol.* 11, R882-R893

Cummings, R., Parinandi, N., Wang, L., Usatyuk, P. & Natarajan, V. (2002a) Phospholipase D/phosphatidic acid signal transduction: role and physiological significance in lung. *Mol. Cell. Biochem.* 234/235, 99-109

Cummings, R.J., Parinandi, N.L., Zaiman, A., Wang, L.X., Usatyuk, P.V., Garcia, J.G. & Natarajan, V. (2002b) Phospholipase D activation by sphingosine 1-phosphate regulates Interleukin-8 secretion in human bronchial epithelial cells. *J. Biol. Chem.* 277, 30227-30235

Czarny, M., Lavie, Y., Fiucci, G. & Liscovitch, M. (1999) Localization of phospholipase D in detergent-insoluble, caveolin-rich membrane domains. Modulation by caveolin-1 expression and caveolin-1_{82-101}. *J. Biol. Chem.* 274, 2717-2724

Czech, M.P. (2000) PIP2 and PIP3: complex roles at the cell surface. *Cell* 100, 603-606

Daniel, L.W., Sciorra, V.A. & Ghosh, S. (1999) Phospholipase D, tumor promoters, proliferation and prostaglandins. *Biochim. Biophys. Acta.* 1439, 265-276

Dascher, C. & Balch W.E. (1994) Dominant inhibitory mutants of ARF1 block endoplasmic reticulum to golgi transport and trigger disassembly of the Golgi apparatus. *J. Biol. Chem.* 269, 1437-1448

Denmat-Ouisse, L.A., Phebidias, C., Honkavaara, P., Robin, P., Geny, B., Min, D.S., Bourgoin, S., Frohman, M.A. & Raymond, M.N. (2001) Regulation of constitutive protein transit by phospholipase D in HT-cl19 A cells. *J. Biol. Chem.* 276, 48840-48846

Dennis, E.A. (1994) Diversity of group types, regulation, and function of phospholipase A_2. *J. Biol. Chem.* 269, 13057-13060

De Vries, L., Zheng, B., Fischer, T., Elenko, E. & Farquhar, M.G. (2000) The regulators of G protein signaling family. *Annu. Rev. Pharmacol. Toxicol.* 40, 235-271

Dhanasekaran, N. & Dermott, J.M. (1996) Signaling by the G_{12} class of G proteins. *Cell. Signal.* 8, 235-245

Divecha, N., Roefs, M., Halstead, J.R., D'Andrea, S., Fernandez-Borga, M., Oomen, L., Saqib, K.M., Wakelam, M.J.O. & D'Santos, C. (2000) Interaction of the type Iα PIP kinase with phospholipase D: a role for the local generation of phosphatidylinositol 4,5-bisphosphate in the regulation of PLD2 activity. *EMBO J.* 19, 5440-5449

Dohlman, H.G. & Thorner, J. (1997) RGS proteins and signaling by heterotrimeric G proteins. *J. Biol. Chem.* 272, 3871-3874

Donaldson, J.G. & Jackson, C.L. (2000) Regulators and effectors of the ARF GTPases. *Curr. Opin. Cell Biol.* 12, 475-482

Donnai, D. & Karmiloff-Smith, A. (2000) Williams syndrome: from genotype through to the cognitive phenotype. *Am. J. Med. Genet.* 97, 164-171

Druey, K.M., Blumer, K.J., Kang, V.H. & Kehrl, J.H. (1996) Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family. *Nature* 379, 742-746

Du, G.W., Altshuller, Y.M., Kim, Y., Han, J.M., Ryu, S.H., Morris, A.J. & Frohman, M.A. (2000) Dual requirement for Rho and protein kinase C in direct activation of phospholipase D1 through G-protein-coupled receptor signaling. *Mol. Biol. Cell* 11, 4359-4368

Edwards, D.C., Sanders, L.C., Bokoch, G.M. & Gill, G.N. (1999) Activation of Lim-kinase by Pak1 couples Rac/Cdc42 GTPase signaling to actin cytoskeleton dynamics. *Nat. Cell Biol.* 1, 253-259

Edwards, D.C. & Gill, G.N. (1999) Structural features of Lim-kinase that control effects on the actin cytoskeleton. *J. Biol. Chem.* 274, 11352-11361

Emoto, M., Klarlund, J.K., Waters, S.B., Hu, V., Buxton, J.M., Chawla, A. & Czech, M.P. (2000) A

role for phospholipase D in GLUT4 glucose transporter translocation. *J. Biol. Chem.* 275, 7144-7151

English, D. (1996) Phosphatidic acid: a lipid messenger involved in intracellular and extracellular signaling. *Cell. Signal.* 8, 341-347

Evellin, S., Nolte, J., Tysack, K., vom Dorp, F., Thiel, M., Oude Weernink, P.A., Jakobs, K.H., Webb, E.J., Lomasney, J.W. & Schmidt; M. (2002) Stimulation of phospholipase C-ε by the M_3 muscarinic acetylcholine receptor mediated by cyclic AMP and the GTPase Rap2B. *J. Biol. Chem.* 277, 16805-16813

Exton, J.H. (1999) Regulation of phospholipase D. *Biochim. Biophys. Acta.* 1439, 121-133

Exton, J.H. (2002) Phospholipase D - structure, regulation and function. *Rev. Physiol. Biochem. Pharmacol.* 144, 1-94

Fahimi-Vahid, M., Gosau, N., Michalek, C., Han, L., Jakobs, K.H., Schmidt, M., Roberts, N., Avkiran, M. & Wieland, T. (2002) Distinct signaling pathways mediate cardiomyocyte phospholipase D stimulation by endothelin-1 and thrombin. *J. Mol. Cell. Cardiol.* 34, 441-453

Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A. & Chen, J. (2001) Phosphatidic-acid mediated mitogenic activation of mTOR signaling. *Science* 294, 1942-1945

Feig, L.A., Urano, T. & Cantor, S. (1996) Evidence for a Ras/Ral signaling cascade. *Trends Biochem. Sci.* 21, 438-441

Fensome, A., Whatmore, J., Morgan, C., Jones, D. & Cockcroft, S. (1998) ADP-ribosylation factor and Rho proteins mediate fMLP-dependent activation of phospholipase D in human neutrophils. *J. Biol. Chem.* 273, 13157-13164

Fields, T.A. & Casey, P.J. (1997) Signalling functions and biochemical properties of pertussis toxin-resistant G-proteins. *Biochem. J.* 321, 561-571

Frank, C., Keilhack, H., Opitz, F., Zschörnig, O. & Böhmer, F.D. (1999) Binding of phosphatidic acid to the protein-tyrosine phosphatase SHP-1 as a basis for activity modulation. *Biochemistry* 38, 11993-12002

Frankel, P., Ramos, M., Flom, J., Bychenok, S., Joseph, T., Kerkhoff, E., Rapp, U.R., Feig, L.A. & Foster, D.A. (1999) Ral and Rho-dependent activation of phospholipase D in v-Raf-transformed cells. *Biochem. Biophys. Res. Commun.* 255, 502-507

Freyberg, Z., Sweeney, D., Siddhanta, A., Bourgoin, S., Frohman, M. & Shields, D. (2001) Intracellular localization of phospholipase D1 in mammalian cells. *Mol. Biol. Cell* 12, 943-955

Frohman, M.A., Sung, T.C. & Morris, A.J. (1999) Mammalian phospholipase D structure and regulation. *Biochim. Biophys. Acta.* 1439, 175-186

Frohman, M.A., Kanaho, Y., Zhang, Y. & Morris, A.J. (2000) Regulation of phospholipase D1 activity by Rho GTPases. *Methods Enzymol.* 325, 177-189

Fruman, D.A., Meyers, R.E. & Cantley, L.C. (1998) Phosphoinositide kinases. *Annu. Rev. Biochem.* 67, 481-507

Fukushima, N., Ishii, I., Contos, J.J.A., Weiner, J.A. & Chun, J. (2001) Lysophospholipid receptors. *Annu. Rev. Pharmacol. Toxicol.* 41, 507-534

Geneste, O., Copeland, J.W. & Treisman, R. (2002) LIM kinase and Diaphanous cooperate to regulate serum response factor and actin dynamics. *J. Cell Biol.* 157, 831-838

Ghosh, S., Strum, J.C., Sciorra, V.A., Daniel, L. & Bell, R.M. (1996) Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-*O*-tetradecanoylphorbol-13-acetate-stimulated Madin-Darby canine kidney cells. *J. Biol. Chem.* 271, 8472-8480

Godi, A., Pertile, P., Meyers, R., Marra, P., DiTullio, G.D., Iurisci, C., Luini, A., Corda, D. & De Matteis, M.A. (1999) ARF mediates recruitment of PtdIns-4-OH kinase-β and stimulates

synthesis of PtdIns(4,5)P_2 on the Golgi complex. *Nat. Cell Biol.* 1, 280-287

Gohla, A., Offermanns, S., Wilkie, T.M. & Schultz, G. (1999) Differential involvement of $G\alpha_{12}$ and $G\alpha_{13}$ in receptor-mediated stress fiber formation. *J. Biol. Chem.* 274, 17901-17907

