Regulation of cytosolic Phospholipase A_2 activity plays a central role in cell responses

De regulatie van de cytosolische fosfolipase A_2 activiteit speelt een centrale rol in de cel respons

(met een samenvatting in het Nederlands)

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Am I right? Am I wrong?

Back off, what have I done

Once in a lifetime ...

David Byrne

Talking Heads *(Once in a lifetime)*

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Abbreviations

ATK	arachidonyl trifluoromethylketone
BEL	bromoenol lactone
4-BPB	4-bromophenacyl bromide
CaLB	calcium-dependent phospholipid binding
Cdk	cyclin-dependent kinase
CHO	chinese hamster ovary
COX	cyclooxygenase
DAG	dipalmitoylglycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	1,4-dithiothreitol
E2F	E2 promoter binding factor
EGF	epidermal growth factor
EGTA	ethyleneglycol-*bis*(β-aminoethyl)-N,N,N',N'-tetraacetic acid
ERK	extracellular regulated kinase
FBS	foetal bovine serum
FGF	fibroblast growth factor
Gö6976	12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo [2,3-a]pyrrolo[3,4-c]carbazole
Gö6983	2-[1-(3-diamethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide
Grb2	growth factor receptor binding protein 2
H_2O_2	hydrogen peroxide
INK4	inhibitors of cdk4
IP3	inositol 1,4,5-trisphosphate
JNK/SAPK	c-jun NH_2-terminal kinase/ stress-activated protein kinase
kDa	kilo dalton
LO	lipoxygenase
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase or ERK kinase
Mnk1	MAP kinase interaction protein kinase 1
N2A	neuroblastoma 2A
NDGA	nordihydroguaiaretic acid
NS-398	N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide
PBS	phosphate buffered saline
PD098059	2-(2'-amino-3'methoxyphenyl)-oxanaphtalen-4-one

PdBu	phorbol-12,13-dibutyrate
PDGF	platelet-derived growth factor
PGHS	prostaglandin H synthase
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
$cPLA_2$	cytosolic phospholipase A_2
$iPLA_2$	calcium-independent phospholipase A_2
$sPLA_2$	secretory phospholipase A_2
PLCγ	phosphoinositide-specific phospholipase Cγ
PMSF	phenylmethylsulfonyl fluoride
Rb	retinoblastoma
Ro31-8220	3-[1-[3(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide
ROS	reactive oxygen species
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gelelecrophoresis
Sos	son of sevenless
U0126	[1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene]

Chapter 1

General introduction

Based on: Gertrude Bunt, Gerda S.A.T. van Rossum, Henk van den Bosch, Arie J. Verkleij and Johannes Boonstra (1999) Regulation of cytosolic phospholipase A_2 in Molecular Mechanisms of Transcellular Signaling, NATO Science Series, Life Sciences, (Thiery, J.P., Ed.), IOS Press, Amsterdam, **309**, 109-125

Gertrude Bunt, Gerda S.A.T. van Rossum, Johannes Boonstra, Henk van den Bosch and Arie J. Verkleij (2000) Regulation of $cPLA_2$ in a new perspective: recruitment of active monomers from an inactive clustered pool. Biochemistry **39**, 7847-7850

Introduction

Phospholipases A_2 are enzymes that hydrolyse fatty acids from the *sn*-2 position of phospholipids, resulting in the release of free fatty acids and lysophospholipids (van den Bosch, 1980; Dennis, 1997). The phospholipase A_2 (PLA_2) superfamily consists of a broad range of enzymes amongst which are the secretory PLA_2s ($sPLA_2$; groups I, II, III, V, IX and X), the Ca^{2+}-independent PLA_2 ($iPLA_2$; group VI) and the cytosolic PLA_2 ($cPLA_2$; group IV) (reviewed in Six & Dennis, 2000). The *sn*-2 position of phospholipids in mammalian cells is enriched with arachidonic acid, which is a substrate for cyclooxygenases, lipoxygenases and cytochrome p450s, giving PLA_2s an important role in the control of the synthesis of prostaglandins, leukotrienes and other eicosanoids. In addition, PLA_2s determine most of the arachidonic acid released in the cell. Arachidonic acid and its metabolites, the eicosanoids, have been implicated in a number of physiological and pathophysiologial processes, including the control of voltage-dependent and Ca^{2+} channels (Keyser & Alger, 1990; Peppelenbosch *et al.,* 1992), modulation and release of neurotransmitters (Lynch *et al.,* 1989), blood vessel tone (Capdevila *et al.,* 2000), inflammation (Heller *et al.,* 1998), mitogenic signalling (Korystov Yu *et al.,* 1998) and cancers (Shappell *et al.,* 2001). Because arachidonic acid is preferentially released by $cPLA_2$, the understanding of its regulation is of great importance.

General aspects of $cPLA_2$

Structure of $cPLA_2$.

The cDNA for human $cPLA_2$ was first cloned by Clark *et al*. and Sharp *et al.* in 1991. The sequence encodes a 85 kDa protein consisting of 749 amino acids which migrates as a 100-110 kDa protein on SDS-PAGE. Within this sequence several domains and motifs have been distinguished (fig. 1). At the N-terminus, a calcium-dependent lipid binding (CaLB) domain is present (Clark, *et al.,* 1991; Nalefski *et al.,* 1994) which serves to bring $cPLA_2$ to the phospholipid substrate. This domain shares homology with the C2 domains first identified in the conventional isoforms of protein kinase C (PKC) (Coussens *et al.,* 1986) and is found in a range of proteins including PLCγ and synaptotagmin (Clark *et al.,* 1995). The CaLB domain, spanning from amino acid 18-141 preferentially binds, unlike other characterised C2 domains, to vesicles comprised of phosphatidylcholine in response to physiological (0.3-1 μM) concentrations of calcium (Nalefski *et al.,* 1998). In contrast to the full length $cPLA_2$, which displays preferential hydrolysis of arachidonoyl-containing phospholipid vesicles, the $cPLA_2$ C2 domain did not show a preference for phospholipid vesicles composed of saturated, unsaturated *sn*-2 fatty acyl chains or the carbonyl oxygens

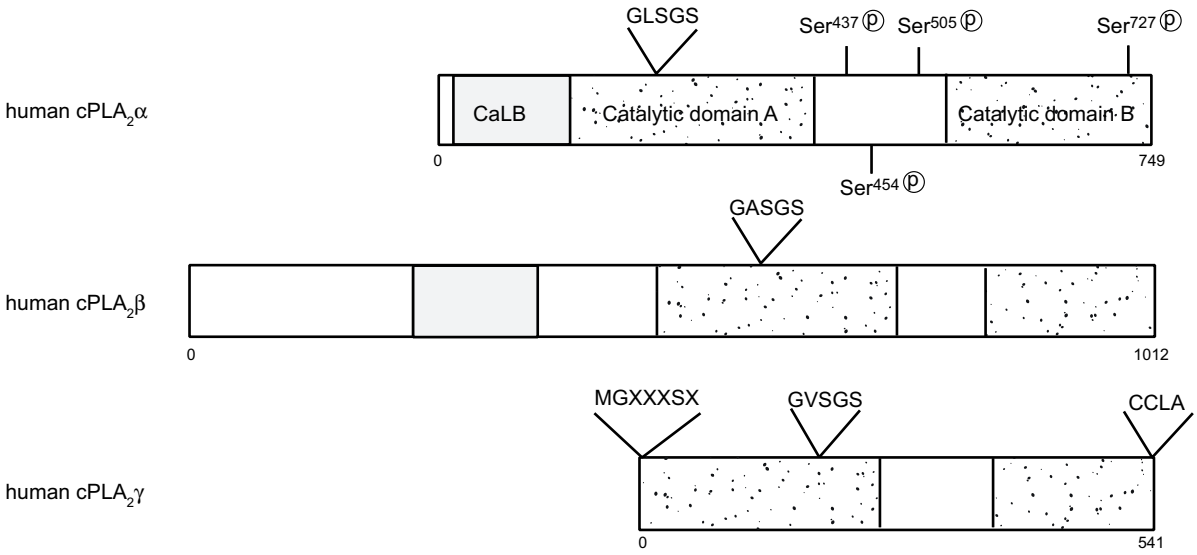

Figure 1. Schematic representation of the primary structure of cPLA$_2$α, β and γ.

at the *sn*-1 or *sn*-2 linkage (Nalefski, *et al.,* 1998). Therefore, the cPLA$_2$ C2 domain was suggested to primarily interact with the headgroup of phosphatidylcholine. The crystal structure of this C2 domain (Perisic *et al.,* 1998) revealed an anti-parallel β-sandwich consisting of two four-stranded sheets and three connecting loops, CBR1, 2 and 3. cPLA$_2$ binds two Ca^{2+}-ions between the three loops (Essen *et al.,* 1997) which is also coordinated by four acidic amino acids (Nalefski & Falke, 1996). Both CBR1 and CBR3 have a prominent cluster of hydrophobic residues and therefore display preferential binding to phospholipids with hydrophobic features of the headgroup like phosphatidylcholine in preference to phosphatidylserine, phosphatidylinositol or phosphatidic acid (Wijkander & Sundler, 1991; Nalefski, *et al.,* 1994; Nalefski and Falke, 1996). In a model for the membrane interaction of the CaLB domain, the hydrophobic CBR3 inserts into the membrane displacing at least two phospholipids, and CBR1 interacts with the hydrophobic regions of the lipid headgroup. Electrostatic interactions between the lipid headgroup and the β-sandwich are weakened by the basic residues of one strand. Binding of calcium renders the surface positively charged whereupon it is directed towards the membrane. One important difference in membrane binding of the cPLA$_2$ C2 domain as compared to other C2 domains like in conventional PKC and synaptotagmin is that hydrophobic interactions prevail over electrostatic interactions (Davletov *et al.,* 1998).

Furthermore, cPLA$_2$ contains the sequence G-L-S^{228}-G-S that closely resembles the lipase consensus motif G-X-S-X-G present in many serine esterases and neutral lipases. Site directed mutagenesis of Ser^{228} to alanine, cysteine or threonine resulted in a complete

loss of activity while $cPLA_2$ remained correctly folded, thus demonstrating the essence of Ser^{228} for catalytic activity of $cPLA_2$ (Sharp *et al.,* 1994; Huang *et al.,* 1996). Besides Ser^{228}, also Arg^{200} and Asp^{549} were shown to be essential for $cPLA_2$ activity (Pickard *et al.,* 1996). $cPLA_2$ has 9 cysteine residues but is, unlike $sPLA_2$ enzymes, stable in the presence of reducing agents showing that these residues are not essential for catalytic activity (Li *et al.,* 1994; Sharp, *et al.,* 1994). However, modification of Cys^{331} with sulfhydryl-modifying reagents resulted in a loss of catalytic activity, suggesting that Cys^{331} is located at a sensitive position near the active site (Li *et al.,* 1996; Pickard, *et al.,* 1996).

In addition to PLA_2 activity, $cPLA_2$ exhibits also lysophospholipase and weak transacylase activity in the presence of lyso-PC micelles (Leslie, 1991; Reynolds *et al.,* 1993; Hanel & Gelb, 1995). No physiological relevance has been ascribed to the transacylase activity, however, lysophospholipase activity has been implicated to play a function in cells to control in this way the levels of potentially cytotoxic lysophospholipids (de Carvalho *et al.,* 1995).

It has been shown that phosphatidylinositol-4,5-bisphosphate (PIP_2) mediates the binding of $cPLA_2$ to lipid vesicles, thereby increasing its activity *in vitro* in a calcium-independent manner (Leslie & Channon, 1990; Buckland & Wilton, 1997; Mosior *et al.,* 1998). At first, Mosior *et al*. proposed a putative pleckstrin homology (PH) domain in $cPLA_2$ since some similarity was observed with a portion of the PH domain of PLCδ1. However, from the crystal structure of $cPLA_2$ protein no clear PH domain was identified (Dessen *et al.,* 1999). Although, addition of PIP_2 to resting cells, or inhibiting the synthesis of PIP_2 correlated with an increase or decrease respectively, of $cPLA_2$ activity (Balsinde *et al.,* 2000). This suggests that $cPLA_2$ can interact directly with PIP_2, generating a binding site for $cPLA_2$ in PIP_2 enriched microdomains to which $cPLA_2$ might translocate upon cell stimulation.

Very recently, investigators demonstrated that ceramide can bind $cPLA_2$ directly via its CaLB domain thereby targeting $cPLA_2$ to its substrate (Huwiler *et al.,* 2001). This binding occurred in a calcium-dependent fashion and increased $cPLA_2$ activity towards ceramide-containing liposomes. Similar results were also observed in calcium ionophore- and epinephrine-stimulated CHO-2B cells. Besides an activation of $cPLA_2$ by ceramide, an inhibition in enzyme activity was measured in liposomal substrates containing sphingomyelin, which could subsequently be restored by addition of cholesterol or ceramide (Klapisz *et al.,* 2000). This is potentially of interest in view of the regulation and subcellular localisation of $cPLA_2$ since ceramide, sphingomyelin and cholesterol are present in rafts and/or caveolea (Brown & London, 1998; Dobrowsky, 2000).

In addition, the human $cPLA_2$ sequence contains several consensus phosphorylation sites for both serine/threonine and tyrosine protein kinases (Sharp, *et al.,* 1991). In Sf9 cells expressing recombinant human $cPLA_2$, four serine residues (Ser^{437},

Ser^{454}, Ser^{505}, Ser^{727}) are phosphorylated upon stimulation with the calcium ionophore A23187 and okadaic acid (de Carvalho *et al.,* 1996), although only Ser^{505} and Ser^{727} are conserved in $cPLA_2$ in other species and have physiological relevance. Ser^{505} is situated in the sequence P-L-S-P which is a consensus mitogen-activated protein kinase (MAPK) phosphorylation site (de Carvalho, *et al.,* 1996), and Ser^{727} lies in a consensus sequence (R-X-S) for basotrophic kinases, such as PKC or PKA.

$cPLA_2$ isoforms.

Recently, two new isoforms of the 85 kDa $cPLA_2$ have been cloned and characterised which are denoted $cPLA_2\beta$ and $cPLA_2\gamma$ (fig. 1) (Underwood *et al.,* 1998; Pickard *et al.,* 1999; Song *et al.,* 1999), giving the name $cPLA_2\alpha$ to the already known 85 kDa $cPLA_2$. $cPLA_2\beta$ and $cPLA_2\gamma$ share an overall sequence identity with $cPLA_2\alpha$ of about 30%. $cPLA_2\beta$ is a 114 kDa protein consisting of 1012 amino acids which is present in most tissues at a low level but is strongly expressed in pancreas and cerebellum (Pickard, *et al.,* 1999), brain and liver (Song, *et al.,* 1999). Both $cPLA_2\beta$ and $cPLA_2\alpha$ have a calcium-dependent lipid binding (CaLB) domain that is lacking in $cPLA_2\gamma$. However, this protein does contain two consensus motifs for lipid modification, a prenylation motif at the C-terminus and a myristoylation site at the N-terminus by which it is bound to the membrane (Underwood, *et al.,* 1998). Because $cPLA_2\gamma$ lacks the CaLB domain, the protein is much shorter, 61 kDa consisting of 541 amino acids. Its mRNA is predominantly expressed in skeletal muscle and heart, suggesting a specific role for $cPLA_2\gamma$ in these tissues. It may well code for the Ca^{2+}-independent PLA_2 that was reported to be highly active under hypoxic conditions, such as ischemia (Hazen *et al.,* 1991; McHowat & Creer, 1997).

The residues at which $cPLA_2\alpha$ can be phosphorylated (Lin *et al.,* 1993; Nemenoff *et al.,* 1993; de Carvalho, *et al.,* 1996) are situated in a region dividing the catalytic domain into two subdomains, A and B. These domains are not conserved in either of the two new isoforms, suggesting that these enzymes are regulated by other mechanisms. All three enzymes share a homologous catalytic region which contains the lipase consensus sequence GXSXG that is located at the N-terminus and the three essential amino acid residues needed for catalytic activity (Sharp, *et al.,* 1991; Pickard, *et al.,* 1996). However, in a liposome assay *sn*-2 cleavage was observed for $cPLA_2\alpha$ but *sn*-1 cleavage was preferred by $cPLA_2\beta$, whereas $cPLA_2\gamma$ was able to cleave at both sites (Song, *et al.,* 1999). Thus the three $cPLA_2$ family members have different regiospecificity towards 1-palmitoyl-2-arachidonyl-PC as a substrate, and they may also have different headgroup specificities.

Regulation of $cPLA_2$ activity

Transcriptional and translational regulation.

$cPLA_2$ is constitutively expressed in most cell types, however, extracellular stimuli, such as interleukin-1, tumor necrosis factor-α (TNF-α), monocyte colony-stimulating factor and epidermal growth factor (EGF) have been shown to induce the prolonged protein expression of $cPLA_2$ in various cell lines (Nakamura *et al.,* 1992; Schalkwijk *et al.,* 1993; Chepenik *et al.,* 1994), although these increases in expression levels are not as large a those for $sPLA_2$ group II. This is probably due to the gene structure of $cPLA_2$ which has features typical of a housekeeping gene (Kramer & Sharp, 1997). The increase in $cPLA_2$ protein expression is a result of enhancing translation of $cPLA_2$ mRNA or by prolonging the half-life of $cPLA_2$ protein through mRNA stabilisation. Suppression of $cPLA_2$ expression is mediated by glucocorticoids, like dexamethasone (Hoeck *et al.,* 1993; Schalkwijk, *et al.,* 1993). Interestingly, the fact that $cPLA_2$ is constitutively expressed may render $cPLA_2$ to be capable of responding rapidly to physiological stimuli, whereas the prolonged increase in $cPLA_2$ protein and activity might be involved in developmental and differentiation processes.

Post-translational regulation of $cPLA_2$ activity.

$cPLA_2$ requires calcium for its activity to translocate from the cytosol to the membrane, where its substrate is located. This has been demonstrated in cells stimulated with extracellular agents that mobilise calcium, including EGF and calcium ionophore A23187, by both cell fractionation or microscopical approaches (Peters-Golden & McNish, 1993; Clark, *et al.,* 1995; Glover *et al.,* 1995; Schalkwijk *et al.,* 1995). The translocation from the cytosol to the membrane is mediated by the CaLB domain that binds two Ca^{2+}-ions. However, the calcium is not directly involved in catalysis (Nalefski, *et al.,* 1994). This is further supported by the observation that a $cPLA_2$ mutant lacking the CaLB domain fails to bind membranes while it is still catalytically active towards monomeric phospholipid substrates.

In addition, a wide variety of agents have been shown to increase $cPLA_2$ phosphorylation and activity (Clark, *et al.,* 1995). The increase in $cPLA_2$ activity is quite modest, about 2-3-fold in agonist-stimulated cells, which can be reversed by phosphatase treatment (Lin *et al.,* 1992; Kramer *et al.,* 1993; Qiu *et al.,* 1993). Subsequently, the phosphorylation of $cPLA_2$ was shown to be mediated by $p42/44^{MAPK}$ and to occur on Ser^{505} (Nemenoff, *et al.,* 1993; Lin, *et al.,* 1993) which is situated in the MAPK consensus site. The importance of this phosphorylation site was demonstrated in Chinese hamster ovary cells overexpressing mutant $cPLA_2$, in which Ser^{505} is substituted by Ala. This mutant could not be phosphorylated and evoke agonist-induced arachidonic acid release (Lin, *et al.,*

1993). However, $cPLA_2$ phosphorylation induced by different stimuli in various cells without a concomitant increase in intracellular calcium does not result in arachidonic acid release, demonstrating that both calcium signalling and phosphorylation are necessary for a full activation of $cPLA_2$ (Murakami *et al.,* 1997). Moreover, phosphorylation of $cPLA_2$ has to precede an increase in intracellular calcium to achieve maximal activity (Schalkwijk *et al.,* 1996) implying that $cPLA_2$ may not be available for phosphorylation when it is first translocated. In line with this is the hypothesis that phosphorylation of Ser^{505} might cause conformational changes in $cPLA_2$ since Ser^{505} is situated between the CaLB and catalytic domains of $cPLA_2$ (Dessen, *et al.,* 1999). It is now becoming clear that $p42/44^{MAPK}$ is not the only MAPK family member involved in $cPLA_2$ phosphorylation and activation. For example, in the human astrocytoma cell line 1321N1, stimulation with thrombin or TNF-α resulted in a phosphorylation and concomitant activation of $cPLA_2$ which is most likely mediated by the c-Jun NH_2-terminal kinase (JNK) (Hernández *et al.,* 1997; Hernández *et al.,* 1999; van Putten *et al.,* 2001). Likewise, $cPLA_2$ activity was blocked in thrombin, collagen or stress-activated platelets that had been treated with an inhibitor for $p38^{MAPK}$ (Waterman *et al.,* 1996; Kramer *et al.,* 1996; Börsch-Haubold *et al.,* 1997; Buschbeck *et al.,* 1999). The phosphorylation sites in agonist-stimulated platelets and HeLa cells were analysed and phosphorylation was found to occur on both Ser^{505} and Ser^{727}, although it was suggested that Ser^{727} is not directly phosphorylated by $p38^{MAPK}$ (Börsch-Haubold *et al.,* 1998).

Ser^{727} lies in the consensus motif for basotrophic kinases such as PKC. The involvement of PKC in $cPLA_2$ activation has been observed in a variety of cell types including macrophages, mesangial cells and thyroid cells (Huwiler & Pfeilschifter, 1993; Qiu & Leslie, 1994; Ekokoski *et al.,* 2000). Furthermore, PKC was shown to phosphorylate recombinant $cPLA_2$ *in vitro* directly, although it activated $cPLA_2$ only minimally (Lin, *et al.,* 1993; Nemenoff, *et al.,* 1993). It is now known, however, that PKC can trigger MAPK activation and thus activate $cPLA_2$ indirectly (Ekokoski, *et al.,* 2000). In contrast, okadaic acid induces $cPLA_2$ phosphorylation of Ser^{727} and arachidonic acid release without elevated calcium levels, and an inhibitor of $p38^{MAPK}$ resulted in a reduced Ser^{505} and Ser^{727} phosphorylation in thrombin-activated cells. The kinase responsible for Ser^{727} phosphorylation of $cPLA_2$ is a downstream substrate of $p38^{MAPK}$ and has recently been identified as the MAP Kinase Interaction Protein Kinase 1 (Mnk1) (Hefner *et al.,* 2000).

Regulation of $cPLA_2$ via a cluster-monomer concept.

Arachidonic acid has been shown to be involved in various cellular responses, such as proliferation (Piomelli, 1993), inflammation (Heller, *et al.,* 1998) and cytotoxicity (Cifone *et al.,* 1993). Since arachidonic acid is preferentially released by $cPLA_2$, its activity has to be tightly controlled. In most inflammatory cells, a significant amount of $cPLA_2$ translocates from the cytosol to the perinuclear region upon its activation (Peters-Golden &

McNish, 1993; Glover, *et al.,* 1995), whereupon arachidonic acid is released and converted into eicosanoids. In other cells, not primarily involved in eicosanoid synthesis, $cPLA_2$ appeared to be localised predominantly in clusters near organellar membranes. Upon stimulation, these clusters did not massively translocate to a specific organel (Bunt *et al.,* 1997). We further investigated this phenomenon, using $cPLA_2$ monomers obtained by gel filtration chromatography as described previously (Spaargaren *et al.,* 1992) and homogenates of Her14 fibroblasts. A 200.000xg particulate fraction was subjected to electron microscopical immunogold detection of $cPLA_2$ complexed to its antibody which revealed the presence of both $cPLA_2$ clusters and monomers, however, only $cPLA_2$ monomers could occasionally be detected at membranes. The calcium-dependent membrane

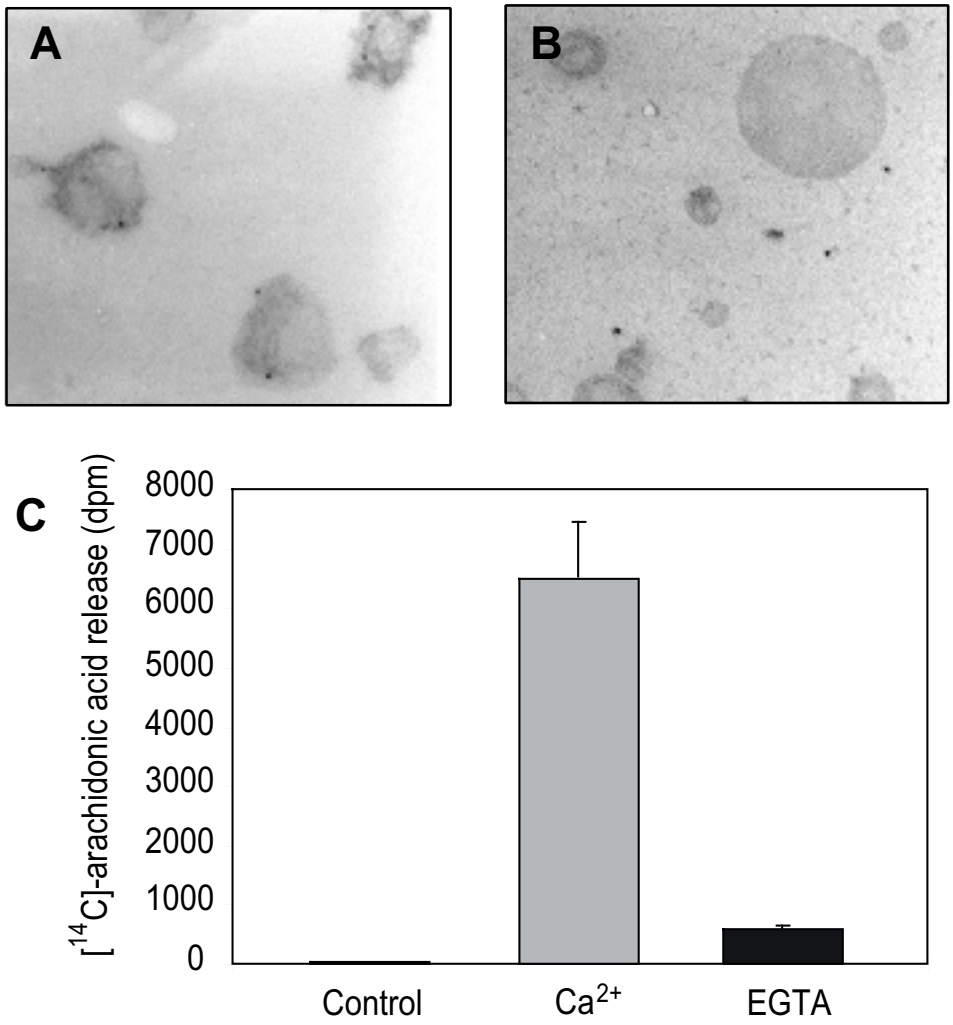

Figure 2. Ca^{2+}-dependent membrane binding by $cPLA_2$ monomers as observed with the electron microsope is related with hydrolytic activity. $cPLA_2$ monomers bind Ca^{2+}-dependently to SAPC MLV's (**A**; 1 mM Ca^{2+}, **B**; 1 mM EGTA) and exhibit Ca^{2+}-dependent arachidonic acid release under the same conditions (**C**).

binding of $cPLA_2$ monomers as well as its activity measured *in vitro*, using the conditions of the electron microscopical studies, was investigated towards multi lamellar vesicles (MLV's) composed of 1-stearoyl-2-arachidonoyl phosphatidylcholine in the presence of Ca^{2+} or EGTA. Figure 2 shows only binding of $cPLA_2$ monomers to MLV's in the presence of Ca^{2+} (fig. 2A) while no binding was observed in the presence of EGTA (fig. 2B). In addition, arachidonic acid release was only detected in the presence of calcium (fig. 2C). From these results we conclude that the clusters represent an inactive pool of $cPLA_2$ from which monomers can be recruited that are only active when bound to membranes. Furthermore, Schalkwijk *et al.* (1996) demonstrated in Her14 fibroblasts that phosphorylation of $cPLA_2$ has to precede an increase in intracellular calcium levels for maximal $cPLA_2$ activation. Collectively, these data lead to the following hypothesis. Phosphorylation of clustered $cPLA_2$ leads to a local release of $cPLA_2$ monomers that subsequently can translocate to the nearby membranes by the increase of intracellular calcium and then become fully active (Bunt *et al.,* 2000). Thus $cPLA_2$ activation by this model does not lead to a massive translocation and activation of $cPLA_2$ but is locally and strictly regulated.

Signal transduction

The p42/44^{MAPK} pathway.

Mammalian cells respond to many extracellular signals thereby activating protein kinase cascades to amplify the signal. Thus allowing the cells to integrate these signals resulting into a cellular response. Components of such a cascade are members of the mitogen-activated protein kinase (MAPK) family, which are serine/threonine protein kinases activated by various stimuli. The MAPK family can be divided into five families: $p42/44^{MAPK}$, $p38^{MAPK}$, JNK, ERK3/4 and ERK5 (Widmann *et al.,* 1999). The $p42/44^{MAPK}$ play an important role in several cellular processes, including cell proliferation, cell cycle regulation, cell survival and differentiation. Furthermore, $p42/44^{MAPK}$ can be activated by various stimuli such as growth factors, cytokines and oxidative stress. The activation of $p42/44^{MAPK}$ by these stimuli is either exerted through protein-tyrosine kinase receptors or G-protein coupled receptors.

Growth factors like EGF, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) bind to the receptors, whereupon the receptors dimerise, resulting in autophosphorylation of tyrosine residues in the intracellular domains of the receptors (Schlessinger & Ullrich, 1992; Fantl *et al.,* 1993). These phosphorylated tyrosine residues may act as high-affinity docking sites for substrates such as pp60c-Src, phosphoinositide-specific phospholipase Cγ (PLCγ), Shc, growth factor receptor-bound protein 2 (Grb2) and

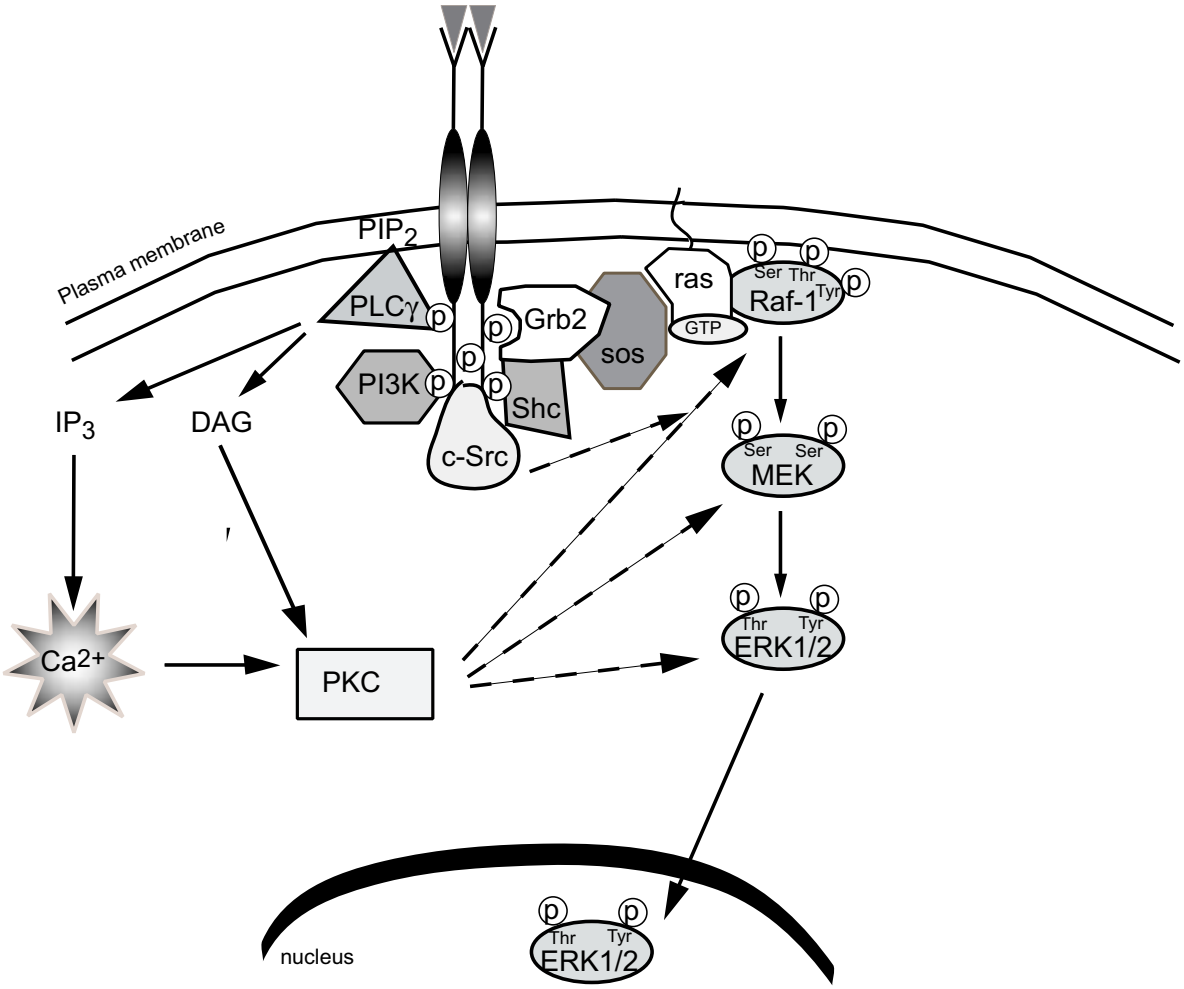

Figure 3. Growth factor-induced signal transduction pathways leading to ERK1/2 activation.

phosphatidylinositol-3-kinase (PI3K) (Anderson *et al.,* 1990; Lowenstein *et al.,* 1992; Hu *et al.,* 1992; Wolf *et al.,* 1995) either to transduce the signal directly further or by recruiting other proteins to the receptor (fig. 3). In case of PLCγ, plasma membrane phosphatidiylinositol-4,5-bisphosphate (PIP_2) becomes hydrolysed into inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 releases calcium from internal stores, which together with DAG can activate certain protein kinase C (PKC) isoforms. PKCs have been implicated in many biological processes including cell morphology, proliferation and differentiation (Hug & Sarre, 1993; Nishizuka, 1995; Jaken, 1996; Livneh & Fishman, 1997).

