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Review

Metabolism and signaling activities of nuclear lipids

A. M. Martelli[a,b,*], F. Falà[a], I. Faenza[a], A. M. Billi[a], A. Cappellini[a], L. Manzoli[a] and L. Cocco[a]

[a] Dipartimento di Scienze Anatomiche Umane e Fisiopatologia dell'Apparato Locomotore, Università di Bologna, via Irnerio 48, 40126 Bologna (Italy), Fax: +39 0512091695, e-mail: amartell@biocfarm.unibo.it
[b] ITOI-CNR, Sezione di Bologna c/o IOR, Bologna (Italy)

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Abstract. Apart from the lipids present in the nuclear envelope, the nucleus also contains lipids which are located further inside and are resistant to treatment with nonionic detergents. Evidence is being accumulated on the importance of internal nuclear lipid metabolism. Nuclear lipid metabolism gives rise to several lipid second messengers that function within the nucleus. Moreover, it is beginning to emerge that nuclear lipids not only act as precursors of bioactive second messengers but may be directly involved in regulation of nuclear structure and gene expression. Over the last 10 years, especially the role of the inositol lipid cycle in nuclear signal transduction has been extensively studied. This cycle is activated following a variety of stimuli and is regulated independently from the inositide cycle located at the plasma membrane. However, the nucleus contain other lipids, such as phosphatidylcholine, sphingomyelin, fatty acids and eicosanoids. There are numerous reports which suggest that these classes of nuclear lipids may play roles in the nucleus as important as those of phosphoinositides. This review aims at highlighting the most important aspects regarding the metabolism and signaling activities of nuclear phosphatidylcholine, sphingomyelin, fatty acids and eicosanoids.

Key words. Phoshatidylcholine; phospholipase D; diacylglycerol; sphingomyelin; sphingomyelinase; phospholipase A_2; nuclear matrix.

Introduction

For many years lipids were thought to be almost inert structural components of cell membranes. However, it is now clear that lipids also play key roles in the regulation of many cell functions because they serve as precursors of several bioactive second messengers generated along multiple signal transduction pathways [1]. Signal transduction events are elicited by the activation of cell-surface receptors by appropriate ligands, causing the generation of second messengers which are released in the cell interior. Phosphoinositides are by far the most intensively investigated lipid class involved in signal transduction [2]. However, other lipid classes participate in cell signaling, such as phosphatidylcholine (PC), sphingolipids and fatty acids [1, 3, 4].

In recent years, an increasing body of evidence has demonstrated that the nucleus is a site of active lipid metabolism for both synthesis and hydrolysis. Nuclear lipids are not only concentrated in the nuclear envelope, as expected, but they are also located further inside the nucleus, given that a significant amount resists treatment with nonionic detergents [5]. Nuclear lipid metabolism is regulated independently from that of the plasma membrane because there are extracellular stimuli which cause the generation of lipid signaling molecules only in the nucleus and not in the plasma membrane [6]. Nuclear lipid second messengers are likely involved in the control of cell proliferation and gene expression [7]. Moreover, nuclear lipids might play roles other than signaling molecules; for example,

* Corresponding author.

they might be somehow involved in the regulation of nuclear structure and transcription [8]. Nuclear phosphoinositides have received a considerable degree of attention. Nevertheless, there are many reports which have dealt with metabolism and signaling activities of nuclear PC, sphingolipids, fatty acids and eicosanoids [9, 10]. These lipids may have functions in the nucleus as important as those of inositol lipids [11–15]. In a recently published review on nuclear lipids, Irvine focused on phosphoinositides and protein kinase C (PKC) [16]. However, much less emphasis was placed on other classes of nuclear lipids. In order to complement this prior review, we shall tackle the issue of highlighting current knowledge about the metabolism and signal transduction activities of nuclear PC, sphingolipids, fatty acids and eicosanoids. However, we will begin with a short discussion of nuclear structure.

Nuclear structure

The nucleus is now recognized as a highly structured organelle with an encompassing nuclear envelope and somewhat diffuse intranuclear domains. The latter are defined as structural components that are dynamically variable in relation to metabolic function [17]. The nuclear envelope is a double membrane whose outer membrane is directly continuous with the endoplasmic reticulum and shares certain properties with the latter. The inner nuclear membrane, with a different lipid and protein composition, is associated with the nuclear lamina and chromatin. The nuclear lamina comprises a meshwork of intermediate filament proteins (referred to as the lamins) located on the inner surface of the inner nuclear membrane [18]. Phospholipids comprise the large bulk (~65%) of nuclear envelope lipids, with lesser amounts of cholesterol and sphingolipids [19]. In contrast to intact nuclear envelope, few studies have focused on the lipid composition of separated outer and inner nuclear membrane. One report, based on filipin-sterol interaction, showed unequal distribution of complexes that suggested higher cholesterol content in the outer compared with the inner nuclear membrane [20]. Differential localization of GM1 ganglioside has also been observed, this being detected in the inner nuclear membrane in association with a Na^+/Ca^{2+} exchanger [21]. It is likely that more complete profiles of lipid composition and function for the two nuclear membranes will emerge following the development of new methods for their isolation. The two membranes of the nuclear envelope are joined at the nuclear pores. The nuclear pores are distributed over the entire nuclear surface and consist of multi-protein assemblies of ~125,000-kDa size that allow passive transfer of small and middle-sized molecules (<50 kDa) between cytoplasm and nucleoplasm. Passage of larger molecules is energy driven and requires a nuclear localization signal (NLS) [22]. It is generally believed that the nucleus contains a sort of skeleton, referred to as the nuclear matrix, whose general function would be to organize nuclear structure. The nuclear matrix is operationally defined as components that remain insoluble after extraction of the nuclei with nonionic detergents and high ionic strength solutions, and treatment with nucleases. Structural and functional links exist between the peripheral lamina and the internal nuclear matrix [23, 24].