Gohla, A. & Bokoch, G.M. (2002) 14-3-3 regulates actin dynamics by stabilizing phosphorylated cofilin. *Curr. Biol.* 12, 1704-1710

Gosau, N., Fahimi-Vahid, M., Michalek, C., Schmidt, M. & Wieland, T. (2002) Signalling components involved in the coupling of α_1-adenoceptors to phospholipase D in neonatal rat cardiac myocytes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 365, 468-476

Gudermann, T., Kalkbrenner, F. & Schultz, G. (1996) Diversity and selectivity of receptor-G protein interaction. *Annu. Rev. Pharmacol. Toxicol.* 36, 429-459

Ha, K.S. & Exton, J.H. (1993) Activation of actin polymerization by phosphatidic acid derived from phosphatidylcholine in IIC9 fibroblasts. *J. Cell Biol.* 123, 1789-1796

Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science* 279, 509-514

Hall, A. (2000) GTPases. Oxford University Press

Hammond, S.M., Altshuller, Y.M., Sung, T.C., Rudge, S.A., Rose, K., Engebrecht, J., Morris, A.J. & Frohman, M.A. (1995) Human ADP-ribosylation factor-activated phosphatidyl-choline specific phospholipase D defines a new and highly conserved gene family. *J. Biol. Chem.* 270, 29640-29643

Hammond, S.M., Jenco, J.M., Nakashima, S., Cadwallader, K., Gu, Q.M., Cook, S., Nozawa, Y., Prestwich, G.D., Frohman, M.A. & Morris, A.J. (1997) Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-α. *J. Biol. Chem.* 272, 3860-3868

He, T.C., Zhou, S., Da Costa, L.T., Yu, J., Kinzler, K.W. & Vogelstein, B. (1998) A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* 95, 2509-2514

Heneweer, C., Kruse, L.H., Kindhäuser, F., Schmidt, M., Jakobs, K.H., Denker, H.W. & Thie, M. (2002) Adhesiveness of human uterine epithelial RL95-2 cells for trophoblast: Rho protein regulation. *Mol. Hum. Reprod.* 8, 1014-1022.

Hess, J.A., Ross, A.H., Qiu, R.G., Symons, M. & Exton, J.H. (1997) Role of Rho family proteins in phospholipase D activation by growth factors. *J. Biol. Chem.* 272, 1615-1620

Hess, J.A., Ji, Q.S., Carpenter, G. & Exton, J.H. (1998) Analysis of platelet-derived growth factor-induced phospholipase D activation in mouse embryo fibroblasts lacking phospholipase C-*g* 1. *J. Biol. Chem.* 273, 20517-20524

Hess, J.A., Buchanan, F.G., Ryder, S. & Exton, J.H. (2000) Altered activation of phospholipase D by lysophosphatidic acid and endothelin-1 in mouse embryo fibroblasts lacking phospholipase C-γ1. *Cell. Signal.* 12, 37-45

Higgs, H.N. & Pollard, T.D. (2001) Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annu. Rev. Biochem.* 70, 649-676

Hirata, M., Kanematsu, T., Takeuchi, H. & Yagisawa, H. (1998) Pleckstrin homology domain as an inositol compound binding module. *Jpn. J. Pharmacol.* 76, 255-263

Hodgkin, M.N., Pettitt, T.R., Martin, A., Michell, R.H., Pemberton, A.J. & Wakelam, M.J.O. (1998) Diacylglycerols and phosphatidates: which molecular species are intracellular messengers? *Trends Biochem. Sci.* 23, 200-204

Hodgkin, M.N., Clark, J.M., Rose, S., Saqib, K. & Wakelam, M.J.O. (1999) Characterization of the regulation of phospholipase D activity in the detergent-insoluble fraction of HL60 cells by protein kinase C and small G-proteins. *Biochem. J.* 339, 87-93

Hodgkin, M.N., Masson, M.R., Powner, D., Saqib, K.M., Ponting, C.P. & Wakelam, M.J.O. (2000) Phospholipase D regulation and localization is dependent upon a phosphatidylinositol 4,5-bisphosphate-specific PH domain. *Curr. Biol.* 10, 43-46

Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A.J., Frohman, M.A. & Kanaho, Y. (1999) Phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* 99, 521-532

Hong, J.H., Oh, S.O., Lee, M., Kim, Y.R., Kim, D.U., Hur, G.M., Lee, J.H., Lim, K., Hwang, B.D. & Park, S.K. (2001) Enhancement of lysophosphatidic acid-induced ERK phosphorylation by phospholipase D1 via the formation of phosphatidic acid. *Biochem. Biophys. Res. Commun.* 281, 1337-1342

Houle, M.G., Kahn, R.A., Naccache, P.H. & Bourgoin, S. (1995) ADP-ribosylation factor translocation correlates with potentiation of GTPγS-stimulated phospholipase D activity in membrane fraction of HL-60 cells. *J. Biol. Chem.* 270, 22795-22800

Houle, M.G. & Bourgoin, S. (1999) Regulation of phospholipase D by phosphorylation-dependent mechanisms. *Biochim. Biophys. Acta.* 1439, 135-149

Huang, C., Hepler, J.R., Gilman, A.G. & Mumby, S.M. (1997) Attenuation of G_i- and G_q-mediated signaling by expression of RGS4 or GAIP in mammalian cells. *Proc. Natl. Acad. Sci. USA* 94, 6159-6163

Hughes, W.E., Larijani, B. & Parker, P.J. (2002) Detecting protein-phospholipid interactions. Epidermal growth factor-induced activation of phospholipase D1b *in situ*. *J. Biol. Chem.* 277, 22974-22979

Hughes, W.E. & Parker, P.J. (2001) Endosomal localization of phospholipase D 1a and 1b is defined by the C-termini of the proteins, and is independent of activity. *Biochem. J.* 356, 727-736

Humeau, Y., Vitale, N., Chasserot-Golaz, S., Dupont, J.L., Du, G., Frohman, M.A., Bader, M.F. & Poulain, B. (2001) A role for phospholipase D1 in neurotransmitter release. *Proc. Natl. Acad. Sci. USA* 98, 15300-15305

Iyer, S.S. & Kusner, D.J. (1999) Association of phospholipase D activity with the detergent-insoluble cytoskeleton of U937 promonocytic leukocytes. *J. Biol. Chem.* 274, 2350-2359

Jackson, C.L. & Casanova, J.E. (2000) Turning on ARF: the Sec7 family of guanine-nucleotide-exchange factors. *Trends Cell Biol.* 10, 60-67

Janmey, P.A., Xian, W. & Flanagan, L.A. (1999) Controlling cytoskeleton structure by phosphoinositide-protein interactions: phosphoinositide binding protein domains and effects of lipid packing. *Chem. Phys. Lipids* 101, 93-107

Jenco, J.M., Rawlingson, A., Daniels, B. & Morris, A.J. (1998) Regulation of phospholipase D2: Selective inhibition of mammalian phospholipase D isoenzymes by α- and β-synucleins. *Biochemistry* 37, 4901-4909.

Jiang, H., Luo, J.-Q., Urano, T., Frankel, P., Lu, Z., Foster, D.A. & Feig, L.A. (1995) Involvement of Ral GTPase in *v*-Src-induced phospholipase D activation. *Nature* 378, 409-412.

Jones, D., Morgan, C. & Cockcroft, S. (1999a) Phospholipase D and membrane traffic potential roles in regulated exocytosis, membrane delivery and vesicle budding. *Biochim. Biophys. Acta.* 1439, 229-244

Jones, D.H., Bax, B., Fensome, A. & Cockcroft, S. (1999b) ADP ribosylation factor 1 mutants identify a phospholipase D effector region and reveal that phospholipase D participates in lysosomal secretion but is not sufficient for recruitment of coatomer 1. *Biochem. J.* 341, 185-192

Jones, D.H., Morris, J.B., Morgan, C.P., Kondo, H., Irvine, R.F. & Cockcroft, S. (2000) Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the Golgi compartment. *J. Biol.*

Chem. 275, 13962-13966

Jones, D.R., Sanjuan, M.A. & Merida, I. (2000) Type Iα phosphatidylinositol 4-phosphate 5-kinase is a putative target for increased intracellular phosphatidic acid. *FEBS Lett.* 476, 160-165

Jones, J.A. & Hannun, Y.A. (2002) Tight binding inhibition of protein phosphatase-1 by phosphatidic acid. Specificity of inhibition by the phospholipid. *J. Biol. Chem.* 277, 15530-15538

Joseph, T., Bryant, A., Frankel, P., Wooden, R., Kerkhoff, E., Rapp, U.R. & Foster, D.A. (2002) Phospholipase D overcomes cell cycle arrest induced by high-intensity Raf signaling. *Oncogene* 21, 3651-3658

Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M. & Aktories, K. (1995) Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* 375, 500-503