Grb2 recruits the guanine-nucleotide exchange factor sos to the plasma membrane to bring sos in close proximity with ras (Egan *et al.,* 1993), a small GTP-binding protein located at the cytoplasmic surface of the plasma membrane (Margolis & Skolnik, 1994).

Sos, subsequently, promotes the release of GDP from the inactive ras-GDP complex, allowing GTP to bind which renders ras active (Bonfini *et al.,* 1992). However, Shc is also able to bind both the receptor and Grb2 serving as another mechanism to recruit sos to the plasma membrane, resulting in ras activation (van der Geer & Pawson, 1995). Once ras is activated it binds to the serine/threonine kinase Raf-1, translocating Raf-1 to the plasma membrane, whereafter it can be tyrosine phosphorylated by membrane-bound tyrosine kinases including c-Src (Marais *et al.,* 1995; Marais & Marshall, 1996). In addition to tyrosine phosphorylation, Raf is phosphorylated on serine residues and on a threonine residue (Marais & Marshall, 1996). There is evidence showing that Raf-1, in cells activated by various growth factors, can be phosphorylated by several different PKC isoforms (Kolch *et al.,* 1993; Morrison *et al.,* 1993; van Dijk *et al.,* 1997; Hausser *et al.,* 2001). Mutation of specific serine phosphorylation sites on Raf does not prevent phorbol ester activation in transfected cells, however (Schönwasser *et al.,* 1998; Whitehurst *et al.,* 1995). When activated, Raf activates MAPK kinase 1 (MEK1) and 2 (MEK 2) by serine phosphorylation (Alessi *et al.,* 1994; Zheng & Guan, 1994), who in turn phosphorylate and thereby activate $p42^{MAPK}$ (or ERK2) and $p44^{MAPK}$ (or ERK1) on threonine and tyrosine residues (Payne *et al.,* 1991; Seger *et al.,* 1992; Wu *et al.,* 1993). Recently, it was demonstrated that both MEK1/2 and ERK1/2 activation was mediated by PKC in growth factor-stimulated cells (Grammer & Blenis, 1997; Adomeit *et al.,* 1999; Hausser, *et al.,* 2001). However, a direct activation or $p42/44^{MAPK}$ is unlikely since PKC is only able to phosphorylate serine and threonine residues and both threonine and tyrosine residues have to be phosphorylated for full activity of $p42/44^{MAPK}$ (Seger & Krebs, 1995). Furthermore, PI3K was found to be involved in both MEK1/2 and ERK1/2 activation (Grammer & Blenis, 1997; Conway *et al.,* 1999).

Cell cycle regulation.

The cell cycle is the set of events responsible for the duplication of the cell and consists of four phases. The S phase, in which the DNA is duplicated; the M phase, in which the duplicated DNA is segregated between the two daughter cells; and two gap phases, G1 before S phase and G2 before M phase. Progression through these phases is regulated by cyclin-dependent kinases (Cdks) and binding with their regulatory subunits, designated cyclins, by multiple phosphorylation and dephosphorylation events (Nigg, 1995; Dirks & Rutka, 1997; Reed, 1997). These cyclin/Cdk complexes regulate passage from one phase to another, for the cell to proliferate, but are also involved in the processes of cell differentiation, senescence and quiescence. Signal transduction pathways activated by signals from the extracellular environment determines the process to be activated. Progression through the cell cycle is dependent both on the presence of growth factors and on cell attachment. For instance, adherent cells in the presence of growth factors continue

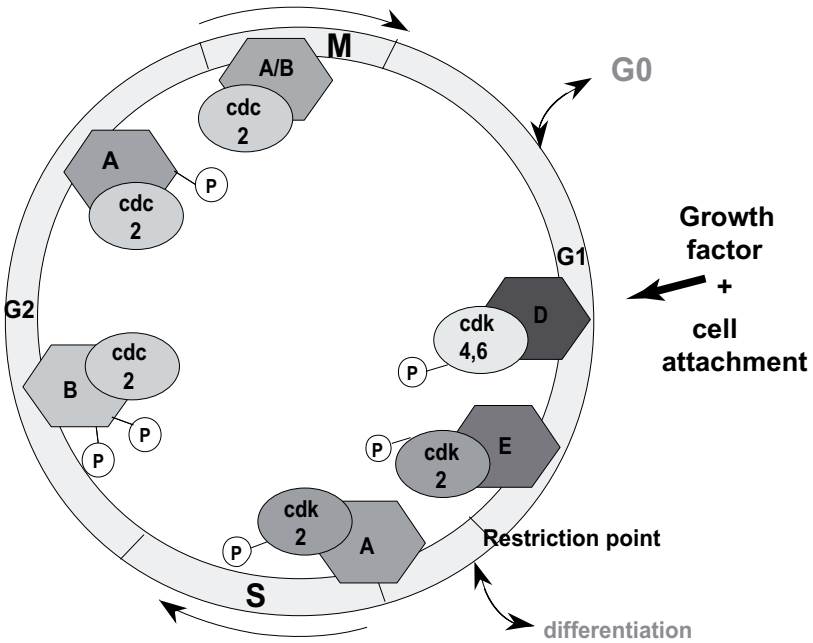

Figure 4. Schematic representation of the regulatory events for cell cycle progression of mammalian cells.

to proliferate. However, if cells are deprived of serum, they will stop progressing through the cell cycle, whereupon exit into the quiescent (G0) state occurs (Pardee, 1974). Normal growing cells, or quiescent cells re-entering the G1 phase of the cell cycle, require growth factors until the restriction point (R), to complete the cell cycle (Pardee, 1974).

Mammalian cells contain at least nine Cdks which are required to regulate the cell cycle (fig. 4) (Nigg, 1995; Bagella *et al.,* 1998). The first cyclin/Cdk complex is activated by growth factors in G1 and consists of a D-type cyclin (cyclin D1, D2 or D3) and Cdk4 or 6 (Sherr, 1995). Activation of the cyclin D/Cdk complex, as well as a c-myc-mediated pathway (Bartek & Lukas, 2001), results in the expression of cyclin E and association with Cdk2 to mediate retinoblastoma (Rb) hyperphosphorylation. Cyclin E/Cdk2 expression and activation is both rate-limiting and essential for S phase entry (Bartek *et al.,* 1996; Sherr & Roberts, 1999). Retinoblastoma phosphorylation results in the release of the transcription factor E2F and the subsequent induction of genes that mediate progression through S phase, like cyclin A and thymidylate synthase (DeGregori *et al.,* 1995). In addition, activation of these complexes is also regulated by a group of proteins called Cdk inhibitors (CKI's) which consists of two families. Members of the INK4 family (*in*hibitors of Cd*k4*; comprising of p15, p16, p18 and p19) bind to Cdk4, whereas members of the CIP/KIP family (comprising of p21, p27 and p57) bind to Cdk2, 4 and 6 (reviewed in Arellano & Moreno, 1997; Hulleman & Boonstra, 2001). Cyclin E/Cdk2 complexes are degraded in early S phase,

whereafter Cdk2 associates with cyclin A. The activity of cyclin A/Cdk2 is necessary for progression through S and is possibly involved in phosphorylation of transcription factors and proteins required for DNA duplication (Cardoso *et al.,* 1993; Fotedar & Fotedar, 1995). Later on, complexes are formed of Cdk1 or Cdc2, with cyclin A, that is required for mitosis until mid prophase (Furuno *et al.,* 1999). Cyclin B/Cdk1 complexes until the G2/M transition are held inactive by phosphorylation of Cdk1 (Mueller *et al.,* 1995), which then is dephosphorylated by cdc25C resulting in Cdk1 activation and progression into mitosis (Kumagai & Dunphy, 1991). Cyclin B degradation is necessary for the cells to exit from mitosis.

The activation of the Cdk by cyclin D depends on cyclin D expression that is synthesised as long as growth factors are present (Sherr, 1994). Furthermore, activated $p42/44^{MAPK}$ was shown to induce the expression of cyclin D and down-regulation of $p27^{KIP}$ to exit from G0, and enter the G1 phase (Ladha *et al.,* 1998). Also other components of the ras-MAPK pathway have been shown to be involved in the regulation of cyclin D and $p27^{KIP}$ (Aktas *et al.,* 1997; Peeper *et al.,* 1997; Cheng *et al.,* 1998). The activation of $p42/44^{MAPK}$ occurs through both growth factor- and extracellular matrix-induced signal transduction pathways (Schwartz *et al.,* 1995; Roovers *et al.,* 1999; Hulleman & Boonstra, 2001), probably to obtain the sustained $p42/44^{MAPK}$ activation that is required to pass the restriction point. Similarly, inhibition of the $p42/44^{MAPK}$ pathway by either antisense constructs, overexpression of kinase-inactive mutants or inhibiting nuclear translocation of $p42/44^{MAPK}$ by inhibitors, blocks DNA synthesis and proliferation (Pagès *et al.,* 1993; Brondello *et al.,* 1995;(Hulleman *et al.,* 1999). On the other hand, activation of this same pathway may also lead to cell cycle arrest, depending on the level of the activated Raf-MAPK pathway (Woods *et al.,* 1997; Sewing *et al.,* 1997; Pumiglia & Decker, 1997). Moreover, overexpression of $p42/44^{MAPK}$ has been found in human breast cancer (Sivaraman *et al.,* 1997), and also Cdc25A, cyclin D and E, myc and Rb are upregulated in several cancers (Gasparotto *et al.,* 1997; Kornmann *et al.,* 1998; Spruck *et al.,* 1999; Hanahan & Weinberg, 2000).

Physiology of the arachidonic acid cascade

Arachidonic acid is the main polyunsaturated fatty acid in the production of eicosanoids, which are oxygenated C_{18}, C_{20} and C_{22} carbon fatty acids. The pathways leading to these eicosanoids is collectively known as the arachidonic acid cascade and consists of three major pathways including cyclooxygenase, lipoxygenase and epoxygenase pathways, named after the first enzymatic step involved. The conversion of arachidonic acid by one of these pathways depends on the cell type (Shimizu & Wolfe, 1990). Eicosanoids

are not stored but synthesised on demand in which the release of arachidonic acid by PLA_2 is the rate-limiting step.

Physiological roles of cPLA$_2$ using gene knock-outs.

To gain more insight in the physiological roles of $cPLA_2$, a homozygous null mouse ($cPLA_2^{-/-}$) was created (Bonventre *et al.,* 1997; Uozumi *et al.,* 1997). The $cPLA_2^{-/-}$ mice appeared to be normal, indicating that $cPLA_2$ is not necessary for normal embryonic development. However, there could be other compensatory influences that maintain a normal development in the absence of $cPLA_2$, although later on it was found that the $cPLA_2^{-/-}$ females were unable to reproduce. Likewise, the COX-deficient mice also showed reproductive abnormalities and mice lacking the $PGF_2\alpha$ receptor did not come into labour (Sugimoto *et al.,* 1997). Furthermore, $cPLA_2^{-/-}$ mice had a reduced brain infarct volume and fewer functional neurogenic deficits after induction of cerebral ischemia and reperfusion. Also the $cPLA_2^{-/-}$ mice were more resistant to MPTP-induced neurotoxicity, that produces a Parkinsonian syndrome in human and non-human primates (Bloem *et al.,* 1990). These results suggest a role for selective $cPLA_2$ inhibitors for the treatment of stroke and Parkinson's disease. $cPLA_2^{-/-}$ mice recover faster from allergen-induced bronchoconstriction and show no airway hyperresponsiveness. Moreover, the eicosanoid generation from peritoneal macrophages and both the immediate and delayed phases of bone marrow-derived mast cells are impaired (Uozumi, *et al.,* 1997; Bonventre, *et al.,* 1997; Fujishima *et al.,* 1999).

Cyclooxygenase pathway.

Arachidonic acid can be converted into prostaglandin H_2 (PGH_2) by the action of cyclooxygenase-1 (COX-1 or prostaglandin H synthase-1 (PGHS-1)) or cyclooxygenase-2 (COX-2 or PGHS-2) that can be further transformed into prostaglandins (PG) or thromboxanes (TX) (fig. 5) (Smith, 1989). Although many different prostanoids exist, their synthesis is cell type specific (Smith *et al.,* 1991). For example thromboxane A_2 (TXA_2) is mainly formed in platelets, while prostacyclin (PGI_2) is the major prostanoid in endothelial cells and prostaglandin E_2 (PGE_2) is mainly present in renal collecting tubule cells. COX-1 is found to be constitutively expressed in most cells and tissues and is involved in cellular housekeeping processes and thrombosis (Patrignani *et al.,* 1994). By contrast, COX-2 is mainly inducibly expressed in activated cells and is amongst others, involved in inflammation, pain and fever (Riendeau *et al.,* 1997; Zhang *et al.,* 1997), various cancers (Kargman *et al.,* 1995; Levy, 1997) and Alzheimer's disease (McGeer & McGeer, 1999). Both enzymes have a similar structure, convert arachidonic acid into PGH_2 with almost identical kinetics and exist often in the same cells and tissues. The reason for the presence of two COX enzymes is now becoming clear from studies of knock-out mice for COX-1 and

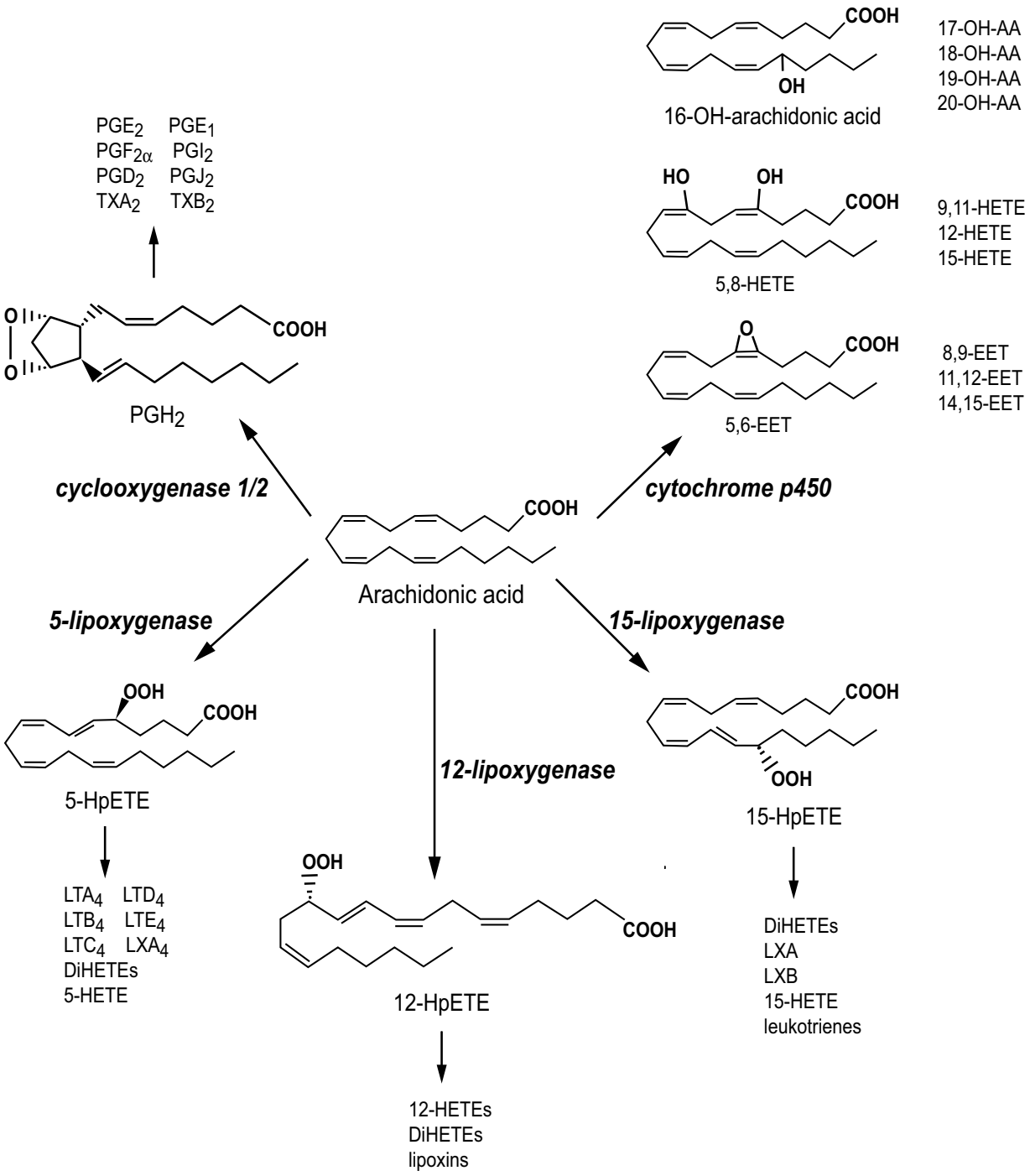

Figure 5. Eicosanoids generated by enzymes of the arachidonic acid cascade.

COX-2 (Langenbach *et al.,* 1999) as well as from studies with isozyme selective inhibitors (DeWitt, 1999). The current idea is that prostanoids formed via COX-2 can signal uniquely through G-protein-coupled receptors and through a nuclear pathway, while products derived via COX-1 most likely only signal through other cell surface receptors. In stimulated cells a biphasic generation of prostanoids occurs in which the initial phase is COX-1 dependent and the delayed phase of slow prostanoid production depends on COX-2 induction (Reddy & Herschman, 1997; Kuwata *et al.,* 1998). Thus, the function of the enzymes is more or less related with the regulation of their expression.

Since COX-1 had been considered a housekeeping cyclooxygenase it was surprising that the knock-out mice were generally healthy. However, these mice showed, for instance, a decreased platelet aggregation in response to arachidonic acid because thromboxanes could not be formed. Also prostaglandins in placenta and/or fetal tissue were found to be critical for parturition (Langenbach *et al.,* 1995).

The COX-2 knock-out ($COX^{-/-}$) mice develop severe nephropathology within the first 6 weeks of life (Morham *et al.,* 1995) which fortunately becomes less severe in later generations (Lim *et al.,* 1999), possibly due to adaptation processes. In contrast to COX-1 knock-out mice, the $COX\text{-}2^{-/-}$ mice display abnormalities in every phase of the reproductive process (Lim *et al.,* 1997). Furthermore, from patient studies a relation between the intake of non-steroidal anti-inflammatory drugs (NSAIDs) and the development of colorectal cancer was found. Indeed, in colorectal cancers the expression of COX-2 but not COX-1 was elevated (Williams *et al.,* 1999). Also breast and lung cancer cells have been found to synthesise large amounts of prostaglandins of which PGE_2 is the major prostaglandin produced by tumour cells (Ara & Teicher, 1996). In animal model studies, cyclooxygenase inhibitors exhibited chemopreventive effects as the number and size of the tumours per animal decreased. Also prostaglandins itself can have anti-tumour activity. For example, a 10 day infusion of PGE_1 in Lewis lung carcinoma mice resulted in a significant reduction in tumour volume, weight, the number of metastasis and the doubling time of the tumour (Ellis *et al.,* 1990).

Lipoxygenase pathway.

Lipoxygenases catalyse the insertion of oxygens in various eicosaenoic acids and in the case of arachidonic acid, this will lead to hydroperoxy-eicosatetraenoic acids (HpETEs). In mammalian cells three major lipoxygenases have been identified according to the oxygenation sites in arachidonic acid, i.e.; 5-lipoxygenase (5-LO), 12-lipoxygenase (12-LO) and 15-lipoxygenase (15-LO) resulting in 5(S)-, 12(S)-, and 15(S)-HpETE (fig. 5). These HpETEs can subsequently undergo several different enzymatic transformations resulting in the production of dihydroxy-eicosatetraenoic acids (DiHETEs), epoxy fatty acids (leukotrienes) and trihydroxy-eicosatetraenoic acids (lipoxins) (Smith *et al.,* 1991).

5-LO is present in a variety of inflammatory cells, among them are leukocytes, neutrophils, basophils and monocytes (Chen *et al.,* 1995; Brock *et al.,* 1995; Pouliot *et al.,* 1996), but is also found in human placenta (Matsumoto *et al.,* 1988). In line with the presence of 5-LO in these cells is that 5-LO has been shown to be involved in inflammation and asthma by the production of the biologically potent leukotrienes (LT) (Ford-Hutchinson, 1990; Lewis *et al.,* 1990). To investigate the function of 5-LO in more detail, knock-out mice were developed. However, the 5-LO deficient mice grew normally, were fertile and no obvious alteration in the phenotype was observed compared to control mice (Chen *et al.,* 1994). Later on it was found that in these mice 5-LO products are required for the development of airway hyperresponsiveness and are partially involved in antigen challenged eosinophil recruitment (Corry *et al.,* 1996; Drazen *et al.,* 1996; Foster *et al.,* 1996). Furthermore, these studies in 5-LO$^{-/-}$ mice revealed the involvement of 5-LO in host defense (Goldhill *et al.,* 1997) and in acute inflammation, although the nature of the stimuli and the location were found to be important.

The functions of the 12- and 15-LO are not well understood since the action of each enzyme can result in the same two products although they have been implicated in intracellular organel degradation, erythrocyte maturation and atherosclerosis (Kühn & Brash, 1990).

Three classes of 12-LOs have been identified which are the platelet type (P12-LO), leukocyte type (L12-LO) and epidermal type (E12-LO). 12-LOs have been found in a variety of tissues including platelets, keratinocytes, leukocytes, A431 epidermal carcinoma cells and other tumour cells (Yoshimoto & Yamamoto, 1995; Funk, 1996). Also the L12-LO$^{-/-}$ mice grew normally and were fertile, indicating that 12-LO is not essential for fetal development. In addition, hardly any 12-HETE and 15-HETE products were detected in 12-LO$^{-/-}$ macrophages but a 5-HETE product instead (Sun & Funk, 1996), indicating that disturbing one pathway can lead to enhancement of another one. 12-HETE modulates neurotransmission (Piomelli *et al.,* 1987) and cell adhesion (Tang *et al.,* 1995) which are fast events, but is also involved in long term events such as platelet aggregation, cell differentiation or survival (Yu *et al.,* 1995; Tang *et al.,* 1996).

The 15-LO exist in reticulocytes, airway epithelium (Funk, 1996) and is upregulated in a number of tumour cells (Kamitani *et al.,* 1998; Shappell, *et al.,* 2001) . 15-LO is not only thought to be involved in inflammation (Samuelsson *et al.,* 1987; Vanderhoek, 1988), but also in reticulocyte differentiation (Schewe & Kühn, 1991; Nadel *et al.,* 1991) and atheroma formation (Feinmark & Cornicelli, 1997). Furthermore, 15-LO can peroxidise membrane lipids thereby changing the structure and function of lipid-protein complexes as is the case in reticulocyte maturation and in the formation of atherosclerotic plaques (Schewe and Kühn, 1991; Nagy *et al.,* 1998).

Epoxygenase pathway.

The epoxygenase pathway is initiated by transforming arachidonic acid via cytochrome p450 mixed-function oxidases finally resulting in cis-epoxy-eicosatrienoic acids (EpEtrEs or EETs), hydroxy-eicosatetraenoic acids (HETEs) and hydroxy-eicosatetraenoic acids (OH-AA) (Capdevila *et al.,* 1992) (fig. 5). As opposed to the cyclooxygenase and lipoxygenase enzymes, the biological and molecular characterisations of the cytochrome p450 preceded the functional studies which have only recently started to be investigated. In animal functions and organs the cytochrome p450 metabolites have been implicated to mediate the release of peptide hormones, to be mediators of salt and water regulation by the kidney and to be involved in vascular tone, although these results are conflicting (Capdevila, *et al.,* 2000). On the cellular level, cytochrome p450 metabolites have been found to be involved in ion channels and transporters and to act as mitogens (Capdevila, *et al.,* 2000). Furthermore, a role for cytochrome p450 has been proposed in hypertension (Rahman *et al.,* 1997).

Eicosanoids can contribute to normal physiologic processes such as inflammation, development and immune function but also to non-physiological processes as in diseases and carcinogenesis. Future experiments are needed to elucidate the specific roles and mechanisms by which these eicosanoids act. However, the biosynthesis of these eicosanoids depends on the availability of free arachidonic acid of which the release is controlled by the action of PLA_2s and especially $cPLA_2$. Thus, it appears that the function of $cPLA_2$ in these processes plays an even more important role.

Scope of this thesis

Arachidonic acid is involved in numerous physiological and pathophysiological processes and is preferentially released by $cPLA_2$, implicating that $cPLA_2$ activity has to be tightly regulated. The aim of this study was to gain more insight in the regulation of $cPLA_2$ in mitogen- and oxidative stress-induced cells, as well as in continuously cycling cells. Furthermore, the possible role of $cPLA_2$ and the downstream arachidonic acid metabolising enzymes, cyclooxygenases and lipoxygenases, in cell cycle progression was investigated.
Chapter 2 deals with the regulation of $cPLA_2$ in serum- and EGF-activated cells as well as in quiescent and proliferating cells. $cPLA_2$ in both serum- and EGF-stimulated cells is activated by $p42/44^{MAPK}$, that in turn is activated differentially involving the Raf-MEK pathway and PKC, but not PI3K. In contrast, $p42/44^{MAPK}$ phosphorylation in quiescent and proliferating cells is solely achieved via the Raf-MEK pathway, but only leads to $cPLA_2$ activation in quiescent cells. These data suggest the activation of different populations of

p42/44^{MAPK} and cPLA$_2$. The results in **chapter 3** show that cPLA$_2$ is activated by different kinds of oxidative stress. H_2O_2-induced cPLA$_2$ activation is partly mediated by phosphorylation through the Raf-MEK-p42/44^{MAPK} pathway and partially by a phosphorylation-independent mechanism, likely involving lipid peroxidation. In **chapter 4** the activation of cPLA$_2$ during the ongoing cell cycle was investigated. cPLA$_2$ activity was high in mitosis, decreasing rapidly in early G1. A small increase was observed in mid/late G1, followed by a strong increase at the G1/S transition. The changes in cPLA$_2$ activity were not due to a difference in cPLA$_2$ expression, but due to phosphorylation of cPLA$_2$ by p42/44^{MAPK}. The possible role of cPLA$_2$ activity in cell cycle progression was studied in **chapter 5**. Inhibiting cPLA$_2$ activity in early G1 using ATK, an inhibitor for cPLA$_2$, resulted in a marked reduction in DNA synthesis. However, no significant difference in total cell number was counted in ATK treated cells for 24h compared to control cells. Furthermore, inhibition of cyclooxygenases at different time points after mitosis did not have any effect on cell cycle progression, whereas inhibition of lipoxygenases result in cell cycle arrest. Moreover, lipoxygenases are required for S phase progression, since no DNA synthesis occurred when lipoxygenase was inhibited. Finally, in **chapter 6** the purpose of different signal transduction pathways regulating cPLA$_2$ activity and the possible functions of cPLA$_2$ and lipoxygenase actions for cell cycle progression will be discussed.

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Chapter 2

Phosphorylation of p42/44^{MAPK} by various signal transduction pathways activates cytosolic phospholipase A_2 to variable degrees

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Abstract

Arachidonic acid has been implicated to play a role in physiological and pathophysiological processes and is selectively released by the 85 kDa cytosolic phospholipase A_2 ($cPLA_2$). The activity of $cPLA_2$ is regulated by calcium, translocating the enzyme to its substrate, and by phosphorylation by a mitogen-activated protein kinase (MAPK) family member and a MAP kinase-activated protein kinase. In this study, the signal transduction pathways in growth factor-induced phosphorylation of $p42/44^{MAPK}$ and $cPLA_2$ activation was investigated in Her14 fibroblasts. $p42/44^{MAPK}$ in response to epidermal growth factor was not only phosphorylated via the Raf-MEK pathway but mainly through protein kinase C (PKC), or a (un)related kinase, in which the phosphorylated $p42/44^{MAPK}$ corresponded with $cPLA_2$ activity. Serum-induced phosphorylation of $p42/44^{MAPK}$ also corresponded with $cPLA_2$ activity but is predominantly mediated via Raf-MEK and partly through PKC, or a (un)related kinase. In contrast, activation of PKC by phorbol ester did not result in increased $cPLA_2$ activity while $p42/44^{MAPK}$ is phosphorylated, mainly via Raf-MEK and through MEK. Moreover, $p42/44^{MAPK}$ phosphorylation is present in quiescent and proliferating cells that is entirely phosphorylated via Raf-MEK but only corresponds to $cPLA_2$ activity in the former cells. Collectively, these data show that $p42/44^{MAPK}$ in proliferating, quiescent and stimulated cells is phosphorylated by various signal transduction pathways suggesting the activation of different populations of $p42/44^{MAPK}$ and $cPLA_2$.

Introduction

Phospholipases A_2 (PLA_2) release fatty acids from the *sn*-2 position of phospholipids. This is of particular interest when arachidonic acid is released since it plays a central role in various cellular processes such as cell migration, mitogenic signalling (Piomelli, 1993; Korystov Yu *et al.,* 1998), inflammation (Heller *et al.,* 1998) and cytotoxicity (Wissing *et al.,* 1997; Grazia Cifone *et al.,* 1997). This arachidonic acid is preferentially released by the 85 kDa cytosolic phospholipase A_2 ($cPLA_2$) (Clark *et al.,* 1995; Sharp *et al.,* 1991) and therefore $cPLA_2$ activity has to be tightly controlled.

$cPLA_2$ activity is regulated by both calcium and phosphorylation. Submicromolar concentrations of calcium are required for $cPLA_2$ to translocate from the cytosol towards phospholipid membranes that is mediated by its calcium-dependent phospholipid binding domain (Nalefski *et al.,* 1994). $cPLA_2$ phosphorylation was found to occur on Ser-505 through a mitogen-activated protein kinase (MAPK) (Clark, *et al.,* 1995 and references

therein) and the importance of cPLA$_2$ phosphorylation was shown in several other studies using a cPLA$_2$ gel shift (Qiu & Leslie, 1994; Sa *et al.,* 1995; Clark, *et al.,* 1995 and references therein). The phosphorylation causes an increase in the intrinsic activity (Kramer *et al.,* 1993; Schalkwijk *et al.,* 1995) and can be reversed by phosphatase treatment (Schalkwijk, *et al.,* 1995; Wijkander & Sundler, 1992). In platelets, p38^{MAPK} has been shown to phosphorylate cPLA$_2$ (Kramer *et al.,* 1996) on Ser-505 (Börsch-Haubold *et al.,* 1998) and for a full activation cPLA$_2$ must also be phosphorylated on Ser-727 by a Mnk1-related protein kinase (Hefner *et al.,* 2000). p42/44^{MAPK} was the first MAPK family member shown to phosphorylate cPLA$_2$ on Ser-505 (Lin *et al.,* 1993) and phosphorylation of cPLA$_2$ has been correlated with p42/44^{MAPK} phosphorylation in many cell models (Clark, *et al.,* 1995; Hirabayashi & Shimizu, 2000).

Stimulation of cells by epidermal growth factor (EGF) results in the activation of cPLA$_2$ (Spaargaren *et al.,* 1992; Bonventre *et al.,* 1990). Subsequently, it was reported that in EGF-activated Herc13 and Her14 fibroblasts cPLA$_2$ was phosphorylated and this phosphorylation had to precede a rise in the intracellular calcium concentration for the enzyme to become maximal active (Schalkwijk, *et al.,* 1995; Schalkwijk *et al.,* 1996). Moreover p42/44^{MAPK} in these cells was also phosphorylated after EGF stimulation in a similar time course. Therefore, we investigated whether EGF-induced p42/44^{MAPK} phosphorylation in Her14 cells results in cPLA$_2$ activation. p42/44^{MAPK}, upon growth factor stimulation of cells is generally thought to become activated through p21ras, following the sequential activation of Raf-1 and MAPK kinase (MEK) (Bokemeyer *et al.,* 1996; Haystead *et al.,* 1992; Marshall, 1995). However, it has recently been demonstrated that p42/44^{MAPK} cannot only be phosphorylated through Raf-MEK but also by phosphatidylinositol-3-kinase in platelet-derived growth factor (PDGF)-stimulated airway smooth muscle cells or Swiss 3T3 fibroblasts (Grammer & Blenis, 1997; Conway *et al.,* 1999). Moreover, activation of p42/44^{MAPK} by protein kinase C has also been reported (Hirabayashi & Shimizu, 2000). Hence, we have established the signal transduction pathways involved in the phosphorylation of p42/44^{MAPK} in EGF-stimulated Her14 fibroblasts.

We observed that the measured activity of cPLA$_2$ in quiescent cells varied between several experiments which may also explain the difference in stimulation of cPLA$_2$ activity in cells upon EGF treatment, as has been reported previously (Kramer, *et al.,* 1993; Lin *et al.,* 1992). In addition, the level of phosphorylated p42/44^{MAPK} in quiescent cells was variable between different experiments and corresponded with cPLA$_2$ activity. This remaining phosphorylation of p42/44^{MAPK} was not due to non-quiescent cells, because, the p42/44^{MAPK}, that was activated via the Raf-MEK pathway in proliferating cells did not result in cPLA$_2$ activation. Furthermore, stimulation of quiescent cells with serum resulted in p42/44^{MAPK} phosphorylation and subsequent cPLA$_2$ activation, as was the case in EGF- but not in phorbol ester-activated Her14 fibroblasts. The signal transduction pathways

leading to p42/44^{MAPK} phosphorylation involve to variable degrees Raf-MEK and protein kinase C (PKC) but not phosphatidylinositol-3-kinase.

Materials and Methods

Materials.

Tissue culture nutrients, Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were purchased from Gibco BRL (Scotland). Ro31-8220, phorbol-12, 13-dibutyrate (PdBu), Gö6983 and Gö6976 were from Calbiochem (La Jolla, USA). PD098059 and U0126 were obtained from Biomol (Playmouth meeting, USA) and Promega (Madison, USA), respectively. LY294002 was from Alexis (Läufelfingen, Switzerland) and Protifar was from Nutricia (Zoetermeer, The Netherlands). Staurosporine, wortmannin and dipalmitoylglycerol were purchased from Sigma (Steinheim, Germany). 1-Stearoyl-2-[1-^{14}C]arachidonoyl-glycero-3-phosphocholine was obtained from Amersham (Buckinghamshire, England) and epidermal growth factor (EGF) was from Harlan Bioproducts (Madison, USA). All other chemicals were either from Sigma or Merck (Darmstadt, Germany).

