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Review

Signaling and interplay mediated by phospholipases A_2, C, and D in LA-N-1 cell nuclei

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Abstract – Phospholipids are integral components of the nuclear membranes and intranuclear domains. Alterations in phospholipid metabolism occur during cellular differentiation, proliferation, and apoptosis, but the molecular mechanism involved in the above processes remains unknown. We propose that the coordinated expression of different genes responsible for the expression of transcription factors, neurotrophins, and cytokines, along with lipid mediators generated by the action of phospholipases A_2, C, and D (PLA_2, PLC, and PLD), play a very important role in differentiation, proliferation, and apoptosis. The purpose of this minireview is to discuss recent developments in PLA_2, PLC, and PLD-mediated signaling in the nucleus of LA-N-1 neuroblastoma cell cultures. In brain tissue, arachidonic acid is mainly released by the action of PLA_2 and phospholipase C/diacylglycerol lipase (PLC/DAG-lipase) pathways. We have used LA-N-1 cell cultures to study activities of PLA_2, C, and D during retinoic acid (RA)-mediated differentiation. The treatment of LA-N-1 cells with RA produces an increase in PLA_2 activity in the nuclear fraction. This increase in PLA_2 activity can be prevented with BMS493, a pan retinoic acid receptor antagonist, suggesting that RA-induced stimulation of PLA_2 activity is a RA receptor-mediated process. The treatment of LA-N-1 cells with 12-O-tetradecanoyl-phorbol-13 acetate (TPA) and RA increases diacylglycerol (DAG) levels indicating the stimulation of PLC activity. This stimulation is blocked by D609, tricyclodecan-9-yl potassium xanthate, a competitive PtdCho-specific PLC inhibitor. LA-N-1 cells also contain DAG-and monoacylglycerol (MAG) lipase activities. Two isoforms of PLD, oleate-dependent and TPA-dependent, are also present in LA-N-1 cell homogenates. RA stimulates the oleate-dependent isoform of PLD, whereas RA does not stimulate the TPA-dependent isoform. Our studies have indicated that lipid mediators generated by the action of PLA_2, PLC, and PLD on nuclear phospholipids markedly affect neuritic outgrowth and neurotransmitter release in cells of neuronal and glial origin. We propose that RA receptors coupled with PLA_2, PLC, and PLD activities in the nucleus may play an important role in the redistribution of arachidonic acid and its metabolites and DAG in nuclear and non-nuclear neuronal membranes during differentiation and growth suppression.

nucleus / phospholipase A_2 / phospholipase C / phospholipase D / arachidonic acid / eicosanoids / peroxisome proliferators activated receptor

Abbreviations: Cer*P*Cho: sphingomyelin; DAG: diacylglycerol(s); IGF: insulin-like growth factor; Ins: inositol; LA-N: Los Angeles neuroblastoma; MAG: monoacylglycerol(s); PAF: platelet-activating factor; PKC: protein kinase C; PL: phospholipase; $cPLA_2$: cytosolic phospholipase A_2; $iPLA_2$: calcium-independent phospholipase A_2; $sPLA_2$: secretory phospholipase A_2; PPAR: peroxisome proliferator-activated receptors; PPRE: peroxisome proliferator response elements;

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PlsEtn: plasmenylethanolamine; PtdCho: phosphatidylcholine; PtdEt: phosphatidylethanol; PtdEtn: phosphatidylethanolamine; PtdOH: phosphatidic acid; PtdIns: phosphatidylinositol; PtdSer: phosphatidylserine; RA: retinoic acid; TNF-α: tumor necrosis factor-α; TPA: 12-O-tetradecanoyl-phorbol-13 acetate.

1. INTRODUCTION

Phospholipids are the major lipid constituents of neural membranes. They provide neural membranes with stability, fluidity, and permeability. Neural membrane phospholipids are a reservoir for bioactive lipid metabolites, second messengers. They are not only involved in neural cell proliferation, differentiation, and apoptosis, but also in the modulation of activities of transporters, ion channels, and membrane-bound enzymes [1]. Local variations in the concentrations of particular phospholipids species mediate the above properties through the formation of lipid rafts and the regulation of lipid-protein interactions. It is well known that phospholipid-mediated signaling involves the generation of bioactive lipid metabolites in response to agonist-receptor interactions at the plasma membrane level [1].

These bioactive lipid metabolites modulate certain genes in the nucleus. The occurrence of phospholipids in nuclei has been documented by biochemical and ultracytochemical procedures [2, 3]. The total phospholipid content of nuclei is reported as 3% by weight compared with 75% for protein and 22% for DNA. Major nuclear phospholipids include phosphatidylinositol (PtdIns), phosphatidylcholine (PtdCho), and sphingomyelin (Cer*P*Cho). Trace amounts of phosphatidylethanolamine (PtdEtn) were also found in the nucleus. This is in contrast to the plasma membranes that contain a considerable amount of PtdCho, PtdEtn, PtdSer (phosphatidylserine), and PtdIns [4, 5]. The phospholipid contents (per mg protein) of the nuclear membrane are approximately nine times that of whole nuclei. Significant information is available on proportions of molecular species in plasma membrane [1, 6], but very little is known about the occurrence of phospholipid molecular species in the nucleus [4]. Collective evidence suggests that nuclear phospholipids have a composition and turnover rate different from phospholipids present in plasma membranes, microsomes, and mitochondria and that the nucleus is a site of an active and autonomous phospholipid metabolism [7–10].