Just, I., Selzer, J., Hofmann, F., Green, G.A. & Aktories, K. (1996) Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. *J. Biol. Chem.* 271, 10149-10153

Kaibuchi, K., Kuroda, S. & Amano, M. (1999) Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu. Rev. Biochem.* 68, 459-486

Kam, Y. & Exton, J.H. (2001) Phospholipase D activity is required for actin stress fiber formation in fibroblasts. *Mol. Cell. Biol.* 21, 4055-4066

Kanoh, H., Williger, B.T. & Exton, J.H. (1997) Arfaptin 1, a putative cytosolic target protein of ADP-ribosylation factor, is recruited to Golgi membranes. *J. Biol. Chem.* 272, 5421-5429

Karnam, P., Standaert, M.L., Galloway, L. & Farese, R.V. (1997) Activation and translocation of Rho (and ADP ribosylation factor) by insulin in Rat adipocytes. Apparent involvement of phosphatidylinositol 3-kinase. *J. Biol. Chem.* 272, 6136-6140

Kavran, J.M., Klein, D.E., Lee, A., Falasca, M., Isakoff, S.J., Skolnik, E.Y. & Lemmon, M.A. (1998) Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains. *J. Biol. Chem.* 273, 30497-30508

Keller, J., Schmidt, M., Hussein, B., Rümenapp, U. & Jakobs, K.H. (1997) Muscarinic receptor-stimulated cytosol-membrane translocation of RhoA. *FEBS Lett.* 403, 299-302

Kim, J.H., Lee, S.D., Han, J.M., Lee, T.G., Kim, Y., Park, J.B., Lambeth, J.D., Suh, P.G. & Ryu, S.H. (1998) Activation of phospholipase D1 by direct interaction with ADP-ribosylation factor 1 and RalA. *FEBS Lett.* 430, 231-235

Kim, Y., Kim, J.E., Lee, S.D., Lee, T.G., Kim, J.H., Park, J.B., Han, J.M., Jang, S.K., Suh, P.G. & Ryu, S.H. (1999) Phospholipase D1 is located and activated by protein kinase Cα in the plasma membrane in 3Y1 fibroblast cells. *Biochim. Biophys. Acta* 1436, 319-330

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. & Kaibuchi, K. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* 273, 245-248

Kishikawa, K., Chalfant, C.E., Perry, D.K., Bielawska, A. & Hannun, Y.A. (1999) Phosphatidic acid is a potent and selective inhibitor of protein phosphatase 1 and an inhibitor of ceramide-mediated responses. *J. Biol. Chem.* 274, 21335-21341

Kiss, Z. (1996) Regulation of phospholipase D by protein kinase C. *Chem. Phys. Lipids* 80, 81-102

Kjøller, L. & Hall, A. (1999) Signalling to Rho GTPases. *Exp. Cell Res.* 253**,** 166-179

Koonin, E.V. (1996) A duplicated catalytic motif in a new superfamily of phosphohydrolases and phospholipid synthases that includes poxvirus envelope proteins. *Trends Biochem. Sci.* 21, 242-243

Kristiansen, S., Nielsen, J.N., Bourgoin, S., Klip, A., Franco, M. & Richter, E.A. (2001) GLUT-4 translocation in skeletal muscle studied with a cell-free assay: involvement of phospholipase D. *Am. J. Physiol. Endocrinol. Metab.* 281, E608-E618

Krugmann, S., Anderson, K.E., Ridley, S.H., Risso, N., McGregor, A., Coadwell, J., Davidson, K.,

Eguinoa, A., Ellson, C.D., Lipp, P., Manifava, M., Ktistakis, N., Painter, G., Thuring, J.W., Cooper, M.A., Lim, Z.Y., Holmes, A.B., Dove, S.K., Michell, R.H., Grewal, A., Nazarian, A., Erdjument-Bromage, H., Tempst, P., Stephens, L.R. & Hawkins, P.T. (2002) Identification of ARAP3, a novel PI3K effector regulating both Arf and Rho GTPases, by selective capture on phosphoinositide affinity matrices. *Mol. Cell* 9, 95-108

Ktistakis, N.T., Brown, H.A., Waters, M.G., Sternweis, P.C. & Roth, M.G. (1996) Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles. *J. Cell Biol.* 134, 295-306

Kuai, J., Boman, A.L., Arnold, R.S., Zhu, X. & Kahn, R.A. (2000) Effects of activated ADP-ribosylation factors on Golgi morphology require neither activation of phospholipase D1 nor recruitment of coatomer. *J. Biol. Chem.* 275, 4022-4032

Kurzchalia, T.V. & Parton, R.G. (1999) Membrane microdomains and caveolae. *Curr. Opin. Cell Biol.* 11, 424-431

Kusano, K.-i., Abe, H. & Obinata, T. (1999) Detection of a sequence involved in actin-binding and phosphoinositide-binding in the N-terminal side of cofilin. *Mol. Cell. Biochem.* 190, 133-141

Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685

Laher, I. & Zhang, J.H. (2001) Protein kinase C and cerebral vasospasm. *J. Cereb. Blood Flow Metab.* 21, 887-906

Lappalainen, P. & Drubin, D.G. (1997) Cofilin promotes rapid actin filament turnover *in vivo*. *Nature* 388, 78-82

Lappalainen, P., Kessels, M.M., Cope, M.J.T.V. & Drubin, D.G. (1998) The ADF homology (ADF-H) domain: a highly exploited actin-binding module. *Mol. Biol. Cell* 9, 1951-1959

Lawler, S. (1999) Regulation of actin dynamics: The LIM-kinase connection. *Curr. Biol.* 9, R800-R802

Lee, C., Kang, H.S., Chung, J.K., Sekiya, F., Kim, J.R., Han, J.S., Kim, S.R., Bae, Y.S., Morris, A.J. & Rhee, S.G. (1997) Inhibition of phospholipase D by clathrin assembly protein 3 (AP3). *J. Biol. Chem.* 272, 15986-15992

Lee, C., Kim, S.R., Chung, J.K., Frohman, M.A., Kilimann, M.W. & Rhee, S.G. (2000) Inhibition of phospholipase D by amphiphysins. *J. Biol. Chem.* 275, 18751-18758

Lee, S., Park, J.B., Kim, J.H., Kim, Y., Kim, J.H., Shin, K.J., Lee, J.S., Ha, S.H., Suh, P.G. & Ryu, S.H. (2001) Actin directly interacts with phospholipase D, inhibiting its activity. *J. Biol. Chem.* 276, 28252-28260

Lee, S., Kim, J.H., Lee, C.S., Kim, J.H., Kim, Y., Heo, K., Ihara, Y., Coshima, Y., Suh, P.G. & Ryu, S.H. (2002) Collapsin response mediator protein-2 inhibits neuronal phospholipase D2 activity by indirect interaction. *J. Biol. Chem.* 277, 6542-6549

Lefkowitz, R.J. (2000) The superfamily of heptahelical receptors. *Nat. Cell Biol.* 2, E133-136

Leiros, I., Secundo, F., Zambonelli, C., Servi, S. & Hough, E. (2000) The first crystal structure of a phospholipase D. *Structure* 8, 655-667

Lemmon, M.A., Ferguson, K.M. & Abrams, C.S. (2002) Pleckstrin homology domains and the cytoskeleton. *FEBS Lett.* 513, 71-76

Le Stunff, H., Dokhac, L., Bourgoin, S., Bader, M.F. & Harbon, S. (2000a) Phospholipase D in rat myometrium: occurrence of a membrane-bound ARF6 (ADP-ribosylation factor 6)-regulated activity controlled by $\beta\gamma$ subunits of heterotrimeric G-proteins. *Biochem. J.* 352, 491-499

Le Stunff, H., Dokhac, L. & Harbon, S. (2000b) The roles of protein kinase C and tyrosine kinases in mediating endothelin-1-stimulated phospholipase D activity in rat myometrium: differential inhibition by ceramides and cyclic AMP. *J. Pharmacol. Exp. Ther.* 292, 629-637

Leung, T., Manser, E., Tan, L. & Lim, L. (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J. Biol. Chem.* 270, 29051-29054

Leung, T., Chen, X.Q., Manser, E. & Lim, L. (1996) The p160 RhoA-binding kinase ROKα is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol. Cell. Biol.* 16, 5313-5327

Lim, L., Manser, E., Leung, T. & Hall, C. (1996) Regulation of phosphorylation pathways by p21 GTPases. The p21 Ras-related Rho subfamily and its role in phosphorylation signalling pathways. *Eur. J. Biochem.* 242, 171-185

Liscovitch, M., Chalifa, V., Pertile, P., Chen, C.S. & Cantley, L.C. (1994) Novel function of phosphatidylinositol 4,5-bisphosphate as a cofactor for brain membrane phospho-lipase D. *J. Biol. Chem.* 269, 21403-21406

Liscovitch, M., Czarny, M., Fiucci, G. & Tang, X.Q. (2000) Phospholipase D: molecular and cell biology of a novel gene family. *Biochem. J.* 345, 401-415