Cell culture.

Her14 cells, which are mouse NIH3T3(0) fibroblasts transfected with the human EGF-receptor cDNA, were grown in DMEM supplemented with 7.5% FBS. Cells were maintained at 37°C in a humidified atmosphere. Cells were grown to a confluency of 30,000 cells/cm^2, whereafter they were serum-starved overnight. Next, the cells were stimulated with FBS (5%), EGF (50 ng/ml) or PdBu (200 nM) for 15 min or for the indicated times. Treating the cells with PD098059 (50 μM) or U0126 (50 μM) 1 h prior to stimulation inhibited MEK activity. Phosphatidylinositol-3-kinase (PI3K) inhibition was performed by treating the cells with wortmannin (10 nM) or LY294002 (10 μM) 30 min prior to stimulation. Protein kinase C (PKC) was inhibited with either Ro31-8220 (10 μM), staurosporine (1 μM), Gö6983 (10 μM) or Gö6976 (10 μM) for a 30 min pre-treatment. Alternatively, it was down-regulated by prolonged treatment with PdBu (200 nM) for 24 h prior to stimulation. After stimulation the cells were washed twice with ice-cold phosphate buffered saline (PBS) whereafter the cells were scraped in homogenisation buffer (50 mM Hepes/NaOH pH 7.4, 0.25 M sucrose, 50 mM NaF, 250 μM Na_3VO_4, 1 mM EGTA, 10 μM leupeptin, 1 μM pepstatin and 1 mM PMSF). The cell lysate was homogenised by 15 strokes through a 26G-needle and sonicated for 3 times 10 seconds. The amount of protein was measured according to Bradford (1976) using a Bio Rad novapath^{TM} microplate reader.

Western blot analysis.

Cell homogenates (10 μg protein) were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane by semi-dry blotting using a BioRad trans-blot SD. The membrane was blocked for 1h at RT with 2% milkpowder in PBST (PBS containing 0.1% (v/v) Tween-20), followed by primary antibody incubations for 1h at RT in 0.2% milkpowder in PBST. Phosphorylated p42/44^{MAPK} or phosphorylated MEK1/2 were detected respectively with a phospho-p42/44 MAPK or a phospho-MEK1/2 rabbit polyclonal antibody at a dilution of 1:1000 (New England Biolabs Incorporated, Beverly, MA). MEK1 (0.25 μg/ml) or ERK2 (0.5 μg/ml) were detected with a monoclonal antibody from Upstate Biotechnology (Lake Placid, NY) or from Transduction Laboratories (Lexington, KY), respectively. Then, the membrane was washed and primary antibodies were detected with goat anti-rabbit and rabbit anti-mouse IgG conjugated to horseradish peroxidase and the bands were visualised with enhanced chemiluminescence (NEN^{TM}, Life science products, Boston).

cPLA$_2$ activity assay.

cPLA$_2$ activity was measured by the release of radiolabeled arachidonic acid from the *sn*-2 position of 1-stearoyl-2-[1-^{14}C]arachidonoyl-glycero-3-phosphocholine as described previously (Schalkwijk, *et al.,* 1996). The radiolabeled phosphatidylcholine was dried under N_2 together with dipalmitoylglycerol and then dispersed in water by sonification for 4 times 15 s under nitrogen to give final concentrations of 4 and 2 μM, respectively. The assay incubation mixture contained 0.2 M Tris pH 8.5, 1 mM $CaCl_2$, 5 mM DTT and 10 μg cell homogenate in a total volume of 200 μl. After incubation for 7-10 min at 37°C the released radiolabeled arachidonic acid was extracted by a modified Dole extraction procedure (van den Bosch *et al.,* 1974) and the radioactivity was determined in a scintillation counter (Tri-Carb 15000, Packard, Meriden, USA). In this way only the activity of cPLA$_2$ was measured (Spaargaren, *et al.,* 1992; Atsumi *et al.,* 1998).

Results

Endogenous cPLA$_2$ activity corresponds with p42/44^{MAPK} phosphorylation in quiescent but not in proliferating cells.

We observed that the cPLA$_2$ activity measured in quiescent cells varied between several experiments that might result in a difference in cPLA$_2$ stimulation (Qiu *et al.,* 1993). In addition, the level of phosphorylated p42/44^{MAPK} in quiescent cells was also subject to alterations. Since it has been previously reported that cPLA$_2$ can be phosphorylated and thereby activated by MAP kinase (MAPK) in a variety of cell models (Lin, *et al.,* 1993;

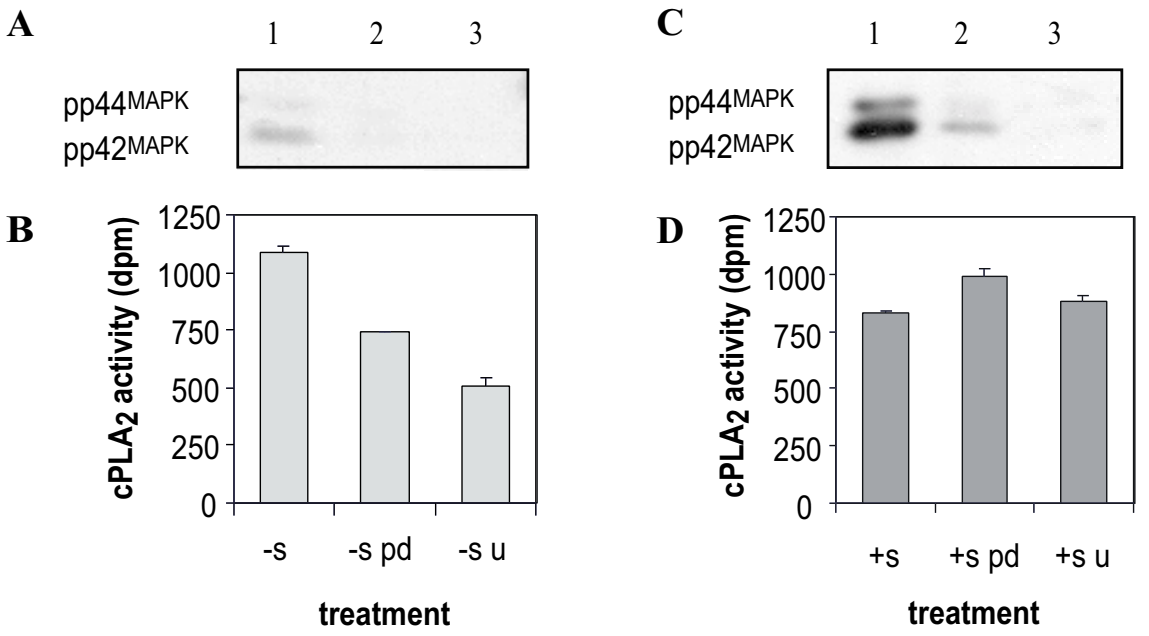

Figure 1. Effect of the MEK inhibitors, PD098059 and U0126, on phosphorylated p42/44^{MAPK} and cPLA$_2$ activity in quiescent and proliferating Her14 cells. Her14 cells were serum-starved overnight (-s) or were continuously grown in medium containing serum (+s). Then these cells were incubated for 1h with 50 μM PD098059 (pd) or U0126 (u), or were left untreated. p42/44^{MAPK} phosphorylation was detected on western blot, using an antibody against phosphorylated p42/44^{MAPK} (A, C) and cPLA$_2$ activity was measured by an *in vitro* assay as described in Materials and Methods of quiescent (B) and proliferating cells (D). Results shown are representative of three independent experiments and S. D. are shown for cPLA$_2$ activity.

Nemenoff *et al.,* 1993), the relation between p42/44^{MAPK} phosphorylation and cPLA$_2$ activity in quiescent cells was investigated. To ensure that the activation range of cPLA$_2$ and p42/44^{MAPK} were identical between each condition within the experiment, the same batch of cells was used for every experiment performed. A low phosphorylation of p42/44^{MAPK} was detected on western blot in quiescent cells (fig. 1A, lane 1), which was almost completely vanished in quiescent cells that were treated for 1h with the MAPK kinase (MEK) inhibitor PD098059 (fig. 1A, lane 2). PD098059 binds directly to the non-phosphorylated isoforms of MEK (MEK-1 and –2), thereby preventing their Raf-mediated activation (Dudley *et al.,* 1995; Favata *et al.,* 1998). Next, another MEK inhibitor, U0126 was used. U0126 inhibits the catalytic activity of MEK (Favata, *et al.,* 1998), although it can still be phosphorylated. Treating quiescent cells for 1h with U0126 resulted in a complete loss of p42/44^{MAPK} phosphorylation (fig. 1A, lane 3). Subsequently, the activity of cPLA$_2$ in these samples was measured as described in Materials and Methods. Figure 1B shows that in quiescent cells cPLA$_2$ activity is present (-s) which gradually decreases upon treatment of these quiescent

cells with PD098059 (-s pd) and U0126 (-s u), respectively. These results show that p42/44^{MAPK} is still phosphorylated in quiescent cells, which was activated via the Raf-MEK pathway and this phosphorylation appears to relate with cPLA$_2$ activation. It might well be that the phosphorylation of p42/44^{MAPK} and the presence of cPLA$_2$ activity in quiescent cells is due to the fact that not all cells have reached the quiescent state. This suggests that the level of phosphorylated p42/44^{MAPK} in proliferating cells (cells that are continuously grown in medium containing serum) would be higher. Indeed, p42/44^{MAPK} phosphorylation was significantly higher in proliferating cells (fig. 1C, lane 1) compared to quiescent cells (fig. 1A, lane 1). Incubating proliferating cells with PD098059 almost completely inhibited the phosphorylation of p42/44^{MAPK} (fig. 1C, lane 2) while it was completely vanished in U0126 treated cells (lane 3). Thus, p42/44^{MAPK} in proliferating cells is predominantly activated via the Raf-MEK pathway as was also observed in quiescent cells. However, cPLA$_2$ activity in proliferating cells (fig. 1D, +s) is not related with p42/44^{MAPK} phosphorylation as the activity in proliferating cells did not decrease in cells treated with PD098059 (+s pd) or U0126 (+s u).

These results show the activation of p42/44^{MAPK} via the Raf-MEK pathway in both quiescent and proliferating cells. However, cPLA$_2$ activity is only related with p42/44^{MAPK} phosphorylation in quiescent cells suggesting that cPLA$_2$ in proliferating cells is activated through other pathways. Therefore, we have studied the signal transduction pathways leading to p42/44^{MAPK} phosphorylation and subsequent cPLA$_2$ activation in EGF- and serum-stimulated cells.

Time-dependent activation of p42/44^{MAPK} and cPLA$_2$ in serum-stimulated cells.

In order to establish whether p42/44^{MAPK} and cPLA$_2$ are activated by serum, quiescent Her14 cells were incubated in the presence of serum for different periods of time. Addition of serum (5%) to the cells resulted in a rapid phosphorylation of p42/44^{MAPK} within 5 min that decreased slowly afterwards (fig. 2A). The activity of cPLA$_2$ in these cells, measured as described in Materials and Methods, demonstrated a transient activation that was already maximal at 5 min after stimulation (fig. 2B). These results demonstrate that serum induces a time-dependent phosphorylation of p42/44^{MAPK} and transient activation of cPLA$_2$.

The Raf-MEK pathway is not the only pathway involved in EGF- and serum-induced p42/44^{MAPK} phosphorylation.

The results in figure 1 demonstrated that p42/44^{MAPK} in quiescent or proliferating cells is predominantly phosphorylated via the Raf-MEK pathway. In addition, Raf-MEK in growth factor-stimulated cells is activated through p21ras, subsequently resulting in p42/44^{MAPK} activation (Haystead, *et al.,* 1992; Marshall, 1995; Avruch *et al.,* 1994). To

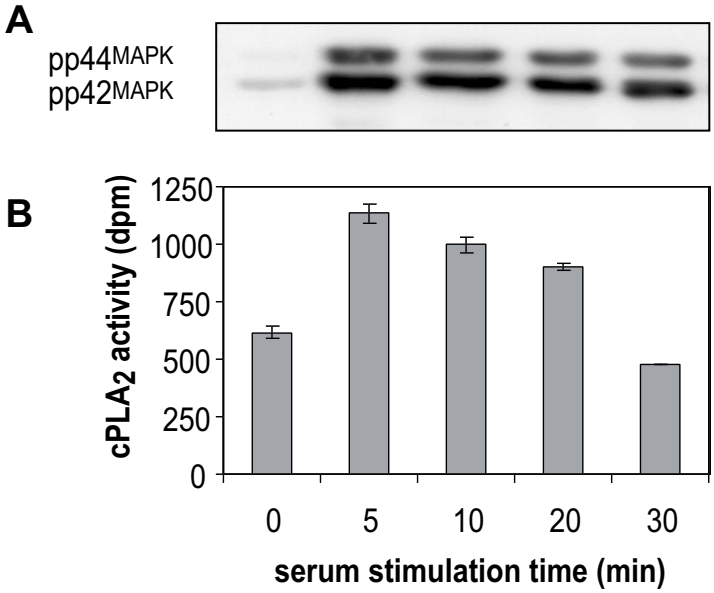

Figure 2. Time course of serum-stimulated Her14 cells. Quiescent Her14 cells were stimulated with 5% serum for the indicated time periods. Thereafter, cells were harvested and p42/44^{MAPK} phosphorylation was detected on western blot (A) and cPLA$_2$ activity was measured (B). A representative experiment and S. D. are shown of three independent ones.

investigate whether this is also true for EGF- and serum-stimulated Her14 cells, experiments were performed using PD098059. As shown on the western blot of figure 3 (lanes 1 and 5), p42/44^{MAPK} is phosphorylated to a low level in quiescent cells, which has almost completely disappeared in quiescent cells treated with PD098059 (lanes 2 and 6). Addition of serum (15 min) or EGF (15 min) to quiescent cells resulted in a strong increase in p42/44^{MAPK} phosphorylation (lanes 3 and 7, respectively). Next, the Raf-MEK pathway was inhibited by treatment of the cells with PD098059 prior to stimulation with serum or EGF. The phosphorylation of p42/44^{MAPK} was largely reduced in serum-activated cells (lane 4) but was hardly decreased in EGF-stimulated cells (lane 8).

Taken together, these results indicate that EGF-induced p42/44^{MAPK} phosphorylation is mainly activated by other pathways than through Raf-MEK while in serum-stimulated cells p42/44^{MAPK} is partly phosphorylated via the Raf-MEK pathway and partly through other signal transduction pathways.

Involvement of Raf-MEK, PI3 kinase and PKC in p42/44^{MAPK} phosphorylation.

In previous studies it was demonstrated that p42/44^{MAPK} can be phosphorylated, in addition to the Raf-MEK pathway, through other well-known signal transduction pathways, including phosphatidylinositol-3-kinase (PI3K) (Bondeva *et al.,* 1998) and protein kinase C

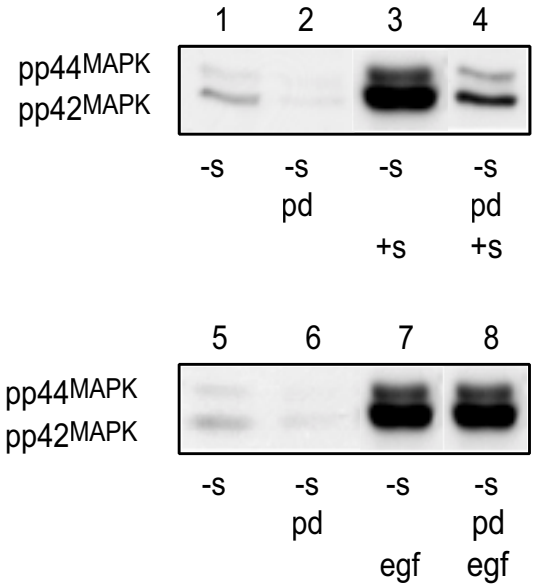

Figure 3. Effect of PD098059 pre-treatment on EGF- and serum-induced p42/44^{MAPK} phosphorylation in Her14 cells. Quiescent cells were left untreated (-s), incubated with PD098059 (pd) for 1h, stimulated afterwards with serum (+s) or EGF (egf), or the cells were treated with PD098059 for 1h prior to stimulation with serum or EGF. The upper panel shows the phosphorylated p42/44^{MAPK} of serum-stimulated cells, and the lower panel of EGF-stimulated cells. Results shown are representative of at least five independent experiments.

(PKC) (Romanelli & van de Werve, 1997). In order to establish whether these signal transduction pathways are involved in serum- and EGF-induced p42/44^{MAPK} phosphorylation in Her14 fibroblasts, the effect of various inhibitors of these pathways was investigated. The possible involvement of PI3K in the phosphorylation of p42/44^{MAPK} was examined using the inhibitors wortmannin and LY294002. Treatment of quiescent cells (fig. 4A, lanes 1 and 5) with wortmannin or LY294002 for 30 min prior to addition of serum (lanes 3 and 4, respectively) or EGF (lanes 7 and 8, respectively) did not result in a significant inhibition of phosphorylated p42/44^{MAPK} as compared to cells stimulated with serum or EGF alone (lanes 2 and 6, respectively). To ensure that wortmannin and LY294002 work in Her14 cells, the phosphorylation of the downstream PI3K enzyme protein kinase B (PKB) was analysed on western blot. No phosphorylation of PKB was observed in the presence of wortmannin or LY294002 (data not shown), as has been demonstrated also in other cell lines (Burgering & Coffer, 1995; Alessi *et al.,* 1996; Berra *et al.,* 1998). These results indicate that PI3K does not play a significant role in serum- or EGF-stimulated phosphorylation of p42/44^{MAPK} in Her14 cells.

PKC activation in stimulated cells was prevented by treatment with the inhibitors: Ro31-8220, a specific common PKC inhibitor (Newby *et al.,* 1995), Gö6983 which inhibits PKCα, β_I, β_{II}, δ and ζ isoforms (Gschwendt *et al.,* 1996), and staurosporine known to inhibit all PKCs and some other kinases (Couldwell *et al.,* 1994). Figure 4B shows that p42/44^{MAPK} was phosphorylated to a low extent in quiescent cells (lanes 1 and 8) which was strongly increased upon stimulation of serum (lane 2) or EGF (lane 9). Incubation of the cells with PD098059 prior to addition of serum, resulted in a considerable reduction in p42/44^{MAPK} phosphorylation (lane 3) but only to a minor reduction in EGF-activated cells (lane 10), which is in agreement with the results presented in figure 3. Pre-treatment of quiescent Her14 cells with staurosporine or Ro31-8220 severely diminished p42/44^{MAPK} phosphorylation upon activation of the cells with either serum (lanes 4 and 5, respectively) or EGF (lanes 11 and 12, respectively). However, Gö6983 treatment did not seem to have any effect on the phosphorylation neither in serum- nor in EGF-stimulated cells (lanes 6 and 13, respectively).

In addition, PKC down-regulation using phorbol ester (PdBu) treatment for 24h was used to investigate the involvement of the conventional (α, β_I, β_{II}, γ) and novel (δ, ε, η, μ, θ) PKCs. NIH3T3 fibroblasts, which are the parental cells of Her14 cells, express PKCα, δ, ζ and λ (Mischak *et al.,* 1993; Akimoto *et al.,* 1994; Doornbos *et al.,* 2000). The down-regulation was confirmed by the absence of PKCα and δ on western blot (data not shown), as was also demonstrated in other cell types (Kramer & Simon, 1999; Huwiler *et al.,* 1993). As shown in figure 4B (lanes 7 and 14), PKC down-modulation had only a slight effect on p42/44^{MAPK} phosphorylation induced by serum or EGF, indicating that the conventional and novel PKCs seem to play a minor role in the signal transduction cascade leading to the phosphorylation of p42/44^{MAPK}. Furthermore, the differences in p42/44^{MAPK} phosphorylation were not due to variations in protein expression levels since p42^{MAPK} was present equally in all lanes (fig. 4B).

Collectively, these data demonstrate that in both serum- and EGF-activated cells p42/44^{MAPK} phosphorylation is mediated via the Raf-MEK pathway and through PKC or a (un)related kinase, that depends on the specificity of Ro31-8220. Furthermore, these data suggest that Ro31-8220- or staurosporine-, but not Gö6983-dependent PKC isoforms are involved in the activation of p42/44^{MAPK} in serum- or EGF-stimulated Her14 cells. Also no conventional or novel PKC isoforms are involved in the phosphorylation of p42/44^{MAPK} since down-regulation of these isozymes did not result in a reduced phosphorylation. Although staurosporine is not a specific PKC inhibitor, the results obtained were similar to those observed with the specific PKC inhibitor Ro31-8220, suggesting that staurosporine in Her14 cells can be used to inactivate PKC. Furthermore, in serum-stimulated cells the Raf-MEK pathway is more important while, in contrast, PKC or a (un)related kinase is more important in EGF-stimulated cells.

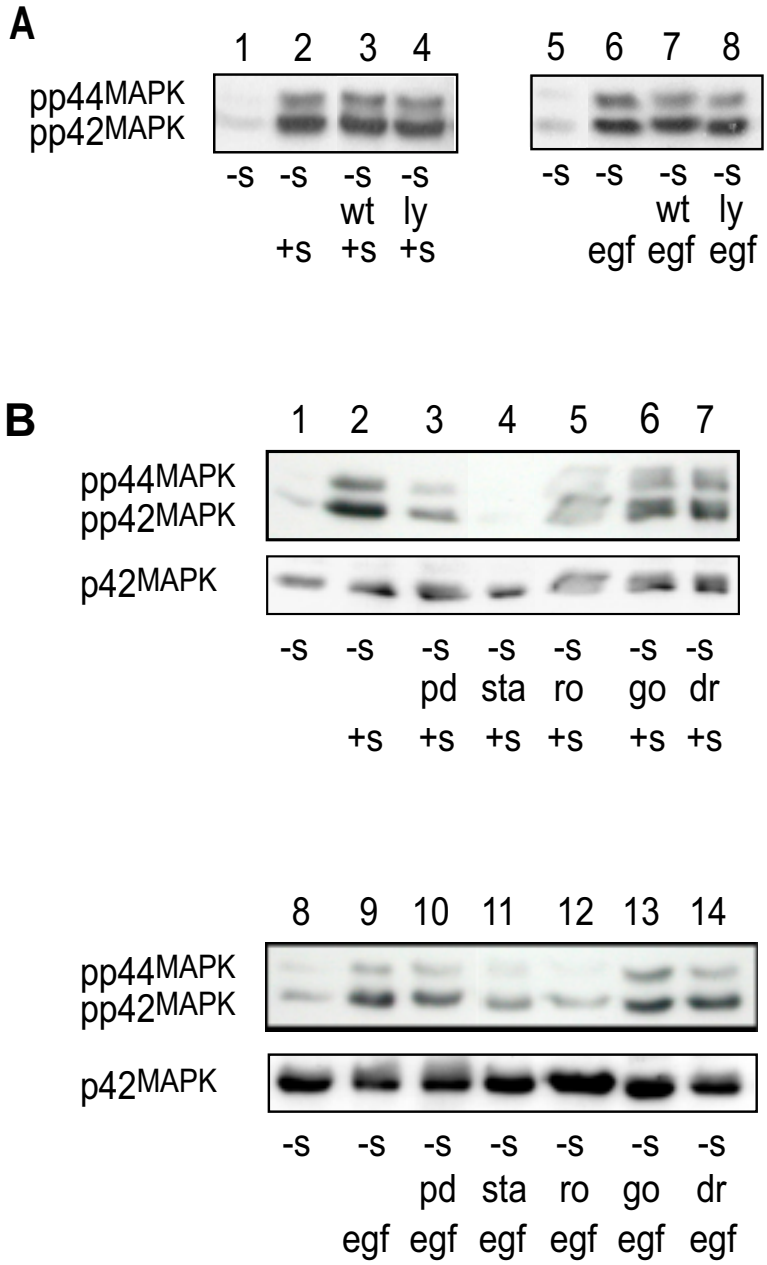

Figure 4. Phosphorylation of p42/44^{MAPK} in the presence of inhibitors for PI3K (A) and PKC in serum- (B, upper panel) and EGF-stimulated cells (B, lower panel). Quiescent cells (-s) were pre-treated for 30 min with wortmannin (wt), LY294002 (ly), staurosporine (sta), Ro31-8220 (ro), or Gö6983 (go), 1h with PD098059 (pd), or for 24h with PdBu (dr). Thereafter, the cells were stimulated with serum (+s) or EGF (egf) for 15 min and p42/44^{MAPK} phosphorylation and p42^{MAPK} expression (B) were detected on western blot. Results shown are representative of four independent experiments.

cPLA$_2$ activity is increased by p42/44^{MAPK} that is phosphorylated via Raf-MEK and through PKC.

The next objective was to investigate whether PKC and Raf-MEK are the two major pathways leading to p42/44^{MAPK} phosphorylation and subsequently to cPLA$_2$ activation. Figure 5A shows a relatively low phosphorylation of p42/44^{MAPK} in quiescent cells (lanes 1 and 7) that was strongly increased in serum- (lane 2) and EGF- stimulated cells (lane 8). As shown earlier a remarkable reduction in p42/44^{MAPK} phosphorylation was observed in quiescent cells treated with PD098059 prior to serum stimulation (lane 3) while only a small reduction was observed in EGF-activated cells (lane 9). However, co-treatment of quiescent cells with PD098059 plus Ro31-8220 either prior to serum (lane 4) or EGF (lane 10) stimulation resulted in a complete inhibition of p42/44^{MAPK} phosphorylation. The involvement of the Raf-MEK pathway and PKC in the phosphorylation of p42/44^{MAPK} was further investigated by down-regulation of PKC, followed by incubation for one hour with PD098059. This treatment did not result in an activation of p42/44^{MAPK} (lanes 5 and 11) indicating that with this treatment no other pathways are induced that activate p42/44^{MAPK}. Next, the phosphorylation of p42/44^{MAPK} in these cells stimulated with serum or EGF was analysed, still demonstrating the presence of some phosphorylated p42/44^{MAPK}, although to a lesser extent in serum- (lane 6) as compared to EGF-stimulated cells (lane 12). This remaining phosphorylation is most likely due to activated PKC, or a (un)related kinase, since these can not be down-regulated. Treatment of both PD098059 and staurosporine prior to stimulation did not result in a phosphorylation of p42/44^{MAPK}, demonstrating only the involvement of the Raf-MEK pathway and PKC, or a (un)related kinase, in serum- or EGF-induced p42/44^{MAPK} phosphorylation. However, PKC, or a (un)related kinase, seems to play a more important role in EGF- compared to serum-stimulated cells. These results are in accordance with the results shown in figure 4.

cPLA$_2$ activity was increased 1.8 in serum- (fig. 5B, column 2) and 2.1 fold in EGF-activated cells (column 8) as compared to the activity in quiescent cells (columns 1 and 7). Inhibition of the Raf-MEK pathway by pre-treating the cells with PD098059 resulted in a complete inhibition of cPLA$_2$ activity in serum- (column 3), but only to a partial inhibition in EGF-activated cells (column 9). Even a further decrease in activity was measured in PD098059 plus Ro31-8220 treated cells, which was more pronounced in EGF- (column 10) than in serum-stimulated cells (column 4). These results demonstrate that cPLA$_2$ activity is mediated via the Raf-MEK pathway and through PKC, or a (un)related kinase, in serum- and EGF-activated cells. Down-regulation of PKC followed by an incubation with PD098059, prior to serum (column 6) or EGF (column 12) stimulation, resulted in an activity of cPLA$_2$ which was found to be at the level that is present in quiescent (columns 1 and 7) and down-regulated plus PD098059 treated cells (column 5 and 11). However, the activity was higher as in PD098059 plus Ro31-8220 treated cells.

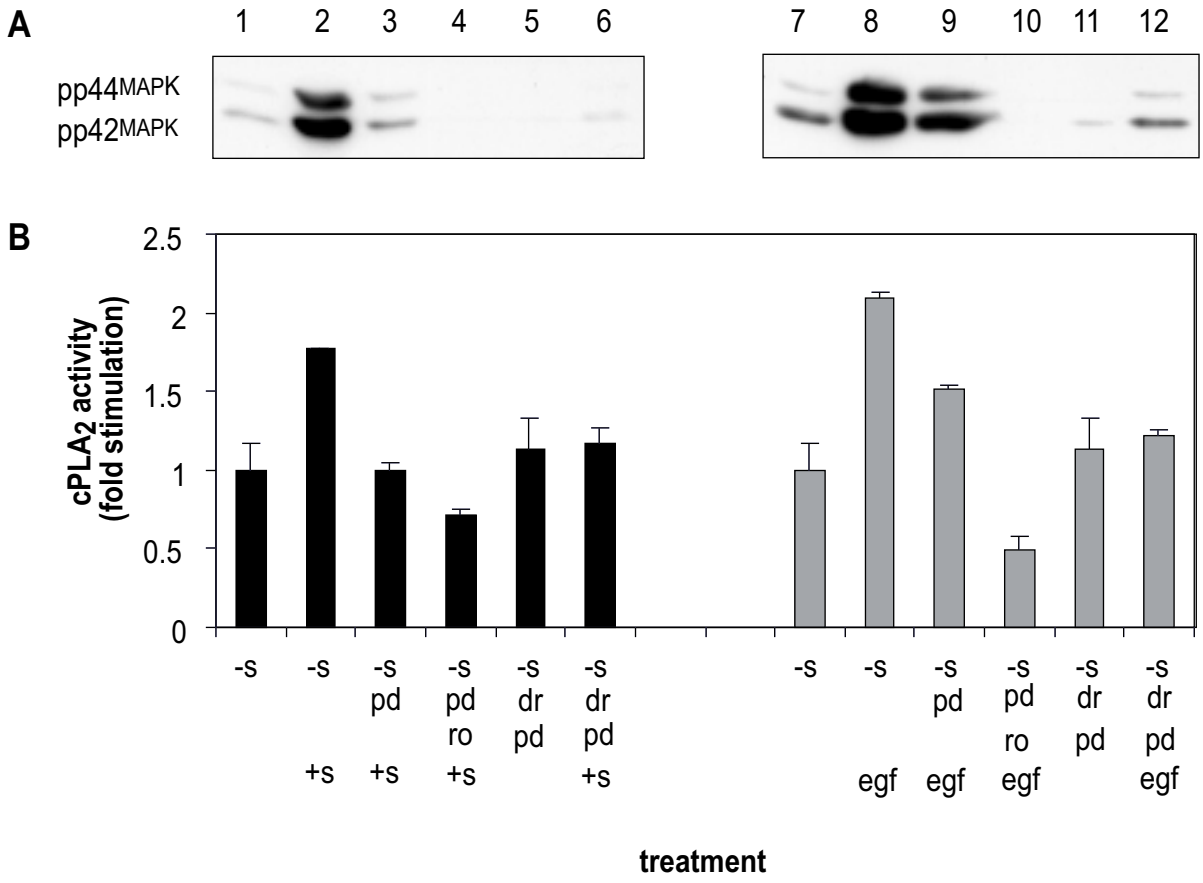

Figure 5. Inhibition of both PKC and the Raf-MEK pathway prohibits p42/44^{MAPK} phosphorylation, and decreases cPLA$_2$ activity at the same time in serum- and EGF-stimulated Her14 cells. p42/44^{MAPK} phosphorylation (A) and cPLA$_2$ activity (B) were determined of quiescent cells treated with vehicle (-s), pre-treated for 1h with PD098059 (pd), 1h PD098059 and 30 min Ro31-8220 (ro), or treated for 24h with PdBu (dr) followed by a 1h incubation with PD098059, and thereafter stimulated with either serum (+s) or EGF (egf). Results and S. D. for cPLA$_2$ activity are shown of two independent experiments.

Taken together cPLA$_2$ activity in serum-activated cells is regulated for the greater part via the Raf-MEK pathway and for a minor part through PKC, or a (un)related kinase, while in EGF-stimulated cells cPLA$_2$ is predominantly regulated via PKC, or a (un)related kinase, and to a much lesser extent through Raf-MEK. Correspondingly, p42/44^{MAPK} is activated via the same pathways as cPLA$_2$ is, thus demonstrating a relation between p42/44^{MAPK} phosphorylation in serum- and EGF-activated Her14 cells and cPLA$_2$ activity.

PKC activates p42/44^{MAPK} through MEK.