The nuclear fraction contains many enzymes of phospholipid metabolism (Tab. I) that generate and regulate the levels of second messengers. These enzymes include phospholipase A_2 (PLA_2), phospholipase C (PLC), phospholipase D (PLD), diacylglycerol kinase (DAG-kinase), diacylglycerol lipase (DAG-lipase), phosphatidylinositol 4-kinase, Mg^{2+}-dependent sphingomyelinase, and CTP: phosphocholine cytidylyltransferase. The second messengers generated by these enzymes modulate neural cell proliferation, differentiation, and apoptosis. These processes involve a coordinated expression of different genes responsible for the expression of transcription factors, neurotrophins, and cytokines [11] along with alterations in activities of protein kinases, phospholipases, and protein phosphatases. During these processes the quantitative ratios among various phospholipids undergo significant changes depending upon the functional state of neurons, astrocytes, and oligodendrocytes. This is modulated by the interaction among them [1, 6, 12, 13]. It is interesting to note that some extracellular stimuli, such as retinoic acid, produce bioactive lipid metabolites, such as diacylglycerol and arachidonic acid, only in the nucleus and not in the plasma membrane. Thus in normal brain agonist-mediated alterations in nuclear fraction are not a duplication of those changes that occur at the plasma membrane level [9, 14–16]. Furthermore, the enzymic properties of phospholipid metabolizing enzymes in the nuclear fraction are different from those found in

Table I. Phospholipid metabolizing enzymes involved in generation and modulation of phospholipid metabolism in the nucleus.

Enzyme	Reference
Phosphatidylinositol synthetase	[109]
Acyl-*sn*-glycero-3-phosphate acyltransferase	[110]
CTP: phosphocholine cytidyl transferase	[111]
Diacylglycerol acyltransferase	[112]
Lysophosphatidic acid phosphohydrolase	[109, 110]
Diacylglycerol kinase	[87, 113]
Sphingosine kinase	[114]
Acetyltransferase	[68]
Diacylglycerol lipase	[29]
Monoacylglycerol lipase	[29, 109, 110]
Isoforms of phosphatidylinositol-specific phospholipase C	[9, 113]
Phosphatidylcholine-specific phospholipase C	[19, 115]
Isoforms of phospholipases A_2	[22, 23, 29, 116]
Phospholipase D	[22, 23, 92]
Phosphatidic acid phosphatase	[92]
Magnesium-dependent neutral sphingomyelinase	[8, 116]
Phosphatidylinositol 4-kinase	[113]
CDP-choline: 1,2-diacylglycerol	[117]
PtdIns 3-, 4-, and 5-kinases	[9, 113]

plasma membrane, microsomes, and cytoplasm [16]. For example, bombesin, a powerful mitogen, stimulates phosphoinositide metabolism at the plasma membrane level but has no effect on phosphoinositide metabolism in the nucleus. Furthermore, insulin-like growth factor-1 (IGF-1) stimulates DAG-kinase activity in the nucleus but not in whole homogenate [9].

Beside their role as reservoirs for second messenger generation, nuclear phospholipids (PtdIns) also stimulate RNA synthesis, not only by modulating RNA polymerase activity but also by affecting chromatin organization [16]. Metabolites of the phosphatidylinositol cycle bind to nuclear skeletal proteins (nuclear lamin B and DNA topoisomerase) and nuclear signaling proteins (phospholipase C) and affect gene expression [10, 17, 18]. Nuclear inositol lipids may also be involved in mRNA transcription and/or processing, DNA replication or repair potentially resulting in cell differentiation, proliferation, or apoptosis. Furthermore, the interaction of phospholipids with histones and non-histone chromosomal proteins suggests that the regulation of RNA polymerase with phospholipids occurs at the level of template availability [13, 17, 18]. The α isoform of CTP: phosphatocholine cytidylyltransferase, the main enzyme of Kennedy pathway [19], also occurs in the nucleus and its activity is involved in temperature-sensitive mutation associated with cell survival. Collective evidence thus suggests that phospholipids and their second messengers play a major role in modulation of differentiation, apoptosis, and growth suppression [10, 20].

Excellent review articles are available on PLC-mediated signaling related to PtdIns breakdown in the nucleus, but very little is known about PLA_2 and PLD-mediated signaling related to PtdCho and PtdEtn [9,

14–16, 18, 21, 22]. We have been studying the effect of retinoic acid-mediated differentiation on phospholipid metabolism in LA-N-1 cells for several years [23, 24]. This cell line has catecholamine characteristics, and like neurons undergoes differentiation in the presence of retinoic acid [25, 26]. Morphologically, this differentiation is characterized by the cessation of mitosis and formation of extended neuritic processes [27]. The purpose of this commentary is to discuss recent developments on PLA_2, PLC, and PLD-mediated signaling in the nucleus of LA-N-1 neuroblastoma cell cultures. It is hoped that this discussion will jump start more studies, not only on cross talk among isoforms of PLA_2, PLC, and PLD in the nucleus, but also on the importance of nuclear PLA_2, PLC, and PLD in modulating the generation of second messengers in other subcellular organelles found in the cytoplasmic compartment.

2. OCCURRENCE OF ISOFORMS OF PHOSPHOLIPASE A_2, PHOSPHOLIPASE C, AND PHOSPHOLIPASE D IN NUCLEAR PREPARATIONS FROM LA-N-1 CELL CULTURES

Isoforms of PLA_2 hydrolyze neural membrane phospholipids at the *sn*-2 position producing lysophospholipids and free fatty acids including arachidonic acid and docosahexaenoic acid [1, 28]. Isoforms of PLC cleave the phosphodiester bond on the glycerol side forming diacylglycerols and a phospho-base, whereas isoforms of phospholipase D hydrolyze the bond between the base and phosphate generating phosphatidic acid and a free base, choline, ethanolamine or inositol [1] (Fig. 1). Major proportions of the PLA_2, PLC, and PLD activities occur mainly in cytoplasm and the subcellular organelles found in cytoplasmic compartment with small amounts (5–10%) of PLA_2, PLC, and PLD localized in the nucleus. The low levels of PLA_2, PLC, and PLD must be viewed in the context that the isolated nuclei contain less than 3% of the total protein present in homogenate.