In ascribing biochemical properties to whole nuclei or nuclear components through the use of the isolated organelle, the question of purity must be rigorously addressed. Purity is usually confirmed by electron microscopy and assay of marker enzymes for potential contaminants, e.g. 5′-nucleotidase (plasma membrane), galactosyltransferase (Golgi apparatus), cytochrome C oxidase (mitochondria), and glucose-6-phosphatase or NADPH-cytochrome C reductase (endoplasmic reticulum). Moreover, the outstanding progress of molecular biology-related techniques has allowed for a further advance in the field of nuclear lipids. The advent of green fluorescent protein (GFP) technology has provided unprecedented opportunities to study the enzymes involved in nuclear lipid metabolism in living cells and to circumvent immunocytochemistry-related problems such as fixation or inadequate antibody penetration.

Furthermore, identification of NLSs and the possibility to express (in some cases as GFP hybrids) complementary DNA of proteins of interest which are mutated, and have thus lost their capacity to localize in the nucleus, represent another extremely valuable tool to unequivocally demonstrate intranuclear localization of lipid metabolism-related enzymes, as we shall see later on.

Nuclear PC metabolism

Like its extranuclear counterpart, nuclear PC comprises the major phospholipid class within that compartment [25]. Quantitative estimates from independent studies of both freshly isolated rat hepatocytes [26] and cultured human IMR-32 neuroblastoma cells [25] have shown that nuclear PC comprises ~6% of the whole cell pool. However, PC that is confined to the nuclear envelope and which is removable by nonionic detergents elevates the proportion of whole cell PC present in the nuclear interior of rat hepatocytes to ~18% of the total [26].

Advances in chromatographic techniques and detection methodologies, and recently the application of mass spectrometry to phospholipids, has permitted elegant molecular species analyses of unparalleled specificity and sensitivity. When combined with deuterated isotope labeling of lipid precursors, tandem electrospray ionization

mass spectrometry (ESI-MS) has rendered the metabolism of nuclear PC molecular species accessible to detailed direct analysis. Application of ESI-MS analyses to interior nuclear PC composition of IMR-32 cells has revealed an unexpectedly high proportion of saturated and monounsaturated molecular species when compared with the composition of PC isolated from the whole cell [25]. Moreover, the saturation enrichment of PC is tightly retained even when whole cell phospholipid is enriched by prolonged exposure to polyunsaturated fatty acids [27]. These findings are consistent with the highly saturated PC found by others in mouse erythroleukemia (MEL) cell nuclei [28]. It has been shown that nuclear PC is not synthesized de novo in this saturated state, but rather is synthesized with a more typical fatty acid profile and subsequently remodeled within the nucleus [25]. It is intriguing that the presence of unesterified fatty acids has been demonstrated in the nucleus of living cells. Remarkably, overexpression of the liver fatty acid binding protein (L-FABP) specifically enhanced uptake and intracellular targeting of long- and medium-chain fatty acids to the nucleus [29].

In the nucleus, PC is synthesized through the de novo pathway (fig. 1) [25], even though a previous report suggested the presence of a choline base-exchange activity in rat liver nuclei [30]. However, the base exchange seems unlikely because no donor endonuclear phospholipid pool is appropriately saturated. The activities of the three enzymes of the de novo pathway, choline kinase (CK), CTP:choline phosphate cytidylyltransferase (CCT) and 1,2-diacylglycerol CDPcholine cholinephosphotransferase (CPT), have been found in the nucleus [25, 31]. Interestingly, treatment of LA-N-1 neuroblastoma cells with phorbol ester resulted in the production of nuclear diacylglycerol (DG), stimulation of nuclear CCT and enhanced nuclear PC synthesis, indicating that DG produced through the hydrolysis of PC in the nucleus is reutilized for the synthesis of nuclear PC [31].

Nuclear localization of CCT has also been demonstrated by immunofluorescent staining of several cell lines (HepG2, CHO, NIH 3T3, L cells) as well as of rat liver tissue [32, 33]. Moreover, GFP-tagging evidence confirmed that the α isoform of CCT is predominantly located with and confined to the nucleus of most cell types [34]. This