It has been reported that PKC is able to activate p42/44^{MAPK} "directly" (Grammer & Blenis, 1997), through MEK (Grammer & Blenis, 1997), or via Raf-MEK (Adomeit *et al.,* 1999; Sozeri *et al.,* 1992). The latter pathway does not occur in our cells since PD098059 did not inhibit serum- or EGF-induced p42/44^{MAPK} phosphorylation completely. In order to establish the pathway in which PKC, or a (un)related kinase, phosphorylates p42/44^{MAPK}, quiescent cells were treated either with PD098059 alone or treated with PD098059 and thereafter stimulated with serum or EGF. The phosphorylation of p42/44^{MAPK} was detected on western blot (fig. 6A), showing no phosphorylation in the presence of PD098059 (lane 4). In cells incubated with PD098059 prior to addition of serum ,some phosphorylation was present (lane 3) which was, as shown earlier, remarkably higher in EGF-stimulated cells (lane 5). Inhibiting MEK activity by incubating the cells with U0126 for 1h prior to stimulation resulted in a complete loss of phosphorylated p42/44^{MAPK}

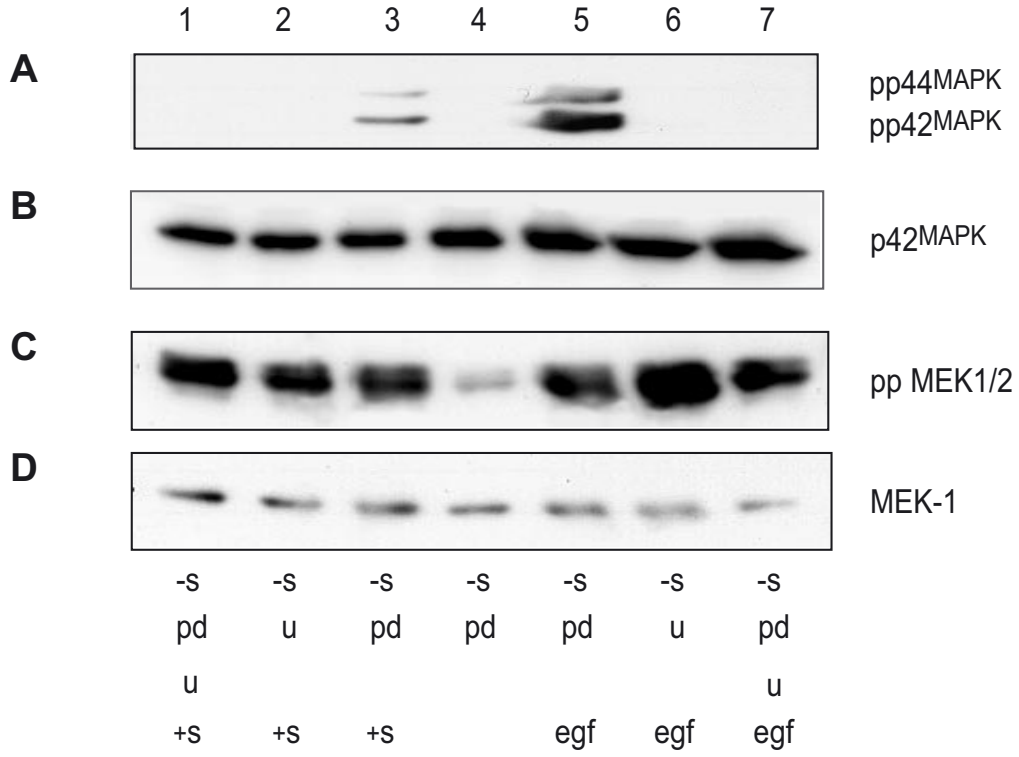

Figure 6. Effect of PD098059 and U0126 on the phosphorylation of p42/44^{MAPK} and MEK1/2 in serum- and EGF-activated cells. Quiescent cells were treated with PD098059 (pd), U0126 (u), or both for 1h and thereafter stimulated with serum or EGF. Cell lysates were separated on gel and (A) p42/44^{MAPK} phosphorylation, (B) p42^{MAPK} expression, (C) MEK1/2 phosphorylation and (D) MEK-1 expression were analysed on western blot as described in Materials and Methods. Results shown are representative of three independent experiments.

(fig. 6A, lanes 2 and 6). The same result was obtained in cells treated with both PD098059 plus U0126 before addition of serum or EGF (lanes 1 and 7, respectively). p42^{MAPK} expression on western blot showed no significant differences in the protein expression level (fig. 6B, lanes 1-7). These results demonstrate that PKC does not phosphorylate p42/44^{MAPK} directly since a combined treatment of PD098059 and U0126 prior to serum or EGF stimulation did not result in phosphorylation of p42/44^{MAPK}. In addition, MEK1/2 phosphorylation was detected on western blot using an antibody directed against phosphorylated MEK-1 and –2 (fig. 6C). The levels of MEK1/2 phosphorylation were comparable in serum- as well as in EGF-stimulated cells pre-treated with PD098059 (lanes 3 and 5, respectively), U0126 (lanes 2 and 6, respectively) and PD098059 plus U0126 (lanes 1 and 7, respectively). Furthermore, these levels were higher compared to quiescent cells treated with PD098059 alone (lane 4). MEK-1 protein expression levels were comparable in all samples (fig. 6D, lanes 1-7), showing that differences observed in MEK phosphorylation were not due to a change in the protein expression levels. These data demonstrate that PKC, or a (un)related kinase, phosphorylates p42/44^{MAPK} through MEK, since U0126 inhibits p42/44^{MAPK} but not MEK phosphorylation in serum- and EGF-activated cells.

Phorbol ester-induced PKC activation subsequently activates p42/44^{MAPK} but not cPLA$_2$.

Since PKC plays a role in the activation of p42/44^{MAPK} in serum- and EGF-stimulated cells that subsequently results in cPLA$_2$ activity, we explored whether the same results could be obtained when PKC was activated directly with phorbol ester. Therefore, quiescent Her14 cells were incubated with PdBu for 15 min resulting in a strong increase in the phosphorylation of p42/44^{MAPK} on western blot (fig. 7A lane 2) compared to non-treated cells (lane 1). Pre-incubation of the cells with PD098059 caused a strong decrease in PdBu-induced p42/44^{MAPK} phosphorylation (lane 3), which was completely inhibited upon U0126 treatment (lane 4). Next, the PdBu activation of PKC was inhibited by a pre-treatment of the cells for 30 min with the PKC inhibitors staurosporine, Ro31-8220, Gö6983 (PKCα, β_I, β_{II}, δ and ζ) or Gö6976 (PKCα, β_I, and μ) (Wenzel-Seifert *et al.,* 1994). Figure 7A shows a complete inhibition of p42/44^{MAPK} phosphorylation in cells pre-treated with staurosporine, Ro31-8220 and Gö6983 (lanes 5, 6 and 7, respectively) but a partial decrease in phosphorylation upon Gö6976 incubation (lane 8). These data indicate that activation of PKC by PdBu results in the phosphorylation of p42/44^{MAPK} which is partially mediated through Raf-MEK and partially proceeds through MEK. Furthermore, this activation of p42/44^{MAPK} is mediated through Gö6983 and partially through Gö6976 dependent PKC isoforms.

In order to correlate PdBu-induced p42/44^{MAPK} phosphorylation with cPLA$_2$ activity, the activity was measured in homogenates of quiescent cells and cells that were stimulated afterwards with PdBu. Hardly any increase in cPLA$_2$ activity was observed in stimulated (fig. 7B column 2) compared to unstimulated cells (fig. 7B column 1). Addition of PdBu to cells treated with PD098059 did not result in a decrease in cPLA$_2$ activity (fig. 7B column 3) and a slight decrease to basal level in U0126 treated cells (fig. 7B column 4). Also no significant decreases in cPLA$_2$ activity were observed in cells incubated with the different PKC inhibitors prior to stimulation with PdBu (fig. 7B columns 5-8), except for a slight decrease in activity in the presence of Ro31-8220. In conclusion, PdBu-induced PKC activation results in the phosphorylation of p42/44^{MAPK} that partially proceeds through MEK and via Raf-MEK but which does not lead to cPLA$_2$ activation.

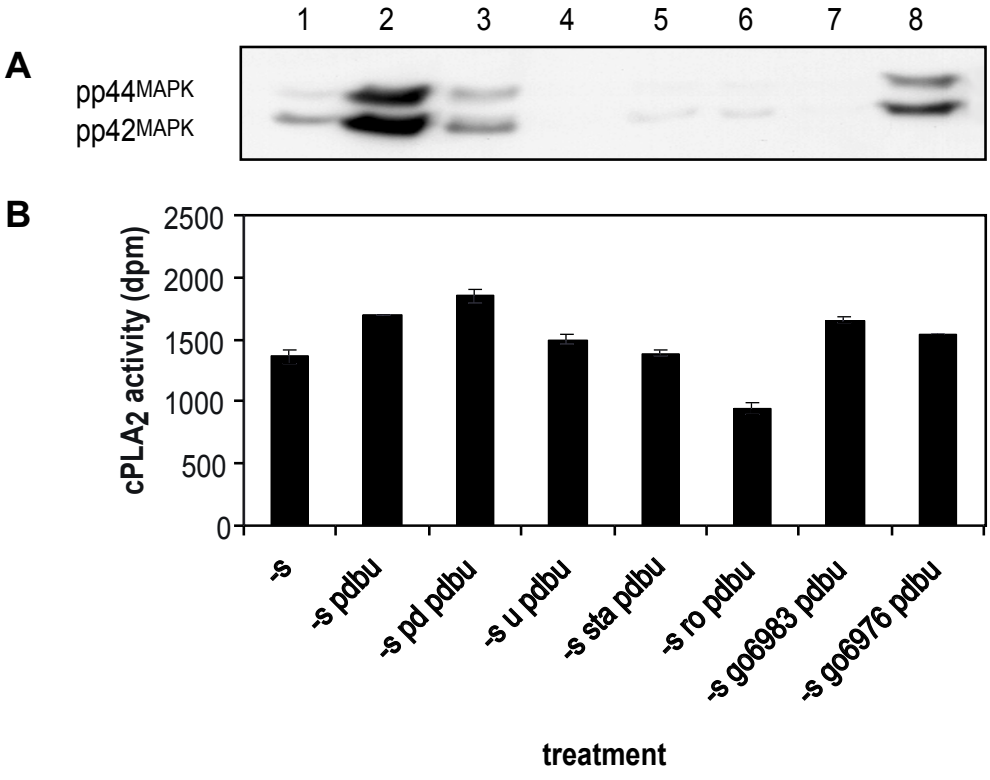

Figure 7. Phorbol ester-induced activation of p42/44^{MAPK} and cPLA$_2$. Quiescent cells (-s) were pre-treated for 1h with PD098059 (pd) or U0126 (u), or for 30 min with staurosporine (sta), Ro31-8220 (ro), Gö6983 (go6983), or Gö6976 (go6976). Thereafter, the cells were stimulated with PdBu (pdbu) for 15 min. p42/44^{MAPK} phosphorylation was analysed on western blot (A) and cPLA$_2$ activity was measured (B) as described in Materials and Methods. Results shown are representative of two independent experiments with S. D. shown for cPLA$_2$ activity.

Discussion

We show here that p42/44^{MAPK} in both quiescent and proliferating cells is predominantly phosphorylated via the Raf-MEK pathway since PD098059 treatment almost completely inhibited this phosphorylation (fig. 1A, C). However, the p42/44^{MAPK} phosphorylation only corresponded with cPLA$_2$ activity in quiescent cells. PD098059 and U0126 did not reduce cPLA$_2$ activity in proliferating cells, suggesting that other signal transduction cascades regulate cPLA$_2$ activity in these cells. For instance, p38^{MAPK} has been shown to activate cPLA$_2$ in platelets, (Kramer, *et al.,* 1996; Hefner, *et al.,* 2000) and c-Jun NH_2-terminal kinase has been implicated to activate cPLA$_2$ in astrocytes (Hernández *et al.,* 1999).

cPLA$_2$ activity was found to be slightly higher in quiescent than in proliferating cells. This is surprising since one would expect the activity in proliferating cells to be higher. All the more because p42/44^{MAPK} was phosphorylated to a higher extent in proliferating cells. This might imply that only a very small fraction of the phosphorylated p42/44^{MAPK} accounts for the cPLA$_2$ activation as measured in quiescent cells. In addition, it has been shown *in vitro* that cPLA$_2$ losses its catalytic activity prematurely when acting on vesicles containing phospholipid substrate (Witmer *et al.,* 1995; Bayburt & Gelb, 1997; Burke *et al.,* 1999). Although, the precise mechanism for this inactivation is not yet established it might explain why cPLA$_2$ activity is lower in proliferating than in quiescent cells. Furthermore, in quiescent cells treated with U0126, cPLA$_2$ was found to be still active although p42/44^{MAPK} phosphorylation was not detected anymore. This remainder is the, so called, basal activity of cPLA$_2$ because treatment with alkaline phosphatase did not reduce this activity (Schalkwijk, *et al.,* 1995).

In contrast to the activation of p42/44^{MAPK} in quiescent and proliferating cells, p42/44^{MAPK} is not solely phosphorylated via the Raf-MEK pathway in EGF- and serum-stimulated Her14 cells. This is because PD098059 pre-treatment did not completely inhibit p42/44^{MAPK} phosphorylation in serum-activated cells, and did not seem to have a big effect at all in EGF-stimulated cells (fig. 3). However, PD098059 treatment does result in the inhibition of the Raf-MEK pathway since p42/44^{MAPK} phosphorylation in serum-activated cells was decreased. In addition, it has been published that p42/44^{MAPK} phosphorylation is almost completely inhibited with PD098059 in hydrogen peroxide-stimulated Her14 cells (van Rossum *et al.,* 1999), indicating that no other signal transduction cascades are influenced by this inhibitor. In addition, p38^{MAPK} phosphorylation was not increased in either EGF- or serum-stimulated cells as compared to unstimulated cells. cPLA$_2$ activity in cells treated with the p38^{MAPK} inhibitor, SB203580, before EGF or serum stimulation did not result in a significant reduction in cPLA$_2$ activity compared to control cells (data not shown). Although, this indicates that p38^{MAPK} in Her14 cells is not involved in cPLA$_2$

activation, an involvement of other p38^{MAPK} isoforms can not be excluded due to the specificity of SB203580 (Cohen, 1997).

By the use of different inhibitors we have investigated whether known signal transduction pathways including PI3K and PKC are involved in serum- or EGF-activated p42/44^{MAPK} and cPLA$_2$ activity in Her14 cells. PI3K did not seem to play a significant role in the phosphorylation of p42/44^{MAPK} in serum- or EGF-stimulated cells since wortmannin and LY294002 did not reduce p42/44^{MAPK} phosphorylation (fig. 4A lanes 3, 4 and 7, 8). Also Conway *et al.* (1999) showed that PI3K plays a minor role in EGF-activated p42/44^{MAPK} phosphorylation in airway smooth muscle cells while in PDGF-stimulated Swiss 3T3 fibroblasts PI3K plays a role in both the early and the late response (Grammer & Blenis, 1997).

The data presented here show that PKC, or a (un)related kinase, depending on the specificity of Ro31-8220, plays a more important role in EGF- as in serum-induced p42/44^{MAPK} phosphorylation. This is supported by the findings that treatment with the common PKC inhibitors Ro31-8220 and staurosporine resulted in a severe inhibition of p42/44^{MAPK} phosphorylation. In addition, Gö6983 dependent and phorbol ester sensitive PKCs were not involved, since Gö6983 treatment and PKC down-regulation did not reduce p42/44^{MAPK} phosphorylation (fig. 4). However, Gö6983 only inhibit a few PKC isoforms in contrast to Ro31-8220 and staurosporine, while prolonged phorbol ester treatment results in the down-regulation of the conventional and novel PKC isotypes, leaving the atypical PKC isotypes unaffected (Huwiler *et al.,* 1992). In accordance with this is the presence of phosphorylated p42/44^{MAPK} in PKC down-regulated and PD098059 treated cells which is a little elevated in EGF-activated compared to serum-stimulated cells. Furthermore, the involvement of PKC, or a (un)related kinase, and the Raf-MEK pathway was also confirmed by treatment of the cells with both PD098059 and Ro31-8220 prior to stimulation.

We show that PKC, or a (un)related kinase, phosphorylates p42/44^{MAPK} through MEK, since PD098059 did not completely reduce the phosphorylation in contrast to U0126 (fig. 6). However, we cannot exclude the possibility that some part of the PKC signal proceeds via Raf-MEK which has been previously reported (Kolch *et al.,* 1993; van Dijk *et al.,* 1997). Moreover, activation of p42/44^{MAPK} by PKC, or a (un)related kinase, independent of either Raf or MEK was excluded because pre-incubation of the cells with PD098059 and U0126 did not result in p42/44^{MAPK} phosphorylation. Although, MEK was phosphorylated and present to a similar level as in PD098059 and U0126 treated cells prior to serum or EGF stimulation. Activation of p42/44^{MAPK} through PKC-MEK has also been observed in Swiss 3T3 fibroblasts stimulated with PDGF (Grammer & Blenis, 1997; Berra *et al.,* 1995), but this activation pathway leads to the prolonged activation of p42/44^{MAPK} while in the initial phase p42/44^{MAPK} could be activated by PKC, independent of Raf or MEK. This is not the case in Her14 fibroblasts, as inhibition of either PKC or a (un)related

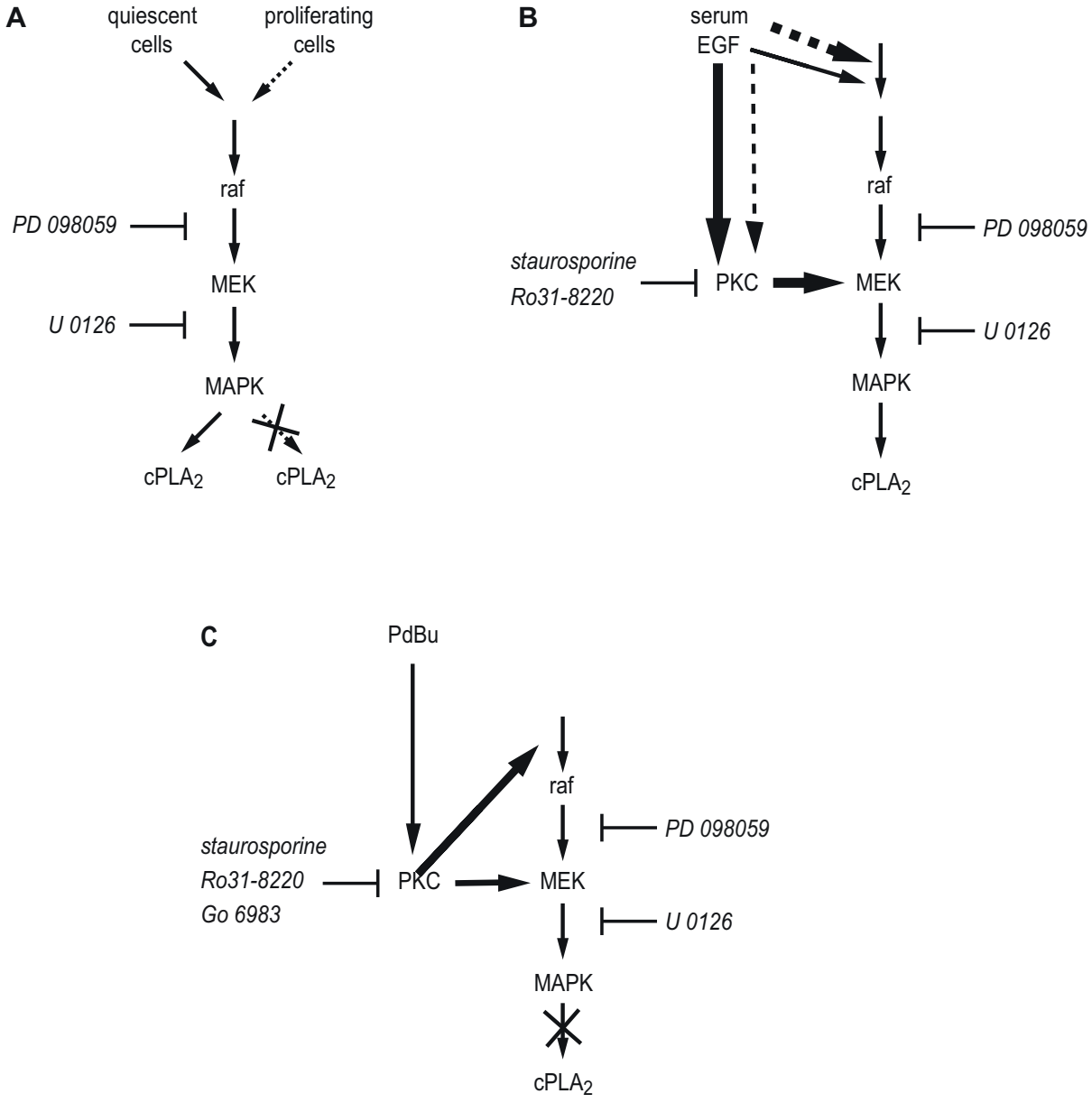

Figure 8. Models summarising the results in this paper for (A) quiescent (solid line) and proliferating (dashed line), (B) EGF- (solid line) and serum- (dashed line) and (C) PdBu-regulated p42/44^{MAPK} and cPLA$_2$ activation in Her14 fibroblasts. (A) Activation of p42/44^{MAPK} in quiescent and proliferating cells proceeds through Raf-MEK leading to cPLA$_2$ activation in the quiescent but not in the proliferating cells. (B) EGF-induced cPLA$_2$ activity is mediated through two pathways: predominantly through PKC-MEK-MAPK and less via Raf-MEK-MAPK while in serum-stimulated cells the signal is for the greater part transduced through Raf-MEK-MAPK and the smaller part via PKC-MEK-MAPK both leading to cPLA$_2$ activity. (C) PdBu-induced PKC activation leads to p42/44^{MAPK} phosphorylation that is partially mediated via MEK and through Raf-MEK but this does not result in cPLA$_2$ activation. italic: effective inhibitors

kinase by Ro31-8220 or staurosporine or the Raf-MEK pathway by PD098059 prior to stimulation for different periods of time did not result in a differential phosphorylation of p42/44^{MAPK} (data not shown). These data suggests that both pathways are activated at the same time and converge to MEK to fully activate p42/44^{MAPK} (see model in fig. 8). In conclusion, the data clearly demonstrate that serum-induced p42/44^{MAPK} phosphorylation is mediated through PKC, or a (un)related kinase, but the majority of the signal proceeds through the Raf-MEK pathway. Both PKC, or a (un)related kinase, and the Raf-MEK pathway are involved in EGF-induced p42/44^{MAPK} phosphorylation, although PKC plays a more important role than the Raf-MEK pathway.

In contrast to serum- or EGF-induced p42/44^{MAPK} phosphorylation, which involves the activation of PKC or a (un)related kinase, direct activation of PKC by phorbol ester (PdBu) incubation resulted in yet another pathway. Direct activation of PKC resulted clearly in the phosphorylation of p42/44^{MAPK} through Raf-MEK and via MEK as shown by the partial inhibition in the presence of PD098059 and a complete inhibition in the presence of U0126 (fig. 7). Moreover, other PKC isoforms are involved in PdBu-stimulated compared to serum- and EGF-activated cells, as Gö6983 completely abolished p42/44^{MAPK} phosphorylation in PdBu-activated cells while it did not had any effect upon serum or EGF addition.

As shown in the models (fig. 8), p42/44^{MAPK} is differentially activated in serum- and EGF- as well as in PdBu-stimulated cells, but also compared to quiescent and proliferating cells. The presence and the level of phosphorylated p42/44^{MAPK} in serum- or EGF-activated cells corresponds with the activity of cPLA$_2$ (fig. 5) as decreases in the level of phosphorylated p42/44^{MAPK} by inhibiting the Raf-MEK, PKC-MEK or both pathways results in a decrease in cPLA$_2$ activity. The data show that in cells stimulated with serum, in which the Raf-MEK pathway plays a more important role than PKC, or a (un)related kinase, in the phosphorylation of p42/44^{MAPK}, cPLA$_2$ activity was reduced in a similar manner. EGF stimulation proceeds for the greater part through PKC, or a (un)related kinase, instead of Raf-MEK, which is observed in the reduction of p42/44^{MAPK} phosphorylation but also in the subsequent cPLA$_2$ activity. While p42/44^{MAPK} is activated via the same pathway only cPLA$_2$ activity corresponds with the presence of phosphorylated p42/44^{MAPK} in quiescent but not in proliferating cells. Additionally the presence of p42/44^{MAPK} phosphorylation and cPLA$_2$ activity were also not related in cells stimulated with PdBu (fig. 7). However, in cat iris sphincter smooth muscle cells PdBu is able to induce an increase in arachidonic acid, which is mediated by cPLA$_2$ (Husain & Abdel-Latif, 1998).

Collectively, these results imply the existence of different pools of p42/44^{MAPK} that have to be phosphorylated at the right time and location to activate cPLA$_2$, unless U0126 is not that specific in preventing p42/44^{MAPK}. The existence of different pools has been suggested for cPLA$_2$. cPLA$_2$ in Her14 fibroblasts was reported to be localised in clusters

near the vicinity of all organellar membranes except for the golgi apparatus and the nucleus (Bunt *et al.,* 1997). Subsequently, it was shown that these clusters represent the inactive form of cPLA$_2$ from which active monomers can be recruited which might occur via phosphorylation by p42/44^{MAPK} (Bunt *et al.,* 2000). Also p42/44^{MAPK} phosphorylation has been reported to occur at different locations induced by different agonists. In EGF-activated cells the EGF receptor was still active when internalised to endosomes (Lai *et al.,* 1989; Wada *et al.,* 1992), suggesting that the active receptor may continue transducing the signal. This has been further stated by the compartimentalisation of phosphorylated EGF receptors and a population of Shc proteins (Baass *et al.,* 1995), and the presence of active Raf-1 and MEK in the early endosomes of hepatocytes (Pol *et al.,* 1998). However, no such mechanism for signal transduction was observed in insulin-induced activation of its receptor in liver parenchyma (Di Guglielmo *et al.,* 1994). In conclusion, p42/44^{MAPK} in quiescent or proliferating cells are differentially activated as in cells stimulated either with serum, EGF or PdBu probably because different pools of p42/44^{MAPK} and cPLA$_2$ are activated.

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Activation of $cPLA_2$ in fibroblasts by H_2O_2: a $p42/44^{MAPK}$ dependent and a phosphorylation-independent mechanism.

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(Submitted)

Abstract

Reactive oxygen species are involved in various cellular processes and diseases. It has been suggested that reactive oxygen species function as mediators of signal transduction, since they can mimic growth factor-induced signalling. H_2O_2 has been reported to activate phospholipase A_2 and hence we investigated the pathway by which reactive oxygen species regulates cytosolic phospholipase A_2 ($cPLA_2$) activity in Her14 fibroblasts. $cPLA_2$ was activated by various concentrations of H_2O_2 in a rapid and transient manner. Also cumene hydroperoxide induced $cPLA_2$ activity. H_2O_2 stimulation of Her14 cells resulted in a partial phosphorylation of $cPLA_2$, which was mediated through the Raf-MEK-p42/44^{MAPK} pathway. Besides, $cPLA_2$ was partially activated through a phosphorylation-independent mechanism. Translocation of $cPLA_2$ to the membrane fraction occurred in H_2O_2-stimulated cells, with a concomitant increase in $cPLA_2$ activity which could not be decreased by removing calcium. Since $cPLA_2$ phosphorylation has to precede its calcium-dependent translocation to membranes, and since H_2O_2 can peroxidise phospholipids resulting in enhanced $cPLA_2$ activity, we suggest that the phosphorylation-independent increase in $cPLA_2$ activity might be due to peroxidation of phospholipids.

Introduction

Most mammalian cells generate reactive oxygen species (ROS) upon stimulation with various ligands, including cytokines, growth factors and hormones (Lo & Cruz, 1995; Sundaresan *et al.,* 1995; Meier *et al.,* 1989). ROS comprises a variety of oxygen free radicals, including superoxide anions, hydroxyl radicals, hydrogen peroxide and nitric oxide. ROS can lead to oxidative stress that has been implicated in cellular processes, such as proliferation (Rao & Berk, 1992; Irani *et al.,* 1997), apoptosis (Buttke & Sandstrom, 1994; Jacobson, 1996; Polyak *et al.,* 1997), but also cellular injury (Chen *et al.,* 1996; Sapirstein *et al.,* 1996) and diseases, such as atherosclerosis and neuronal degenerative diseases (Crawford & Blankenhorn, 1991; Schubert *et al.,* 1995). It has been suggested that ROS may function as mediators of signal transduction processes (Sundaresan, *et al.,* 1995; Chen *et al.,* 1995). Additionally, exogenous administration of hydrogen peroxide (H_2O_2) induced phosphorylation of the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptor (Miller *et al.,* 1994; Gamou & Shimizu, 1995), suggesting that oxygen free radicals mimic growth factor-induced signalling events. Indeed H_2O_2-induced tyrosine phosphorylation of the EGF receptor resulted in complex formation with the signalling molecules Shc, Grb2 and sos followed by ras activation in vascular smooth

muscle cells (VSMC) (Rao, 1996). Although H_2O_2 was shown to inhibit EGF receptor internalisation and ubiquitination (de Wit *et al.,* 2001), this does not necessarily contradict a role of ROS in growth factor-mediated signalling. Furthermore, oxidative stress was shown to induce Raf-1 activation (Kasid *et al.,* 1996; Abe *et al.,* 1998) and activation of p42/44^{MAPK} as well as the c-Jun NH_2-terminal kinase (JNK) (Stevenson *et al.,* 1994; Sundaresan, *et al.,* 1995; Guyton *et al.,* 1996), p38^{MAPK} (Buschbeck *et al.,* 1999) and PKC family members (Konishi *et al.,* 1997). ROS were also able to increase the expression of cyclin D and the immediate early genes c-fos and c-jun (Naveilhan *et al.,* 1994; Martínez-Muñoz *et al.,* 2001). It has been suggested that phospholipase A_2 (PLA_2) could be involved in these events, since a non-specific PLA_2 inhibitor, mepacrine, blocked H_2O_2-induced c-fos and c-jun mRNA expression in VSMC (Rao *et al.,* 1993; Rao *et al.,* 1993). Also, PLA_2 and ROS have been implicated in cellular injury (Weinberg, 1991; Bonventre, 1993). Moreover, *in vitro* studies showed that the presence of peroxidated phospholipids in substrate vesicles enhanced PLA_2 activity and the subsequent release of arachidonic acid (Rashba-Step *et al.,* 1997; Chaitidis *et al.,* 1998).

The 85-kDa cytosolic PLA_2 ($cPLA_2$) preferentially generates the oxidant sensitive arachidonic acid from membrane phospholipids and both calcium and phosphorylation regulate its activity. Submicromolar concentrations of calcium are required for $cPLA_2$ to translocate from the cytosol to the membrane, where its substrate is located, and this translocation is mediated by its calcium-dependent phospholipid binding domain (Nalefski *et al.,* 1994). Phosphorylation of $cPLA_2$ has been shown to occur through p42/44^{MAPK} (Lin *et al.,* 1993; Qiu & Leslie, 1994; van Rossum *et al.,* 2001), p38^{MAPK} (Kramer *et al.,* 1996; Börsch-Haubold *et al.,* 1998) and JNK (Hernández *et al.,* 1999; van Putten *et al.,* 2001). Moreover, $cPLA_2$ is maximally activated when it is first phosphorylated followed by a calcium-dependent translocation to membranes (Abdullah *et al.,* 1995; Schalkwijk *et al.,* 1996). The purpose of the present study was, to examine whether H_2O_2 is able to activate $cPLA_2$ in Her14 fibroblasts and, more importantly, to determine the pathway through which $cPLA_2$ is activated. It is demonstrated that $cPLA_2$ is activated by various concentrations of H_2O_2 and cumene hydroperoxide. H_2O_2 induced a transient activation of $cPLA_2$. $cPLA_2$ was partially activated by phosphorylation through the Raf-MEK-p42/44^{MAPK} pathway, and partially through a phosphorylation-independent mechanism. H_2O_2 can peroxidise phospholipids thereby enhancing $cPLA_2$ activity, that might be responsible for the phosphorylation-independent increase in $cPLA_2$ activity, since $cPLA_2$ translocated to the membrane fraction under calcium free conditions, which resulted in an increased $cPLA_2$ activity.

Materials and Methods

Materials.

Tissue culture nutrients, Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were purchased from Gibco BRL (Scotland). PD098059 and U0126 were obtained from Biomol (Playmouth meeting, USA) and Promega (Madison, USA), respectively. Protifar was from Nutricia (Zoetermeer, The Netherlands) and alkaline phosphatase was from Roche diagnostics (Mannheim, Germany). Hydrogen peroxide, cumene hydroperoxide and dipalmitoylglycerol were purchased from Sigma (Steinheim, Germany). 1-Stearoyl-2-[1-^{14}C]arachidonoyl-glycero-3-phosphocholine was obtained from Amersham (Buckinghamshire, England). All other chemicals were either from Sigma or Merck (Darmstadt, Germany).

Cell culture and stimulation.

Her14 cells, which are mouse NIH3T3(0) fibroblasts transfected with the human EGF-receptor cDNA, were grown in DMEM supplemented with 7.5% FBS. Cells were maintained at 37°C in a humidified atmosphere. Cells were grown to a confluency of 30,000 cells/cm^2, whereafter they were serum-starved overnight. After washing the cells twice with phosphate buffered saline (PBS), the cells were incubated for the indicated periods in PBS supplemented with 5 mM glucose in the absence or presence of the indicated concentrations hydrogen peroxide (H_2O_2) at 37°C. The same procedure was performed for cells stimulated with cumene hydroperoxide. When appropriate, the cells were treated with PD098059 (50 μM) or U0126 (50 μM) 1 h prior to stimulation to inhibit MEK activity.

Cell lysis and cell fractionation.

After stimulation, the cells were washed twice with ice-cold PBS whereafter the cells were scraped in homogenisation buffer (50 mM Hepes/NaOH pH 7.4, 0.25 M sucrose, 50 mM NaF, 250 μM Na_3VO_4, 1 mM EGTA, 10 μM leupeptin, 1 μM pepstatin and 1 mM PMSF). The cells were homogenised by 15 strokes through a 26G-needle and sonicated for 3 times 10 seconds. The amount of protein was measured according to Bradford (1976) using a Bio Rad novapath^{TM} microplate reader. Alternatively, the homogenate was centrifuged at 200,000xg for 30 min at 4°C to obtain the membrane fraction and the cytosolic fraction.

Western blot analysis.

Cell homogenates (10 μg protein) were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane by semi-dry blotting using a BioRad trans-blot SD. The membrane was blocked for 1h at RT with 2% milkpowder in PBST (PBS

containing 0.1% (v/v) Tween-20), followed by primary antibody incubations for 1h at RT in 0.2% milkpowder in PBST. Phosphorylated p42/44^{MAPK} was detected with a phospho-p42/44^{MAPK} rabbit polyclonal antibody at a dilution of 1:1000 (New England Biolabs Incorporated, Beverly, MA). ERK2 (0.5 μg/ml) and cPLA$_2$ (0.2 μg/ml) were detected with a monoclonal antibody from Transduction Laboratories (Lexington, KY) or from Santa Cruz Biotechnology (Heidelberg, Germany). Then, the membrane was washed and primary antibodies were detected with goat anti-rabbit or rabbit anti-mouse IgG conjugated to horseradish peroxidase and the bands were visualised with enhanced chemiluminescence (NEN^{TM}, Life science products, Boston).

cPLA$_2$ activity assay.

cPLA$_2$ activity was measured by the release of radiolabeled arachidonic acid from the *sn*-2 position of 1-stearoyl-2-[1-^{14}C]arachidonoyl-glycero-3-phosphocholine as described previously (Schalkwijk, *et al.,* 1996). The radiolabeled phosphatidylcholine was dried under N_2 together with dipalmitoylglycerol and then dispersed in water by sonification for 4 times 15 s under nitrogen to give final concentrations of 4 and 2 μM, respectively. The assay incubation mixture contained 0.2 M Tris pH 8.5, 1 mM $CaCl_2$, 5 mM DTT and 10 μg cell homogenate in a total volume of 200 μl. After incubation for 7-10 min at 37°C the released radiolabeled arachidonic acid was extracted by a modified Dole extraction procedure (van den Bosch *et al.,* 1974) and the radioactivity was determined in a scintillation counter (Tri-Carb 15000, Packard, Meriden, USA). In this way only the activity of cPLA$_2$ was measured (Spaargaren *et al.,* 1992; Atsumi *et al.,* 1998) and always less than 5% of the substrate was hydrolysed. Measuring the activity of non-phosphorylated cPLA$_2$ was performed as described previously (Schalkwijk *et al.,* 1995) by addition of 10 units alkaline phosphatase to an assay mixture containing 10 μg homogenate, 1 mM $MgCl_2$, 0.25 M Tris pH 8.5, 1.25 mM $CaCl_2$ and 6.25 mM DTT in a final volume of 160 μl. After a 20 min incubation at 37°C, 40 μl substrate was added and the cPLA$_2$ activity assay was performed as described above.