In general, the function of the signal transduction network is to convey extracellular signals from the cell surface to the nucleus to induce a biological response at the gene level. Brain PLA_2, PLC, and PLD are major signaling enzymes involved not only in neural cell proliferation, differentiation, and growth under normal conditions, but also in neuroinflammation, oxidative stress, and cell death under pathological situations. These enzymes are linked to various receptors with different coupling mechanisms at the plasma membrane and nuclear domain levels. PLA_2, PLC, and PLD control the intensity and duration of the signal transduction process by modulating the levels of second messengers including arachidonic acid, diacylglycerols, platelet activating factor, and eicosanoids [1, 24, 29, 30].

The kinetic properties of PLA_2, PLC, and PLD in the nuclear fraction from LA-N-1 cells are shown in Table II. The response of nuclear PLA_2, PLC, and PLD to their inhibitors is different from that of the PLA_2, PLC, and PLD associated with non-nuclear compartment [9, 31].

2.1. Nuclear PLA_2 activities

More than 14 isoforms of PLA_2 occur in mammalian tissues. These enzymes have been subdivided into several groups depending upon their structure, enzymic properties, subcellular localization, and cellular function [29, 30, 32, 33]. PLA_2 isoforms include secretory phospholipase A_2 ($sPLA_2$), cytosolic phospholipase A_2 ($cPLA_2$), plasmalogen-selective phospholipase A_2 (PlsEtn-PLA_2), and calcium independent phospholipase A_2 ($iPLA_2$). Genes coding for $sPLA_2$, $cPLA_2$, and $iPLA_2$ occur in different regions of brain and in both neurons and glial cells [34–37]. Some of these isoforms of PLA_2 have been partially purified and characterized from brain tissue. However, none of the above isoforms have been cloned and

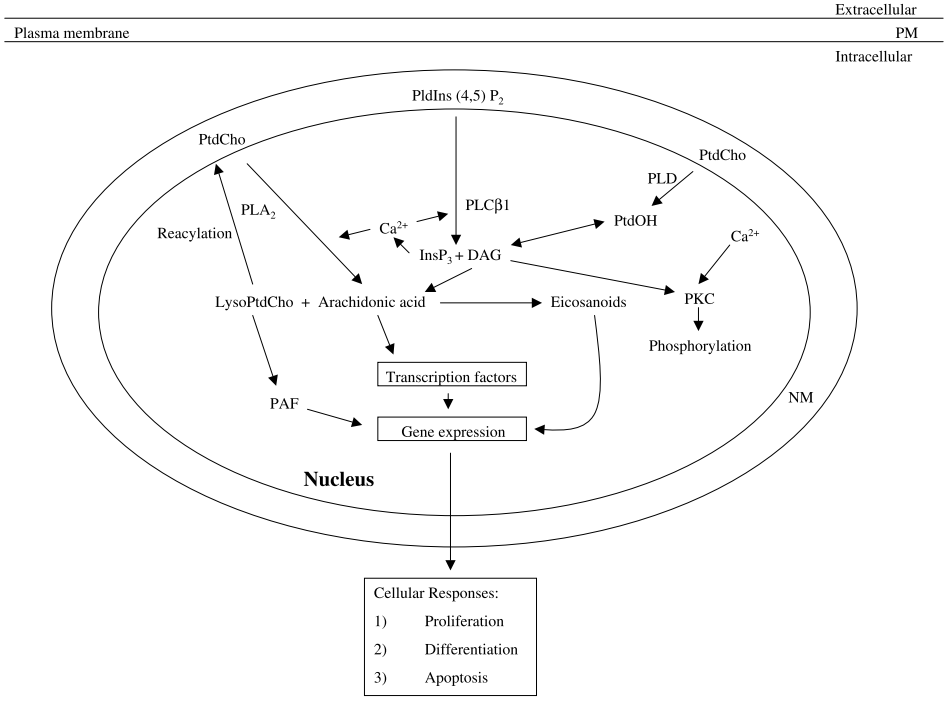

Figure 1. A hypothetical diagram showing the interactions among second messengers generated by PLA_2, PLC, and PLD in the nucleus. PtdCho, phosphatidylcholine; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; LysoPtdCho, lysophosphatidylcholine; $InsP_3$, inositol 1,4,5-trisphosphate; PtdOH, phosphatidic acid; PAF, platelet activating factor; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C, NM, nuclear membrane, and PM, plasma membrane.

fully characterized from brain tissue and considerable information is available on the PLA_2 activities in cytoplasmic fraction and non-nuclear subcellular organelles [1, 28].

Our studies on the subcellular distribution of PLA_2 activity in LA-N-1 cells indicate that the nuclear fraction contains at least two calcium-independent PLA_2 activities. The 110 kDa PLA_2 hydrolyzes 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PtdEtn), whereas the 39 kDa PLA_2 selectively acts on plasmenylethanolamine (PlsEtn). It is well known that retinoic acid (RA) easily diffuses through cellular membranes, transported to the nucleus by retinal-binding proteins, and produces biological effects through RA receptors [30]. Thus the treatment of LA-N-1 cell cultures with retinoic acid results in a marked stimulation of PLA_2 activities hydrolyzing PtdEtn and PlsEtn. The specific activity of PtdEtn-PLA_2 increases during the first 5 days, whilst that of PlsEtn-PLA_2 increases rapidly during the first 2 days after retinoic acid treatment [23, 24]. The specific activities of PLA_2 hydrolyzing PtdEtn and PlsEtn increase about 5- and 3.5-fold respectively, between 6 and 10 h in the nuclei while those of the cytosolic PLA_2 enzymes are elevated only 1.3- and 1.7-fold during the same period [23, 24] This indicates that the effect of retinoic acid in LA-N-1 cells is mediated through the nuclear phospholipids. The collective evidence suggests that nuclear

Table II. Kinetics parameters of nuclear PLA_2, PLC, and PLD in LA-N-1 cells.