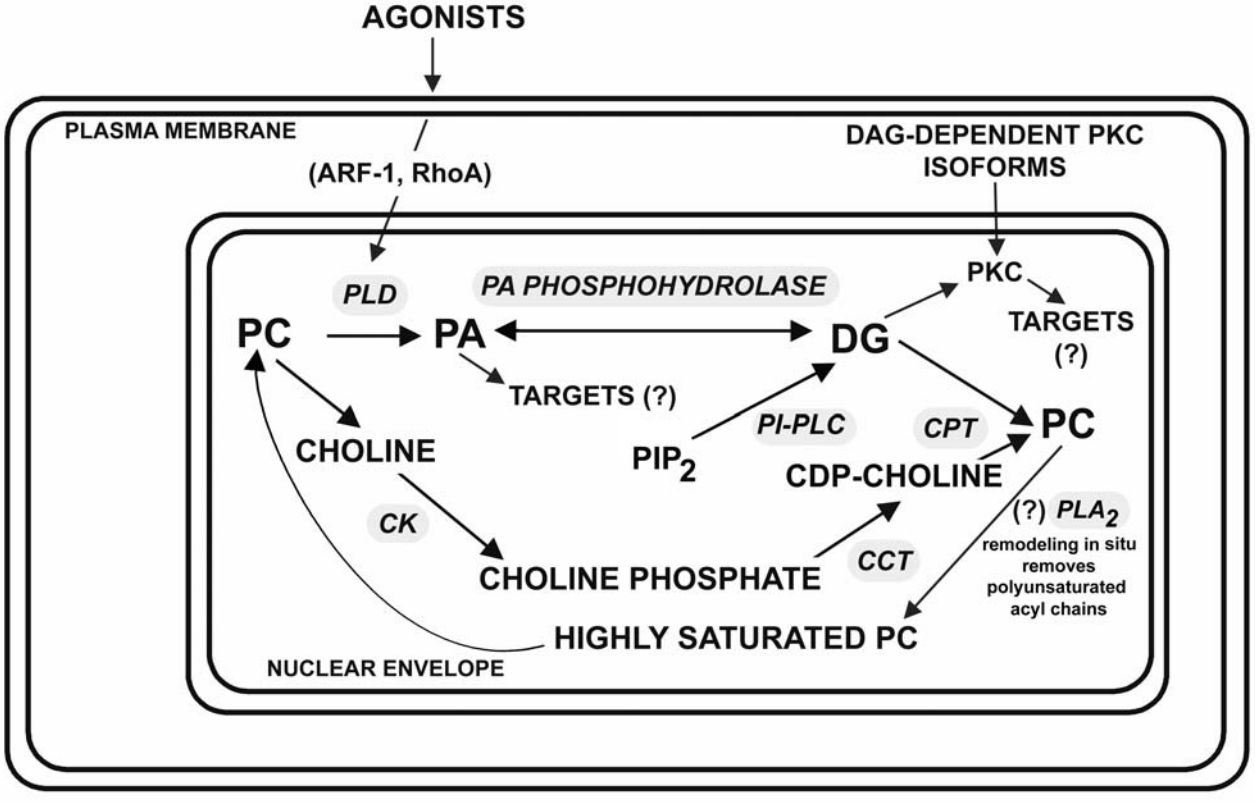

Figure 1. Proposed model of nuclear PC metabolism and signaling activity. Agonists upregulate nuclear PLD, at least in some cases by promoting migration to the nucleus of activating molecules such as ARF-1 and RhoA. The DG produced through PLD attracts to the nucleus DG-dependent PKC isoforms which target nuclear proteins which remain to be identified. Note, however, that the presence in the nucleus of PA phosphohydrolase has yet to be demonstrated by immunocytochemistry. DG may be reused for the synthesis of PC. A lack of specificity of incorporation of DG into nuclear PC has been demonstrated [25], so some DG might derive, at least in theory, also from inositol lipid hydrolysis. Intranuclear remodeling of PC by PLA_2 might lead to generation of the highly saturated PC typical of the nuclear compartment.

contrasts with β1 and β2 isoforms, which show extranuclear localization. Elucidation of the mechanism responsible for the nuclear localization of CCTα has come from the molecular analysis of its amino terminus region. Indeed, in rat liver tissue it has been identified a 21-residue NLS (^{8}KVNSRKRRKEVPGPNGATEED^{28}) whose deletion disrupted CCTα nuclear localization [35]. It is interesting that a caspase-6 and/or -8cleavage site has been mapped to TEED^{28} ↓G, the final residue of the NLS [36], so that exclusion of CCTα from the nucleus is a feature of some types of apoptosis. However, the consequences of this exclusion remain to be elucidated.

An outstanding issue is the structure of intranuclear PC. CPT is an integral membrane protein [37]; and the demonstrated endonuclear CPT would be expected to reside within a similar membrane environment. There are reports showing the presence in the nucleus of internal membranous structures continuous with the endoplasmic reticulum [38, 39]. Therefore, endonuclear PC might be present in these membranous channels. However, this is in contrast to resistance to Triton X-100 treatment. Therefore, it could not be ruled out that intranuclear lipids are in a diverse physicochemical state, perhaps bound to nuclear proteins. These proteins may be chromatin proteins or constituents of the nuclear matrix [31, 32], such as ING2, a very recently identified intranuclear receptor for inositol lipids [40].

Interestingly, in rat liver nuclear matrix the content of cholesterol and sphingomyelin (SM) is respectively five and three times higher than in chromatin, whereas the amount of PC is lower, indicating a less fluid structure [41, 42].

Signaling through nuclear PC and phospholipase D (PLD)

The enzyme PLD (PC phosphatidohydrolase) catalyzes the hydrolysis of the terminal diester bond of glycerophospholipids (mostly PC), resulting in the formation of phosphatidic acid (PA) plus a related base. PA is subsequently converted into lyso-PA by phospholipase A_2 (PLA_2) or to DG by PA phosphohydrolase [43] (fig. 1).

The mammalian PLDs cloned to date include PLD1 (~120 kDa) and PLD2 (~105 kDa). PLD1 exists as two splice variants, referred to as a and b. Splice variants of PLD2 have also been described [44, 45]. PLD1 is activated by the ADP ribosylation factor (ARF), Ral and Rho family of GTPases, as well as by PKC-α. Both PLD1 and PLD2 have an absolute requirement for phosphatidylinositol (4,5) bisphosphate (PIP_2). PLD2 activity is elevated by fatty acids such as oleate, and its in vitro activity is unaffected by either GTPases or PKC-α, even though in vivo PLD2 activity may be elevated by PKC-α. There are, however, reports showing that PLD2 can be inhibited by oleate [46, 47]. Moreover, the presence of a 90-kDa PLD distinct from PLD1 or PLD2 has been reported in human neutrophils [48]. This form is inhibited by oleate.

Using immunofluorescence and immunogold labeling, PLD1 has been localized to the nucleus of mammalian cells and, upon Golgi apparatus collapse in response to brefeldin A, intranuclear staining increased significantly [49]. The same group has also investigated the intracellular distribution of PLD2 and found that it is localized to the rims of the Golgi apparatus. Similarly to PLD1, PLD2 translocated to the nucleus when the Golgi apparatus was disrupted by treatment with brefeldin A [50]. The significance of the translocation of PLD isoforms from the Golgi apparatus to the nucleus is at present unclear.

Others, however, have reported the intranuclear presence of PLD2 in renal carcinoma cells and have suggested that this isoform might somehow be involved in tumorigenesis because its amount was very low in the nucleus of normal renal cells [51].

There are several reports in which nuclear PLD has been assayed under basal conditions or following a variety of stimuli, as summarized in table 1. There is general consensus over the fact that activation of nuclear PLD leads to increased levels of DG in the nucleus, which serves as a chemoattractant molecule for DG-dependent isoforms

Table 1. Experimental systems in which a nuclear PLC activity was assayed.