Liu, B.P. & Strittmatter, S.M. (2001) Semaphorin-mediated axonal giudance via Rho-related G proteins. *Curr. Opin. Cell Biol.* 13, 619-626

Lou, Z.K., Billadeau, D.D., Savoy, D.N., Schoon, R.A. & Leibson, P.J. (2001) A role for RhoA/Rock/LIM-kinase pathway in the regulation of cytotoxic lymphocytes. *J. Immunol.* 167, 5749-5757

Lu, Z.M., Hornia, A., Joseph, T., Sukezane, T., Frankel, P., Zhong, M., Bychenok, S., Xu, L.Z., Feig, L.A. & Foster, D.A. (2000) Phospholipase D and RalA cooperate with the epidermal growth factor receptor to transform 3Y1 rat fibroblasts. *Mol. Cell. Biol.* 20, 462-467

Lucas, L., del Peso, L., Rodriguez, P., Penalva, V. & Lacal, J.C. (2000) Ras protein is involved in the physiological regulation of phospholipase D by platelet-derived growth factor. *Oncogene* 19, 431-437

Lukowski, S., Lecomte, M.C., Mira, J.P., Marin, P., Gauterol, H., Russo-Marie, F. & Geny, B. (1996) Inhibition of phospholipase D activity by fodrin. An active role for the cytoskeleton. *J. Biol. Chem.* 271, 24164-24171

Lukowski, S., Mira, J.P., Zachowski, A. & Geny, B. (1998) Fodrin inhibits phospholipase A2, C, D by decreasing polyphosphoinositide cell content. *Biochem. Biophys. Res. Commun.* 248, 278-284

Luo, J.Q., Liu, X., Hammond, S.M., Colley, W.C., Feig, L.A., Frohman, M.A., Morris, A.J. & Foster, D.A. (1997) RalA interacts directly with the Arf-responsive, PIP_2-dependent phospholipase D1. *Biochem. Biophys. Res. Commun.* 235, 854-859.

Luo, J.Q., Liu, X., Frankel, P., Rotunda, T., Ramos, M., Flom, J., Jiang, H., Feig, L.A., Morris, A.J., Kahn, R.A. & Foster, D.A. (1998) Functional association between Arf and RalA in active phospholipase D complex. *Proc. Natl. Acad. Sci. USA* 95, 3632-3637

Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K. & Narumiya, S. (1999) Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and Lim-kinase. *Science* 285, 895-898

Majerus, P.W., Kisseleva, M.V. & Norris, F.A. (1999) The role of phosphatases in inositol signaling reactions. *J. Biol. Chem.* 274, 10669-10672

Malcolm, K.C., Elliott, C.M. & Exton, J.H. (1996) Evidence for Rho-mediated agonist stimulation of phospholipase D in Rat1 fibroblasts. Effects of *Clostridium botulinum* C3 exoenzyme. *J. Biol. Chem.* 271, 13135-13139

Manifava, M., Thuring, J.W.J.F., Lim, Z.Y., Packman, L., Holmes, A.B. & Ktistakis, N.T. (2001) Differential binding of traffic-related proteins to phosphatidic acid- or phosphatidylinositol (4,5)-bisphosphate-coupled affinity reagents. *J. Biol. Chem.* 276, 8987-8994

Marcil, J., Harbour, D., Naccache, P.H. & Bourgoin, S. (1997) Human phospholipase D1 can be

tyrosine-phosphorylated in HL-60 granulocytes. *J. Biol. Chem.* 272, 20660-20664

Marinissen, M.J. & Gutkind, J.S. (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol. Sci.* 22, 368-376.

Martin, A., Brown, F.D., Hodgkin, M.N., Bradwell, A.J., Cook, S.J., Hart, M. & Wakelam, M.J.O. (1996) Activation of phospholipase D and phosphatidylinositol 4phosphate 5-kinase in HL60 membranes is mediated by endogenous Arf but not Rho. *J. Biol. Chem.* 271, 17397-17403

Martin, T.F.J. (1998) Phosphoinositide lipids as signaling molecules: common themes for signal transduction, cytoskeletal regulation, and membrane trafficking. *Annu. Rev. Cell Dev. Biol.* 14, 231-264

Martin, T.F.J. (2001) $PI(4,5)P_2$ regulation of surface membrane traffic. *Curr. Opin. Cell Biol.* 13, 493-499

Matsui, S., Matsumoto, S., Adachi, R., Kusui, K., Hirayama, A., Watanabe, H., Ohashi, K., Mizuno, K., Yamaguchi, T., Kasahara, T. & Suzuki, K. (2002) LIM kinase 1 modulates opsonized zymosan-triggered activation of macrophage-like U937 cells. Possible involvement of phosphorylation of cofilin and reorganization of actin cytoskeleton. *J. Biol. Chem.* 277, 544-549

Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A. & Kaibuchi, K. (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for the small GTP binding protein Rho. *EMBO J.* 15, 2208-2216

McPhail, L.C., Waite, K.A., Regier, D.S., Nixon, J.B., Qualliotine-Mann, D., Zhang, W.X., Wallin, R. & Sergeant, S. (1999) A novel protein kinase target for the lipid second messenger phosphatidic acid. *Biochim. Biophys. Acta* 1439, 277-290

Meacci, E., Vasta, V., Moorman, J.P., Bobak, D.A., Bruni, P., Moss, J. & Vaughan, M. (1999) Effect of Rho and ADP-ribosylation factor GTPases on phospholipase D activity in intact human adenocarcinoma A549 cells. *J. Biol. Chem.* 274, 18605-18612

Meberg, P.J., Ono, S., Minamide, L.S., Takahashi, M. & Bamburg, J.R. (1998) Actin depolymerizing factor and cofilin phosphorylation dynamics: response to signals that regulate neurite extension. *Cell Motil. Cytoskeleton* 39, 172-190

Melendez, A., Floto, R.A., Gillooly, D.J., Harnett, M.M. & Allen, J.M. (1998) FcγRI coupling to phospholipase D initiates sphingosine kinase-mediated calcium mobilization and vesicular trafficking. *J. Biol. Chem.* 273, 9393-9402

Meng, Y., Zhang, Y., Tregoubov, V., Janus, C., Cruz, L., Jackson, M., Lu, W.Y., MacDonald, J.F., Wang, J.Y., Falls, D.L. & Jia, Z. (2002) Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron* 35, 121-133

Michaely, P.A., Mieno, C., Ying, Y.S. & Anderson, R.G.W. (1999) Polarized distribution of endogenous Rac1 and RhoA at the cell surface. *J. Biol. Chem.* 274, 21430-21436

Min, D.S., Kim, E.G. & Exton, J.H. (1998) Involvement of tyrosine phosphorylation and protein kinase C in the activation of phospholipase D by H_2O_2 in Swiss 3T3 fibroblasts. *J. Biol. Chem.* 273, 29986-29994

Minamide, L.S., Striegl, A.M., Boyle, J.A., Meberg, P.J. & Bamburg, J.R. (2000) Neurodegenerative stimuli induce persistent ADF/cofilin-actin rods that disrupt distal neurite function. *Nat. Cell Biol.* 2, 628-636

Miura, K., Jacques, K.M., Stauffer, S., Kubosaki, A., Zhu, K., Hirsch, D.S., Resau, J., Zheng, Y. & Randazzo, P.A. (2002) ARAP1: a point of convergence for Arf and Rho signaling. *Mol. Cell* 9, 109-119

Mitchell, R., McCulloch, D., Lutz, E., Johnson, M., MacKenzie, C., Fennell, M., Fink, G., Zhou, W. & Sealfon, S.C. (1998) Rhodopsin-family receptors associate with small G proteins to activate phospholipase D. *Nature* 392, 411-414

Mizuno, K., Okano, I., Ohashi, K., Nunone, K., Kuma, K., Miyata, T. & Nakamura, T. (1994)

Identification of a human cDNA encoding a novel protein kinase with two repeats of LIM/double zinc finger motif. *Oncogene* 9, 1605-1612

Morris, A.J., Engebrecht, J. & Frohman, M.A. (1996) Structure and regulation of phospholipase D. *Trends Pharmacol. Sci.* 17, 182-185

Morris, A.J., Frohman, M.A. & Engebrecht, J. (1997) Measurement of phospholipase D activity. *Anal. Biochem.* 252, 1-9

Morris, A.J. & Malbon, C.G. (1999) Physiological regulation of G protein-linked signaling. *Physiol. Rev.* 79, 1373-1430

Moss, J. & Vaughan, M. (1998) Molecules in the ARF orbit. *J. Biol. Chem.* 273, 21431-21434

Mukai, H., Toshimori, M., Shibata, H., Takanaga, H., Kitagawa, M., Miyahara, M., Shimakawa, M. & Ono, Y. (1997) Interaction of PKN with α-actinin. *J. Biol. Chem.* 272, 4740-4746

Mullins, R.D., Heuser, J.A. & Pollard, T.D. (1998) The interaction of the Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl. Acad. Sci. USA* 95, 6181-6186