Kinetic parameter	Value	Reference
PtdEtn-PLA_2		
pH optimum	7.4	[22]
Km value (μM)	35.0 ± 5.0	[22]
Vmax (pmol/min/mg)	14.65 ± 1.5	[22]
PlsEtn-PLA_2		
pH optimum	8.0	[22]
Km value (μM)	50.0 ± 7.0	[22]
Vmax (pmol/min/mg)	25.7 ± 2.0	[22]
PLD		
pH optimum	6.5	[84]
Km value (μM)	330	[84]
Vmax (pmol/min/mg)	3.8	[84]

Nuclei were prepared and assayed as described earlier [19, 22, 23]. Nothing is known about the kinetic parameters of PtdIns-PLA_2 and PtdCho-PLC in LA-N-1 cell nuclei.

Table III. Effect of BMS493 and cycloheximide on PlsEtn-PLA_2 and PtdEtn-PLA_2 in the nuclear fraction from LA-N-1 cell cultures.

Treatment	PlsEtn-PLA_2 (pmol/min/mg protein)	PtdEtn-PLA_2 (pmol/min/mg protein)
Control	4.82 ± 0.39	3.57 ± 0.27
RA (1.0 μM)	11.83 ± 0.47	6.32 ± 0.25
BMS493 (10.0 μM)	4.62 ± 0.37	3.37 ± 0.35
BMS493 (10.0 μM) + RA (1.0 μM)·mL^{-1})	4.92 ± 0.55	3.13 ± 0.42
Cycloheximide (0.5 μg·mL^{-1}) + RA (1.0 μM)	7.92 ± 0.43	4.95 ± 0.37

Modified from [23].

PLA_2 activities are stimulated first, then cytosolic PLA_2 activities, while membrane PLA_2 activities are not changed as a result of retinoic acid treatment [22, 24, 30].

The pretreatment of LA-N-1 cells with the pan retinoic acid receptor antagonist, BMS493, blocks the stimulation of nuclear PLA_2 activities suggesting that the nuclear PLA_2 stimulation by retinoic acid is a receptor-mediated process (Tab. III). The retinoic acid-mediated stimulation of PtdEtn-PLA_2 and PlsEtn-PLA_2 activities in the nuclear fraction is also inhibited by a low concentration of cycloheximide but the extent of inhibition produced by cycloheximide is lower than that of BMS493. This observation suggests that the cycloheximide-induced inhibition of PLA_2 activities is due to a general decrease in protein synthesis. In contrast, the BMS493-mediated inhibition is due to its specific antagonistic activity towards retinoic acid receptor [24, 30].

The stimulation of PLA_2 activities in nuclei by RA is in agreement with labeling studies. Thus the treatment of prelabeled LA-N-1 cells with RA has indicated that arachidonic acid release in the nuclear fraction is a biphasic process. A rapid release within the first minute is followed by a sustained arachidonic acid release at 30 min that continues even after 10 h [24]. This

observation is supported by studies on the effect of PLA_2 inhibitors that are known to exert a biphasic effect over several days on elongation in dorsal root ganglion axon, enhancing outgrowth at low concentrations and inhibiting outgrowth at higher concentration [38]. It is interesting to note here that $cPLA_2$ inhibitors delay the initial outgrowth of neurites on laminin and exogenous arachidonic acid enhances neurite outgrowth [39]. Also, interphase cells release arachidonic acid in response to agonists, and this release is strongly inhibited in mitotic cells [40].

The addition of $cPLA_2$ inhibitors suppresses proliferation of cells by inducing apoptosis [41]. Assays of $cPLA_2$ activities in various phases of the cell cycle indicate that $cPLA_2$ activity is high in mitosis, decreases afterwards, and is increased again in G1 and following the G1/S transition phases. During these phases, elevations in $cPLA_2$ activity are due to increased phosphorylation rather than increased $cPLA_2$ protein expression. This suggestion is supported by studies in which phosphatase treatment of $cPLA_2$ reduces its activity [42]. The inhibition of $cPLA_2$ with arachidonyl trifluoromethylketone in early G1 phase markedly reduces DNA synthesis suggesting that $cPLA_2$ plays an important role in progression of the cell cycle. Cyclooxygenase inhibitors have no effect on cell cycle progression into S phase [42], indicating that the $cPLA_2$-dependent progression is not mediated by arachidonic acid metabolites generated by cyclooxygenase. However, the lipoxygenase inhibitors caffeic acid and nordihydroguaiaretic acid inhibit DNA synthesis when added in early G1 phase [42].

PLA_2 isoforms are not only involved in the cell cycle but also in apoptotic cell death [30]. Inhibitors of PLA_2 activity block apoptosis [43, 44]. In permeabilized HeLa cells, PLA_2 isoforms play an important role in nuclear shrinkage during hypoxic injury. Bromoenol lactone, a potent inhibitor of $iPLA_2$ [45], blocks the hypoxia-mediated nuclear shrinkage not only in HeLa cells but also in PC12 cell cultures. These observations suggest that PLA_2 isoforms are required for nuclear shrinkage in caspase-independent apoptotic cell death [30, 46].

Retinoic acid inhibits apoptosis in some cell populations [47], whereas it induces apoptosis in others [48]. The mechanism of retinoic acid-mediated apoptosis remains unknown. However, two mechanisms have been proposed [49]. According to the first mechanism, retinoic acid induces apoptosis through mitochondrial dysfunction [50]. The second mechanism involves cytokine-mediated stimulation of PLA_2 activity [49], resulting in the generation of excess arachidonic acid. This excess arachidonic acid affects the transcription of many genes, not only through the generation of eicosanoids, but also directly [51, 52]. At present, the genes involved at various stages of proliferation, differentiation, and apoptosis cannot be specified [53–55]. However, these genes may include the genes controlling synaptic plasticity, cytoskeleton, and membrane association, signal transduction, and energy metabolism [56]. In addition, arachidonic acid also modulates activities of certain isoforms of protein kinase C and PLC in the nucleus [30]. Both these enzymes are involved in signal transduction processes associated with cell proliferation and differentiation [48, 57]. Arachidonic acid also gives rise to reactive oxygen species (ROS) that may induce oxidative stress leading to either cell proliferation or apoptotic cell death depending on cell type [47]. Both arachidonic acid and ROS are known to modulate gene expression through the activation of the redox-sensitive transcription factors such as nuclear factor κB (NFκB) [11, 58] and activator protein-1 (AP-1) [59]. NFκB has binding sites on the $cPLA_2$ promotor. This binding may induce expression of other cytokines such as IL-1β and TNF-α (Fig. 2) and neurotrophins [60, 61]. AP-1 transcription factors are among the best characterized DNA-binding proteins in the brain [62]. They consist of either jun-jun homodimer or a jun-Fos heterodimer