Results

H$_2$O$_2$ induces activation of cPLA$_2$.

In order to investigate whether cPLA$_2$ is activated by oxidative stress, quiescent Her14 cells were stimulated with different concentrations H_2O_2 for 10 min. The cells were subsequently harvested and cPLA$_2$ activity was measured as described in Materials and Methods. Figure 1A shows that H_2O_2 is able to increase cPLA$_2$ activity dose dependently, 1.6-2 fold, being maximal around 2 mM H_2O_2. The effect of H_2O_2 on cell integrity was

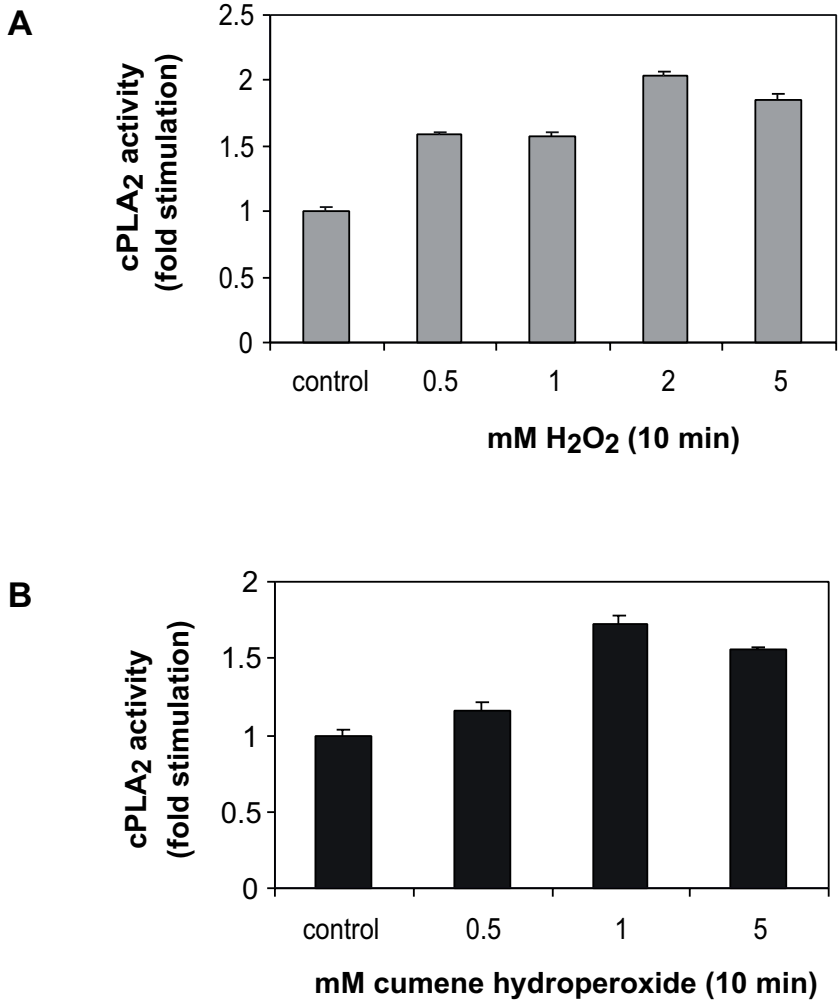

Figure 1. Concentration-dependent activation of $cPLA_2$ by (A) H_2O_2 and (B) cumene hydroperoxide. Quiescent Her14 cells were stimulated for 10 min with different concentrations of (A) H_2O_2 or (B) cumene hydroperoxide. Thereafter, the cells were harvested and $cPLA_2$ activity was measured as described in Materials and Methods. A representative experiment ± S. D. are shown of, respectively, 3 and 2 independent experiments.

determined by the release of lactate dehydrogenase (LDH). Exposure of Her14 cells with increasing concentrations of H_2O_2 up to 10 mM did not result in a significant release of LDH as compared to control cells which was shown previously (de Wit *et al.,* 2000). Next, we established whether $cPLA_2$ can be activated by another oxidant, which has different molecular and physicochemical properties than H_2O_2. Therefore, quiescent Her14 cells were treated with different concentrations of cumene hydroperoxide for 10 min. This resulted in a similar activation pattern of $cPLA_2$ to that observed with H_2O_2, being dose dependent and maximally activated around 1 mM (fig. 1B).

Subsequently, the time course of cPLA$_2$ activation induced by 1 mM H_2O_2 was examined. H_2O_2 induced a rapid activation of cPLA$_2$ within 5 min after stimulation, which appears to be transient, reaching near basal levels again after 60 min of H_2O_2 stimulation (fig. 2). These data show that cPLA$_2$ in Her14 fibroblasts was transiently activated by different concentrations of oxidative stress and in a time-dependent manner.

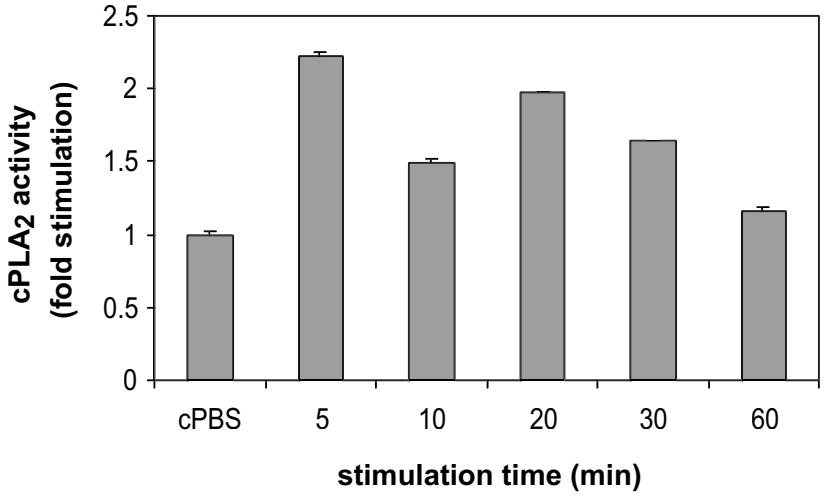

Figure 2. Time course of H_2O_2-stimulated Her14 cells. Quiescent Her14 cells were stimulated for the indicated times with 1 mM H_2O_2 whereafter, the cells were harvested and cPLA$_2$ activity was measured as described in Materials and Methods. Data are shown as means ± S. D. (n=2).

Regulation of cPLA$_2$ activity in H_2O_2-stimulated Her14 fibroblasts.

We recently showed in Her14 cells stimulated with EGF or serum that p42/44^{MAPK} was activated via Raf-MEK and through PKC-MEK, subsequently leading to cPLA$_2$ activation (van Rossum *et al.,* 2001). H_2O_2 has also been demonstrated to induce activation of p42/44^{MAPK} (Rao, 1996; Abe, *et al.,* 1998; de Wit *et al.,* 1998) and hence we investigated the possible involvement of p42/44^{MAPK} in H_2O_2-induced cPLA$_2$ activation. Figure 3A (left columns) shows in quiescent cells treated for 5 and 30 min with H_2O_2 an increase in cPLA$_2$ activity of about 2 and 1.5 fold respectively, as compared to control cells, which is in agreement with the results shown in figure 2. H_2O_2 stimulation for 5 and 30 min of cells pre-treated for 1h with the MAPK kinase (MEK) inhibitor PD098059 resulted in an inhibition of cPLA$_2$ activity (fig. 3A, middle columns) to the level present in quiescent cells. PD098059 binds directly to the non-phosphorylated isoforms of MEK (MEK-1 and –2), thereby preventing their Raf-mediated activation (Dudley *et al.,* 1995; Favata *et al.,* 1998). However, cPLA$_2$ activity was even further decreased in quiescent cells incubated with

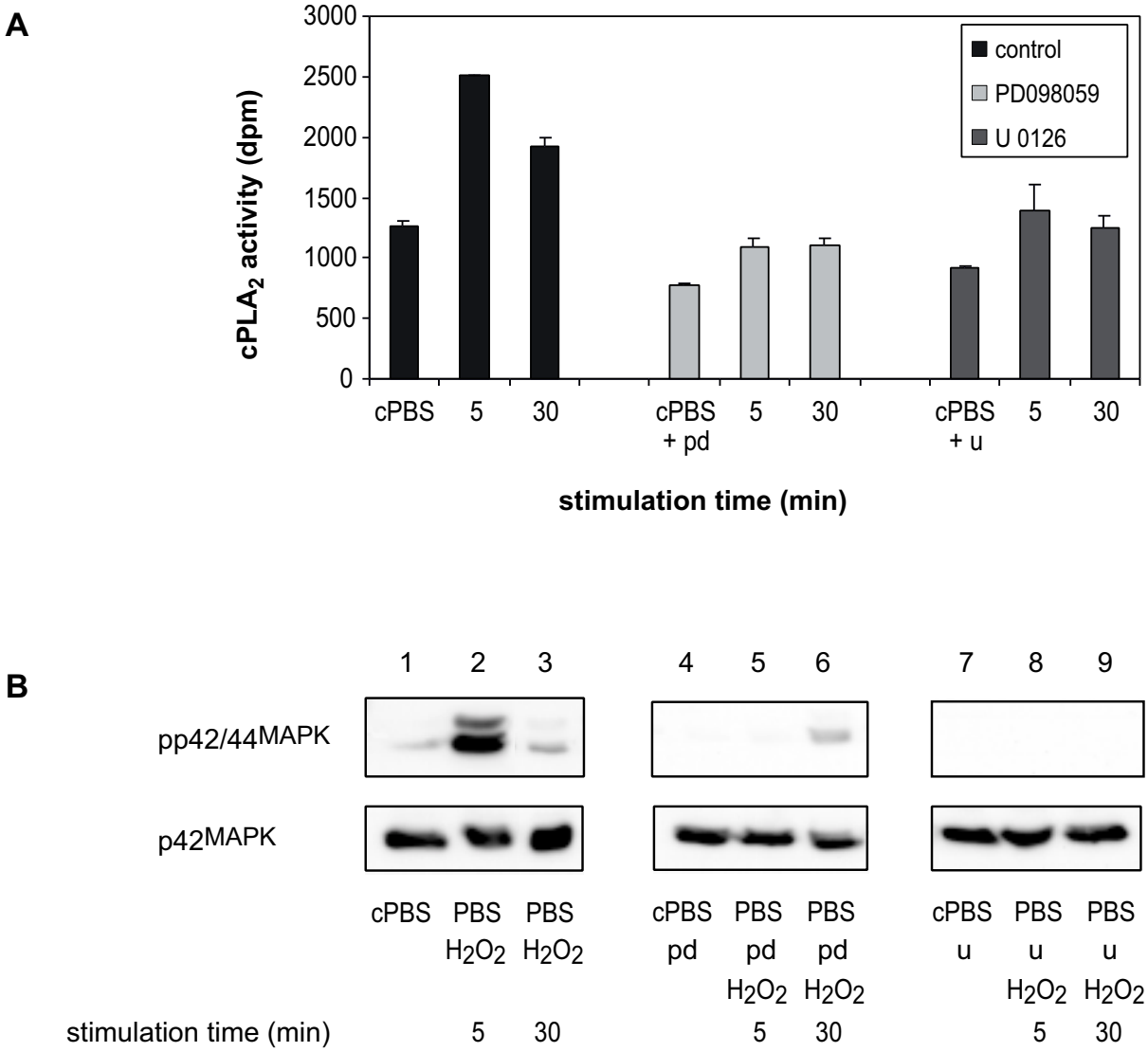

Figure 3. Effect of the MEK inhibitors, PD098059 and U0126, on (A) $cPLA_2$ activity and (B) $p42/44^{MAPK}$ phosphorylation in H_2O_2-stimulated Her14 cells. Quiescent Her14 cells were left untreated, incubated for 1h with PD098059 or U0126 and then stimulated for 5 and 30 min with H_2O_2. (A) Cell lysates were prepared in which $cPLA_2$ activity was measured and (B) $p42/44^{MAPK}$ phosphorylation and $p42^{MAPK}$ expression were analysed on western blot as described in Materials and Methods. Results shown are representative of 2 independent experiments

PD098059 compared to untreated cells. Similar results were obtained with another MEK inhibitor (fig. 3A, right columns), U0126, that inhibits the catalytic activity of MEK (Favata, *et al.,* 1998). These data indicate that H_2O_2 activates $cPLA_2$ via the Raf-MEK-$p42/44^{MAPK}$ pathway in Her14 fibroblasts.

However, we have recently demonstrated that inhibiting the phosphorylation of p42/44^{MAPK} did not always lead to a reduced cPLA$_2$ activity (van Rossum, *et al.,* 2001). Therefore, the phosphorylation of p42/44^{MAPK} in the same samples was analysed on western blot, showing phosphorylated p42/44^{MAPK} in quiescent cells (fig. 3B, lane 1) which is increased upon H_2O_2 stimulation, and is significantly higher after 5 min (lane 2) than after 30 min (lane 3). This is in line with the cPLA$_2$ activity measured (fig. 3A, left columns) in these cells. No, p42/44^{MAPK} phosphorylation was present in quiescent cells treated with PD098059 (lane 4), as in cells stimulated afterwards for 5 min with H_2O_2 (lane 5). However, at 30 min, p42/44^{MAPK} phosphorylation was present again (lane 6), which has to be due to a pathway independent of Raf-MEK. p42/44^{MAPK} phosphorylation was not detectable in quiescent cells treated with U0126 (lane 7) as well as in cells stimulated afterwards with H_2O_2 for 5 or 30 min (lanes 8, 9, respectively). p42^{MAPK} levels were analysed on western blot showing that p42^{MAPK} was present in equal amounts in all samples. Taken together, these results show activation of p42/44^{MAPK} via the Raf-MEK pathway in quiescent cells that correlated with cPLA$_2$ activity and which both can be decreased by PD098059 treatment. However, H_2O_2-induced cPLA$_2$ activation is only partially mediated by p42/44^{MAPK}, because both PD098059 and U0126 were unable to completely inhibit the increased cPLA$_2$ activity. p42/44^{MAPK} phosphorylation in H_2O_2-stimulated cells is activated via the Raf-MEK pathway, and in addition, a secondary response in p42/44^{MAPK} activation occurs through a Raf-MEK-independent pathway.

cPLA$_2$ activation induced by H_2O_2 is partially independent of its phosphorylation.

From figure 3A it is concluded that H_2O_2-induced cPLA$_2$ activity is not completely mediated by p42/44^{MAPK}. This could be mediated by one of the other MAPK family members, since JNK in astrocytes and p38^{MAPK} in platelets have been shown to phosphorylate and activate cPLA$_2$ (Kramer, *et al.,* 1996; Hernández, *et al.,* 1999; van Putten, *et al.,* 2001; Börsch-Haubold, *et al.,* 1998). Therefore, Her14 cells were stimulated either with EGF or with H_2O_2 and cPLA$_2$ activity was measured showing an increase in cPLA$_2$ activity as compared to unstimulated cells (fig. 4). To evaluate whether the increase in cPLA$_2$ activity was entirely due to phosphorylation of the enzyme, the homogenates were incubated with alkaline phosphatase, whereafter cPLA$_2$ activity was measured. This treatment revealed a complete reduction to basal level of cPLA$_2$ activity in EGF-stimulated cells (fig. 4), as has been demonstrated previously (Schalkwijk, *et al.,* 1995). However, no complete reduction was measured in H_2O_2-stimulated cells, suggesting that, in addition to phosphorylation, cPLA$_2$ was activated by another mechanism.

It has been shown *in vivo* that ROS can peroxidise phospholipids (Davies, 1995; Drummen *et al.,* 1999). cPLA$_2$ activity was shown to be increased when vesicles containing peroxidised phospholipids were used as a substrate. In these experiments, also the calcium

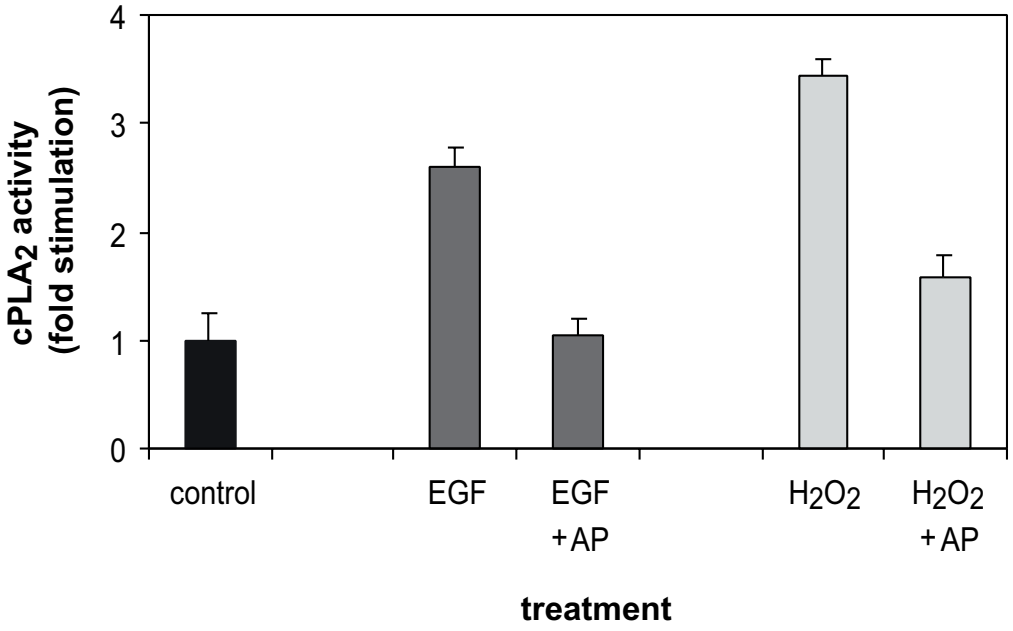

Figure 4. Effect of phosphatase treatment on $cPLA_2$ activity in EGF- and H_2O_2-stimulated Her14 cells. Quiescent Her14 cells were either left untreated or stimulated with EGF (5 min) or H_2O_2 (20 min). The obtained cell lysates were either left untreated or incubated with alkaline phosphatase whereafter $cPLA_2$ activity was measured as described in Materials and Methods.

requirement for $cPLA_2$ was decreased (Rashba-Step, *et al.,* 1997). To determine whether H_2O_2 treatment of the cells resulted in an increased, calcium-independent, translocation of $cPLA_2$ to cellular membranes, Her14 cells were stimulated for 20 min with H_2O_2. Next, the cells were homogenised in homogenisation buffer containing EGTA to create calcium free conditions. The homogenate of control and H_2O_2-stimulated cells were subjected to ultracentrifugation to obtain a cytosolic and a membrane fraction. $cPLA_2$ protein was analysed on western blot and despite the calcium free conditions, a significant amount of $cPLA_2$ was translocated to the membrane fraction upon H_2O_2 stimulation as compared to control cells (fig. 5A). This is interestingly since no translocation of $cPLA_2$ occurred in calcium ionophore-stimulated Her14 cells under calcium free conditions (Bunt *et al.,* 1997). No significant difference was detected in the amount of $cPLA_2$ present in the cytosolic fractions between control and stimulated cells (fig. 5A). This indicates a tight binding of $cPLA_2$ to the membranes under calcium free conditions.

Next, $cPLA_2$ activity was measured in the homogenates of control and H_2O_2-stimulated cells to ensure that $cPLA_2$ was activated (fig. 5B, left columns). No difference in $cPLA_2$ activity was measured in the cytosolic fractions (fig. 5B, middle columns), while a significant increase in $cPLA_2$ activity in the membrane fraction of H_2O_2 treated versus

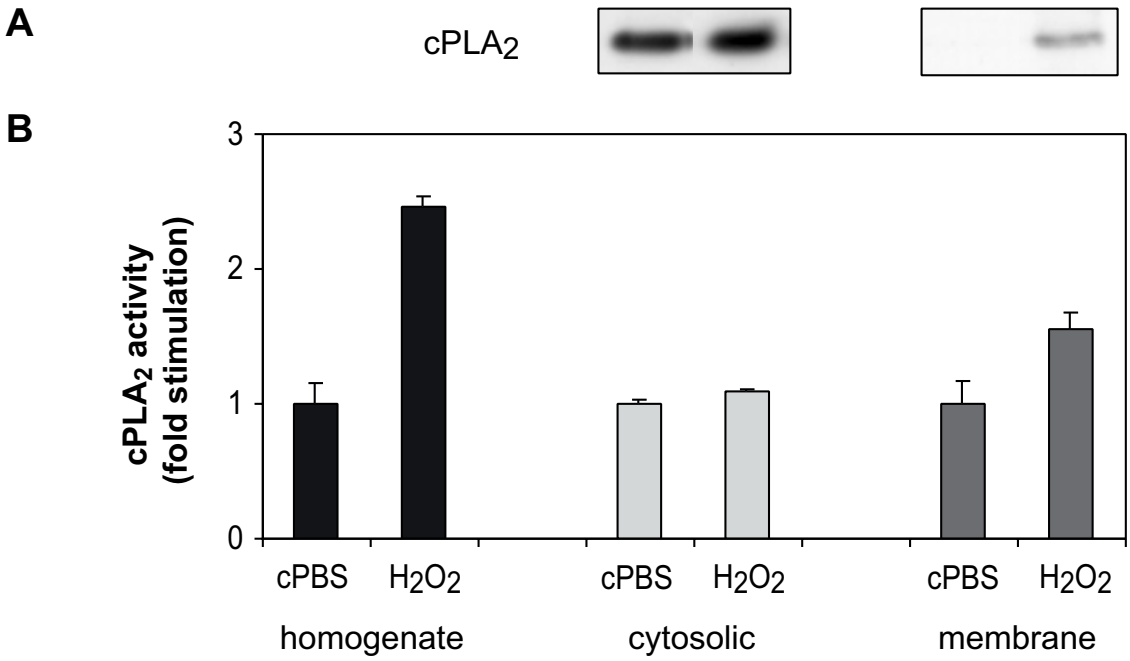

Figure 5. H_2O_2-induced $cPLA_2$ translocation (A) and activity (B) in Her14 cells. Quiescent cells were stimulated with H_2O_2 for 20 min, whereafter the cells were harvested under calcium free conditions. Subsequently, part of the homogenate was centrifuged at 200,000xg to obtain the particulate and cytosolic fraction. Ten µg of protein was used for electrophoresis and $cPLA_2$ protein was analysed on western blot (A). $cPLA_2$ activity was measured in homogenate, cytosolic and particulate fractions of stimulated and control cells (B).

control cells was measured (fig. 5B, right columns). Thus, these data suggest that H_2O_2 might peroxidise membrane phospholipids *in vivo* thereby enhancing $cPLA_2$ activity.

Discussion

In this study, we examined whether $cPLA_2$ is activated by H_2O_2 and determined the pathways by which $cPLA_2$ is activated. First we showed that $cPLA_2$ is similarly activated by the oxidants H_2O_2 and cumene hydroperoxide in a dose dependent manner in Her14 fibroblasts, being maximal at around 2 and 1 mM, respectively. $cPLA_2$ was rapidly and transiently activated in response to H_2O_2 and returned to basal level after 60 min.

An important step in $cPLA_2$ activity regulation is phosphorylation of $cPLA_2$, thereby increasing its intrinsic activity by 2-3 fold (Nemenoff *et al.,* 1993; Kramer *et al.,* 1993). In many cells, activation of $p42/44^{MAPK}$ has been shown to mediate $cPLA_2$ phosphorylation and activation (for reviews Leslie, 1997; Murakami *et al.,* 1997). In

addition, $p38^{MAPK}$ has been demonstrated (Kramer, *et al.,* 1996; Börsch-Haubold, *et al.,* 1998) and JNK has been implicated in $cPLA_2$ activation (van Putten, *et al.,* 2001). H_2O_2-induced $cPLA_2$ activity in Her14 cells is partially mediated by $p42/44^{MAPK}$, because both PD098059 and U0126 were unable to inhibit $cPLA_2$ activity completely. $p42/44^{MAPK}$ was activated via the Raf-MEK pathway while, in addition, a secondary response in $p42/44^{MAPK}$ phosphorylation occurred after 30 min, which was Raf-MEK independent. Furthermore, treatment of quiescent cells with PD098059 completely inhibited $p42/44^{MAPK}$ phosphorylation, that corresponded with a decrease in $cPLA_2$ activity, showing that $p42/44^{MAPK}$ and $cPLA_2$ were activated via Raf-MEK, as has been shown previously (van Rossum, *et al.,* 2001). The remaining $cPLA_2$ activity that was not inhibited by either PD098059 or U0126 could be mediated by one of the other MAPK family members. However, alkaline phosphatase treatment of H_2O_2-stimulated cells did not reduce $cPLA_2$ activity to basal levels, in contrast to EGF-stimulated cells, which has been reported previously (Schalkwijk, *et al.,* 1995). Thus the remaining $cPLA_2$ activity is not mediated by phosphorylation through $p38^{MAPK}$, JNK or any other kinase.

It has been demonstrated that ROS are able to peroxidise lipids from biomembranes *in vivo* (Drummen, *et al.,* 1999). This can lead to changes in ion permeability, surface charge (Kühn *et al.,* 1983), cell signalling (Keller & Mattson, 1998; Uchida *et al.,* 1999) and altered membrane fluidity (Imai *et al.,* 2000) of the cell. Changes in membrane fluidity due to oxidised lipids have been shown to increase PLA_2 activity (Salgo *et al.,* 1993; van den Berg *et al.,* 1993), that was suggested to be due to a change in molecular surface area and structural composition of peroxidised versus unperoxidised membranes. Alternatively, H_2O_2 peroxidised lipids may create a polar nature, which facilitates the displacement of the released arachidonic acid from the membrane, thereby prolonging the catalytic activity of $cPLA_2$, which usually is subject to product inhibition (Reynolds *et al.,* 1993; Bayburt & Gelb, 1997; Burke *et al.,* 1999). H_2O_2 is able to raise the intracellular calcium concentration (Golconda *et al.,* 1993), which might result in $cPLA_2$ translocation and binding to membranes, whereupon $cPLA_2$ is maximal activated (Abdullah, *et al.,* 1995; Schalkwijk, *et al.,* 1996). Additionally, lipid peroxidation is known to increase the negative surface charge of membranes allowing increased calcium binding (Vladimirov *et al.,* 1980). Furthermore, peroxidised lipids also decrease the calcium requirement for $cPLA_2$ activity (Rashba-Step, *et al.,* 1997), suggesting that $cPLA_2$ translocation and binding to membranes is facilitated. Indeed, the H_2O_2-induced $cPLA_2$ activity reside in the membrane fraction, even under calcium free conditions, which did not occur in calcium-ionophore-stimulated Her14 cells (Bunt, *et al.,* 1997). Rahsba-Step *et al.* (1997) suggested that the effects of membrane peroxidation and phosphorylation on $cPLA_2$ activity are additive. We suggest that the increased activity of $cPLA_2$ due to membrane peroxidation might merely be due to an increase in substrate availability for $cPLA_2$.

Acknowledgments

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Chapter 4

Cytosolic phospholipase A_2 activity during the ongoing cell cycle.

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Abstract

Cytosolic phospholipase A_2 ($cPLA_2$) is of special interest because it selectively releases arachidonic acid from membrane phospholipids. Arachidonic acid has been implicated to play an important role in various cellular responses. Recently arachidonic acid release and prostaglandin synthesis have been shown to be cell cycle dependent and therefore the activity of $cPLA_2$ during the ongoing cell cycle was investigated, using the mitotic shake off method for cell synchronisation. $cPLA_2$ activity was high in mitotic cells and decreased rapidly in the early G1 phase. A strong increase in activity was measured following the G1/S transition in both neuroblastoma and Chinese hamster ovary cells. The changes in activity were not due to a difference in $cPLA_2$ expression but due to phosphorylation of $cPLA_2$. Phosphorylation of $cPLA_2$ occurs through MAPK since the use of a specific MAPK kinase inhibitor and serum depletion of synchronised cells inhibited $cPLA_2$ activity.

Introduction

The 85 kDa cytosolic phospholipase A_2 ($cPLA_2$) preferentially catalyses the hydrolysis of phospholipids at the *sn*-2 position generating arachidonic acid. The released arachidonic acid can be oxygenated either by cyclooxygenase to form prostaglandins and thromboxanes or by lipoxygenase to form leukotrienes. Arachidonic acid and/or its metabolites have been implicated to play an important role in various cellular responses such as cell migration, mitogenic signalling (Piomelli, 1993; Korystov Yu *et al.,* 1998), inflammation (Heller *et al.,* 1998) and cytotoxicity (Cifone *et al.,* 1993; Grazia Cifone *et al.,* 1997). $cPLA_2$ activity is regulated by phosphorylation in addition to calcium-dependent binding to the membrane substrate (Clark *et al.,* 1995). Phosphorylation of $cPLA_2$ increases its intrinsic enzyme activity by 2 to 3-fold (Nemenoff *et al.,* 1993; Kramer *et al.,* 1993) and was shown to be mediated by $p42/44^{MAPK}$ (ERK1/2 or MAPK) on Ser^{505} upon cell activation (Schalkwijk *et al.,* 1995; de Carvalho *et al.,* 1996). Furthermore, for full activation of $cPLA_2$, the enzyme needs first to be phosphorylated followed by translocation to membranes by calcium (Schalkwijk *et al.,* 1996; Abdullah *et al.,* 1995).

The cell cycle can be divided into four phases: the first gap phase (G1), DNA synthesis (S), the second gap phase (G2) and mitosis (M). At a point in G1, the restriction point (R), the cell is committed to progress into S phase independently of growth factors (Pardee, 1974). ERK1/2 activation plays an important role in cell cycle progression in both G0 stimulated cells but also during the ongoing cell cycle (Pagès *et al.,* 1993; Le Gall *et al.,* 1998). Both growth factor signalling and signal transduction routes resulting from cell

attachment can activate MAPK (Seger & Krebs; 1995; Giancotti, 1997; Hulleman *et al.,* 1999a).

Recently MAPK expression and phosphorylation was reported during G1 phase of the cell cycle in Chinese hamster ovary (CHO) cells (Hulleman *et al.,* 1999b). In addition, phosphorylated ERK1/2 translocated to the nucleus during mid/late G1 of the cell cycle. As pointed out earlier, MAPK is able to phosphorylate cPLA$_2$, which upon translocation to membranes releases arachidonic acid. It has been reported that arachidonic acid was released from interphase cells in response to hormones, and that this release becomes strongly inhibited in mitotic cells (Berlin & Preston, 1995). The use of phospholipase A$_2$ inhibitors in tumour cells resulted in a suppressed proliferation of the cells by inducing apoptosis (Korystov Yu *et al.,* 1998). Therefore we investigated whether cPLA$_2$ activity is cell cycle dependent since ERK1/2 activity alters and arachidonic acid release differs during the cell cycle.

We report that cPLA$_2$ activity is high in mitosis, decreases afterwards and is increased again in G1 and following the G1/S transition. At these periods, cPLA$_2$ activity is due to increased phosphorylation rather than by increased cPLA$_2$ protein expression, since phosphatase treatment of cPLA$_2$ reduced its activity. Moreover, preventing phosphorylation of MAPK either by inhibiting the upstream activator MEK, or by serum depletion of synchronised cells resulted in a decrease in cPLA$_2$ activity. The knowledge of cPLA$_2$ activity during the ongoing cell cycle is of great importance since cPLA$_2$ generates arachidonic acid, which has in some tumour cells proliferative (Adachi *et al.,* 1996; Tang *et al.,* 1997) and in other tumour cells apoptotic effects (Korystov Yu *et al.,* 1998; Chan *et al.,* 1998). Also the cyclooxygenase and lipoxygenase enzymes which can convert arachidonic acid into bioactive lipids have been implicated to play a role in tumorigenesis (Ikawa *et al.,* 1999; Sawaoka *et al.,* 1999).

Materials and Methods

Cell culture.

Neuroblastoma (N2A) or Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Scotland) supplemented with 25 mM HEPES and 7.5% foetal bovine serum (FBS, Gibco BRL, Scotland). Cells were maintained at 37°C in a humidified atmosphere.

Mitotic shake off.

One day prior to shake off, cells were cultured at 5x10^6 cells per 175 cm^2 flask. Each hour flasks were shaken for 1 min at 37°C to obtain mitotic cells, which were replated

on tissue culture dishes as described previously (Boonstra *et al.,* 1981). The cells were harvested at different hours after mitosis, by removing the medium and washing the cells twice with ice-cold phosphate buffered saline (PBS). Thereafter, the cells were scraped in homogenisation buffer (50 mM Hepes/NaOH pH 7.4, 0.25 M sucrose, 50 mM NaF, 250 μM Na_3VO_4, 1 mM EGTA, 10 μM leupeptin, 1 μM pepstatin and 1 mM PMSF). Cells were homogenised by 15 strokes through a 26G needle and sonicated for 3 times 10 seconds. Mitotic cells were centrifuged at 600xg for 7 min whereafter the cells were washed twice and either harvested, or replated on tissue culture dishes in serum-free medium. Then the cells were harvested at the indicated times. In other experiments mitotic cells were replated on tissue culture dishes and at different hours after mitosis incubated for 10 min with 50 μM of the MEK inhibitor U0126 (Promega, Madison, USA), whereafter the cells were harvested as described above.