Muthalif, M.M., Parmentier, J.H., Benter, I.F., Karzoun, N., Ahmed, A., Khandekar, Z., Adl, M.Z., Bourgoin, S. & Malik, K.U. (2000) Ras/mitogen-activated protein kinase mediates norepinephrine-induced phospholipase D activation in rabbit aortic smooth muscle cells by a phosphorylation-dependent mechanism. *J. Pharmacol. Exp. Ther.* 293, 268-274

Nagaoka, R., Kusano, K.-i., Abe, H. & Obinata, T. (1995) Effects of cofilin on actin filamentous structures in cultured muscle cells. Intracellular regulation of cofilin action. *J. Cell Sci.* 108, 581-593

Nakagawa, O., Fujisawa, K., Ishizaki, T., Saito, Y., Nakao, K. & Narumiya, S. (1996) ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett.* 392, 189-193

Nakashima, S. & Nozawa, Y. (1999) Possible role of phospholipase D in cellular differentiation and apoptosis. *Chem. Phys. Lipids* 98, 153-164

Neer, E.J. (1995) Heterotrimeric G Proteins: Organizers of transmembrane signals. *Cell* 80, 249-257

Neill, J.D., Duck, L.W., Sellers, J.C., Musgrove, L.C., Scheschonka, A., Druey, K.M. & Kehrl, J. (1997) Potential Role for a regulator of G protein signaling (RGS3) in gonadotropin-releasing hormone (GnRH) stimulated desensitization. *Endocrinology* 138, 843-846

Newton, A.C. (1997): Regulation of protein kinase C. *Curr. Opin. Cell Biol.* 9, 161-167

Nishida, E., Maekawa, S. & Sakai H. (1984) Cofilin, a protein in porcine brain that binds to actin filaments and inhibits their interactions with myosin and tropomyosin. *Biochemistry* 23, 5307-5313

Nishita, M., Aizawa, H. & Mizuno, K. (2002) Stromal cell-derived factor 1α activates LIM kinase 1 and induces cofilin phosphorylation for T-cell chemotaxis. *Mol. Cell. Biol.* 22, 774-783

Nishizuka, Y. (1995): Protein kinase C and lipid signaling for sustained cellular responses. *FASEB. J.* 9, 484-496

Niwa, R., Nagata-Ohashi, K., Takeichi, M., Mizuno, K. & Uemura, T. (2002) Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. *Cell* 108, 233-246

Offermanns, S., Wieland, T., Homann, D., Sandmann, J., Bombien, E., Spicher, K., Schultz, G. & Jakobs, K.H. (1994) Transfected muscarinic acetylcholine receptors selectively couple to G$_i$-type G proteins and G$_{q/11}$. *Mol. Pharmacol.* 45, 890-898

Ohashi, K., Nagata, K., Maekawa, M., Ishizaki, T., Narumiya, S. & Mizuno, K. (2000) Rho-associated kinase ROCK activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop. *J. Biol. Chem.* 275, 3577-3582

Ohguchi, K., Banno, Y., Nakashima, S., Kato, N., Watanabe, K., Lyerly, D.M. & Nozawa, Y. (1996a) Effects of *Clostridium difficile* toxin A and toxin B on phospholipase D activation in human promyelocytic leukemic HL60 cells. *Infect. Immun.* 64, 4433-4437

Ohguchi, K., Banno, Y., Nakashima, S. & Nozawa, Y. (1996b) Regulation of membrane-bound phospholipase D by protein kinase C in HL60 cells. Synergistic action of small GTP-binding protein RhoA. *J. Biol. Chem.* 271, 4366-4372

Ohguchi, K., Nakashima, S., Tan, Z., Banno, Y., Dohi, S. & Nozawa, Y. (1997) Increased activity of small GTP-binding protein-dependent phospholipase D during differentiation in human promyelocytic leukemic HL60 cells. *J. Biol. Chem.* 272, 1990-1996

Oishi, K., Takahashi, M., Mukai, H., Banno, Y., Nakashima, S., Kanaho, Y., Nozawa, Y. & Ono, Y. (2001) PKN regulates phospholipase D1 through direct interaction. *J. Biol. Chem.* 276, 18096-18101

Ojio, K., Banno, Y., Nakashima, S., Kato, N., Watanabe, K., Lyerly, D.M., Miyata, H. & Nozawa, Y. (1996) Effect of *Clostridium difficile* toxin B on IgE receptor-mediated signal transduction in rat basophilic leukemia cells: Inhibition of phospholipase D activation. *Biochem. Biophys. Res. Commun.* 224, 591-596

Okamoto, T., Schlegel, A., Scherer, P.E. & Lisanti, M.P. (1998) Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J. Biol. Chem.* 273, 5419-5422

Ono, S. & Ono, K. (2002) Tropomyosin inhibits ADF/cofilin dependent actin filament dynamics. *J. Cell Biol.* 156, 1065-1076

Oshiro, N., Fukata, Y. & Kaibuchi, K. (1998) Phosphorylation of moesin by Rho-associated kinase (Rho-kinase) plays a crucial role in the formation of microvilli-like structures. *J. Biol. Chem.* 273, 34663-34666

Ott, D.E., Coren, L.V., Kane, B.P., Busch, L.K., Johnson, D.G., Sowder II, R.C., Chertova, E.N., Arthur, L.O. & Henderson, L.E. (1996) Cytoskeletal proteins inside human immunodeficiency virus type 1 virions. *J. Virol.* 70, 7734-7743

Oude Weernink, P.A., Guo, Y.J., Zhang, C., Schmidt, M., von Eichel-Streiber, C. & Jakobs, K.H. (2000a) Control of cellular phosphatidylinositol 4,5-bisphosphate levels by adhesion signals and Rho GTPases in NIH 3T3 fibroblasts. Involvement of both phosphatidylinositol-4-phosphate 5-kinase and phospholipase C. *Eur. J. Biochem.* 267, 5237-5246

Oude Weernink, P.A., Schulte, P., Guo, Y.J., Wetzel, M., Amano, M., Kaibuchi, K., Haverland, S., Voß, M., Schmidt, M., Mayr, G.W. & Jakobs, K.H. (2000b) Stimulation of phosphatidylinositol-4-phosphate 5-kinase by Rho-kinase. *J. Biol. Chem.* 275, 10168-10174

Park, S.K., Min, D.S. & Exton, J.H. (1998) Definition of the protein kinase C interaction site of phospholipase D. *Biochem. Biophys. Res. Commun.* 244, 364-367

Park, J.B., Kim, J.H., Kim, Y., Ha, S.H., Kim, J.H., Yoo, J.S., Du, G., Frohman, M.A., Suh, P.G. & Ryu, S.H. (2000) Cardiac phospholipase D2 localizes to sarcolemmal membranes and is inhibited by α-actinin in an ADP-ribosylation factor-reversible manner. *J. Biol. Chem.* 275, 21295-21301

Parmentier, J.H., Muthalif, M.M., Saeed, A.E. & Malik, K.U. (2001) Phospholipase D activation by norepinephrine is mediated by 12(*S*)-, 15(*S*)-, and 20-hydroxyeicosatetraenoic acids generated by stimulation of cytosolic phospholipase A_2. Tyrosine phosphorylation of phospholipase D_2 in response to norepinephrine. *J. Biol. Chem.* 276, 15704-15711

Peralta, E.G., Ashkenazi, A., Winslow, J.W., Ramachandran, J. & Capon, D.J. (1988) Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. *Nature* 334, 434-437

Pertile, P., Liscovitch, M., Chalifa, V. & Cantley, L.C. (1995) Phosphatidylinositol 4,5-bisphosphate synthesis is required for activation of phospholipase D in U937 cells. *J. Biol. Chem.* 270, 5130-

5135

Pettitt, T.R., Martin, A., Horton, T., Liossis, C., Lord, J.M. & Wakelam, M.J.O. (1997) Diacylglycerol and phosphatidate generated by phospholipase C and D, respectively, have distinct fatty acid compositions and functions. Phospholipase D-derived diacylglycerol does not activate protein kinase C in porcine aortic endothelial cells. *J. Biol. Chem.* 272, 17354-17359

Pierce, K.L., Premont, R.T. & Lefkowitz, R.J. (2002) Seven-transmembrane receptors. *Nat. Rev. Mol. Cell Biol.* 639-650

Plonk, S.G., Park, S.K. & Exton, J.H. (1998) The α-subunit of the heterotrimeric G protein G_{13} activates a phospholipase D isozyme by a pathway requiring Rho family GTPases. *J. Biol. Chem.* 273, 4823-4826

Ponting, C.P. & Kerr, I.D. (1996) A novel family of phospholipase D homologues that includes phospholipid synthases and putative endonucleases: identification of duplicated repeats and potential active site residues. *Protein Sci.* 5, 914-922

Porcelli, A.M., Ghelli, A., Hrelia, S. & Rugolo, M. (2002) Phospholipase D stimulation is required for sphingosine-1-phophate activation of actin stress fibre assembly in human airway epithelial cells. *Cell. Signal.* 14, 75-81