Cell type	Stimulus	Stimulatory factors	Inhibitory factors	PLD isoform	References
MDCK	none	phorbol esters, ATP, RhoA	chelerytrine, calphostin C	PLD1 (?)	[45, 46]
Rat liver, AH 7974	hepatectomy	ARF-1, RhoA, GTP-γ-S, PIP_2	n.d.	PLD1 (?)	[47]
Rat liver, AH 7974	hepatectomy	fatty acids (oleate)	n.d.	PLD2 (?)	[47]
Rat macrophages	α2-macroglobulin	ARF-1, GTP-γ-S	n.d.	PLD1 (?)	[48]
HL60	camptothecin	n.d.	n.d.	(?)	[49]
IIC9	α-thrombin	RhoA	n.d.	PLD1b	[50–54]
Rat brain, kidney, spleen, heart liver, LA-N-1	ATRA	oleate	PA, phosphatidylserine, phosphatidylglycerol, cardiolipin	PLD2(?)	[55–57]
LA-N-1	phorbol esters	ATP, GTP-γ-S	n.d.	PLD1(?)	[22]
HL60	DMSO	ATP, PIP_2	oleate, GTP-γ-S	90-kDa PLD	[58]

n.d., not determined.

of PKC (fig. 1), as originally proposed by Irvine and co-workers for DG originated from inositol lipids hydrolysis through the action of a phosphoinositide-specific phospholipase C (PI-PLC) [52].

A PLD-mediated pathway for generating DG has been found in nuclei prepared from Madin-Darby canine kidney (MDCK) cells. PLD activity was enhanced by either phorbol esters or ATP [53]. Further studies demonstrated that activation of this nuclear PLD was regulated by the small GTP-binding protein RhoA and was downstream of PKC, as evidenced by the fact that PKC inhibitors, such as chelerytrine and calphostin C, could prevent stimulation of PLD activity [54]. Because of these features, the measured nuclear PLD activity might be PLD1. Banno and co-workers [55] reported the presence of two distinct nuclear PLD activities in rat liver cells (resting, cycling and neoplastic). One of the activities was markedly stimulated by ARF-1 in the presence of GTP-γ-S and PIP_2, whereas it was insensitive to ATP and phorbol esters. This activity might well correspond to PLD1. The other activity was stimulated by fatty acids, especially by oleate, and might tentatively be identified as PLD2. It is very interesting that the ARF-1-dependent nuclear PLD activity increased during the S-phase of rat liver regeneration and was also much higher in ascites hepatoma AH 7974 cells than in the resting liver. In contrast, oleate-dependent PLD activity remained constant throughout the cell cycle. These authors also demonstrated that the intranuclear levels of the stimulating proteins of the nuclear PLD activity (ARF-1, RhoA) increased during S-phase of liver regeneration. Moreover, the amount of nuclear PKC-δ (but not PKC-β_{II}) rose during S-phase, and it could be connected to the increase in the DG levels in nuclei, presumably due to upregulation of PLD activity.

Further evidence suggests the existence in rat macrophages of ARF-1-dependent nuclear PLD activity which was upregulated by receptor-recognized forms of α2-macroglobulin [56].

An increase in PLD-generated nuclear DG mass has been demonstrated by our laboratory in HL60 human promyelocytic leukemia cells treated with the apoptosis inducer camptothecin. In this case, the increase in nuclear DG mass was related to enhanced nuclear activity of PKC-α [57].

The issue of nuclear PLD has long been explored by Raben and co-workers, using, as an experimental system, IIC9 fibroblasts challenged with α-thrombin. In these cells, α-thrombin treatment resulted in an immediate (2.5–5 min) rise in nuclear DG mass which remained elevated for at least 30 min. In cells labeled with [^{3}H]myristic acid, α-thrombin induced an increase in radiolabeled nuclear DG, suggesting that they derived, at least in part, from PC. In parallel, the same group also observed a 10-fold increase in nuclear PKC-α levels [58]. Subsequently, they devoted their efforts to precisely identifying the phospholipid source(s) of the stimulated nuclear DG. They examined the molecular species profiles of the induced DG by derivatizing them, followed by conversion to tert-butyldimethylsilyl esters and analysis by capillary gas chromatography. The profiles of the nuclear DG resembled the species profiles of PC but not of inositol lipid species [59]. In IIC9 cells α-thrombin exposure resulted in stimulation of nuclear PLD activity, which followed translocation to the nucleus of RhoA [60]. The nuclear PLD isoform, whose activity increases in IIC9 cells in response to α-thrombin, was subsequently identified as the 1b isoform [61, 62]. Significantly, this isoform was activated in the nucleus but not in the Golgi apparatus.

An oleate-dependent PLD activity residing in the nuclei of rat brain neurons was characterized by Kanfer and co-workers [63]. The enzyme activity was cation independent, required a slightly acidic pH optimum (6.5) and was stimulated by unsaturated, but not saturated, fatty acids. A similar activity was also detected in nuclei from rat kidney, spleen, heart and liver. The same group demonstrated that acidic phospholipids and PA inhibited the neuronal nuclear PLD activity, suggesting the possible existence of cross-talk between different signal transduction pathways which operate in the nucleus [64]. The oleate-dependent PLD activity of neuroblastoma cell nuclei was specifically activated by all-*trans* retinoic acid (ATRA) [65], whereas another nuclear PLD activity, dependent on G proteins, was elevated in response to phorbol ester treatment of LA-N-1 cells [31].

Finally, our laboratory recently demonstrated that in HL60 human leukemia cells treated with the differentiating agent dimethylsulfoxide (DMSO), there was an increase in the amount of a 90-kDa PLD which might be the same as that identified by Horn and co-workers [48] because its activity was inhibited by oleate [66]. This activity was responsible for increased nuclear DG levels and migration to the nucleus of PKC-α.

In all of the aforementioned investigations, PLD activity was assayed in nuclei retaining their envelope, and at least in one case [55] it has been shown that Triton X-100 extracted nuclear PLD activity, suggesting it was located in the nuclear membrane. However, the study by Freyberg and co-workers [49] has shown that PLD1 is also localized in the interior regions of the nucleus.

At present there are no data regarding a possible role played by PA produced in the nucleus by PLD. In the cytoplasm PA has been implicated in the regulation of the actin cytoskeleton by inducing actin polymerization and formation of stress fibers. Moreover, PA can activate phosphatidylinositol 4-phosphate 5-kinase, increasing the synthesis of PIP_2 [67]. Intriguingly, actin may be one of the most important constituents of the nuclear matrix [68], while phosphatidylinositol 4-phosphate 5-kinase is present in the speckle domains of the nucleus [69, 70].

Thus, in the future it will be of fundamental importance to investigate the role (if any) of PA generated within the nucleus.