Western blot analysis.

Proteins of $1x10^5$ cells per time point were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane by semi-dry blotting using a BioRad trans-blot SD. The membrane was blocked for 1 h at RT with 2% milk powder in PBST (PBS containing 0.1% (v/v) Tween-20) following primary antibody incubations for 1 h in 0.2% milk powder in PBST. Cyclin A (Calbiochem, Cambridge, UK) and $p42^{MAPK}$ (Upstate Biotechnology, Lake Placid, NY) were detected with a monoclonal antibody at a dilution of 2.5 μg/ml and 0.5μg/ml, respectively. Phosphorylated ERK1/2 was detected with a phospho-p42/44 MAPK rabbit polyclonal antibody (dilution of 1:8000) from New England Biolabs Incorporated (Beverly, MA). $cPLA_2$ was detected with a rabbit polyclonal antibody (0.2 μg/ml) obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Subsequently, the membrane was washed and primary antibodies were detected with rabbit anti-mouse and donkey anti-rabbit IgG conjugated to horseradish peroxidase and the bands were visualised with enhanced chemiluminescence (NEN^{TM} Life science products, Boston).

$cPLA_2$ activity assay.

$cPLA_2$ activity was measured by the release of radiolabeled arachidonic acid from the *sn*-2 position of 1-stearoyl-2-[1-^{14}C]arachidonoyl-glycero-3-phosphocholine (Amersham, Buckinghamshire, England) as described previously (Schalkwijk *et al.,* 1996). Briefly, aliquots of the radiolabeled phosphatidylcholine was dried under N_2 together with dipalmitoylglycerol (Sigma, Steinheim, Germany) and then dispersed in water by sonification for 4 times 15 sec under nitrogen to give final concentrations of 4 μM and 2 μM, respectively, in the assay. The assay incubation mixture contained 0.2 M Tris pH 8.5, 1 mM $CaCl_2$, 5 mM DTT and the homogenate of $2x10^5$ cells in a total volume of 200 μl. After incubation for 7-10 min at 37°C the released radiolabeled arachidonic acid was extracted by

a modified Dole extraction procedure (van den Bosch *et al.,* 1974) and the radioactivity was determined in a scintillation counter (Tri-Carb 15000, Packard, Meriden, USA). Measuring the activity of non-phosphorylated cPLA$_2$ was performed as described previously (Schalkwijk *et al.,* 1995) by addition of 10 units alkaline phosphatase (Roche diagnostics, Mannheim, Germany) to an assay mixture containing the homogenate of $2x10^5$ cells, 1 mM $MgCl_2$, 0.25 M Tris pH 8.5, 1.25 mM $CaCl_2$ and 6.25 mM DTT in a final volume of 160 µl. After a 20 min incubation at 37°C, 40 µl substrate was added and the cPLA$_2$ activity assay was performed as described above. Under these assay conditions always less than 5% of the substrate was hydrolysed.

Results

cPLA$_2$ activity during the cell cycle.

It has been previously reported that in HeLa cells arachidonic acid release was cell cycle independent (Lahoua *et al.,* 1989) but in fibroblasts arachidonic acid was released from interphase cells which could be the result of activated phospholipases A$_2$ (Berlin & Preston, 1995). Since cPLA$_2$ is known to selectively liberating arachidonic acid, we examined whether cPLA$_2$ activity changes during the G1 and/or S phase of the ongoing cell cycle in neuroblastoma cells (N2A), by using the mitotic shake off method for cell synchronisation (Boonstra *et al.,* 1981). Detection of the S phase cyclin A on western blot or ^{3}H-thymidine incorporation into the DNA of the cells as described by Hulleman *et al.,* (1999a) was used to assess the G1/S-phase transition of N2A cells. Synchronised N2A cells were harvested at different hours after mitosis and cPLA$_2$ activity of an identical amount of cells was determined using an *in vitro* assay as described in Materials and Methods in which only the activity of cPLA$_2$ was measured (Spaargaren *et al.,* 1992; Schalkwijk *et al.,* 1992; Atsumi *et al.,* 1998). Figure 1A shows in N2A cells a decrease in cPLA$_2$ activity during the first hours after mitosis, followed by a small peak in G1 and a strong increase starting at the G1/S phase transition. In Chinese hamster ovary (CHO) cells the activity of cPLA$_2$ remained constant during the early G1 (fig. 1B). Thereafter, the activity slightly decreased to increase transiently, which at the G1/S phase transition significantly increased again. Except for the decrease in activity after mitosis a similar cPLA$_2$ activity pattern was determined in CHO cells as observed in synchronised N2A cells, implying that this pattern is not cell type specific. However, there are slight differences at the timing of the peaks between experiments, which is mostly due to variations in the length of the G1 phase (Zetterberg & Larsson, 1991).

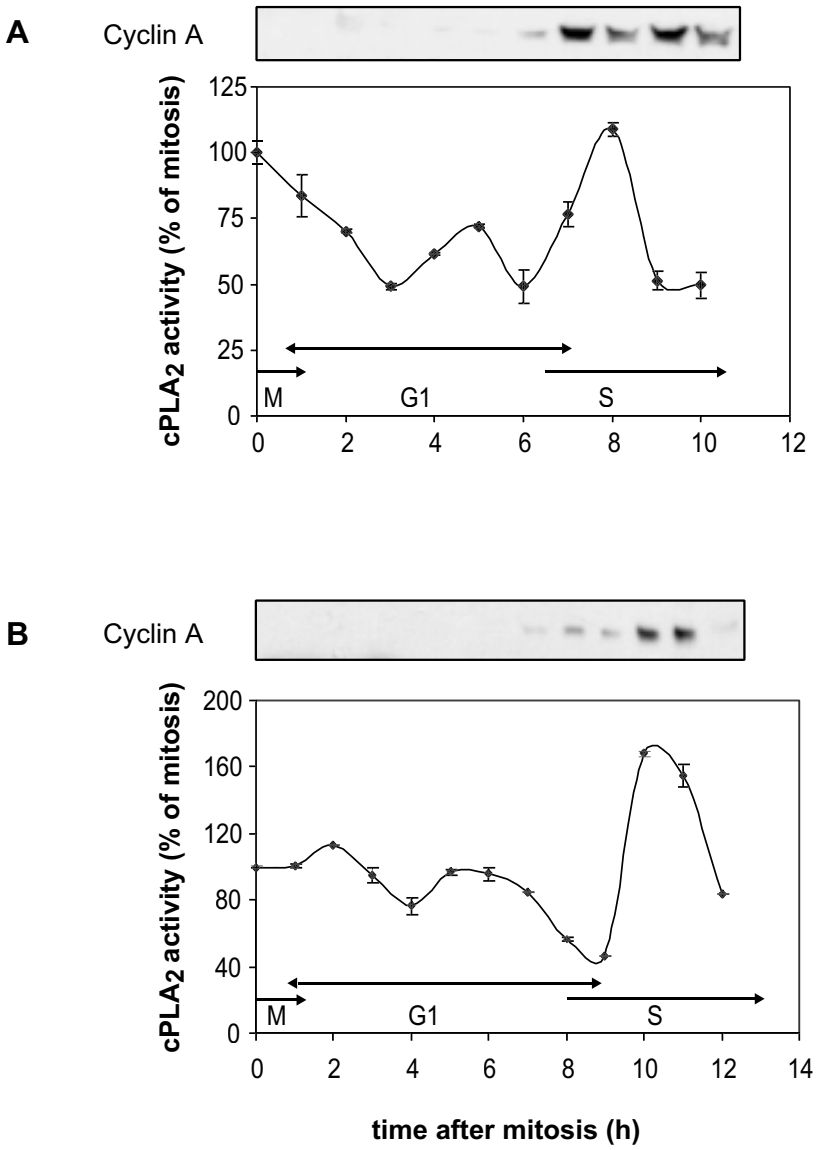

Figure 1. Activity of $cPLA_2$ in (A) N2A and (B) CHO cells. Cells were synchronised by mitotic shake off and were harvested at different hours after mitosis. $cPLA_2$ activity was measured of $2x10^5$ cells using an *in vitro* assay as described in Materials and Methods. G1/S phase transition was detected on western blot by cyclin A expression. Similar data were obtained in respectively 8 (A) and 2 (B) separate experiments, each performed in duplicate.

To investigate whether these changes in $cPLA_2$ activity are the result of differences in expression level, the total amount of $cPLA_2$ protein was analysed on western blot (fig. 2A), showing small differences in $cPLA_2$ protein expression during the cell cycle. In order to establish the significance of the cell cycle related changes in $cPLA_2$ expression the bands of four different experiments were quantified and the average percentage of expression,

relative to mitotic cells, was calculated. As shown in figure 2D the cPLA$_2$ expression gradually increases about two-fold during the cell cycle, with a slightly stronger increase between 4 and 6 hours after mitosis. cPLA$_2$ protein expression is nearly constant until 3 hours after mitosis while cPLA$_2$ activity is decreased by approximately 50%. A rise in protein expression was observed between 4-6 hours but does not result in grossly different cPLA$_2$ activities. By contrast, the cPLA$_2$ expression does not change in the time period from 6 to 9 hours, while the cPLA$_2$ activity increases over 2-fold between 6 to 8 hours and decreases again from 8-9 hours (fig. 1). These data suggest that cPLA$_2$ activity during the cell cycle, in particular the increase in early S-phase, is regulated by other factors, such as phosphorylation.

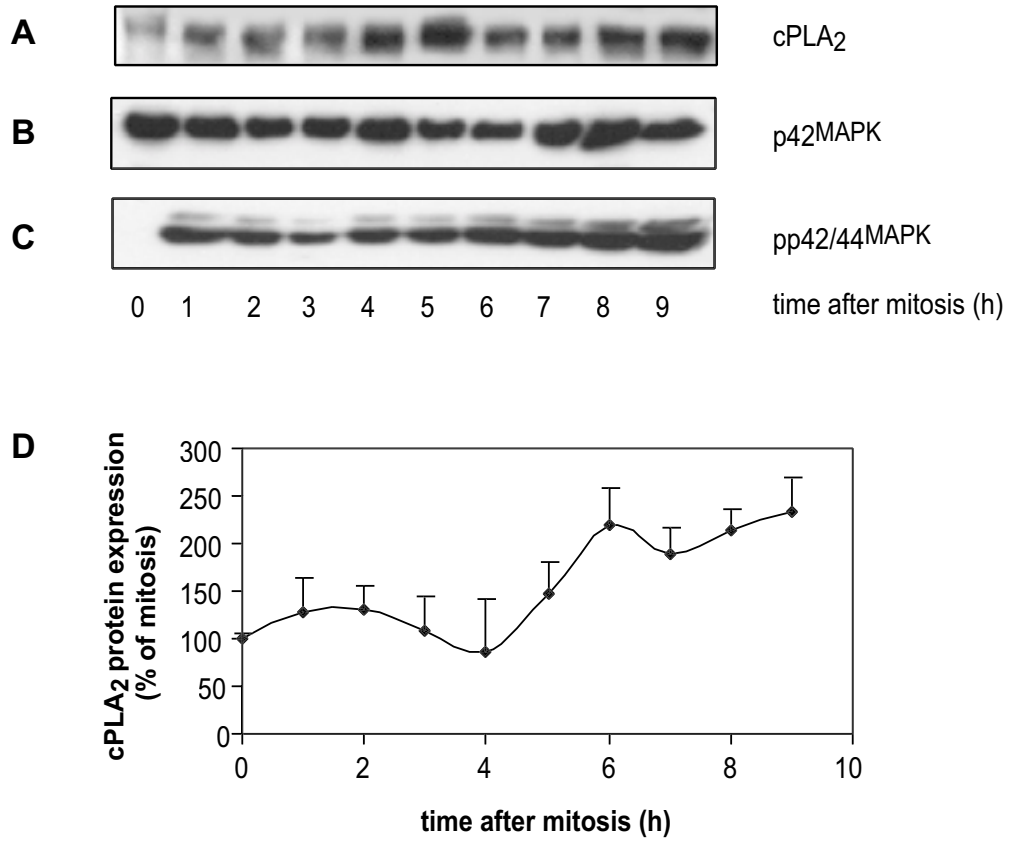

Figure 2. Expression of (A) cPLA$_2$; (B) p42^{MAPK}; (C) phosphorylated p42/44^{MAPK} and (D) quantification of cPLA$_2$ protein expression during the cell cycle. Synchronised N2A cells were harvested at the indicated hours after mitosis whereafter, 1x10^5 cells were used for electrophoresis and the following proteins were analysed on western blot. A: Expression of cPLA$_2$. B: Expression of p42^{MAPK}. C: Phosphorylation of p42/44^{MAPK}. D: cPLA$_2$ protein expressions of four separate experiments were quantified and the average percentage of cPLA$_2$ protein and S.E.M relative to mitotic cells was calculated.

In stimulated cells, the phosphorylation of $cPLA_2$ by ERK1/2 has been shown to play an important role in the release of arachidonic acid (Clark *et al.,* 1995; Murakami *et al.,* 1997) and is required for maximal activation of $cPLA_2$ (Lin *et al.,* 1993; Kramer *et al.,* 1996; Qiu *et al.,* 1998). So far, nothing is known about the involvement of MAPK in the activation of $cPLA_2$ during the ongoing cell cycle. Recently it has been demonstrated in CHO cells that MAPK is continuously expressed during the cell cycle and that only a minor part is phosphorylated in the G1 and S phase (Hulleman *et al.,* 1999b). The expression of MAPK (fig. 2B) was examined to serve as an internal control. In addition, MAPK phosphorylation was analysed on western blot (fig. 2C), using an antibody directed against phosphorylated $p42/44^{MAPK}$. MAPK protein expression remained fairly constant during the cell cycle while ERK1/2 is phosphorylated during the G1 and even more so in S phase but not in mitosis. From these results no direct relationship between ERK1/2 phosphorylation and $cPLA_2$ activity could be drawn. The gradual changes in MAPK phosphorylation do not account for the more sudden variations in $cPLA_2$ activity.

Regulation of cytosolic phospholipase A_2 by phosphorylation during the ongoing cell cycle.

$cPLA_2$ activity is regulated by calcium since $cPLA_2$ binds to membranes in a calcium dependent manner. Another important factor that enhances and regulates $cPLA_2$ activity is phosphorylation of the enzyme. Phosphorylation of $cPLA_2$ can occur through different MAPKs, like $p38^{MAPK}$ in thrombin-stimulated platelets (Kramer *et al.,* 1996) and ERK1/2 (Nemenoff *et al.,* 1993; Qiu *et al.,* 1998), of which the latter one has been found to phosphorylate and activate $cPLA_2$ in many cell models, and which has also been shown to be essential for progression through the cell cycle (Pagès *et al.,* 1993; Le Gall *et al.,* 1998). In order to establish the role of ERK1/2 in $cPLA_2$ activation, ERK1/2 activation was prevented using the MAPK kinase (MEK) inhibitor U0126. U0126 inhibits MEK directly by inhibiting the catalytic activity of the enzyme and thus the activation of ERK1/2 (Favata *et al.,* 1998). Synchronised N2A cells were either left untreated or were, at different hours after mitosis, treated for 10 min with U0126 whereafter the cells were harvested (fig. 3). In this way it is unlikely that the MEK inhibitor affects cell cycle progression. The activity of $cPLA_2$ in control cells showed again a decrease after mitosis, followed by a peak in G1 and a peak after the G1/S transition (fig. 3A). In cells treated with the MEK inhibitor, $cPLA_2$ activity is lower as compared to control cells, however the pattern is similar. Furthermore, in mitosis, at the end of G1 and following the G1/S transition a reduction of approximately 50% in $cPLA_2$ activity was observed between control and U0126 treated cells. In agonist-stimulated cells an increase of 2-3 fold in $cPLA_2$ activity by MAPK was measured (Lin *et al.,* 1993) which might well be the activity that is reduced upon U0126 treatment. The effects of calcium that might influence $cPLA_2$ activity during the cell cycle was eliminated by determining the activity *in vitro* under constant calcium concentrations and thus the

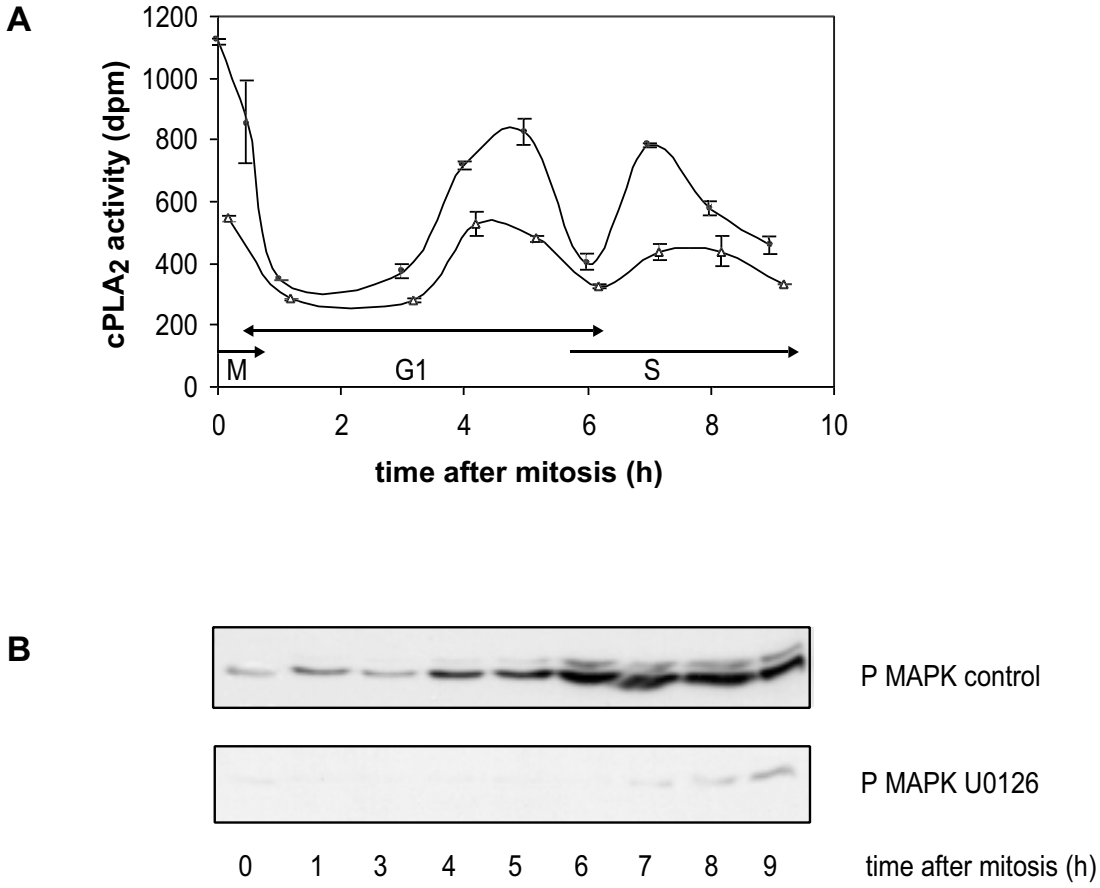

Figure 3. cPLA$_2$ activity (A) and MAPK phosphorylation (B) in the absence (●) or presence(△) of the MEK inhibitor U0126. N2A cells synchronised by mitotic shake off were replated on tissue culture dishes. Cells were harvested at the indicated time points or were then treated for 10 min with the specific MEK inhibitor, U0126, whereafter these cells were harvested. cPLA$_2$ activity was measured as described in Materials and Methods (A) and phosphorylated MAPK was analysed on western blot (B).

activities measured are not affected by calcium fluctuations. This suggests that at these periods cPLA$_2$ is activated by MAPK. In addition, western blot analysis displays phosphorylated MAPK in control cells while this appears to be virtually completely inhibited in U0126 treated cells (fig. 3B) showing that with a 10 min treatment of this inhibitor MAPK was not phosphorylated anymore. Despite this nearly complete inhibition of MAPK phosphorylation the cPLA$_2$ activity is not completely inhibited. This can be due to the residual activity of either the non-phosphorylated form of cPLA$_2$ (Lin *et al.,* 1993) or of some residual active and phosphorylated enzyme that was present at the moment of addition of U0126 and has not yet been completely de-phosphorylated during the 10 min treatment with the inhibitor.

The results from figure 3 suggest that during the cell cycle $cPLA_2$ is at least in part phosphorylated by MAPK, thereby increasing its activity at distinct periods. To evaluate whether the increase in $cPLA_2$ activity at these periods is indeed due to phosphorylation of the enzyme, the effect of phosphatase treatment on $cPLA_2$ activity was investigated. $cPLA_2$ activity of synchronous cells at different hours after mitosis was measured resulting in the characteristic activity pattern during the ongoing cell cycle. Next, mitotic cells, cells from early and late G1 and of S phase were treated with alkaline phosphatase whereafter $cPLA_2$ activity was measured. Figure 4 shows in mitotic cells as well as in late G1 and S phase cells a remarkable reduction in $cPLA_2$ activity of approximately 64, 49 and 45% respectively as compared to control, while only a marginal decrease of 21% in activity was measured in early G1 cells. This is also the period in which $cPLA_2$ has a reduced activity during the cell cycle. These results are in agreement with the reduced activities measured in synchronous cells treated with the MEK inhibitor U0126 (fig. 3), indicating that indeed $cPLA_2$ activity during the cell cycle is enhanced through phosphorylation by MAPK.

In order to further establish the involvement of MAPK in $cPLA_2$ activation a more *in vivo* approach was used. MAPK in stimulated cells can be activated either by growth factors or via cell attachment (Seger & Krebs, 1995; Lin *et al.,* 1997; Giancotti, 1997), but has recently been shown to be growth factor dependent during the ongoing cell cycle (Hulleman *et al.,* 1999a). Therefore, mitotic cells were replated either on tissue culture

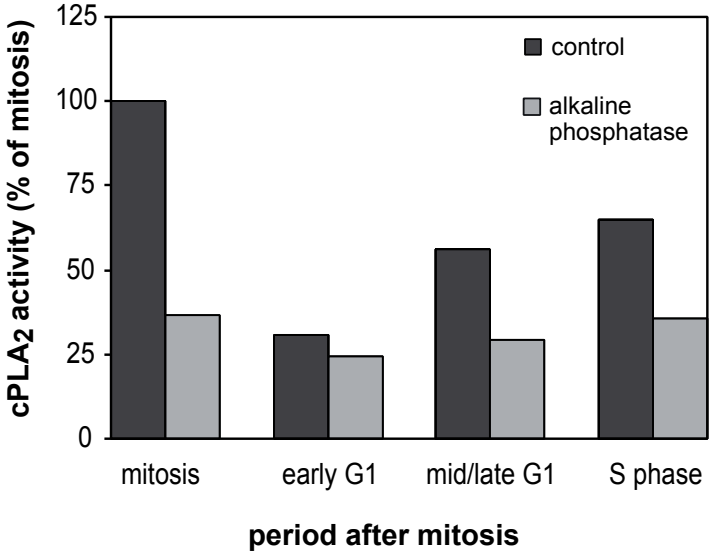

Figure 4. Effect of phosphatase treatment on $cPLA_2$ activity. $cPLA_2$ activity of synchronised N2A cells at different hours after mitosis was measured and mitotic cells, cells from early and late G1, and of S phase were treated with alkaline phosphatase whereafter the activity was measured as described in Materials and Methods.

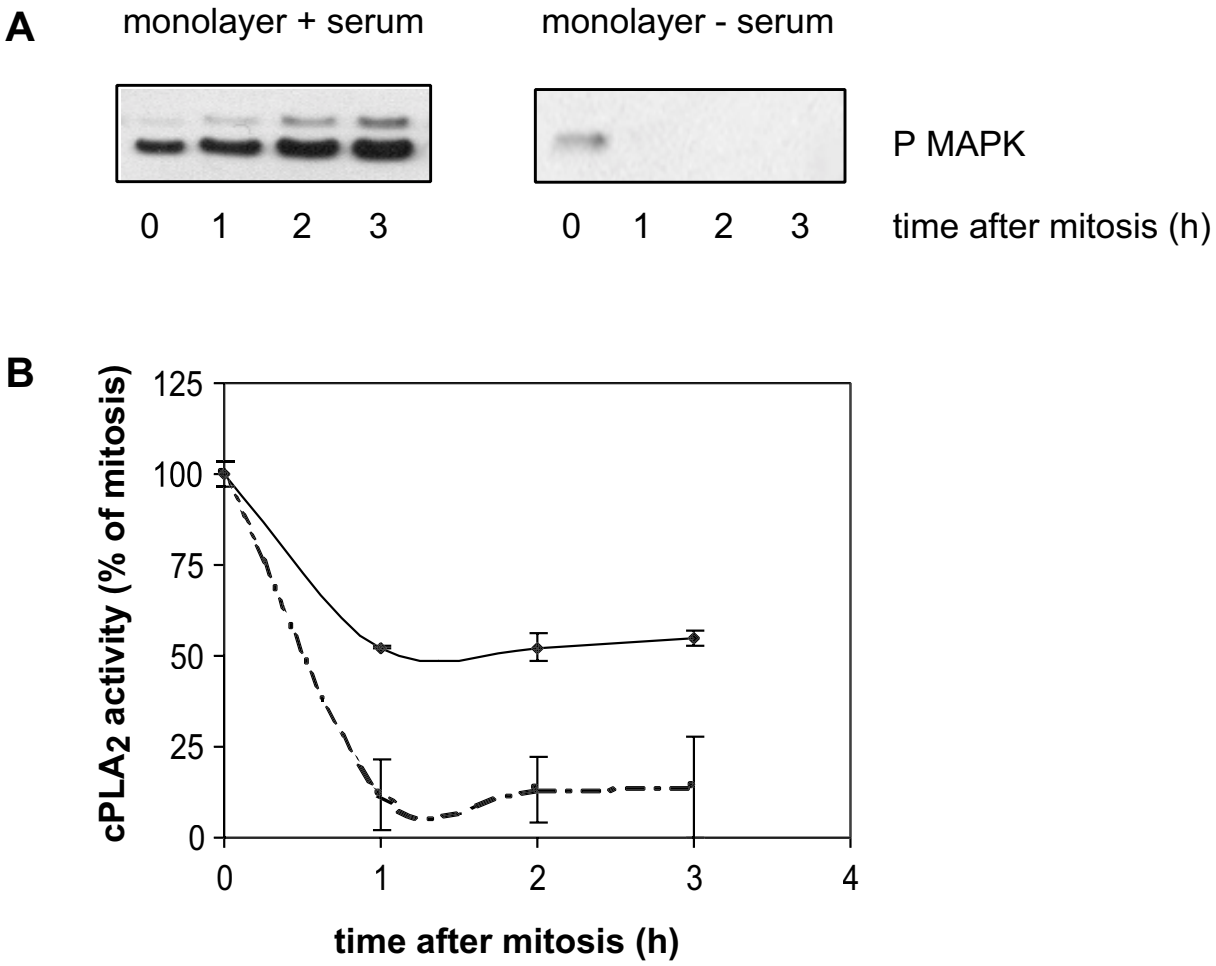

Figure 5. Effect of serum depriviation on (A) MAPK phosphorylation and (B) cPLA$_2$ activity. Mitotic N2A cells obtained with the mitotic shake off method were either directly replated on a tissue culture dish in the presence of serum (monolayer + serum; solid line) or in the absence of serum (monolayer – serum; dashed line). After harvesting at the indicated time points, MAPK phosphorylation was analysed on western blot and cPLA$_2$ activity was measured *in vitro* as described in Materials and Methods.

dishes in the presence or absence of serum for the indicated hours (fig. 5). In this way MAPK that is activated through growth factors is eliminated (Hulleman *et al.,* 1999a). Western blot analysis confirmed that cells replated in serum free medium did not exhibit phosphorylated ERK1/2 in contrast to control cells (fig. 5A). In addition, cPLA$_2$ activity of serum-deprived cells rapidly decreased after mitosis and remained at a low level for at least 3 hours as compared to control cells (fig. 5B). Measurements of longer time points were not performed since serum-deprived cells will stop cell cycle progression. All together, these results also demonstrate that cPLA$_2$ activity is increased through phosphorylation by MAPK.

Discussion

Arachidonic acid is known to be able to fulfil different cellular responses and is selectively released by $cPLA_2$. In order to establish the possible role of $cPLA_2$ in the cell cycle, $cPLA_2$ activity was measured during the cell cycle of N2A and CHO cells synchronised by mitotic shake off. $cPLA_2$ activity during the phases of the cell cycle was measured by an *in vitro* assay showing that $cPLA_2$ is highly active in mitosis which is decreased rapidly afterwards. However, Berlin and Preston (1995) found no arachidonic acid release in metaphase-arrested HeLa cells stimulated with calcium releasing agents. This was surprisingly, since the activity measured *in vitro* and protein expression of mitotic and interphase cells were comparable and even though mitotic $cPLA_2$ was constitutively phosphorylated in unstimulated cells (Berlin & Preston, 1995). However, these authors used an anti-mitotic drug to synchronise the cells, that may have effects on cell cycle progression (Schmid-Alliana *et al.,* 1998). Moreover, arachidonic acid release was not measured during the ongoing cell cycle.

We measured an elevated transient activity of $cPLA_2$ during the G1 phase that is followed by a strong increase in the S phase. This activation pattern of $cPLA_2$ has not only been observed in neuroblastoma cells but also in CHO cells, demonstrating that this pattern is not cell type specific and can also be found in other cell types**.** No correlation between $cPLA_2$ activity during the ongoing cell cycle with variations in $cPLA_2$ expression levels was found suggesting that the activity is regulated by other factors. In addition, in cryosections of Her 14 fibroblasts it was shown that $cPLA_2$ exists as monomers but is, next to this, predominantly present in clusters (Bunt *et al.,* 1997). These clusters then represent the inactive form of $cPLA_2$ of which small amounts of active monomers are recruited which are localised to the membrane (Bunt *et al.,* 2000). Furthermore, although the expression levels vary, nothing is known about the percentage of active monomers that account for the $cPLA_2$ activity pattern during the cell cycle.

$cPLA_2$ activity is predominantly regulated by calcium and phosphorylation, of which the latter one has to precede the calcium-dependent translocation for its full activation (Schalkwijk *et al.,* 1996). In this study we investigated the regulation of $cPLA_2$ activity by phosphorylation during the ongoing cell cycle. Western blot analysis showed no direct relationship between phosphorylated MAPK and $cPLA_2$ activity. Therefore, MAPK activation was prevented by inhibiting MEK activity with a specific inhibitor showing that there is indeed a relation between ERK1/2 phosphorylation and $cPLA_2$ activity during the ongoing cell cycle. A clear inhibition of $cPLA_2$ activity using U0126 was only observed in mitosis, late G1 and S phase where $cPLA_2$ is found to be highly active in control cells**.** In addition, treatment of $cPLA_2$ at these periods with alkaline phosphatase resulted also in a reduction of approximately 50% in $cPLA_2$ activity as was also observed when MAPK

phosphorylation was inhibited. Almost no reduction in activity was measured in periods where cPLA$_2$ activity during the cell cycle was low suggesting that this represents the basal activity. It seems as if the changes in cPLA$_2$ activity during the ongoing cell cycle in both N2A and CHO cells are modest. It has been published that cPLA$_2$ could only be activated about 2-3 times above basal level upon phosphorylation (Qiu *et al.,* 1993; Kramer *et al.,* 1993; Schalkwijk *et al.,* 1995) which resembles the decrease in activity upon phosphatase treatment or MAPK inhibition in our cells.

The relation between phosphorylated MAPK and cPLA$_2$ activity was further established by preventing the existence of phosphorylated MAPK in cells by growing mitotic cells in the absence of serum (Hulleman *et al.,* 1999a). In these cells cPLA$_2$ activity was markedly decreased as compared to control cells in which MAPK was phosphorylated. All together, these results indicate that MAPK phosphorylates cPLA$_2$ thereby activating the enzyme in a growth factor dependent way in the G1 and S phase of the ongoing cell cycle.

Our findings are of particular interest in view of cell cycle progression since both MAPK and especially cPLA$_2$ are involved in tumorigenesis (Adachi *et al.,* 1996; Korystov Yu *et al.,* 1998). Firstly, Lahoua *et al.* (1989) showed that biosynthesis of PGE_2 and $PGF_{2\alpha}$, metabolites of arachidonic acid, was increased in S phase, i.e. in the same phase in which we measured a high cPLA$_2$ activity. Secondly, in hepatocytes quinacrine, a commonly used phospholipase A$_2$ inhibitor, prevented hepatocyte growth factor induced arachidonic acid release and ^{3}H-thymidine incorporation (Adachi *et al.,* 1996). An effect that was also found in tumour cells treated with phospholipase A$_2$ and lipoxygenase inhibitors (Korystov Yu *et al.,* 1998).

Acknowledgments

We wish to thank Esther Hulleman and José Bijvelt for their stimulating discussions and technical assistance.

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Chapter 5

$cPLA_2$ and lipoxygenase are required for cell cycle progression in neuroblastoma cells.

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(Submitted)

Abstract

Arachidonic acid has been implicated in regulating cellular proliferation, and is preferentially released by the 85 kDa cytosolic phospholipase A_2 ($cPLA_2$). Recently, we demonstrated that $cPLA_2$ is activated in distinct periods during the ongoing cell cycle of neuroblastoma cells. The purpose of the present study was to establish the role of these $cPLA_2$ activity peaks in cell cycle progression. Inhibition of $cPLA_2$ activity with arachidonyl trifluoromethylketone (ATK) in early G1 phase resulted in a marked reduction in DNA synthesis, whereas inhibitors for $sPLA_2$ and $iPLA_2$ had no remarkable effect. A 24h incubation of neuroblastoma cells with ATK, in which $cPLA_2$ activity was still inhibited, revealed no significant difference in cell number as compared to untreated cells. This suggests redundancy of the different PLA_2 enzymes. Lipoxygenase inhibition in early G1 resulted in G1 phase arrest, whereas inhibitors for cyclooxygenase had no effect. Furthermore, cells stopped progressing through S phase when lipoxygenase was inhibited in early S phase, demonstrating the requirement of lipoxygenase products for S phase progression.