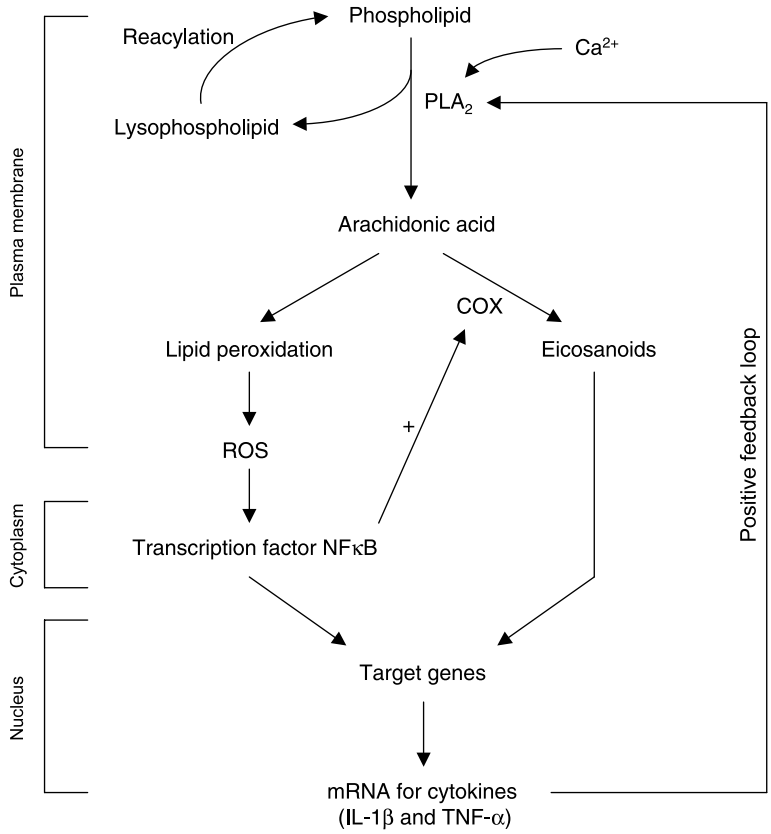

Figure 2. Relationships between neural membrane phospholipid metabolism and ROS-mediated stimulation of NFκB modulates target genes in the nucleus. Target genes include not only genes involved in the generation of cytokines (IL-1β and TNF-α), but also genes controlling synaptic plasticity, cytoskeleton, and membrane association, signal transduction, and energy metabolism [11, 56]. Lipid peroxidation and generation of ROS occurs in neural membranes, the ROS stimulate NFκB in the cytosol, and NFκB modulates target genes in the nucleus, including PLA_2, cytosolic PLA_2 ($cPLA_2$), reactive oxygen species (ROS), cyclooxygenase-1 and-2 (COX).

and bind to a specific site present in the promoter region of a wide variety of genes implicated in cell proliferation and tumorigenesis [63]. These observations strongly suggest that the nucleus is an important site of control for the incorporation and redistribution of arachidonic acid from nuclear phospholipids into other cellular membranes and nuclear PLA_2 activities and transcriptional factors like NFκB and AP-1 play important roles in the modulation of various stages cellular proliferation, differentiation and apoptosis.

Cytosolic $cPLA_2$ translocates to nuclear membranes during cellular stimulation [64]. This enzyme contains a calcium-dependent lipid-binding domain. In response to intracellular calcium, this domain facilitates the binding of $cPLA_2$ to endoplasmic reticulum and perinuclear membranes [65, 66]. A

transient calcium flux causes reversible translocation without the release of arachidonic acid, whilst a sustained calcium flux results in a prolonged perinuclear translocation with accompanying arachidonic acid release. During hypoxic injury, the depletion of ATP results in translocation of $cPLA_2$ to the nucleus. This translocation is partially blocked by okadaic acid, a phosphatase 2A inhibitor [67]. This observation suggests the involvement of phosphorylation/dephosphorylation processes in the translocation of $cPLA_2$.

At present, nothing is known about the relationship between the $cPLA_2$ translocated to the perinuclear membrane and the activities of intrinsic nuclear PLA_2 isoforms. In contrast, $sPLA_2$ translocates from the inside to the outside of cells during stimulation and hydrolyzes fatty acids from outer cellular membranes. These observations suggest that different multiple forms of PLA_2 act on different phospholipid molecular species which reside at different subcellular locations by showing that the appropriate subcellular locations are enriched with the phospholipid substrate proposed to be degraded by $sPLA_2$ and $cPLA_2$ [1, 28].

Lysophosphatidylcholine, the other product of PLA_2 catalyzed reactions, is either reacylated to phosphatidylcholine (Fig. 1), further hydrolyzed, or metabolized to platelet activating factor (PAF). A neuronal nuclear acetyltransferase was found in young rat brain [68, 69]. In nuclear membranes these enzymes are involved in the generation of platelet activating factor (PAF) [70, 71]. PAF is known to induce the expression of c-fos and c-jun and prostaglandin synthase. The expression of c-fos, c-jun, and prostaglandins is blocked by BN-50730, a specific PAF antagonist [72, 73].