Pröschel, C., Blouin, M.J., Gutowski, N.J., Ludwig, R. & Noble, M. (1995) Limk1 is predominantly expressed in neural tissues and phosphorylates serine, threonine and tyrosine residues *in vitro*. *Oncogene* 11, 1271-1281

Rameh, L.E. & Cantley, L.C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem.* 274, 8347-8350

Redina, O.E. & Frohman, M.A. (1998) Organization and alternative splicing of the murine phospholipase D2 gene. *Biochem. J.* 331, 845-851

Regier, D.S., Greene, D.G., Sergeant, S., Jesaitis, A.J. & McPhail, L.C. (2000) Phosphorylation of $p22^{phox}$ is mediated by phospholipase D-dependent and –independent mechanisms. Correlation of NADPH oxidase activity and $p22^{phox}$ phosphorylation. *J. Biol. Chem.* 275, 28406-28412

Ren, X.D. & Schwartz, M.A. (2000) Determination of GTP loading on Rho. *Methods Enzymol.* 325, 264-272

Reuther, G.W. & Der, C.J. (2000) The Ras branch of small GTPases: Ras family members don't fall far from the tree. *Curr. Opin. Cell Biol.* 12, 157-165

Rhee, S.G. (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* 70, 281-312

Rizzo, M.A., Shome, K., Vasudevan, C., Stolz, D.B., Sung, T.C., Frohman, M.A., Watkins, S.C. & Romero, G. (1999) Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent Raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. *J. Biol. Chem.* 274, 1131-1139

Rizzo, M.A., Shome, K., Watkins, S.C. & Romero, G. (2000) The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. *J. Biol. Chem.* 275, 23911-23918

Rosenblatt, J. & Mitchison, T.J. (1998) Actin, cofilin and cognition. *Nature* 393, 739-740

Ross, E.M. & Wilkie, T.M. (2000) GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu. Rev. Biochem.* 69, 795-827

Rudge, S.A., Morris, A.J. & Engebrecht, J. (1998) Relocalization of phospholipase D activity mediates membrane formation during meiosis. *J. Cell Biol.* 140, 81-90

Rümenapp, U., Geiszt, M., Wahn, F., Schmidt, M. & Jakobs, K.H. (1995) Evidence for ADP-ribosylation-factor-mediated activation of phospholipase D by m3 muscarinic acetylcholine receptor. *Eur. J. Biochem.* 234, 240-244

Rümenapp, U., Schmidt, M., Wahn, F., Tapp, E., Grannass, A. & Jakobs, K.H. (1997) Characteristics of protein-kinase-C- and ADP-ribosylation-factor-stimulated phospho-lipase D activities in human embryonic kidney cells. *Eur. J. Biochem.* 248, 407-414.

Rümenapp, U., Schmidt, M., Olesch, S., Ott, S., von Eichel-Streiber, C. & Jakobs, K.H. (1998) Tyrosine-phosphorylation-dependent and Rho-protein-mediated control of cellular phosphatidylinositol 4,5-bisphosphate levels. *Biochem. J.* 334, 625-631

Rümenapp, U., Asmus, M., Schablowski, H., Woznicki, M., Han, L., Jakobs, K.H., Fahmi-Vahid, M., Michalek, C., Wieland, T. & Schmidt, M. (2001) The M_3 muscarinic acetylcholine receptor expressed in HEK-293 cells signals to phospholipase D via G_{12} but not G_q-type G proteins. Regulators of G proteins as tools to dissect pertussis toxin-resistant G protein in receptor-effector coupling. *J. Biol. Chem.* 276, 2474-2479

Sakisaka, T., Itoh, T., Miura, K., Takenawa, T. (1997) Phosphatidylinositol-4,5-bisphosphate phosphatase regulates the rearrangement of actin filaments. *Mol. Cell. Biol.* 17, 3841-3849

Sambook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Sato, T.K., Overduin, M. & Emr, S.D. (2001) Location, Location, Location: membrane targeting directed by PX domains. *Science* 294, 1881-1885

Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases. *Cell* 103, 211-225

Schmidt, A. & Hall, M.N. (1998) Signaling to the actin cytoskeleton. *Annu. Rev. Cell. Dev. Biol.* 14, 305-338

Schmidt, M., Hüwe, S.M., Fasselt, B., Homann, D., Rümenapp, U., Sandmann, J. & Jakobs, K.H. (1994) Mechanisms of phospholipase D stimulation by m3 muscarinic acetylcholine receptors. Evidence for involvement of tyrosine phosphorylation. *Eur. J. Biochem.* 225, 667-675

Schmidt, M., Bienek, C., Rümenapp, U., Zhang, C., Lümmen, G., Jakobs, K.H., Just, I., Aktories, K., Moos, M. & von Eichel-Streiber, C. (1996a) A role for Rho in receptor- and G protein-stimulated phospholipase C. Reduction in phosphatidylinositol 4,5-bisphosphate by *Clostridium difficile* toxin B. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 354, 87-94

Schmidt, M., Rümenapp, U., Bienek, C., Keller, J., von Eichel-Streiber, C. & Jakobs, K.H. (1996b) Inhibition of receptor signaling to phospholipase D by *Clostridium difficile* toxin B. Role of Rho proteins. *J. Biol. Chem.* 271, 2422-2426

Schmidt, M., Rümenapp, U., Nehls, C., Ott, S., Keller, J., von Eichel-Streiber, C. & Jakobs, K.H. (1996c) Restoration of C*lostridium difficile* toxin-B-inhibited phospholipase D by phosphatidylinositol 4,5-bisphosphate. *Eur. J. Biochem.* 240, 707-712

Schmidt, M., Voß, M., Thiel, M., Bauer, B., Grannaß, A., Tapp, E., Cool, R.H., de Gunzburg, J., von Eichel-Streiber, C. & Jakobs KH. (1998) Specific inhibition of phorbol ester-stimulated phospholipase D by *Clostridium sordellii* lethal toxin and *Clostridium difficile* toxin B-1470 in HEK-293 cells. Restoration by Ral GTPases. *J. Biol. Chem.* 273, 7413-7422

Schmidt, M., Voß, M., Oude Weernink, P.A., Wetzel, J., Amano, M., Kaibuchi, K. & Jakobs, K.H. (1999) A role for Rho-kinase in Rho-controlled phospholipase D stimulation by the m3 muscarinic acetylcholine receptor. *J. Biol. Chem.* 274, 14648-14654

Schmidt, M., Frings, M., Mono, M.-L., Guo, Y., Oude Weernink, P.A., Evellin, S., Han, L. & Jakobs, K.H. (2000) G protein-coupled receptor-induced sensitization of phospholipase C stimulation by receptor tyrosine kinases. *J. Biol. Chem.* 275, 32603-32610

Schmitz, A.A.P., Govek, E.E., Böttner, B. & Van Aelst, L. (2000) Rho GTPases: signaling, migration, and invasion. *Exp. Cell Res.* 261, 1-12

Schürmann, A., Schmidt, M., Asmus, M., Bayer, S., Fliegert, F., Koling, S., Massmann, S., Schilf, C., Subauste, M.C., Voß, M., Jakobs, K.H. & Joost, H.G. (1999) The ADP-ribosylation factor (ARF)-related GTPase ARF related protein binds to the ARF-specific guanine nucleotide

exchange factor cytohesin and inhibits the ARF-dependent activation of phospholipase D. *J. Biol. Chem.* 274, 9744-9751

Sciorra, V.A. & Morris, A.J. (1999) Sequential actions of phospholipase D and phosphatidic acid phosphohydrolase 2b generate diglyceride in mammalian cells. *Mol. Biol. Cell* 10, 3863-3876

Sciorra, V.A., Rudge, S.A., Prestwich, G.D., Frohman, M.A., Engebrecht, J. & Morris, A.J. (1999) Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes. *EMBO J.* 20, 5911-5921

Shen, Y., Xu, L. & Foster, D.A. (2001) Role for phospholipase D in receptor-mediated endocytosis. *Mol. Cell. Biol.* 21, 595-602

Shome, K, Vasudevan, C. & Romero, G. (1997) ARF proteins mediate insulin-dependent activation of phospholipase D. *Curr. Biol.* 7, 387-396

Shome, K., Nie, Y. & Romero, G. (1998) ADP-ribosylation factor proteins mediate agonist-induced activation of phospholipase D. *J. Biol. Chem.* 273, 30836-30841

Simonsen, A., Wurmser, A.E., Emr, S.D. & Stenmark, H. (2001) The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol.* 13, 485-492

Skippen, A., Jones, D.H., Morgan, C.P., Li, M. & Cockcroft, S. (2002) Mechanism of ADP ribosylation factor-stimulated phosphatidylinositol 4,5-bisphosphate synthesis in HL60 cells. *J. Biol. Chem.* 277, 5823-5831