Introduction

The factors that determine whether cells continue to proliferate, arrest growth or differentiate, are activated by signals from the extracellular environment and operate predominantly during the G1 phase of the cell cycle. Progression through the cell cycle is regulated by cyclin-dependent kinases (Cdks) that are activated upon binding with their cyclins, and by multiple phosphorylation and dephosphorylation steps (Nigg, 1995; Dirks & Rutka, 1997; Reed, 1997). The activity of Cdks is negatively regulated by Cdk inhibitory proteins that constists of two families of which the INK4 family specifically inhibits cyclin D/Cdk4,6 complexes, while the Kip/Cip family inhibits most cyclin/Cdk2,4 and 6 complexes (Sherr & Roberts, 1995; Hulleman & Boonstra, 2001). The first cyclin/Cdk complex in the G1 phase is activated by growth factors and consists of cyclin D and Cdk4 or 6 (Sherr, 1995), resulting in retinoblastoma phosphorylation and the subsequent activation of cyclin E/Cdk2. Furthermore, activated $p42/44^{MAPK}$ was shown to induce cyclin D expression and down-regulation of $p27^{Kip}$ (Aktas *et al*., 1997; Cheng *et al*., 1998). In addition, a sustained activation of $p42/44^{MAPK}$ is required to pass the restriction point, whereas inhibition of $p42/44^{MAPK}$ blocks DNA synthesis and proliferation (Pagès *et al*., 1993; Hulleman *et al*., 1999; Roovers *et al*., 1999). Also, $p42/44^{MAPK}$ overexpression was observed in human breast cancer cells (Sivaraman *et al*., 1997), showing the importance of $p42/44^{MAPK}$ activity in cell proliferation.

Recently, it was demonstrated that cytosolic phospholipase A_2 (cPLA$_2$) activity was cell cycle dependent and furthermore, cPLA$_2$ phosphorylation in these periods was mediated by p42/44^{MAPK} (van Rossum *et al*., 2001). cPLA$_2$ releases preferentially arachidonic acid from membrane phospholipids. The released arachidonic acid can be metabolised by cyctochrome p450s, cyclooxygenases, or by lipoxygenases to produce eicosanoids. Arachidonic acid and/or its metabolites appear to have an important role in growth-dependent signalling pathways and are involved in mitogenic signalling, cell migration and cytotoxicity (Piomelli, 1993; Sa & Fox, 1994; Grazia Cifone *et al*., 1997). Furthermore, most tumour cells produce elevated levels of eicosanoids that result in an induced growth and invasiveness of the tumours (Reich & Martin, 1996). Accordingly, in oncogenic ras transformed lung cancer cells, cPLA$_2$ expression was found to be constitutively high (Heasley *et al*., 1997).

To gain more insight in the mode of action of cPLA$_2$ in cell proliferation we studied the role of cPLA$_2$ activity in cell cycle progression. We present for the first time that cPLA$_2$ activity in G1 phase was required for progression into S phase. Neither iPLA$_2$ nor sPLA$_2$ are involved in progression to S phase. cPLA$_2$ inhibition for 24h resulted in a comparable cell number of ATK treated versus untreated cells, suggesting redundancy of the different PLA$_2$ enzymes. By using inhibitors we assessed the involvement of lipoxygenase, but not cyclooxygenase in cell cycle progression into S phase. Moreover, lipoxygenase inhibition in early G1 resulted in G1 phase arrest. DNA synthesis and S phase progression is blocked when lipoxygenase is inhibited in early S phase, demonstrating that lipoxygenase metabolites are required for S phase progression.

Materials and Methods

Materials.

Tissue culture nutrients, Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES (DMEM-HEPES) and foetal bovine serum (FBS) were purchased from Gibco BRL (Scotland). Methyl-[^{3}H]-thymidine (2 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). Arachidonyl trifluoromethylketone (ATK), bromoenol lactone (BEL), 5(S)-HpETE and 12(S)-HpETE were acquired from Cayman chemical (Ann Arbor, USA). Manoalide was from Biomol (Playmouth meeting, USA) and, NS-398 and caffeic acid were obtained from Calbiochem (La Jolla, USA). 4-Bromophenacyl bromide (4-BPB), indomethacin, nordihydroguaiaretic acid (NDGA) and arachidonic acid were purchased from Sigma-Aldrich chemie (Steinheim, Germany). All other chemicals were either from Sigma or Merck (Darmstadt, Germany).

Cell culture and cell synchronisation.

Neuroblastoma (N2A) cells were grown in DMEM-HEPES supplemented with 7.5% foetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere. One day prior to shake off, cells were cultured at $5x10^6$ cells per 175 cm^2 flask. Each hour flasks, containing asynchronous cells, were shaken for 1 min at 37°C to obtain mitotic cells as described previously (Boonstra *et al*., 1981).

[^{3}H]-thymidine incorporation.

Mitotic cells were plated in 24-wells plates at a density of $3x10^4$ cells per well in DMEM-HEPES containing 7.5 % FBS and 1 μCi [^{3}H]-thymidine/well. At the indicated times, the cells were washed twice with phosphate-buffered saline (PBS) whereafter, the cells were dissolved in 0.1 M NaOH and the incorporated [^{3}H]-thymidine was counted in a scintillation counter (LS 6000 SE, Beckman Instruments, Fullerton, CA). In other experiments, as indicated, [^{3}H]-thymidine incorporation was started 3 hours ahead of the actual experiment as a control. Then at the indicated times after mitosis half of the cells were incubated with ATK (10 μM) to inhibit $cPLA_2$ or with NDGA (10 μM) to inhibit lipoxygenase. After 30 min, [^{3}H]-thymidine was added to both control and ATK or NDGA treated cells. Fifteen min later the cells were washed twice with PBS, then washed with 10% trichloroacetic acid, washed again twice with PBS, whereafter the cells were dissolved in 0.1 M NaOH and [^{3}H]-thymidine incorporation was measured.

Western blot analysis.

Mitotic cells were replated and at the indicated times after mitosis the medium was removed and the cells were washed twice with ice-cold PBS. Subsequently, the cells were scraped in homogenisation buffer (50 mM Hepes/NaOH pH 7.4, 0.25 M sucrose, 50 mM NaF, 250 μM Na_3VO_4, 1 mM EGTA, 10 μM leupeptin, 1 μM pepstatin and 1 mM PMSF). Cells were homogenised by 15 strokes through a 26G needle and sonicated for 3 times 10 seconds. Proteins of $1x10^5$ cells per time point were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane by semi-dry blotting using a BioRad trans-blot SD. The membrane was blocked for 1 h at RT with 2% milk powder in PBST (PBS containing 0.1% (v/v) Tween-20) following primary antibody incubations overnight at 4°C in 0.2% milk powder in PBST. Cyclin A (Calbiochem, Cambridge, UK) was detected with a monoclonal antibody at a concentration of 2.5 μg/ml. Subsequently, the membrane was washed and primary antibodies were detected with rabbit anti-mouse IgG conjugated to horseradish peroxidase and the bands were visualised with enhanced chemiluminescence (NEN^{TM} Life science products, Boston).

Results

cPLA$_2$ activity in G1 phase is required for cell cycle progression.

We have previously demonstrated that cPLA$_2$ activity is cell cycle dependent in neuroblastoma (N2A) and Chinese hamster ovary (CHO) cells (van Rossum, *et al.,* 2001), being high in mitosis, thereafter decreasing in early G1. A small increase in activity was

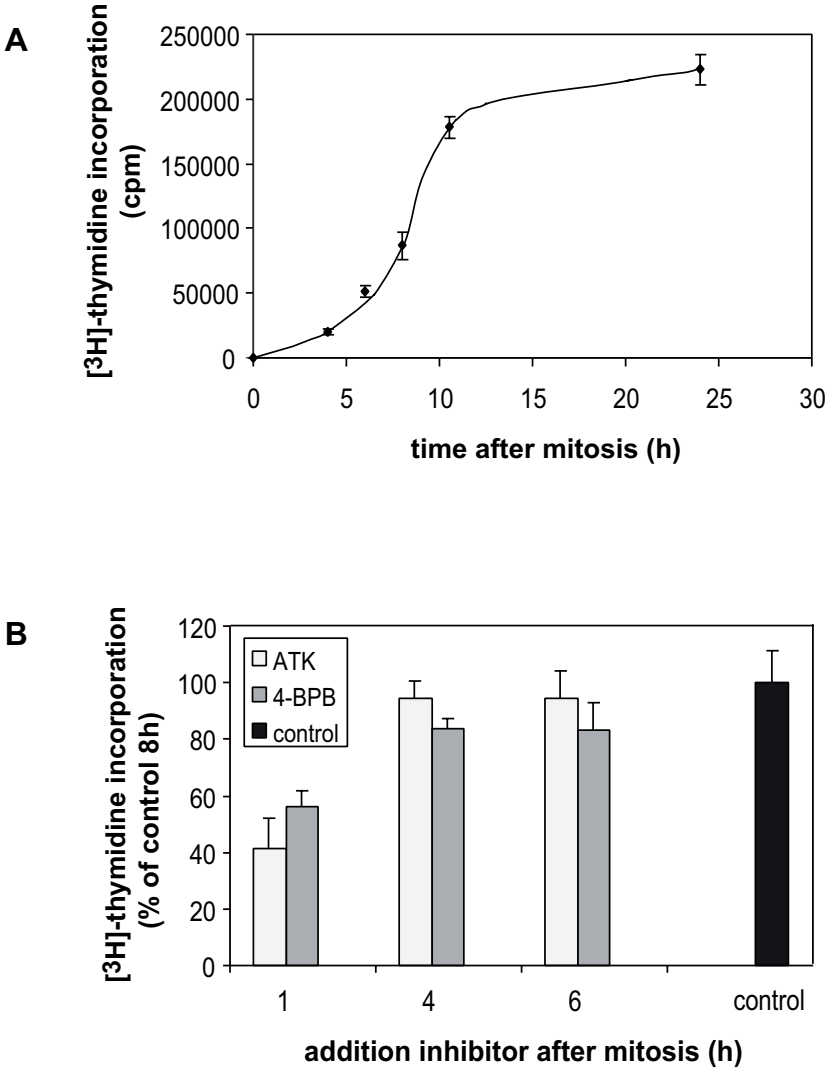

Figure 1. [^{3}H]-Thymidine incorporation (A) and effect of ATK and 4-BPB on DNA synthesis (B) in N2A cells, synchronised by mitotic shake off. (A) N2A cells were synchronised using the mitotic shake off method. [^{3}H]-Thymidine (1 μCi/well) was added to the mitotic cells and at the indicated times after mitosis the incorporated radiolabel was quantified using a liquid scintillation counter. (B) ATK (10 μM) and 4-BPB (10 μM) were added to synchronous cells at 1, 4 and 6h after mitosis and left to incubate until 8h after mitosis, whereafter the incorporated [^{3}H]-thymidine into the DNA was measured. Data are means ± SD (n=3).

measured during mid/late G1, and a strong increase was measured following the G1/S transition. In order to investigate whether $cPLA_2$ activity is required for cell cycle progression to S phase, the activity of $cPLA_2$ was inhibited at different time points in G1 phase in N2A cells, which were synchronised by using the mitotic shake off method (Boonstra, *et al.,* 1981). Therefore, a non-specific PLA_2 (4-bromophenacyl bromide; 4-BPB) (Chang *et al*., 1987) and a potent reversible $cPLA_2$ (arachidonyl trifluoromethyl ketone; ATK) inhibitor (Street *et al*., 1993; Trimble *et al*., 1993) were used. Synchronous cells were either left untreated, or were incubated with ATK (10 μM) or 4-BPB (10 μM) from 1, 4 and 6h after mitosis. At 8h, at which most cells have entered the S phase (fig. 1A), DNA synthesis was determined by measuring [^{3}H]-thymidine incorporation of the cells as described in Materials and Methods. As shown in figure 1B, [^{3}H]-thymidine incorporation was reduced approximately 60% as compared to control cells, if ATK is added 1h after mitosis, while no reduction is measured when ATK is added at later time points. Apart from the effect on DNA synthesis, inhibition of $cPLA_2$ activity did not influence the total number of cells, indicating that the inhibition of DNA synthesis was not due to cytotoxic effects of ATK (data not shown). In addition, a similar pattern of [^{3}H]-thymidine incorporation was obtained in synchronous N2A cells treated with 4-BPB. These results indicate the requirement of $cPLA_2$ activity in G1 for cell cycle progression.

However, ATK has also been reported to be able to inhibit the Ca^{2+}-independent PLA_2 ($iPLA_2$) in macrophages, although at higher concentrations (Ackermann *et al*., 1995). Therefore, it was investigated whether the reduction in [^{3}H]-thymidine incorporation was solely due to $cPLA_2$ or due to other PLA_2 isoforms as well. To discriminate between $cPLA_2$ and $iPLA_2$, bromoenol lactone (BEL) was used, which is a potent irreversible inhibitor of $iPLA_2$, but not of $cPLA_2$ (Zupan *et al*., 1993; Ackermann, et al., 1995). The involvement of $sPLA_2$ was investigated by the use of manoalide (Jacobson *et al*., 1990). BEL (2.5 μM) or manoalide (0.1 μM) were added to synchronously growing cells at 2, 4 and 6h after mitosis, or the cells were left untreated. The [^{3}H]-thymidine incorporation measured at 8h was only slightly reduced when BEL was added at 2 and 4h after mitosis as compared to control cells (fig. 2). No effect was observed in cells incubated with BEL from 6h after mitosis. Manoalide only marginally affected the [^{3}H]-thymidine incorporation of the cells when added at 2, but not at 4 or 6h as compared to control cells. This indicates that $sPLA_2$ is not and $iPLA_2$ is at best only marginally involved in cell cycle progression to S phase.

It is, however, not clear whether $cPLA_2$ inhibition results in cell cycle arrest or whether it is just delayed. Therefore, cell count experiments were performed in which cells were left untreated, or to which ATK was added at 0, 1 or 2h after mitosis. At 24h after mitosis the total number of cells in ATK treated cells was similar to untreated cells (fig. 3) and moreover was increased as compared to the total number of cells present at 8h, showing that cells have completed their cell cycle. In addition, ATK was still functional, since $cPLA_2$

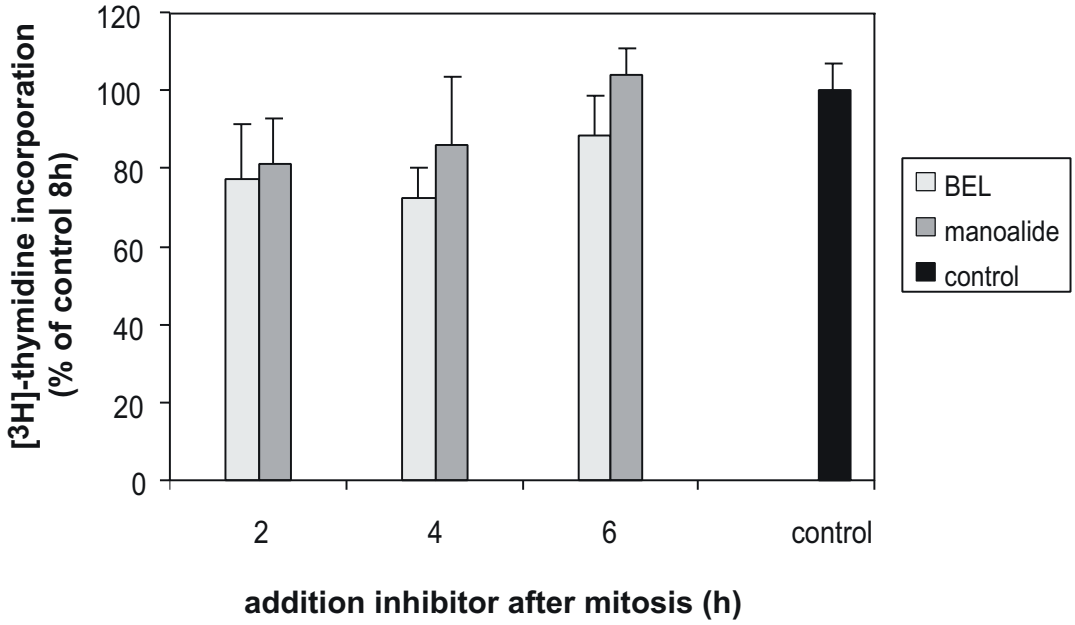

Figure 2. Effects of PLA$_2$ inhibitors on cell cycle progression into S phase in N2A cells. Synchronous N2A cells were left untreated, or incubated with ATK (10 μM), BEL (2.5 μM) and manoalide (0.1 μM) from 2, 4 and 6h after mitosis. [^{3}H]-Thymidine incorporation was measured at 8h after mitosis. Data are the means ± SD (n=3).

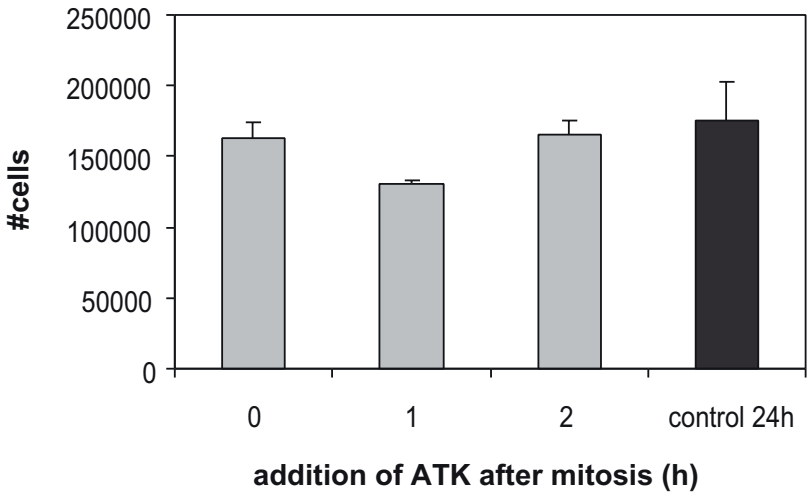

Figure 3. Long term cPLA$_2$ inhibition does not result in cell cycle arrest. To synchronous N2A cells, ATK (10 μM) was added at 0, 1 and 2h after mitosis and cells were left to incubate until 24h after mitosis. Then, the total numbers of cells were counted. Data are means ± SD (n=2).

activity in synchronous cells treated for 8 or 24h with ATK was inhibited as compared to untreated cells (data not shown).

Collectively, these results show the requirement of $cPLA_2$, but not $iPLA_2$ or $sPLA_2$, in cell cycle progression, since 4-BPB did not reduce DNA synthesis any further than ATK, whereas inhibitors for $iPLA_2$ and $sPLA_2$ displayed no significant effect. In addition, $cPLA_2$ inhibition in early G1 results in a temporal inhibition of cell cycle progression.

Lipoxygenase, but not cyclooxygenase are involved in cell cycle progression.

The arachidonic acid released by $cPLA_2$, can be further metabolised by cyclooxygenases or lipoxygenases into a large family of eicosanoids that have been implicated amongst others, in mitogenic signalling, cytotoxicity and cancers (Dethlefsen *et al*., 1994; Hsi & Eling, 1998; Shappell *et al*., 2001). To examine the role of cyclooxygenase (COX) and lipoxygenase (LO) on cell cycle progression, we evaluated the effects of COX and LO inhibitors on DNA synthesis. Indomethacin (Indo, 10 μM), a non-selective COX inhibitor (Laneuville *et al*., 1994), and NS 398 (10 μM), a selective COX-2 inhibitor (Copeland *et al*., 1994), were added at 2, 4 and 6h after mitosis and [^{3}H]-thymidine

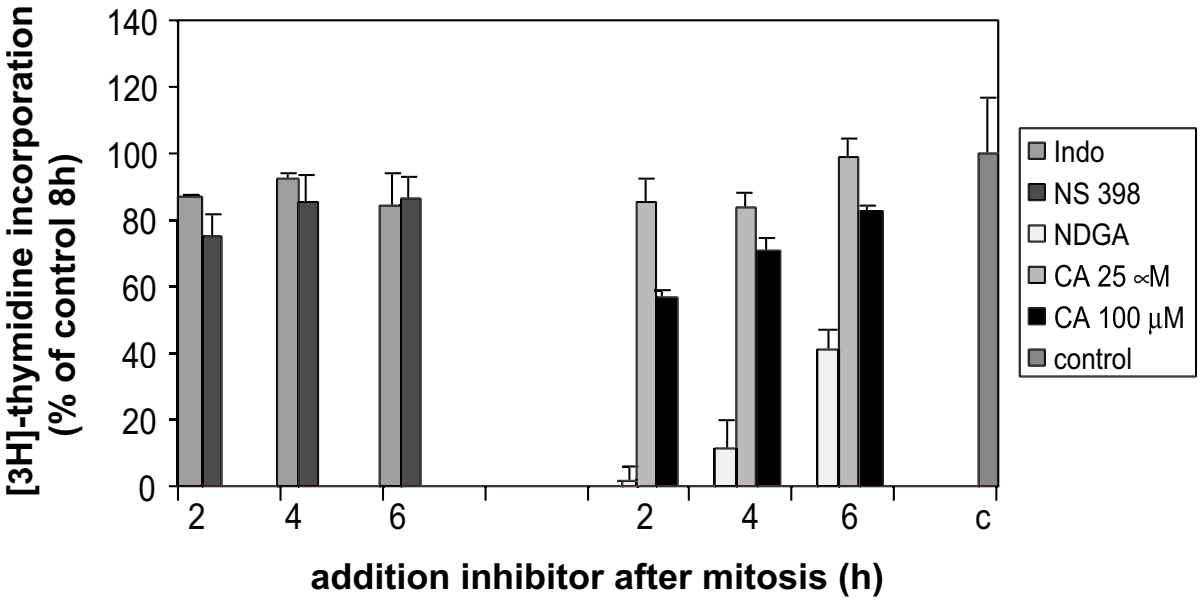

Figure 4. Effects of cyclooxygenase and lipoxygenase inhibitors on cell cycle progression. Synchronous N2A cells were left untreated, or incubated with the cyclooxygenase inhibitors Indo (10 μM) and NS 398 (10 μM), and the lipoxygenase inhibitors NDGA (10 μM) and CA (25 and 100 μM) from 2, 4 and 6h after mitosis. The amount of [^{3}H]-thymidine incorporated into the DNA was measured at 8h after mitosis. Data are means ± SD (n=3).

incorporation was measured at 8h after mitosis. These inhibitors did not cause a significant reduction in the [^{3}H]-thymidine incorporation relative to control cells (fig. 4), demonstrating that neither COX-1 nor COX-2 plays a role in G1/S phase progression. On the other hand, a complete inhibition in [^{3}H]-thymidine incorporation was measured at 8h when lipoxygenase was inhibited from 2 or 4h after mitosis with 10 μM nordihydroguaiaretic acid (NDGA), a potent inhibitor of lipoxygenases (Hope *et al*., 1983; Salari *et al*., 1984). Still an inhibition of approximately 60% was observed upon addition of NDGA at 6h after mitosis. These inhibitory effects of NDGA were not due to cytotoxic effects of NDGA, since the total number of cells was similar to untreated cells (data not shown). In contrast, 25 μM caffeic acid (CA), which predominantly inhibits 5- and 12-LO but at higher IC_{50} values than the common lipoxygenase inhibitor NDGA (Koshihara *et al*., 1984; Rao *et al*., 1993), was not effective in inhibiting [^{3}H]-thymidine incorporation. A significant inhibition of about 40 and 30%, respectively, was measured when 100 μM caffeic acid was added only at 2 and 4h. These results show that lipoxygenase, is involved in cell cycle progression to S phase and that inhibition of lipoxygenase in early G1 may result in cell cycle arrest. We further investigated whether lipoxygenase inhibition in G1 results in G1 phase arrest by

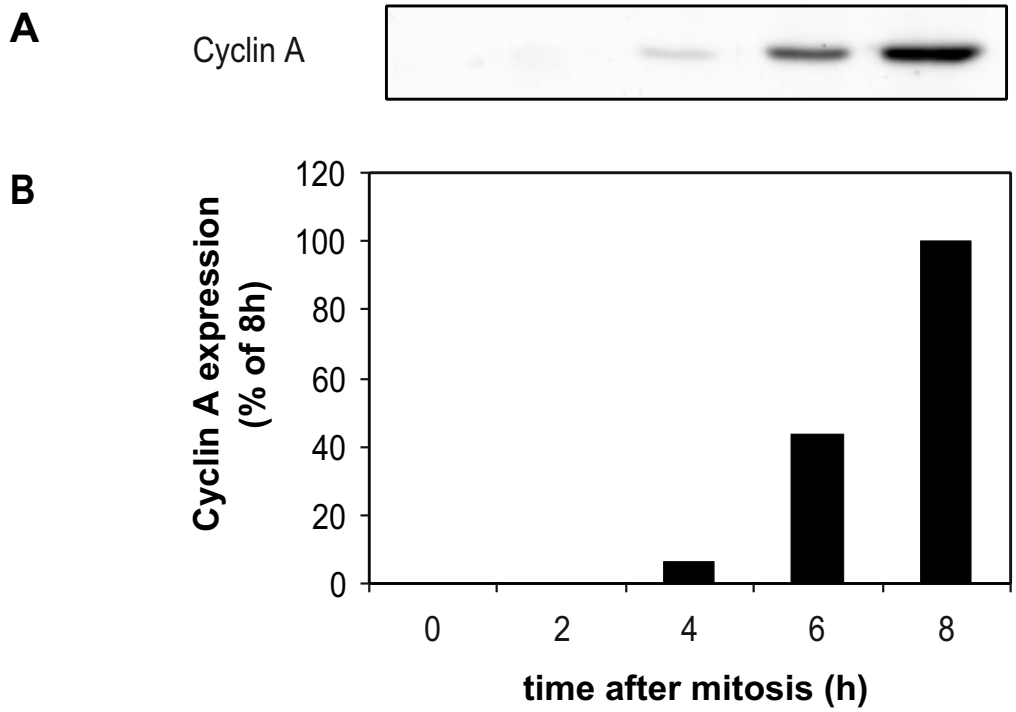

Figure 5. Expression of cyclin A during the ongoing cell cycle. Cells were synchronised via mitotic shake off, replated and harvested at the indicated times after mitosis as described in Materials and Methods. Cell lysates of 1x10^5 cells were separated on SDS-PAGE whereafter, cyclin A was analysed on western blot (A) and cyclin A expression was quantified (B).

determination of cyclin A expression, since cyclin A is expressed in late G1 prior to DNA synthesis (Schulze *et al*., 1995). The western blot in figure 5A shows that during the ongoing cell cycle cyclin A is a little expressed at 4h after mitosis, which represents mid/late G1 (fig. 1A), while at 6h, in early S phase, and at 8h, in S phase, a significant increase in cyclin A is observed. These results were evaluated by quantification of the bands and presented as a percentage of cyclin A expressed at 8h after mitosis (fig. 5B).

Incubating synchronous N2A cells from 0 or 2h after mitosis with NDGA and left to incubate until 8h, expressed cyclin A only to a low extent, as compared to cyclin A expression in control cells at 8h (fig. 6A). The densitrometrical evaluations of the blots show that cyclin A expression of NDGA treated cells at 0 or 2h did not reach the expression present at 8h after mitosis (fig. 6B), but remained at the level present at 4h after mitosis of normal cycling cells (fig. 5B). Thus demonstrating that the cells are arrested in G1. Similarly, treating cells from 4 or 6h after mitosis with NDGA and analysing cyclin A at 8h did not result in a full expression as compared to control cells at 8h (fig. 6). Cyclin A

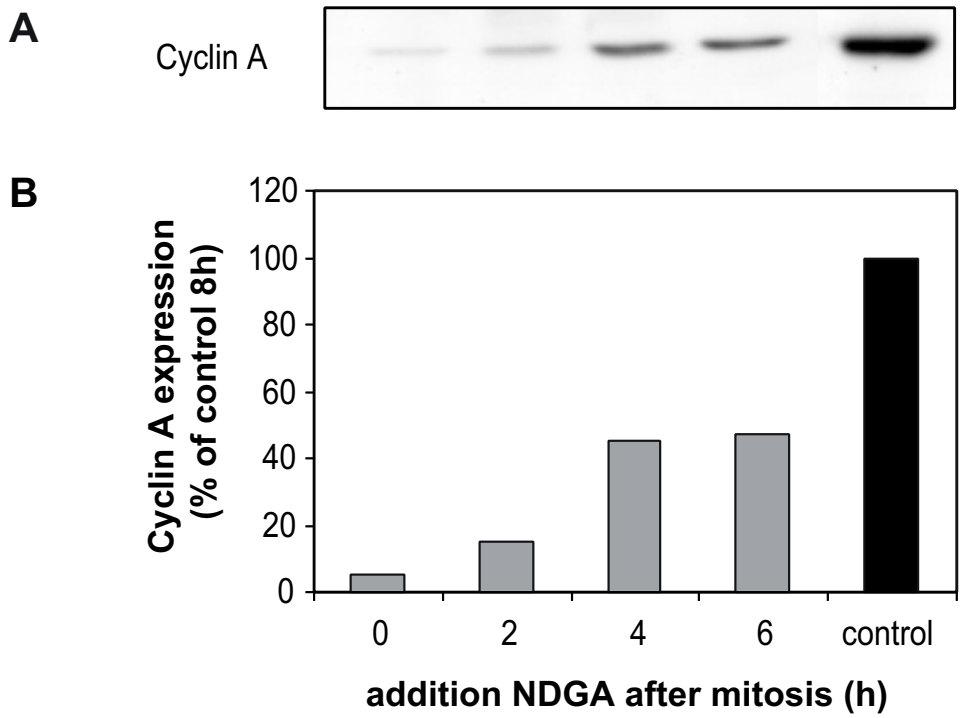

Figure 6. Lipoxygenase inhibition results in cell cycle arrest. Synchronised N2A cells were left untreated or were incubated with NDGA (10 μM) from 0, 2, 4 and 6h after mitosis and harvested at 8h after mitosis. Thereafter, $1x10^5$ cells were used for electrophoresis and cyclin A was analysed on western blot (A). Quantification of cyclin A expression of cells incubated with NDGA, as percentage of untreated cells at 8h after mitosis.

expression remained at the level present at 6h of untreated cycling cells, as can also be drawn from the densitometrical evaluations (fig. 6B versus 5B). To more specifically examine cPLA$_2$ mediated regulation of G1/S phase progression, synchronous cells were treated with ATK at different hours after mitosis whereafter arachidonic acid (25 μM) was added back. At 8h after mitosis [^{3}H]-thymidine incorporation was measured but no increase in DNA synthesis was measured as compared to ATK treated cells (data not shown). Also no increase was measured when using higher or lower concentrations of arachidonic acid. However, arachidonic acid can be broken down in the β-oxidation to generate energy or reacylated into phospholipids by iPLA$_2$. Therefore, similar experiments were performed in which lipoxygenase was inhibited with NDGA at different hours after mitosis. One or ten μM of 5(S)-, 12(S)-, 15(S)-HpETE or 15(S)-HETE was added back, but no increase in [^{3}H]-thymidine incorporation was measured as compared to NDGA treated cells. These data demonstrate that lipoxygenase inhibition in early G1 results in G1 phase arrest which, in our experimental set- up, cannot be overcome by the first synthesised products of lipoxygenase, and that lipoxygenase may also be involved in S phase progression.

Effect of cPLA$_2$ and lipoxygenase inhibition on S phase progression.

Since cPLA$_2$ activity during the cell cycle is high in S phase, we examined the possible role of cPLA$_2$ during the S phase. Therefore, each hour after mitosis, the [^{3}H]-thymidine incorporation was measured as a control, and ATK was added at 6h after mitosis, which is in S phase, to inhibit cPLA$_2$ activity. No inhibition in [^{3}H]-thymidine incorporation was observed (fig. 7 triangles), as compared to control cells (fig. 7, diamonds), showing that cPLA$_2$ activity in S phase in not necessary for DNA synthesis.

Next, we investigated whether lipoxygenase products play a role in S phase progression. Therefore, NDGA was added at 3 and 5.30h after mitosis, and [^{3}H]-thymidine incorporation measurements were started for different time periods. As shown in figure 8, 3h after mitosis is still in the G1 phase (triangles) of the cell cycle, while at 5.30 the cells are starting to progress through S phase (squares). When inhibiting lipoxygenase at 3h, a full inhibition in the [^{3}H]-thymidine incorporation was observed, showing that the cells are not able to progress into S phase any longer. These data are in agreement with the previous results of figure 6 in that inhibition of lipoxygenase in early G1 resulted in G1 phase arrest. Furthermore, the addition of NDGA at 5.30h resulted in a complete inhibition in DNA synthesis that was also confirmed by cyclin A expression (fig. 6), thus demonstrating the importance of lipoxygenase products in the progression through S phase.

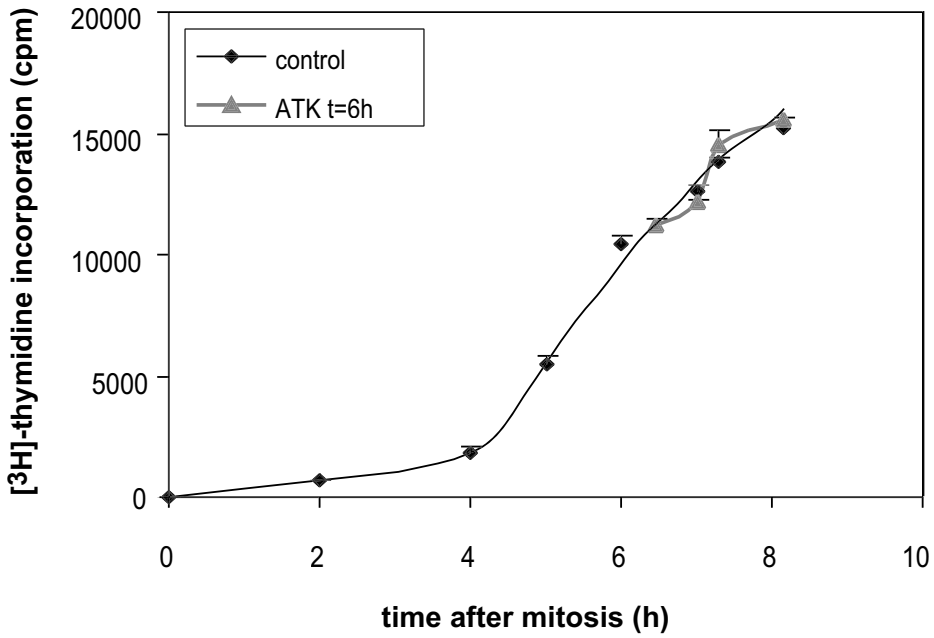

Figure 7. cPLA$_2$ activity is necessary for S phase progression. ATK (10 μM) was added to half of the cells at 6h (triangles) after mitosis. After 30 min [^{3}H]-thymidine (1 μCi/well) was added to both untreated and ATK treated cells and 15 min later [^{3}H]-thymidine incorporation was measured at the indicated times after mitosis as described in Materials and Methods. Data are represented as means ± SD (n=3).