Signaling through nuclear PC and PC-specific phospholipase C (PC-PLC)

There still is no unambiguous demonstration for the existence a mammalian PC-PLC, and despite efforts in a number of laboratories, it has not yet been cloned. Some findings point to the likelihood that PC:ceramide phosphocholinetransferase (also referred to as SM synthase), which transfers the phosphocholine group from PC to ceramide to generate SM (see fig. 2), may account for the putative mammalian PC-PLC [71]. It should be emphasized that many of the studies on PC-PLC have relied on the purported PC-PLC-selective inhibitor, D609. However, PC:ceramide phosphocholinetransferase is sensitive to D609.

Nuclear PC-PLC activity has been invoked to explain the mechanisms leading to DG generation in MEL cells [28]. In this study, the authors used the primary alcohol propanol to switch the preference of PLD from water during the cleavage of PC, leading to formation of phosphatidylpropanol, which cannot be dephosphorylated to produce DG. Thus, incubation with propanol would lead to decrease of nuclear DG if it was derived from this pathway. However, no changes were found, suggesting that nuclear DG derived from PC is generated by the action of PC-PLC. It has been claimed that PC-PLC activity is bound to chromatin in rat liver [72], but PC:ceramide phosphocholinetransferase activity was also found to be chromatin associated in hepatocyte nuclei [73]. Finally, it should be mentioned that immunostaining of human natural killer lymphocytes with antibodies to PC-PLC decorated the nuclear interior; however, these antibodies had been generated to bacterial PC-PLC [74].

Other functions of nuclear PC

The role of endonuclear PC might not be restricted to signaling. PC might act as a 'contra-flow' phospholipid to permit net import of inositol lipids into the nucleus through phosphatidylinositol transfer protein (PITP). Indeed, at variance with PC, endonuclear inositide de novo synthesis has not been demonstrated [75]. The PITP isoform that has both endonuclear and extranuclear locations is PITPα [76]. Endonuclear PC fits the necessary requirements, and in appropriate conditions is efficiently transported on PITPα [77].

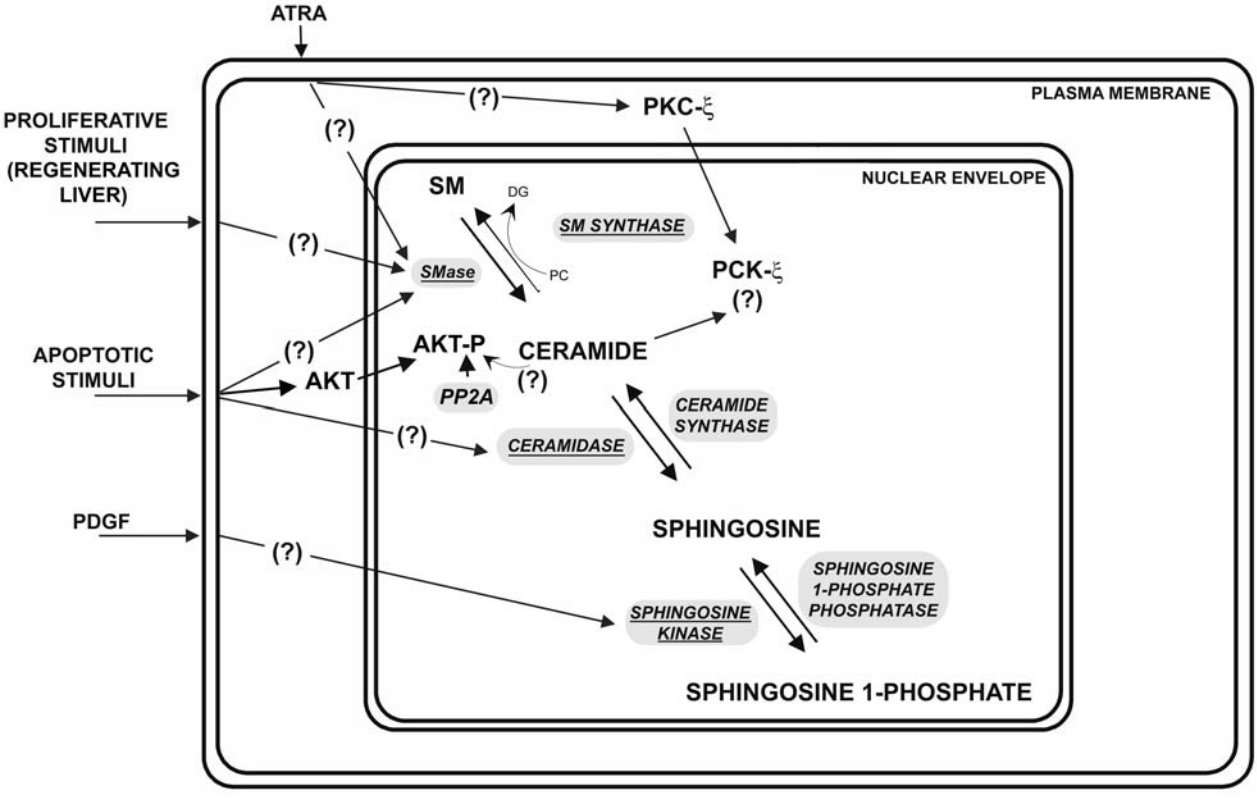

Figure 2. Proposed model of nuclear SM metabolism and signaling activity. SM-metabolizing enzymes so far identified at the nuclear level are underlined. It is completely unknown how various kinds of stimuli target these enzymes. Nuclear ceramide might be involved in the regulation of PKC-ζ or might play a role in the downregulation of intranuclear Akt.

Given the abundance of PC within the nucleus and also its high enrichment in saturated fatty acids, there is a tempting parallel with the saturated (mostly dipamitoyl) PC found in lung surfactant, a material whose unusual physical properties are attributed to dipalmitoyl PC [78]. It could be that PC forms an important part of chromatin structure by some kind of liquid crystalline phase.

Nuclear SM metabolism

SM is an important lipid responsible for the generation of bioactive ceramide, sphingosine and sphingosine 1-phosphate that exert numerous biological effects [79]. Even though the major sites of SM metabolism are generally considered to be the plasma membrane, the Golgi and intracellular acidic compartments, such as endosomes and lysosomes, the nucleus is now established as another site for the generation of bioactive sphingolipids.