2.2. Nuclear PLC activities

Enzymes of the phosphoinositide cycle are present in the nucleus (Fig. 1) and the nuclear phosphatidylinositol cycle is completely independent of that found at the plasma membrane and at the cytoskeleton [9, 13–15]. Isoforms of PtdIns-specific PLC occur in nuclear fractions prepared from neural and non-neural tissues. There are four isoforms of the PtdIns-specific PLC in brain tissue [74, 75], but PLCδ4 and PLCβ1 are the only isoforms specific to the nucleus. The PLCβ1 is constitutively expressed in the nucleus and is activated very early during nuclear signaling while PLCδ4 is expressed later during the transition phase. The intranuclear phosphatidylinositol cycle is modulated by extracellular stimuli, such as retinoic acid, affecting cell proliferation, differentiation, or neoplastic transformation. Thus the amount and activity of PtdIns-PLCβ is increased in the nucleus following the incubation of HL60 cells with RA [76].

The hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC isozymes generates diacylglycerols and inositol 1,4,5-trisphosphate. Diacylglycerols stimulate nuclear protein kinase C isoforms and inositol 1,4,5-trisphosphate mobilizes calcium via its receptors on inner nuclear membrane (Fig. 1). Thus, inositol phosphates have a role in calcium homeostasis within the nucleus. Nuclear calcium homeostasis is known to modulate a number of critical nuclear events such as the regulation of transcription factors, cell cycle regulation, gene transcription, DNA replication, and nuclear envelope breakdown. Furthermore, inositol 1,4,5-trisphosphate generated during the intranuclear phosphatidylinositol cycle can also be converted via successive kinases to inositol tetrakisphosphate ($InsP_4$), inositol pentakisphosphate ($InsP_5$), and inositol hexakisphosphate ($InsP_6$). $InsP_4$ inhibits chloride channel conductance, $InsP_5$ inhibits signal transduction pathway involving phosphatidylinositol 3-kinase, and $InsP_6$ has been proposed to play an important role in mRNA transport and regulation of the transcription of some genes [9, 77]. This suggests that nuclear phosphatidylinositol metabolites may not just act as substrates for second messenger generation but they may be directly involved in mRNA splicing, regulation of transcription factors, cell

cycle regulation, gene transcription, and DNA replication [9, 21]. Furthermore, highly phosphorylated inositols have also been implicated in chromatin remodeling [78]. These observations strongly support the hypothesis that lipid mediators of inositol metabolism play an essential role in modulation of nuclear activities.

Other phospholipids, such as PtdCho and trace amounts of PtdEtn, also occur in neuronal nuclei [79, 80], but little is known about their metabolism in neural cell nuclei [5]. The occurrence of PtdCho-specific PLC in neural cells is well established, but attempts to purify and clone it from mammalian tissues have failed [81, 82]. Antony et al. have determined the activities of the PLC hydrolyzing PtdCho in the nuclear fraction obtained from LA-N-1 cells [19, 23, 24, 83, 84]. The kinetic properties of nuclear PtdCho-PLC are shown in Table II. The treatment of LA-N-1 cells with TPA as well as RA produces an increase in nuclear DAG levels. This increase, including the stimulation of PLC and CTP: phosphocholine cytidylyltransferase activities, is inhibited by D609, a phosphatidylcholine PLC inhibitor. This not only suggests that the stimulation of PLC by RA is a receptor-mediated process, but also indicates that the PtdCho cycle occurs in nuclear preparations from LA-N-1 cells. D609 also blocks ceramide phosphocholine transferase activity. Thus, D609 is not a specific inhibitor of PtdCho-specific PLC. This may complicate the analysis of PLC activity in the presence of D609 at the nuclear level. Thus DAG generated by the hydrolysis of phosphatidylcholine in the nuclei can be reutilized for the synthesis of nuclear phosphatidylcholine and is required for the activation of CTP:phosphocholine cytidylyltransferase [83].

PLC isoforms also play an important role in cell cycle progression. The two subtypes of PLCβ1, when overexpressed in the nucleus, cause the overexpression of cyclin D3 and its kinase (cdk4). Based on various cell biology studies, nuclear PLCβ1 signaling is involved in the G1 progression phase

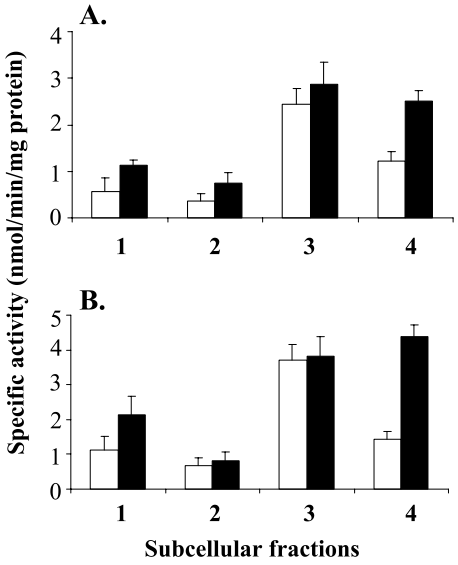

Figure 3. Activities of diacylglycerol lipase (**A**) and monoacylglycerol lipase (**B**) in subcellular fractions prepared from LA-N-1 cell cultures. (**1**) Homogenate, (**2**) cytosol, (**3**) non-nuclear pellet and (**4**) nucleus. Control (clear bars) and treated with retinoic acid (filled bars). Enzymic activities were determined using thioester substrates [108]. This information is modified from [29]. Each point is the mean of 3 experiments with the S.E.M. indicated by the bar.

of the cell cycle through cyclin D3 and its kinase [85]. In contrast, the cytosolic enzyme PLCγ1 does not fluctuate during the cell cycle. Stimulation of quiescent 3T3 cells with IGF-1 increases the activity of the phosphatidylinositol-specific PLC-β1 by 2- to 3-fold within a few minutes. This suggests that phosphatidylinositol-specific PLC-β1 may be involved in control of cell proliferation.