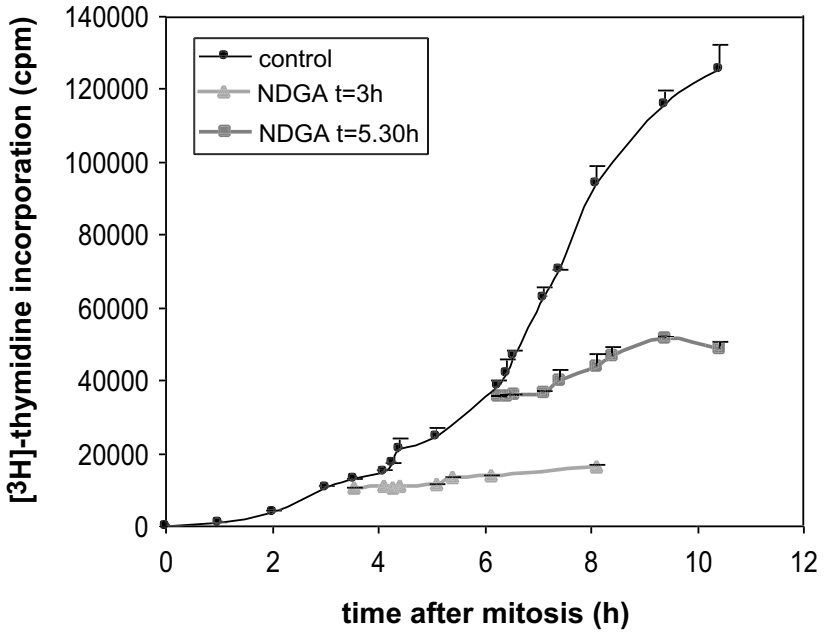

Figure 8. Lipoxygenase is required for S phase progression. At 3 (triangles) and 5.30h (squares) after mitosis NDGA (10 μM) was added to the cells. After 30 min [^{3}H]-thymidine (1 μCi/well) was added to both untreated and NDGA treated cells and 15 min later [^{3}H]-thymidine incorporation was measured at the indicated times after mitosis as described in Materials and Methods. Data are represented as means ± SD (n=3).

Discussion

Although previous studies demonstrated that PLA_2 inhibition reduced cell proliferation (Adachi *et al*., 1996; Martinez *et al*., 1997; Korystov Yu *et al*., 1998), the present study is the first to show that $cPLA_2$ activity at distinct periods in the cell cycle is required for cell cycle progression. By using [^{3}H]-thymidine incorporation as a marker for DNA synthesis and progression from G1 to S phase, inhibition of $cPLA_2$ in early G1, using ATK resulted in a reduced DNA synthesis. This reduction was measured only when $cPLA_2$ was inhibited until 2-3h after mitosis. Interestingly, after 2-3h the small increase in $cPLA_2$ activity during the G1 phase occurs (van Rossum, *et al.,* 2001). Inhibition of $cPLA_2$ at 4h, or at 6h, which is in G1 or S phase, respectively, did not result in a reduced DNA synthesis. In addition, similar results were obtained with the non-specific PLA_2 inhibitor, 4-BPB. Manoalide only slightly reduced DNA synthesis at 2h and BEL at 2 and 4h, the latter reduction might be the result of phosphatidate phosphohydrolase inhibition (Balsinde & Dennis, 1996). These data show that in N2A cells, neither $iPLA_2$ nor $sPLA_2$ play an important role in cell cycle progression. In N2A cells the activity of $cPLA_2$ in mid/late G1 phase is important for cell cycle progression into S phase, while $cPLA_2$ activity in S phase is not required for DNA synthesis.

Since $cPLA_2$ activity was required for G1/S phase progression we evaluated whether this inhibition resulted in cell cycle arrest. The total cell number of synchronised N2A cells after a 24h treatment with ATK was comparable to untreated cells and moreover, was increased to the total number of cells present at 8h. This shows that the cells have completed their cell cycle, and may result in an increased doubling time of the ATK treated cells. It has been previously demonstrated that proliferation of asynchronous human coronary artery vascular smooth muscle cells growing to confluency was inhibited by ATK, but no phase-specific arrest of the cell cycle was observed (Anderson *et al*., 1997). We also find that, although $cPLA_2$ activity is required for G1/S phase progression, the cells do complete their cell cycle, while $cPLA_2$ activity was still inhibited after a 24h treatment of ATK. It is, however, also possible that $cPLA_2$ function is taken over by other enzymes, like the $sPLA_2$s, which then become active to finally generate the arachidonic acid that is necessary for progression through the cell cycle. Redundancy of PLA_2 enzymes has been observed in $P388D_1$ macrophages in which a transient accumulation of arachidonic acid produced by $cPLA_2$ resulted in phospholipid hydrolysis which was probably mediated by activated $sPLA_2$ (Balsinde & Dennis, 1996).

The mechanism by which $cPLA_2$ influences cell cycle progression remains to be determined. Cyclooxygenase inhibitors had no effect on cell cycle progression into S phase, indicating that the $cPLA_2$-dependent progression is not mediated by arachidonic acid metabolites converted by cyclooxygenase. However, our findings demonstrate that

lipoxygenase is involved in cell cycle progression. Since the lipoxygenase inhibitors, caffeic acid and NDGA, respectively partially and completely inhibited DNA synthesis when added in early G1, which was not due to cytotoxic effects of the inhibitors (data not shown). Additionally, cells in which lipoxygenase was inhibited from 3h after mitosis did not result in DNA synthesis (fig. 8). This was also confirmed by the low expression of cyclin A in cells incubated with NDGA from 0 or 2h after mitosis as compared to control cells. These data show an arrest in G1 phase of lipoxygenase inhibited cells. In line with this are the results of Korystov *et al.* (1998) who demonstrated a suppression of cell proliferation of lympholeukemic cells with NDGA, also by blocking the G1/S transition. However, the mechanism by which NDGA blocks the G1/S transition is still unknown. Re-addition of arachidonic acid or the lipoxygenase metabolites 5(S)-, 12(S)- or 15(S)-HpETE, or 15(S)-HETE at various times and concentrations after mitosis could not overcome the inhibitory effect of ATK or NDGA. It might be that NDGA inhibits a protein through the inhibition of lipoxygenase, which is required for S phase entry. It has been reported that lipoxygenase was involved in mediating arachidonic acid-induced $p42/44^{MAPK}$ activation (Rao *et al*., 1994; Chang & Wang, 2001). However, lipoxygenase inhibition by NDGA did not affect $p42/44^{MAPK}$ phosphorylation in N2A cells (data not shown), indicating that lipoxygenase exerts its effects downstream of $p42/44^{MAPK}$. Since eicosanoids are synthesised on demand at the time and location needed, it is possible that the concentrations used are still to high or to low. The products may even not reach the location at which they act, like for arachidonic acid that, next to its action, can be broken down to generate energy or can be re-incorporated into phospholipids.

Lipoxygenase inhibition in early S phase also resulted in a block in DNA synthesis. Furthermore, cells in which lipoxygenase was inhibited from 4 or 6h after mitosis were arrested in early S phase as judged by cyclin A expression. Taken together, $cPLA_2$ activity in G1 phase is necessary for cell cycle progression into S phase, while $cPLA_2$ activity in S phase is not involved in DNA synthesis. Furthermore, lipoxygenase is required for both G1/S and S phase progression.

Acknowledgments

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Chapter 6

Summarising Discussion

Signal transduction pathways activating cPLA$_2$.

The studies described in chapters 2, 3 and 4 show that cPLA$_2$ is activated through different signal transduction pathways depending on the stimulus in the extracellular environment. In response to EGF, cPLA$_2$ is predominantly activated through PKC-MEK-p42/44^{MAPK}, while serum-induced cPLA$_2$ activity is mainly mediated via the Raf-MEK-p42/44^{MAPK} pathway. In contrast, direct activation of PKC by phorbol ester (PdBu) did not result in increased cPLA$_2$ activity, while p42/44^{MAPK} was activated via Raf-MEK and through MEK. Activation of cPLA$_2$ by the oxidant H_2O_2 is partly mediated via Raf-MEK-p42/44^{MAPK} and partly through a phosphorylation-independent mechanism involving peroxidised phospholipids.

These results suggest that activation of cPLA$_2$ is not only governed by post-translational modifications but, more importantly, by localisation of the signal transduction components at a certain time that determines whether cPLA$_2$, at which place and what time cPLA$_2$ will be activated. Cells appear to respond to diverse stimuli resulting in the activation of different signal transduction pathways which might lead to different cellular processes, such as differentiation, proliferation and apoptosis. This shows that cells are very flexible in adapting to changes in the extracellular environment. This is illustrated by an increasing number of evidence indicating that p42/44^{MAPK} can be targeted to specific sites within the cell. For example, p42/44^{MAPK} is able to associate with the cytoskeleton, is present at different locations in and phases of mitotic cells, can phosphorylate several proteins in the cytoplasm, or translocate to the nucleus to activate transcription factors (reviewed in Schaeffer & Weber, 1999; Hulleman & Boonstra, 2001). Nerve growth factor stimulation of PC12 cells resultes in a sustained activation of p42/44^{MAPK}, leading to exit of the cell cycle and differentiation of the cells. In contrast, EGF induces a transient p42/44^{MAPK} activation leading to proliferation of the cells (reviewed in Marshall, 1995).

We propose in serum- and EGF-activated Her14 cells, activation of p42/44^{MAPK} throughout the entire cell, since the EGF receptor is still active when internalised in endosomes (Lai *et al*., 1989; Wada *et al*., 1992). Additionally, compartimentalisation of phosphorylated EGF receptors with Shc proteins, but also active Raf and MEK in early endosomes have been shown (Baass *et al*., 1995; Pol *et al*., 1998). The activated p42/44^{MAPK} then phosphorylates and activates cPLA$_2$. In contrast, activation of PKC by PdBu probably occurs at the plasmamembrane, because of the hydrophobic nature of PdBu. Thereupon translocation of PKC to the membrane occurs, resulting in p42/44^{MAPK} activation near the plasmamembrane. However, no activation of cPLA$_2$ was observed, likely because p42/44^{MAPK} and cPLA$_2$ are not present at exactly the same place. This implies the activation of another population of p42/44^{MAPK}, which might indeed be the case, since PdBu activates another set of PKC isoforms, in contrast to EGF and serum stimulation of Her14 cells (chapter 2). Exposing cells to H_2O_2 can activate cPLA$_2$ within the entire cell at membranes.

It has been shown that H_2O_2 inhibits internalisation of receptors, like for the EGF and growth hormone receptor (Strous *et al*., 1996; de Wit *et al*., 2001). Also the ubiquitination of the receptors is inhibited, therefore, the receptor will not be degraded by the proteasome pathway and thus will remain active. Because of this, signalling molecules are activated at the plasmamembrane or any other membrane within the cell. This results in $cPLA_2$ activation in the vicinity of the membranes, whereupon $cPLA_2$ translocates to the membrane due to H_2O_2-induced peroxidation of membrane lipids. Arachidonic acid release from the membrane is facilitated due to these peroxidised lipids thereby prolonging $cPLA_2$ activity, which it is usually subject to product inhibition (Reynolds *et al*., 1993; Bayburt & Gelb, 1997; Burke *et al*., 1999). Over time, $cPLA_2$ has to be dephosphorylated to inactivate the enzyme, probably by phosphatases. Phosphatases can be inactivated through the action of H_2O_2 (Sullivan *et al*., 1994; Caselli *et al*., 1998), leaving $cPLA_2$ active for a longer period of time.

$cPLA_2$ in Her14 cells is predominantly localised as clusters near all organellar membranes, except for the golgi apparatus (Bunt *et al*., 1997). No translocation of $cPLA_2$ to (particular) membranes was observed in EGF- as well as in A23187-stimulated cells. We have proposed a cluster-monomer model in which these clusters represent the inactive form of $cPLA_2$ from which active monomers can be recruited upon stimulation (Bunt *et al*., 2000), that might occur via phosphorylation of $cPLA_2$ by $p42/44^{MAPK}$. This means that, in principle, $cPLA_2$ can be activated at every membrane within the cell, but the presence of $p42/44^{MAPK}$ to phosphorylate $cPLA_2$ is a prerequisite. As described above, $p42/44^{MAPK}$ and other components of the signal transduction pathways differ in localisation, depending on the stimuli and therefore, is $cPLA_2$ only activated at certain locations within the cell, otherwise the cell will be destroyed. Thus, to acquire a good insight in the cellular functions of $cPLA_2$, one should actually focus on the localisation of active $cPLA_2$ monomers.

Cell cycle regulation.

The cellular localisation of signal transduction components determines whether $cPLA_2$ will be activated. However, understanding the function of $cPLA_2$ in cells requires also knowledge of the activation of $cPLA_2$ in a temporal manner. Therefore, the activity of $cPLA_2$ was investigated during the ongoing cell cycle as described in chapters 4 and 5. $cPLA_2$ activity was high in mitosis, decreasing afterwards, peaking at mid/late G1 and a subsequent increase in activity was measured following the G1/S transition. These changes in $cPLA_2$ activity were not due to differences in $cPLA_2$ protein expression, but due to $p42/44^{MAPK}$ mediated phosphorylation of the enzyme. Inhibition of $cPLA_2$ in early G1 phase resulted in a reduced progression into S phase.

The high activity of $cPLA_2$ in mitotic cells suggests a function for $cPLA_2$ during M phase. At prometaphase the spindle microtubules have to interact with the chromosomes. To accomplish this, the nuclear envelope has to be broken down that might be mediated by $cPLA_2$. Immunofluorescence studies showed that a small pool of total $p42/44^{MAPK}$ is also active during M phase where it is associated with tubulin and MAP-2 (Morishima-Kawashima & Kosik, 1996). Interestingly, it was reported that $cPLA_2$ in mitotic cells obtained by colcemid treatment was suppressed compared to interphase cells (Berlin & Preston, 1995). This seems to be conflicting; however, colcemid disrupts microtubuli leaving $p42/44^{MAPK}$ and $cPLA_2$ inactive. $p42/44^{MAPK}$ is also inactive when cyclin B is degraded, to exit from mitosis, which might result in the decrease in $cPLA_2$ activity after mitosis.

The next period of increased $cPLA_2$ activity is during mid/late G1. In this period $p42^{MAPK}$ was shown to translocate to the nucleus (Hulleman *et al*., 1999), and $cPLA_2$ activation was mediated by $p42/44^{MAPK}$. This suggests that $cPLA_2$ is activated at the nuclear envelope, or is also translocated to the nucleus, since the arachidonic acid released by $cPLA_2$ is involved in G1/S progression. Lipoxygenase inhibition in early G1 arrested N2A cells in G1. This indicates that the arachidonic acid released in mid/late G1 is converted by lipoxygenase into lipoxygenase products, which are actually involved in G1/S phase progression. However, the precise mechanism remains to be dissolved.

Lipoxygenase inhibition in S phase was demonstrated to block DNA synthesis. This process is probably not mediated through the conversion of arachidonic acid released by $cPLA_2$, since $cPLA_2$ inhibition in S phase did not result in a reduced DNA synthesis. In addition, also inhibition of $cPLA_2$ at 4 or 6h after mitosis did not reduce DNA synthesis, although this is not completely clear. $cPLA_2$ and especially lipoxygenase play an important role in the progression of the cell cycle. This is supported by the observation that lipoxygenase and $cPLA_2$ are overexpressed in cancer cells (Funk, 1996; Heasley *et al*., 1997; Ikawa *et al*., 1999; Shappell *et al*., 2001). Thus it is important to understand the function and mechanism by which lipoxygenase and $cPLA_2$ regulate cell cycle progression for the development and therapeutic use of anti-cancer drugs.

Phospholipase A_2 and reactive oxygen species in cellular injury

At present, it is commonly known that ROS, such as $O_2^{\bullet -}$ and H_2O_2, are produced in a variety of cells in response to growth factors, agonists of G-protein-coupled receptors (GPCRs) and cytokines, and are required for the mitogenic response. For example, EGF (Bae *et al*., 1997), PDGF (Sundaresan *et al*., 1995), thrombin (Patterson *et al*., 1999), lysophosphatidic acid (Sekharam *et al*., 2000), Interleukin-1, Interferon-γ (Krieger-Brauer & Kather, 1995) and TNF-α (Lo & Cruz, 1995). Upon the release of H_2O_2, the receptor

becomes tyrosine phosphorylated, resulting in complex formation with signalling molecules, such as Shc, Grb2 and sos (Rao, 1996). Macrophages and neutrophils also produce ROS to destroy microorganisms in the extracellular environment. An inappropriate release of these agents into the surrounding tissues and cells can result in cellular injury. This depends on the concentration and duration of the stress, on the cell type and on the cell cycle phase of the cells. For instance brain function and kidney epithelial cells are highly sensitive to ROS (Sussman & Bulkley, 1990; Schubert *et al*., 1995; Sapirstein *et al*., 1996). On the other hand, astroglial cells as well as Her14 cells appear to be more resistant to oxidants (Café *et al*., 1995), whereas CHO cells arrest in G1 and in G2/M phase (Clopton & Saltman, 1995).

PLA_2 and ROS have been implicated in various forms of cellular injury. The increase in $cPLA_2$ activity is suggested to occur through a mechanism as discussed earlier, in which $cPLA_2$ activity is increased due to phosphorylation of the enzyme and due to peroxidation of membrane lipids. Because of this increase in (c)PLA_2 activity and the concomitant release of arachidonic acid, it was first thought that (c)PLA_2 would have a repair function by releasing peroxidised arachidonic acid. However, in many of these studies, no discrimination was made between oxidised and unoxidised arachidonic acid. It was shown later that $cPLA_2$ still preferentially liberates unoxidised over oxidised arachidonic acid (Nigam & Schewe, 2000), thus indicating that $cPLA_2$ is not involved in a repair function. Recent studies show the involvement of $cPLA_2$ in cell death (Wissing *et al*., 1997; de Valck *et al*., 1998; Hornfelt *et al*., 1999). The injured cells have to be broken down, and although the precise mechanism of action is unknown, it might involve a massive activation of $cPLA_2$.

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Samenvatting

Samenvatting

Het menselijk lichaam is opgebouwd uit zeer veel cellen. Deze cellen bestaan uit verschillende kamertjes (organellen), die in het celvocht (cytoplasma) liggen dat omgeven wordt door een muur (de celmembraan). De organellen hebben allemaal hun eigen functie. Zo is er de kern waarin het erfelijk materiaal (het DNA) ligt opgeslagen en de mitochondriën zorgen voor de energieproduktie in de cel. In de lysosomen worden niet meer functionerende organellen en grote molekulen afgebroken, terwijl de peroxisomen zorgen voor de afbraak van verschillende vetten. Nieuwe eiwitten worden gemaakt in het endoplasmatisch reticulum die daarna naar het golgi-apparaat gaan waar de eiwitten gesorteerd en gelabeld worden zodat ze op de goede plek in de cel terechtkomen. Al deze organellen zijn omgeven door een membraan dat voornamelijk opgebouwd is uit vetachtige molekulen, fosfolipiden genaamd. Fosfolipiden bestaan uit een "ruggegraat" waaraan op de 1- (*sn*-1) en 2- (*sn*-2) plaats een vetzuurstaart vastzit en op de 3^{e} plaats bevindt zich de kopgroep.

In de cel zijn bepaalde enzymen (eiwitten die een reaktie sneller kunnen laten verlopen) die het vetzuur van de 2-plaats van membraan fosfolipiden vrijmaken (hydrolyseren), dat zijn de fosfolipases A_2. Dit hydrolyseren is heel belangrijk wanneer het vrije vetzuur arachidonzuur is, omdat arachidonzuur bij veel fysiologische processen en ziektes betrokken is, zoals bij ontstekingen, neurodegeneratieve ziektes als Alzheimer en Parkinson, het doodgaan van cellen, het doorgeven van boodschappen in een cel (signaal transductie) en kanker. Arachidonzuur wordt preferentieel vrijgemaakt door het cytosolische fosfolipase A_2 ($cPLA_2$) dat 85 kDa groot is. De activiteit van het $cPLA_2$ moet goed gereguleerd worden in de cel, anders ontstaan er pathologische verschijnselen. $cPLA_2$ kan alleen arachidonzuur vrijmaken als het aan membranen gebonden is, omdat hier zich het arachidonzuur bevindt. Hydrolysering van arachidonzuur kan alleen als het $cPLA_2$ actief is, want in een inactieve toestand bevindt het $cPLA_2$ zich in het cytoplasma. $cPLA_2$ activiteit wordt verhoogd wanneer een fosfaatgroep wordt geplaatst op het aminozuur serine nummer 727 en/of 505 door een signaal transductie eiwit behorende tot de MAPK familie, meestal $p42/44^{MAPK}$. Daarna kan onder invloed van een verhoogde calcium concentratie in de cel het $cPLA_2$ aan membranen binden en vervolgens arachidonzuur vrijmaken.

In hoofdstuk 1 worden de karakteristieken van het $cPLA_2$ beschreven, wat er al bekend is over de regulatie van $cPLA_2$ en hoe het proces van celdeling is gereguleerd. Verder wordt er beschreven wat het belang is van $cPLA_2$, en enzymen die het arachidonzuur kunnen omzetten, voor ontwikkeling en groei, en in ziektes.

In hoofdstuk 2 is beschreven hoe het $cPLA_2$ wordt geactiveerd als cellen van buitenaf worden gestimuleerd met verschillende signalen (prikkels), bijvoorbeeld groeifactoren. Deze signalen gaan op een voor hen specifieke antenne (receptor) zitten die

zich in de membraan bevindt. Daarna wordt dit signaal in de cel doorgegeven via allerlei signaal transductie eiwitten totdat het uiteindelijke doeleiwit geactiveerd is. Cellen die we eerst in een rusttoestand gebracht hadden, werden vervolgens met verschillende groeifactoren gestimuleerd. Ook is gekeken naar de regulatie van $cPLA_2$ in rustende of constant groeiende cellen. Met behulp van remmers voor verschillende signaal transductie eiwitten werd gevonden dat het $cPLA_2$ via verschillende routes kan worden geactiveerd, zoals zo vele wegen naar Rome leiden. Zo blijkt $cPLA_2$ in rustende cellen geactiveerd via de Raf-MEK-p42/44^{MAPK} signaal transductie route, terwijl deze route in groeiende cellen ook actief is maar dit niet leidt tot $cPLA_2$ activering. In rustende cellen die gestimuleerd werden met serum werd voornamelijk de Raf-MEK-p42/44^{MAPK} route actief en voor een klein gedeelte de PKC-MEK-p42/44^{MAPK} route. Beide routes resulteerde in verhoogde $cPLA_2$ activiteit. Als de cellen met epidermale groeifactor gestimuleerd worden, wordt $cPLA_2$ hoofdzakelijk via de PKC-MEK-p42/44^{MAPK} route geactiveerd en voor een heel klein gedeelte via de Raf-MEK-p42/44^{MAPK} route. Echter directe activatie van PKC door cellen phorbol ester te geven, leidt wel tot p42/44^{MAPK} activatie zowel via de PKC-MEK als de PKC-Raf-MEK route, alleen leidt dit niet tot $cPLA_2$ activering.

In hoofdstuk 3 is gekeken of $cPLA_2$, en hoe $cPLA_2$ geactiveerd wordt wanneer rustende cellen blootgesteld worden aan oxidatieve stress door waterstofperoxide (H_2O_2) aan de cellen te geven. We spreken van oxidatieve stress als cellen worden blootgesteld aan een verhoogde concentratie zuurstofradicalen. Zuurstofradicalen zijn tussenprodukten die worden gevormd bij de omzetting van zuurstof naar water welke erg reaktief zijn. Deze zuurstofradicalen kunnen reageren met (en daarmee schade aanrichten aan) DNA, lipiden en eiwitten. Cellen hebben hiertegen afweermechanismen. Als deze mechanismen niet goed werken, of er is een overproductie van zuurstofradicalen ontstaan dan leidt dit tot oxidatieve stress. Oxidatieve stress speelt een rol bij zowel cellulaire processen als celdeling, celdood en celschade alswel bij arteriosclerose en neurodegeneratieve ziektes. Onder normale omstandigheden zijn kleine hoeveelheid zuursofradicalen nodig voor een goed verloop van signaal transductie routes. Wij vonden dat $cPLA_2$ snel geactiveerd kan worden door H_2O_2 op een concentratie en tijdsafhanklijke manier. H_2O_2 stimulatie van rustende cellen resulteerde in een gedeeltelijke activering van het $cPLA_2$ door fosforylatie via de Raf-MEK-p42/44^{MAPK} signaal transductie route. Verder werd $cPLA_2$ ook gedeeltelijk geactiveerd via een fosforylatie-onafhankelijke mechanisme. Dus, in H_2O_2 geactiveerde cellen gebeurt $cPLA_2$ activering op een andere manier dan in groeifactor gestimuleerde cellen. Zoals eerder gezegd is voor $cPLA_2$ activiteit ook calcium nodig om $cPLA_2$ aan de membraan te laten binden, maar in dit geval was $cPLA_2$ nog steeds actief in de membraan fractie te vinden, ook als het calcium weggevangen was. Uit de literatuur is bekend dat, in cellen, zuurstofradicalen lipiden kunnen beschadigen door deze te peroxideren (reactief zuurstof in de lipiden in te bouwen). Hierdoor treden er veranderingen op in de membranen van de cel,

waardoor het $cPLA_2$ beter bij zijn substraat arachidonzuur kan en dus meer arachidonzuur kan vrijmaken uit het membraan. Door deze membraanveranderingen is er minder calcium nodig om $cPLA_2$ naar de membraan te brengen en is deze binding misschien ook steviger. Dit alles leidt ertoe dat het $cPLA_2$ naast de activering door fosforylatie extra actief wordt door H_2O_2-geïnduceerde veranderingen in de membranen. Uit de resultaten van de hoofdstukken 2 en 3 volgt dat de route die na een bepaalde stimulatie actief is afhangt van de plaats van verschillende signaal transductie eiwitten op een bepaald tijdstip in de cel, wat al dan niet leidt tot $cPLA_2$ activering. Cellen kunnen zich op deze manier sneller aanpassen aan veranderingen in hun omgeving.

Tot nu toe is de activering van het $cPLA_2$ op één tijdstip bekeken. Om de functie van het $cPLA_2$ beter te begrijpen moet ook naar de activering van het $cPLA_2$ in de loop van de tijd bekend zijn. Dit is gedaan door de activiteit te meten gedurende de celcyclus. Celcyclus is het proces dat leidt tot celdeling welke bestaat uit 4 fasen: de mitose waarin het verdubbelde DNA verdeeld wordt over de twee nieuw ontstane cellen, de G1 fase, de S fase waarin het DNA verdubbeld (gerepliceerd) wordt en de G2 fase. De voortgang (progressie) van de celcyclus wordt goed gereguleerd. In elke fase checkt de cel of alle factoren optimaal zijn alvorens de overgang naar de volgende fase te maken. Hierbij zijn bepaalde eiwitten van belang, de cyclines. Elke fase heeft zo zijn eigen specifieke cyclines die weer met een ander eiwit, de cycline-afhankelijke kinase (Cdk) een actief complex kunnen vormen. Een cycline/Cdk complex komt via actieve signaal tranductie routes tot stand, maar kan zelf het signaal ook weer doorgeven. Als ergens in de route iets ontregeld is, stopt de cel, en optimaliseert eerst alle factoren alvorens verder te gaan. Wanneer die factoren binnen een bepaalde tijd niet geoptimaliseerd kunnen worden kan de cel besluiten “zelfmoord” (apoptose) te plegen. De cel kan ook dusdanig ontregeld zijn dat het ongeremd kan delen wat kan leiden tot tumorvorming.

In hoofdstuk 4 is beschreven dat $cPLA_2$ activiteit verandert gedurende de celcyclus. De activiteit van het $cPLA_2$ is hoog in mitotische cellen en neemt daarna snel af in de vroege G1 om weer toe te nemen in het midden-late G1. Na de G1/S overgang neemt de activiteit van het $cPLA_2$ weer sterk toe. De verandering in de activiteit van het $cPLA_2$ werd niet veroorzaakt door het aantal molekulen $cPLA_2$ ($cPLA_2$ eiwit expressie), maar doordat het $cPLA_2$ in deze actieve perioden gefosforyleerd was door $p42/44^{MAPK}$.

Vervolgens hebben we in hoofdstuk 5 onderzocht wat de functie van deze verhoogde $cPLA_2$ activiteits pieken betekenen voor de progressie van de celcylcus. Celcyclus progressie werd bepaald door de hoeveelheid radioactief gelabeld thymidine te meten dat ingebouwd wordt in het DNA ($[^3H]$-thymidine incorporatie of DNA synthese). Dit gebeurt alleen als cellen zich in de S fase bevinden en zo kun je meten of cellen van de G1 naar de S fase gaan. Als de activiteit van $cPLA_2$ vroeg in de G1 fase geremd wordt door ATK (een gemodificeerd arachidonzuur molekuul) blijkt de DNA synthese met ongeveer 50%

verminderd te zijn. Dit effect is niet toe te schrijven aan andere leden van de fosfolipase A_2 familie. Het vrijgekomen arachidonzuur kan ook omgezet worden door cyclooxygenases tot onder andere prostaglandines en thromboxanen, of door lipoxygenases tot onder andere leukotriënen en HETEs. Remming van cyclooxygenases op verschillende tijdstippen in de G1 resulteerde niet in een remming in DNA synthese. Daarentegen leidde remming van lipoxygenases wel tot verminderde thymidine incorporatie. Wanneer lipoxygenase vroeg in de G1 werd geremd stopte de cellen later in de G1 fase. Dus, zowel $cPLA_2$ als lipoxygenase zijn betrokken bij celcyclus progressie van de G1 naar de S fase. $cPLA_2$ activiteit is ook verhoogd in de S fase en dus werd onderzocht of $cPLA_2$ ook een rol speelt bij S fase progressie. Dit bleek niet het geval te zijn, omdat er geen verminderde DNA synthese werd gemeten wanneer $cPLA_2$ was geremd in cellen die zich in de S fase bevonden. Vervolgens werden lipoxygenases geremd in cellen die zich vroeg in de S fase bevonden, maar er vond geen DNA synthese meer plaats. Dit wil zeggen dat de activiteit van $cPLA_2$ in de S fase niet belangrijk is voor S fase progressie. Het kan wel een andere functie hebben in de S fase of zelfs later in de celcyclus, maar dat moet nog verder onderzocht worden. Verder blijkt dat lipoxygenases heel belangrijk zijn voor S fase progressie.

Tenslotte wordt in hoofdstuk 6 de resultaten van de verschillende hoofdstukken bediscussieerd. Wat is nu de fysiologische relevantie van al deze resultaten. Het is belangrijk te weten wat de functie van $cPLA_2$ is. We weten dat $cPLA_2$ overal in de cel aanwezig is en dus overal geactiveerd kan worden. Om de functie te weten te komen moet er onderzocht worden hoe $cPLA_2$ gereguleerd wordt en vooral waar het zich in actieve vorm in de cel bevindt. Verder moet er ook bekend zijn hoe $cPLA_2$ in de tijd wordt geactiveerd. Pas dan kan er op deze signaal transductie routes ingegrepen worden en kunnen nieuwe specifieke remmers gemaakt worden met een therapeutisch doel.

Nawoord

Ja, en dan nu het nawoord. Nou mensen, het wordt eindelijk weer rustig op de 5e verdieping, en omstreken, en daaruit blijkt dat ik een fijne tijd heb gehad bij de MCB. Daarvoor wil ik alle (ex)collega's en (ex)studenten bedanken voor de prettige werksfeer, maar een aantal mensen wil ik toch extra bedanken.

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Curriculum vitae

Gerda van Rossum werd geboren op 10 januari 1972 te Zegveld. In 1989 behaalde zij het HAVO diploma aan de Rijksscholengemeenschap "F. A. Minkema" te Woerden, waarna in 1991 aan dezelfde scholengemeenschap het VWO diploma werd behaald. Vervolgens begon zij in hetzelfde jaar aan de studie Scheikunde aan de Universiteit Utrecht. Het doctoraal examen werd afgelegd in augustus 1996 met als klein bijvak Medische Microbiologie (Prof. Dr. J. Verhoef en Dr. W. van Wamel) aan het Eijkman-Winkler instituut in het Academisch Ziekenhuis Utrecht, groot bijvak Farmacologie (Prof. Dr. J. Pfeilschifter en Dr. A. Huwiler) aan het Biozentrum in Basel, Zwitserland en als hoofdvak Biochemie van Lipiden (Prof. Dr. H. van den Bosch en Dr. M. J. B. M. Vervoordeldonk) aan de Universiteit Utrecht. Met ingang van januari 1997 begon zij als onderzoekster in opleiding in dienst van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (gebied Chemische Wetenschappen) aan de Universiteit Utrecht bij de vakgroep Moleculaire Celbiologie van de faculteit Biologie onder leiding van Prof. Dr. A. J. Verkleij, Prof. Dr. J. Boonstra en Prof. Dr. H. van den Bosch (Biochemie van Lipiden, faculteit Scheikunde). Tijdens deze periode werd het in dit proefschrift beschreven onderzoek verricht.

Vanaf september 2001 zal Gerda werkzaam zijn als post-doctoraal onderzoeker aan het Vrije Universiteits Medisch Centrum in Amsterdam, onder leiding van Prof. Dr. J. Lankelma, Dr. K. Hoekman (afdeling medische oncologie) en Prof. Dr. V.W.M. van Hinsbergh (Gaubius Laboratorium TNO-PG te Leiden).