The SM cycle initiates when various types of sphingomyelinase (SMase) are activated, resulting in cleavage of SM to ceramide and phosphorylcholine. Ceramide can be catabolized to sphingosine and sphingosine 1-phosphate, or can be consumed by SM synthase. If this occurs, the result is resynthesis of SM and completion of the cycle (fig. 2).

The presence of SM in isolated nuclei has been documented in several reports (e. g. [80, 81]). In the nucleus of rat liver and rat AH 7974 ascites hepatoma cells SM represents between 22 and 35% of that found in the plasma membrane, although the relative amount (SM/mg protein) of nuclear SM is much lower than the plasma membrane value. The above-mentioned studies have highlighted that nuclear SM, when compared with plasma membrane SM, contains more C16- and C18-fatty acids at the expense of C24-fatty acids in both rat liver and AH 7974 cells. On the other hand, there are no differences as far as the sphingosine base composition is concerned. When SM is considered, there are also some compositional differences, depending on its subnuclear location. Indeed, in AH 7974 cell nucleus, chromatin SM contains higher levels of C16:0 and C18:0 at the expense of C24:0 and C24:1 when compared with either nuclear envelope or nuclear matrix SM. Moreover, the ratio of dihydrosphingosine to sphingosine was found to be 2 in chromatin, whereas both bases exist in almost equal amounts in SM from other sites. Therefore, because of its different composition, the chromatin-associated SM might have a unique function [82].

SM might be involved in protecting RNA from RNase action. Indeed, there is a report demonstrating the isolation of a nuclear complex containing SM, neutral SMase (nSMase), SM synthase, PC, a putative PC-PLC and RNase-resistant RNA [73]. This RNA became sensitive to RNase after heat treatment, as happens for double-stranded RNA, as well as after SMase digestion, suggesting an interaction between SM and the two RNA strands. The functions of double-stranded RNA are still unclear, but it is now beginning to emerge that it gives rise to small RNAs, which control gene expression [83, 84].

SM metabolizing enzymes found in the nucleus are a Mg^{2+}-dependent nSMase [85], ceramidase [86], sphingosine kinase [87] and SM synthase [73, 88, 89] (fig. 2).

The presence of nSMase activity in nuclei of rat ascites hepatoma cells was demonstrated for the first time by Tamiya-Koizumi and co-workers [85]. The same group reported recently that this nSMase is the type 1 species and is selectively bound to the nuclear matrix [90]. In this investigation, intranuclear localization of nSMase was demonstrated by immunogold labeling, fluorescent immunocytochemistry and immunoblotting.

However, other investigators reported the presence of a Mg^{2+}-independent nSMase activity in hepatocyte nuclei, associated to either the nuclear envelope [91] or the nuclear matrix [92]. In resting hepatocytes, nSMase (Mr 92 kDa) was bound to the nuclear envelope. However, during liver regeneration the enzyme moved to the internal nuclear matrix. During compensatory hepatic growth, nuclear SM decreased in reverse proportion to an increase in the levels of nSMase activity. In contrast, there was a corresponding increase in the nuclear mass of both ceramide and sphingosine [91]. Subsequently, a chromatin-bound nSMase activity, with biochemical properties different from that present in the nuclear membrane, has been identified [93]. When the behavior of this chromatin-bound activity was compared with the DNA synthesis rate during liver regeneration, it was observed that maximal enzyme activity corresponded to the beginning of the S-phase, while nuclear envelope-associated activity did not change.

Signaling though nuclear SM

Sphingolipid breakdown products, particularly ceramide, are now being recognized as important players in apoptosis [94–96]. There are some findings which point to the likelihood that increased SM metabolism in the nucleus could be related to apoptotic cell death. Using the portal vein branch ligation system of rat liver, the upregulation of both nSMase and ceramidase activity has been observed, with concomitant increases in their reaction products, ceramide and sphingosine, in the hepatocyte nuclei of ligated lobes prior to the onset of apoptosis [86]. It should be emphasized that enzyme activation was detected neither in the proliferating hepatocyte nuclei of nonligated lobes, nor in the plasma membranes of either ligated or nonligated lobes. Moreover, it has been shown that nSMase activation and ceramide generation in the nucleus occur in an ionizing radiation-sensitive TF-1 cell clone. However, they were not detected in the nucleus of

radio-resistant TF-1 cells or in nucleus-free cell lysates [97]. What might be the role of intranuclear ceramide during apoptosis? There are several putative downstream targets of ceramide which may link this lipid with apoptosis: they include ceramide-activated protein kinase, cathepsin D, and serine/threonine protein phosphatases PP1 and PP2 A (reviewed in [95]). It is intriguing that ceramide induces dephosphorylation of the powerful survival agent Akt (also known as PKB, protein kinase B), with a concomitant loss of function (see [95] and references therein). Since active (phosphorylated) Akt migrates to the nucleus [98] and once there can be dephosphorylated by PP2 A [99], it might be envisaged that nuclear ceramide is somehow involved in downregulating Akt activity in the nucleus during apoptosis (fig. 2).

Another target for nuclear ceramide may be PKC-α. Indeed, the differentiation of HL60 human leukemia cells induced by ATRA was paralleled by an increase within the nucleus in both ceramide and catalytically active PKC-α [100] (fig. 2). Interestingly, PKC-ζ displays a cysteine-rich domain that has been hypothesized to play a role in the binding of ceramide to this kinase [101]. Ceramide has been demonstrated to increase PKC-ζ activity towards hnRNPA1, a known regulator of alternative splicing [102]. Increased phosphorylation of hnRNPA1 will increase its RNA binding affinity. Thus, mechanisms that increase ceramide levels within the nucleus may affect the alternative splicing of genes required for cell differentiation.

However, others have found that during apoptosis of rat liver cells there is an increase of chromatin-bound SM as a consequence of upregulation of SM synthase activity. In contrast, it was found that chromatin-bound SM synthase was depressed, while SMase reached a peak of activity during liver proliferation induced by ciprofibrate. It was thus hypothesized that chromatin SM may have a role in cell duplication by influencing chromatin structure stability [103].

Moreover, sphingolipids such as sphingosine might be involved in regulating intranuclear Ca^{2+} concentration, and the existence of a sphingosine-gated Ca^{2+}-permeable channel in liver nuclei has been hypothesized [104].