Slaaby, R., Jensen, T., Hansen, H.S., Frohman, M.A. & Seedorf, K. (1998) PLD2 complexes with the EGF receptor and undergoes tyrosine phosphorylation at a single site upon agonist stimulation. *J. Biol. Chem.* 273, 33722-33727

Slaaby, R., Du, G., Altshuller, Y.M., Frohman, M.A. & Seedorf, K. (2000) Insulin-induced phospholipase D1 and phospholipase D2 activity in human embryonic kidney-293 cells mediated by the phospholipase Cγ and protein kinase Cα signaling cascade. *Biochem. J.* 351, 613-619

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C. (1985) Measurement of protein using bicinchonic acid. *Anal. Biochem.* 150, 76-85

Sotiropoulos, A., Gineitis, D., Copeland, J. & Treisman, R. (1999) Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell* 98, 159-169

Steed, P.M. & Chow, A.H.M. (2001) Intracellular signaling by phospholipase D as a therapeutic target. *Curr. Pharm. Biotechnol.* 2, 241-256

Stuckey, J.A. & Dixon, J.E. (1999) Crystal structure of a phospholipase D family member. *Nat. Struct. Biol.* 6, 278-284

Sumi, T., Matsumoto, K. & Nakamura, T. (2001a) Specific activation of LIM-kinase 2 via phosphorylation of threonine 505 by ROCK, a Rho-dependent protein kinase. *J. Biol. Chem.* 276, 670-676

Sumi, T., Matsumoto, K., Shibuya, A. & Nakamura, T. (2001b) Activation of LIM-kinase by myotonic dystrophy kinase-related Cdc42-binding kinase α. *J. Biol. Chem.* 276, 23092-23096

Sung, T.C., Roper, R.L., Zhang, Y., Rudge, S.A., Temel, R., Hammond, S.M., Morris, A.J., Moss, B., Engebrecht, J. & Frohman, M.A. (1997) Mutagenesis of phospholipase D defines a superfamily including a trans-Golgi viral protein required for poxvirus pathogenicity. *EMBO J.* 16, 4519-4530

Sung, T.C., Altshuller, Y.M., Morris, A.J. & Frohman, M.A. (1999a) Molecular analysis of mammalian phospholipase D2. *J. Biol. Chem.* 274, 494-502

Sung, T.C., Zhang, Y., Morris, A.J. & Frohman, M.A. (1999b) Structure analysis of human phospholipase D1. *J. Biol. Chem.* 274, 3659-3666

Takai, Y., Sasaki, T. & Matozyki, T. (2001) Small GTP-binding proteins. *Physiol. Rev.* 81, 153-208

Tassabehji, M., Metcalfe, K., Fergusson, W.D., Carette, M.J., Dore, J.K., Donnai, D., Read, A.P.,

Pröschel, C., Gutowski, N.J., Mao, X. & Sheer, D. (1996) LIM-kinase deleted in Williams syndrome. *Nat. Genet.* 13, 272-273

Teal, S.B., Hsu, V.W., Peters, P.J., Kluasner, R.D. & Donaldson, J.G. (1994) An activating mutation in ARF1 stabilizes coatamer binding to Golgi membranes. *J. Biol. Chem.* 269, 3135-3138

Teruel, M.N. & Meyer, T. (2000) Translocation and reversible localization of signaling proteins: a dynamic future for signal transduction. *Cell* 103, 181-184

Toker, A. (1998) The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. *Curr. Opin. Cell Biol.* 10, 254-261

Toker, A. (2002) Phosphoinositides and signal transduction. *Cell. Mol. Life Sci.* 59, 761-779

Toshima, J., Toshima, J.Y., Amano, T., Yang, N., Narumiya, S. & Mizuno, K. (2001) Cofilin phosphorylation by protein kinase testicular protein kinase 1 and its role in integrin-mediated actin reorganization and focal adhesion formation. *Mol. Biol. Cell* 12, 1131-1145

Towbin, H., Stadelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350-4354

Tsai, S.C., Adamik, R., Hong, J.X., Moss, J., Vaughan, M., Kanoh, H. & Exton, J.H. (1998) Effects of arfaptin 1 on guanine nucleotide-dependent activation of phospholipase D and cholera toxin by ADP-ribosylation factor. *J. Biol. Chem.* 273, 20697-20701

Ushio-Fukai, M., Alexander, R.W., Akers, M., Lyons, P.R., Lassegue, B. & Griendling, K.K. (1999) Angiotensin II receptor coupling to phospholipase D is mediated by the βγ subunits of heterotrimeric G proteins in vascular smooth muscle cells. *Mol. Pharmacol.* 55, 142-149

van Dijk, M.C.M., Postma, F., Hilkmann, H., Jalink, K., van Blitterswijk, W.J. & Moolenaar, W. H. (1998) Exogenous phospholipase D generates lysophosphatidic acid and activates Ras, Rho and Ca^{2+} signaling pathways. *Curr. Biol.* 8, 386-392

Vanhaesebroeck, B., Leevers, S.J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P.C., Woscholski, R., Parker, P.J. & Waterfield, M.D. (2001) Systhesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* 70, 535-602

Venable, M.E. & Obeid, L.M. (1999) Phospholipase D in cellular senescence. *Biochim. Biophys. Acta.* 1439, 291-298

Vitale, N., Caumont, A.S., Chasserot-Golaz, S., Du, G., Wu, S., Sciorra, V.A., Morris, A.J., Frohman, M.A. & Bader, M.F. (2001) Phospholipase D1: a key factor for the exocytotic machinery in neuroendocrine cells. *EMBO J.* 20, 2424-2434

Vojtek, A.B. & Der, C.J. (1998) Increasing complexity of the Ras signaling pathway. *J. Biol. Chem.* 273, 19925-19928

Voß, M., Oude Weernink, P.A., Haupenthal, S., Möller, U., Cool, R.H., Bauer, B., Camonis, J.H., Jakobs, K.H. & Schmidt, M. (1999) Phospholipase D stimulation by receptor tyrosine kinases mediated by protein kinase C and a Ras/Ral signaling cascade. *J. Biol. Chem.* 274, 34691-34698

Wang, L.X., Cummings, R., Usatyuk, P., Morris, A., Irani, K. & Natarajan, V. (2002) Involvement of phospholipase D1 and D2 in sphingosine 1-phosphate-induced ERK activation and interleukin-8 secretion in human bronchial epithelial cells. *Biochem. J.* in press.

Wang, M.S., Schinzel, A., Kotzot, D., Balmer, D., Casey, R., Chodirker, B.N., Gyftodimou, J., Petersen, M.B., Lopez-Rangel, E. & Robinson, W.P. (1999) Molecular and clinical correlation study of Williams-Beuren syndrome: no evidence of molecular factors in the deletion region or imprinting affecting clinical outcome. *Am. J. Med. Genet.* 86, 34-43

Watling, K.J. (2001) *The Sigma-RBI handbook of receptor classification and signal transduction.* Sigma-Aldrich Research Biochemicals Incorporated, USA.

West, S.C. (1994) The processing of recombination intermediates: mechanistic insights from studies of

bacterial proteins. *Cell* 76, 9-15

Wieland, T. & Chen, C.K. (1999) Regulators of G-protein signalling: a novel protein family involved in timely deactivation and desensitization of signalling via heterotrimeric G proteins. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 360, 14-26

Wilde, C., Barth, H., Sehr, P., Han, L., Schmidt, M., Just, I. & Aktories, K. (2002) Interaction of the Rho-ADP-ribosylating C3 exoenzyme with RalA. *J. Biol. Chem.* 277, 1477-14776

Williger, B.T., Ho, W.T. & Exton, J.H. (1999a) Phospholipase D mediates matrix metalloproteinase-9 secretion in phorbol ester-stimulated human fibrosarcoma cells. *J. Biol. Chem.* 274, 735-738

Williger, B.T., Ostermann, J. & Exton, J.H. (1999b) Arfaptin 1, an ARF-binding protein, inhibits phospholipase D and endoplasmic reticulum/Golgi protein transport. *FEBS Lett.* 443, 197-200

Wu, Y.Q., Sutton, V.R., Nickerson, E., Lupski, J.R., Potocki, L., Korenberg, J.R., Greenberg, F., Tassabehji, M. & Shaffer, L.G. (1998) Delineation of the common critical region in Williams syndrome and clinical correlation of growth, heart defects, ethnicity, and parental origin. *Am. J. Med. Genet.* 78, 82-89

Xie, Z., Ho, W.T. & Exton, J.H. (2000) Association of N- and C-terminal domains of phospholipase D. Contribution of the conserved HKD motifs to the interaction and the requirement of the association for Ser/Thr phosphorylation of the enzyme. *J. Biol. Chem.* 275, 24962-24969

Xie, Z., Ho, W.T., Spellman, R., Cai, S. & Exton, J.H. (2002) Mechanisms of regulation of phospholipase D1 and D2 by the heterotrimeric G proteins G_{13} and G_q. *J. Biol. Chem.* 277, 11979-11986