DAG and MAG-lipase activities are present in subcellular fractions prepared from LA-N-1 cell homogenates (Fig. 3). Non-nuclear pellets that contained plasma membranes, mitochondria, and microsomes displayed the highest specific activity for DAG-and MAG-lipases. The nuclear fraction had significant DAG-and MAG-lipase activities. These enzymes hydrolyze DAG which is

generated in LA-N-1 cells by the action of PLC and PLD on nuclear phospholipids [19]. DAG cannot be regarded as a single entity, as there are as many as 50 different DAG molecular species that are present in LA-N-1 cells [12]. Many of these molecular species are involved in the activation of nuclear protein kinase C isozymes [12, 86]. In the nucleus, DAG is also a substrate for DAG-kinase [18, 87]. This enzyme phosphorylates DAG to phosphatidic acid (PtdOH). Thus nuclear DAG-lipase and DAG-kinase attenuate the DAG generated signal by regulating the amount of nuclear DAG available for the activation of protein kinase C isozymes. It has been suggested that DAG derived from PtdIns are shuttled directly to a DAG kinase present in the nucleus, but DAG derived from PtdCho are not accessible to DAG-kinase. This suggests that nuclei contain at least two distinct pools of DAG that are generated by the action of two distinct phospholipases.

In brain tissue, DAG are a fundamental lipid second messenger that is generated not only at the plasma membrane level but also in the nucleus. At both subcellular levels, DAG are distributed in several pools that may be involved in modulating PKC activity in signal transduction processes associated with cellular proliferation and differentiation. The levels of nuclear DAG fluctuate during the cell cycle progression indicating that DAG molecular species have an important regulatory role not only in signal transduction but also in cell division [18]. In contrast, the cytoplasmic levels of DAG remain constant. A major increase in DAG and marked decrease of phosphatidylinositol in the nucleus coincides with the S phase of the cell cycle [88].

3. NUCLEAR PLD ACTIVITIES

PLD hydrolyzes PtdCho to generate membrane-bound phosphatidic acid (PtdOH) and soluble choline (Fig. 1). Whereas choline formation may be important for some aspects of PLD function in the nucleus, it is thought that the generation of PtdOH (and its downstream metabolites, DAG) constitutes the crucial contribution of the enzyme to PLD-dependent signaling pathways. PtdOH can also be metabolized to lysophosphatidic acid (lyso-PtdOH). This metabolite interacts with its receptor in the nucleus and modulates neural cell proliferation and differentiation [89, 90]. In neural cells, PLD isozymes activate other signaling enzymes, facilitate membrane vesicle fusion events, or serve as a lipid anchor for membrane-associated proteins [1, 91]. The kinetic properties of PLD in LA-N-1 nuclei are shown in Table II. Rat brain neuronal nuclei contain a PLD that is activated by unsaturated fatty acids, particularly oleic acid [92]. PLD, in addition to having PtdCho hydrolyzing activity, also catalyzes a transphosphatidylation reaction generating a phosphatidylalcohol. GTPγS, ATPγS, phosphatidic acid, and phosphatidylethanol inhibit the neuronal nuclear PLD. The PLD activity of neuronal nuclei is higher than that detected in the nuclei of glial cells or extracellular neural cells.

The incubation of LA-N-1 cell nuclei with TPA and GTPγS in the presence of 1.0% ethanol results in formation of PtdEt. This observation suggests the presence of a G-protein linked phospholipase D in LA-N-1 cell nuclei [19]. Recent studies on LA-N-1 cells have indicated that LA-N-1 cell nuclei contain two PLD isoforms, a G protein-dependent isoform that is stimulated by phorbol ester, and an oleate-dependent isoform that is stimulated by RA [84]. In contrast, non-nuclear PLD (PLD_1 and PLD_2) activities are stimulated by PKC-α, phosphatidylinositol 4, 5-bisphosphate, ADP-ribosylation factor, and Rho-family proteins in a GTP-dependent manner. PLD_2, however, only requires the presence of phosphatidylinositol 4, 5-bisphosphate for optimal activity to be expressed in assays in vitro. On the other hand, the basal activity of PLD_1 is increased 14-fold in the presence of phorbol 12-myristate [93]. Distinct isoforms of PLD in the nucleus may be involved in the generation of different

molecular species of PtdOH and ultimately DAG by the action of phosphatidate phosphohydrolase during cellular proliferation and differentiation [86]. A transient increase in the ADP-ribosylation factor-dependent nuclear PLD is reported to occur in the S-phase of regenerating rat hepatocytes [91], indicating that PLD activity is also associated with the cell cycle.

The stimulation of PLA_2, C, and D activities in nuclear preparations induced by RA may be useful for metabolic processes in LA-N-1 cells because (a) it aids the generation of eicosanoids and these metabolites may directly bind and activate nuclear transcription factors [57], (b) it accelerates the acylation-deacylation cycle for maintenance of essential phospholipids in the nuclear membrane (remodeling), (c) it protects the nuclear membrane from lipid peroxidation [22], and (d) it induces neuritogenesis not only by activating protein kinase C isozymes [94], but also by generating and modulating the levels of arachidonic acid and its metabolites [30].

4. INVOLVEMENT OF PEROXISOME PROLIFERATOR RECEPTORS IN RETINOIC ACID-MEDIATED PLA_2, PLC, AND PLD SIGNALING

Recently, a critical role of peroxisome proliferator-activated receptors (PPARs) in the regulation of phospholipid metabolism and inflammatory processes has become increasingly apparent. PPARs are members of the nuclear receptor superfamily of ligand-dependent transcription factors that, upon heterodimerization with the RA receptor, bind to specific DNA sequence elements (PPREs), thus regulating the expression of target genes [95]. PPREs have been identified in the regulatory regions of a variety of genes associated with rat $sPLA_2$ [96]. Three different types of PPARs, α, γ, and δ have been reported in non-neural cells. PLA_2 activity is coupled to a PPAR receptor and clofibrate, an activator of the PPAR-α receptor, regulates the expression of $cPLA_2$ and cyclooxygenase-2 activities [97]. Also, PPAR-γ modulates the synthesis of $sPLA_2$ by interfering with the activity of transcription factors such as NFκB [98].