Nuclear phospholipase A_2

Phospholipase A_2s (PLA_2s) comprise a family of lipolytic enzymes which cleave the sn-2 ester bond in glycerophospholipids, releasing free fatty acids and lysophospholipids [105]. PLA_2 is the enzyme activity involved in the first step of arachidonic acid (AA) metabolism. Various forms of PLA_2 have been characterized, purified and cloned from diverse sources. A single mammalian cell type often contains more than one form of PLA_2. A recent classification [106, 107] has divided the various types of PLA_2 into three categories: the secretory PLA_2s ($sPLA_2$s), which require Ca^{2+} for their activities at millimolar levels; cytosolic PLA_2s ($cPLA_2$s), which are sensitive to micromolar levels of Ca^{2+}; Ca^{2+}-independent PLA_2s ($iPLA_2$s), which are completely independent of Ca^{2+} for their activities.

$cPLA_2$s, which cleave mostly PC but in some cases also phosphatidylethanolamine (PE) containing AA at the s n-2 position, have been described to be involved in cell proliferation and in gene transcription [108].

The presence of a neutral PLA_2 at the nuclear level was originally demonstrated in rat ascites hepatoma cells [109]. This nuclear matrix-associated enzyme activity, which hydrolyzed PC and PE with the same efficiency at neutral pH, was stimulated by acidic phospholipids, in particular by phosphoinositides, which also lowered the Ca^{2+} concentration required for its half-maximal activation. Nuclear matrix-bound PLA_2 activity, which did not show specificity towards PC and PE, was subsequently also identified in rat liver by another group [92]. Several investigations have hinted that nuclear PLA_2 could be linked to cell proliferation. Indeed, in subconfluent bovine endothelial cells, the 85-kDa $cPLA_2$ resided prominently in the nucleus, whereas in confluent cultures it became primarily cytoplasmic [110].

Recently, the molecular mechanisms which regulate the nuclear import and export of 85-kDa $cPLA_2$ ($cPLA_2$-α) were partly elucidated [111]. This study confirmed that intranuclear localization of $cPLA_2$ is dependent on the proliferative state also in EA.hy.926 human endothelial cells. Moreover, it has been found that a broad-range protein kinase inhibitor, staurosporine, caused a decrease in the nuclear level of $cPLA_2$, whereas the protein phosphate inhibitor okadaic acid increased the amount of intranuclear $cPLA_2$. Since treatment with selective inhibitors for mitogen-activated protein (MAP) kinases (both p42/44 and p38 MAP kinase) resulted in a dramatic decrease in the levels of nuclear $cPLA_2$, it was suggested that import of $cPLA_2$ may require phosphorylation. One of the potential $cPLA_2$ phosphorylation sites, Ser505, lies within the PXPS motif, which represents a consensus MAP kinase phosphorylation site. However, it is unlikely that this site is involved in the control of nuclear import of $cPLA_2$, because previous results obtained in CHO cells demonstrated that a mutation of Ser505 did not at all affect nuclear migration of $cPLA_2$ even though it strongly diminished its enzymatic activity [112]. This report showed instead that deletion of the Ca^{2+}-dependent phospholipid binding (C2) domain negatively affected nuclear migration of $cPLA_2$.

However, $cPLA_2$ contains at least one other residue which can be phosphorylated by MAP kinases, i.e. Ser515 [113]. Therefore, this residue might be important for the control of nuclear import of $cPLA_2$. Analysis of the amino acid sequence of $cPLA_2$-α reveals the existence of two potential NLSs: amino acids 54–60 (PDSRKRT) and amino acids

269–283 (PQKVKRYVESLWKKK), which may be involved in targeting of this protein. The first putative NLS lies within the C2, and this is consistent with the results reported above. Phosphorylation-mediated regulation of NLSs is a common phenomenon (reviewed in [114]), hence it might be that phosphorylation of cPLA$_2$-α regulates not only its activity but also its subcellular localization.

Grewal and co-workers [111] have found also that nuclear export of cPLA$_2$-α is sensitive to leptomycin B, an indication of the existence of a typical nuclear export signal (NES) which should correspond to amino acids 552–563 (LTFNLPYPLIL). This hypothesis is supported by the evidence that the proteolytic fragment of this enzyme generated by caspase cleavage during apoptosis (amino acids 1–522) was found to be exclusively intranuclear [115].

cPLA$_2$-α is not the only type of PLA$_2$ found in the nucleus, however. In U_{III} cells, a stromal cell line derived from normal rat uterus, pancreatic PLA$_2$ (PLA2-I, which belongs to sPLA$_2$s) has a stimulating effect on DNA synthesis. Endogenous PLA$_2$-I (which is first secreted then internalized via its membrane receptor) was mainly located in the nucleus in highly proliferative cells, whereas its location was cytoplasmatic in nonproliferative cells. When nonconfluent cells were incubated with exogenous PLA$_2$-I, the enzyme was internalized and, in the majority of cells, appeared within the nucleus [116].

Furthermore, nuclear iPLA$_2$s (Mr 110 and 39 kDa) are activated by ATRA in LA-N-1 neuroblastoma cells. Interestingly, these enzymes hydrolyzed ethanolamine phospholipids to produce AA and lysoglycerophospholipids [117, 118]. Very recently, iPLA$_2$ was shown by immunoblotting and immunofluorescent labeling to migrate to the nucleus in response to hypoxia, and it has been demonstrated that iPLA$_2$ activity is required for nuclear shrinkage in caspase-independent cell death. Therefore, it has been hypothesized that activated iPLA$_2$ may disrupt nuclear architecture [119].