Xu, Y., Seet, L.F., Hanson, B. & Hong, W. (2001) The phox homology (PX) domain, a new player in phosphoinositide signaling. *Biochem. J.* 360, 513-530

Yamaguchi, Y., Katoh, H., Mori, K. & Negishi, M. (2002) Ga_{12} and Ga_{13} interact with Ser/Thr protein phosphatase type 5 and stimulate its phosphatase activity. *Curr. Biol.* 12, 1353-1358

Yamazaki, M., Miyazaki, H., Watanabe, H., Sasaki, T., Maehama, T., Frohman, M.A. & Kanaho, Y. (2002) Phosphatidylinositol 4-phosphate 5-kinase is essential for ROCK-mediated neurite remodeling. *J. Biol. Chem.* 277, 17226-17230

Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E. & Mizuno, K. (1998) Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* 393, 809-812

Yonezawa, N., Nishida, E. & Sakai, H. (1985) pH control of actin polymerization by cofilin. *J. Biol. Chem.* 260, 14410-14412

Young, L.S. & Mautner, V. (2001) The promise and potential hazards of adenovirus gene therapy. *Gut* 48, 733-736

Zajchowski, L.D. & Robbins, S.M. (2002) Lipid rafts and little caves. Compartmentalized signalling in membrane microdomains. *Eur. J. Biochem.* 269, 737-752

Zhang, C., Schmidt, M., von Eichel-Streiber, C. & Jakobs, K.H. (1996) Inhibition by toxin B of inositol phosphate formation induced by G protein-coupled and tyrosine kinase receptors in N1E-115 neuroblastoma cells: involvement of Rho proteins. *Mol. Pharmacol.* 50, 864-869

Zhang, Y., Altshuller, Y.M., Hammond, S.M., Morris, A.J. & Frohman, M.A. (1999) Loss of receptor regulation by a phospholipase D1 mutant unresponsive to protein kinase C. *EMBO J.* 18, 6339-6348

Zheng, Y. (2001) Dbl family guanine nucleotide exchange factors. *Trends Biochem. Sci.* 26, 724-732

8. Appendix

Original Publications:

Wilde, C., Barth, H., Sehr, P., **Han, L.**, Schmidt, M., Just, I. & Aktories, K. (2002) Interaction of the Rho-ADP-ribosylating C3 exoenzyme with RalA. *J. Biol. Chem*. 277, 14771-14776

Fahimi-Vahid, M., Gosau, N., Michalek, C., **Han, L.**, Jakobs, K. H., Schmidt, M., Roberts, N., Avkiran, M. & Wieland, T. (2002) Distinct signaling pathways mediate cardiomyocyte phospholipase D stimulation by endothelin-1 and thrombin. *J. Mol. Cell Cardiol.* 34, 441-453

Rümenapp, U., Asmus, M., Schablowski, H., Woznicki, M., **Han, L.**, Jakobs, K. H., Fahimi-Vahid, M., Michalek, C., Wieland, T. & Schmidt, M. (2001) The M_3 muscarinic acetylcholine receptor expressed in HEK-293 cells signals to phospholipase D via G_{12} but not G_q-type G proteins. Regulators of G proteins as tools to dissect pertussis toxin-resistant G proteins in receptor-effector coupling. *J. Biol. Chem.* 276, 2474-2479

Schmidt, M., Frings, M., Mono, M. L., Guo, Y., Oude Weernink, P.A., Evellin, S., **Han, L.** & Jakobs, K. H. (2000) G protein-coupled receptor-induced sensitization of phospholipase C stimulation by receptor tyrosine kinases. *J. Biol. Chem.* 275, 32603-32610

Abstracts and Posters in Conferences/Workshops:

Wieland, T., Hoeppner, G., **Han, L.**, Rümenapp, U., Schmidt, M. & Jakobs, K. H. (2002) Gβγ-mediated activation of Rho by RGS3. *Circulation*, in press.

Han, L., Kindhäuser, F., Caracciola, P., Oude Weernink, P.A., Jakobs, K. H., Schmidt, M., Heneweer, C., Kruse, L. H. , Thie, M. & Mizuno, K. (2002) Involvement of cofilin in the Rho-kinase/Lim-kinase-dependent stimulation of phospholipase D (PLD) by M_3 muscarinic acetylcholine receptor. *Naunyn-Schmiedeberg's Arch Pharmacol* 365, R50

Han, L., Oude Weernink, P.A., Jakobs, K. H. & Schmidt, M. (2002) Stimulation of phospholipase D by the M_3 muscarinic acetylcholine receptor mediated by LIM-kinase

and cofilin. Goethe University/Aventis Workshop in Chemical Biology. Wiesbaden, Germany

Wieland, T., Höppner, G., **Han, L.**, Rümenapp, U., Schmidt, M. & Jakobs, K. H. (2002) Gβγ-mediated activation of Rho by RGS3. *Naunyn-Schmiedeberg's Arch Pharmacol* 365, R45

Han, L., Woznicki, M., Limper, B., Eicken, S., Caracciola, P., Oude Weernink P.A., Jakobs, K. H., Mizuno, K. & Schmidt, M. (2001) Involvement of Lim-kinase in stimulation of phospholipase D by Rho and Rho-kinase. *Naunyn-Schmiedeberg's Arch Pharmacol* 363, R61

Schmidt, M., Evellin, S., Frings, M., **Han, L.**, Mono, M. L., Pieper, N., Oude Weernink, P. A. & Jakobs, K. H. (2001) Long term potentiation of phospholipase C and Ca^{2+} signaling induced by muscarinic and bradykinin receptors in neuronal cells. *Naunyn-Schmiedeberg's Arch Pharmacol* 363, R53

Acknowledgements

I am most grateful to Prof. Dr. Karl H. Jakobs for the opportunity to perform my dissertation work successfully in the Institute of Pharmacology, University Hospital Essen, for his scientific supervision as well as his human-touched understanding to several subtle things.

Especially, I appreciate the great help from Priv.-Doz. Dr. Martina Schmidt. Under her direct supervision, I solved many theoretical and experimental problems during my dissertation work. I also thank Prof. Dr. Thomas Wieland for his support in generating the PLD adenoviruses. I would also like to mention the support from Dr. Michael Thie, Carola Heneweer and Lars Kurse at the confocal laser microscope. Finally, I thank Drs. Paschal A. Oude Weernink and Frank vom Dorp for their patience to correct my thesis.

Furthermore, I express my thanks to Kirsten Baden, Helgard Geldermann, Monika Hagedorn and Doris Petermeyer for their excellent technical assistance, to our secretaries, Gertrud Sieberg and Annette Görtz, for their help, and to all other colleagues and doctoral students of the Institute of Pharmacology for their friendly help and, most important, happy communication.

Finally, I am thankful in particular to my family, my wife and my parents. Their love, understanding and support encourage me always to the fulfillment of my dissertation and my goals in the future.

Curriculum Vitae

Persönliche Daten:

Name:	Li Han
Geburtsdatum:	29. Oktober 1973
Geburtsort:	Binzhou, China
Wohnort:	Hohlweg 24, 45147 Essen
Familienstand:	verheiratet
Nationalität:	Chinesisch

Schulausbildung:

1984-1987	Dritte Grundschule (Binzhou, ShanDong)
1987-1990	Gymnasium (Binzhou, ShanDong)
Mai 1990	Abitur (sehr gut)

Universitätsausbildung:

1990-1994	Studium der molekularen Mikrobiologie, Universität ShanDong Abschluß: *Bachelor of Science*, mit Auszeichnung Bachelorarbeit: Isolierung, Aufreinigung, Charakterisierung von Glukosedehydrogenase und Regeneration von Coenzymen. Prof. Gaoxiang Li, Chinesisches Nationales Institut für Mikrobiologie, Beijing.
1994-1997	*Master of Medicine*. Masterarbeit: Genome macrorestriction analysis of common nosocomial infection strains. Experimental conditions for producing the protoplast of *Rhizomucor pusillus*. Prof. Shiping Chen, Medical Postgraduate College of Chinese People's Liberation Army, Beijing.
seit 2000	Erstellung der Doktorarbeit bei Prof. Dr. K. H. Jakobs am Institut für Pharmakologie, Universitätsklinikum Essen.

Berufliche Tätigkeiten:

1997–1999	Wissenschaftlicher Assistent, Institut für Krankenhaus-Hygiene, Zentrum für Chinesische Nationale Pathologie und Toxikologie Fungi Medical Postgraduate College of Chinese People's Liberation Army, Beijing.

Essen, den Li Han

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., daß ich das Arbeitsgebiet, dem das Thema „G Protein-Coupled Receptor Signaling to Phospholipase D1 mediated by G_{12}-Type G Proteins, LIM-kinase and Cofilin“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Herrn Li Han befürworte.

Essen, den 13.11.2002

Prof. Dr. Karl H. Jakobs

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., daß ich die vorliegende Dissertation selbständig verfaßt und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den 13.11.2002

Li Han

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., daß ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, und daß diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, den 13.11.2002

Li Han