Signaling through PLA$_2$

The overall feeling is that nuclear PLA$_2$s are involved in cell growth regulation. Increased lyso-PC production has been shown to lead to proliferation of U937 cells [120], while increased levels of AA have been linked with proliferation of A549 cells [121]. The need for a nuclear PLA$_2$ in proliferation is further supported by the high levels of cPLA$_2$ in the nucleus of cancerous cells which are continuously proliferating [110]. However, we do not know how these lipid second messengers, generated within the nucleus, might influence proliferation rates. A clue about the role played by a nuclear PLA$_2$ has come by a series of studies performed on smooth muscle cells. At present, much interest surrounds these cells because their proliferation is increasingly seen to be intimately tied to the etiology of both atherosclerosis and hypertension (e. g. [122, 123]). These cells are sensitive to angiotensin II, which provokes a proliferating response [124]. Freeman and co-workers [125] reported that angiotensin II was capable of inducing a nuclear translocation of a cPLA$_2$ in vascular smooth muscle cells, with a subsequent release and accumulation of AA within the nucleus, which peaked at 10 min after stimulation and was downregulated by 20 min. Interestingly, a short (12 min) exposure to angiotensin II in these cells caused intranuclear migration of PKC-α [126], a phenomenon that could be related to production of AA in the nucleus. Indeed, it should be recalled that PKC-α can be activated by AA, albeit only in the presence of Ca^{2+} [127]. It is intriguing that AA seems to be involved in the regulation of nuclear Ca^{2+} mobilization [128]. Therefore, the effects of upregulated nuclear PLA$_2$ activity could be mediated by PKC isoforms, which are important players in cell proliferation [129].

Nuclear PLA$_2$ activity might also be related to changes in the fatty acid composition of nuclear phospholipids (PC, PE, inositol lipids and phosphatidylserine), which are associated with liver regeneration. Indeed, it has been shown that at 28 h post-hepatectomy, oleic acid at the 2 position increased transiently at the expense of AA and docasahexaenoic acid. In contrast, changes at the 1-position were quite limited [130]. In this connection, it should be emphasized that there also is evidence that fatty acids may directly control gene expression by modulating the activity of transcription factors such as hepatocyte nuclear factor-4α [131, 132]. Moreover, nuclear PLA$_2$ activity might be essential for PC remodeling.

Eicosanoid metabolism in the nucleus

The oxygenated metabolites of AA, leukotrienes, prostaglandins and thromboxane (collectively referred to as eicosanoids), are a group of potent bioactive lipid metabolites that regulate normal homeostatic processes and also promote acute inflammatory responses [133].

The first step in the production of leukotrienes involves conversion of AA to leukotriene A_4 (LTA_4) by 5-lipoxygenase (5-LO). 5-LO may found either in the cytoplasm or in the nucleus [134]. Several recent studies have begun to define the structural determinants in 5-LO required for its localization inside the nucleus. Using stringent and functional criteria, a NLS comprising amino acids 518–530 of 5-LO has been identified [135]. Mutations of the basic residues within this region abolished nuclear import of 5-LO but had no effect on enzymatic activity. However, additional data suggest that there may be other sites (at Arg112 and Lys158) required for nuclear localization of 5-LO [136]. There is also evidence that nuclear localization of 5-LO is regulated by a NLS and a NES, although

the signal for export remains to be identified [137]. LTA_4 is a highly reactive electrophilic compound that has been shown to covalently bind to nucleosides and nucleotides [138]. Thus, it might be that LTA_4 reacts with either DNA or RNA (or both) and affects transcriptional processes. LTA_4 is unstable, and it can be converted to leukotriene B_4 (LTB_4) by LTA_4 hydrolase. The subcellular localization of LTA_4 hydrolase has recently been studied, and cell type-dependent differences were apparent [139]. It was localized to the soluble compartment of the nucleus along with 5-LO in resting alveolar macrophages and rat basophilic leukemia cells. LTB_4 produced inside the nucleus might have a regulatory role, because it has been shown to be an activating ligand for the transcription factor peroxisome proliferator-activated receptor α (PPARα) [140]. PPARα is a nuclear hormone receptor for fatty acids and hypolipidemic drugs which regulates genes involved in lipid homeostasis [141].

Cyclooxygenases (COXs) are the first committed enzymes for the production of prostaglandins. The metabolism of AA by COXs is a two-step process involving an initial reaction in which AA is complexed with two molecules of O_2 to form prostaglandin G2, which is then reduced to prostaglandin H2 by the peroxidase activity of COXs [142]. Both COX1and COX2 have been localized to the nuclear envelope [143]. However, in resting endothelial cells COX2 is mainly localized within the nucleus in the perichromatin zone. Treatment of these cells with interleukin-1 increases expression of COX2 protein in the nucleus [144]. At present, however, it is unclear what functional role(s) intranuclear COXs may have, even though prostaglandins have also been implicated as ligands for PPARα [145].

Concluding remarks

Having established the existence of several signaling pathways operating in the nuclear compartment which are based on PC, SM, fatty acids and eicosanoids, we are still faced with a formidable number of problems which await resolution. Some of these problems have been at least partially solved in the case of nuclear inositol lipid metabolism and signaling. Indeed, in this case we know some of the signals linking receptor stimulation at the cell surface to activation of the nuclear lipid cycle [12]. Moreover, we have begun to elucidate the ways in which some nuclear enzymes involved in inositide metabolism are regulated [5, 11, 12]. Evidence has also been collected that this class of phospholipids is involved in messenger RNA (mRNA) synthesis and chromatin structure [8]. We also know the precise subnuclear localization of some phosphoinositides, because specific monoclonal antibodies to these molecules have been generated [146].

Our overall knowledge about the other classes of nuclear phospholipids is still sketchy at best. We are just beginning to unravel the complex mechanisms which regulate nuclear import of the enzymes involved in the metabolism of these phospholipids, but we still do not know whether their regulation is similar to their cytoplasmic counterparts. The precise localization of PC, SM and fatty acids is still problematic, because no reliable antibodies to these molecules are available, even though 'anti-phospholipid' antibodies seem to be a distinctive feature of a myriad of human disorders (reviewed in [147]). In some investigations, mapping of nuclear phospholipids has been obtained by means of the PLA_2-colloidal gold complex technique, but obviously this method lacks selectivity (e. g. [148]).

Downstream signaling in the nucleus is another facet which requires in-depth investigation, because we know very little of the physiological targets of some nuclear lipid second messengers. In addition, the study of possible cross-talk between different lipid signaling pathways present at the nuclear level has never been undertaken in a systematic manner. For example, we do not know whether there is any linkage between nuclear PIP_2 and PLD.

The technological advances of recent years should enable us to address many of these issues in the near future.

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