In SK-N-BE neuroblastoma cells, RA not only induces the typical changes in cell morphology and cellular growth associated with differentiation, but also modifies polyunsaturated fatty acid metabolism [99], strongly indicating that the generation of polyunsaturated fatty acids by $cPLA_2$ is closely associated with the process of differentiation. In addition, arachidonic acid also modulates protein kinase C and phospholipase C γ activities in nucleus. Both enzymes are involved in signal transduction processes associated with cell proliferation and differentiation [29]. These observations strongly suggest that the nucleus is an important site of control for the incorporation and redistribution of arachidonic acid and DAG from nuclear phospholipids into other cellular membranes. The amount of arachidonic acid associated with the nuclei of proliferating cells is greatly diminished compared to non-proliferating cells [100], suggesting that PLA_2 isozymes are closely associated with cell proliferation processes. In contrast, docosahexaenoic acid inhibits the transcriptional activity and DNA binding of PPAR [101], indicating that specific fatty acids exert different responses in different cells at different subcellular levels. Eicosanoid-mediated activation of PPARs is a key mechanism by which prostaglandins govern the expression of genes involved in cellular differentiation [102]. The potency of the eicosanoid-mediated activation of PPARs varies with the type of prostaglandin and the type of PPAR. For example, PPARγ, which is relatively insensitive to fatty acid activation, is activated 80-90-fold by PGD_1 and PGD_2 [103]. Lyso-PtdOH interacts with PPARγ and promotes growth and differentiation [90]. The collective evidence suggests that PPARs are also coupled to phospholipases and may be involved in the regulation and distribution of arachidonic acid and its

metabolites from nucleus to non-nuclear compartments during differentiation and cell growth [104].

5. INTERPLAY AMONG NUCLEAR AND NON-NUCLEAR PLA_2, PLC, AND PLD ACTIVITIES

Transcripts and immunoreactive proteins for PLA_2, PLC, and PLD are widely expressed throughout the brain tissue, but very little is known about the interactions among nuclear and non-nuclear PLA_2, PLC, and PLD [30, 105]. Studies on this topic are not only complicated by the occurrence of isoforms of these enzymes, but also by the presence of similar, but not identical, isoforms of PLA_2, PLC, and PLD in cytoplasm and other subcellular organelles. The multiplicity of PLA_2, PLC, and PLD in brain tissue provides diversity in function and specificity of various isoforms in the regulation of enzymic activity in response to a wide range of extracellular signals. This complicates the analysis of their function at cellular and subcellular levels. The complexity of this problem becomes obvious when one considers the coupling mechanisms of various isoforms of PLA_2, PLC, and PLD with different receptors in a single neural cell in nuclear and non-nuclear membrane fractions and then tries to associate PLA_2, PLC and PLD activities with neuronal function.

The different isoforms of PLA_2, PLC, and PLD are not only regulated by calcium ions but also by covalent modification mediated by intrinsic protein tyrosine kinases, mitogen-activated kinases, and protein kinases C. Some isoforms of these enzymes are inducible to a further extent with cytokines and growth factors such as IL-1, IL-3, tumor necrosis factor-α (TNF-α), and nerve growth factor. Isoforms of PLA_2, PLC, and PLD may not function interchangeably but act in parallel to transducer signals [30]. It is likely that various isoforms of PLA_2, PLC, and PLD act on different cellular pools of phospholipids located in different subcellular organelles of various types of neural cells and these isoforms may be regulated by different coupling mechanisms generating common second messengers.

Free unsaturated fatty acids, the product of a PLA_2 reaction, and DAG, the product of PLC and PLD catalyzed reactions, act synergistically to stimulate PKC activity. Coordination and integration of these second messengers in nuclear and non-nuclear compartments is necessary for optimal functioning of signal transduction processes. The PLC- and PLD-mediated release of DAG results in the stimulation of various isoforms of protein kinase C. This leads to the activation of both PLA_2 as well as PLD [106]. Similarly, activation of nuclear PLA_2 isoforms generates arachidonic acid and eicosanoids and these lipid mediators have been shown to activate isoforms of PLC, PLD, and PKC [107].

The interactions among metabolites generated by PLA_2, PLC, and PLD at subcellular levels may provide neural cells and brain tissue with great versatility in ensuring that DAG, arachidonic acid, and eicosanoids are efficiently utilized. Thus, the cross talk among PLA_2, PLC, and PLD isozymes is essential for maintaining normal neuronal and glial cell growth. In the nucleus, signaling mediated by PLA_2, PLC, and PLD has an advantage over plasma membrane signaling in that second messengers generated by these enzymes during differentiation may directly interact with nuclear factors producing physiological and morphological changes [30]. In brain tissue, the activity of PLA_2, PLC, and PLD isoforms may depend not only on structural, physicochemical, and dynamic properties of neural membranes, but also on the type and metabolic state of neural cells. The activation of PLA_2, PLC, and PLD isoforms at the subcellular level in neural cell is the rate-limiting step for the production of lipid mediators such as arachidonic acid, eicosanoids, and DAG. Therefore, tight regulation of PLA_2, PLC, and PLD isozymes is

very important for normal brain function. As stated above, the regulation of PLA_2, PLC, and PLD activities is quite complex and is mediated by several factors such as translocation and phosphorylation and mechanisms such as gene expression and cross-talk among isoforms of these enzymes at cellular and subcellular level [30]. The collective evidence thus suggests that formation of PLA_2, PLC, and PLD-generated second messengers in the nucleus may be linked to neuronal growth and differentiation via the activation of certain PKC isozymes and subsequent phosphorylation [30].

6. CONCLUSION AND DIRECTION FOR FUTURE STUDIES

The nucleus harbors intrinsic signal transduction pathways as indicated by the presence of nuclear phospholipases A_2, C, and D and their phospholipid substrates. Treatment with retinoic acid (RA) produces a marked increase in the activities of the PLA_2 hydrolyzing PtdEtn and PlsEtn, the PLC specific for PtdCho, and the PLD in LA-N-1 cell nuclei. Also, RA stimulates DAG-lipase and MAG-lipase activities. RA had no effect on the activities of PLA_2, PLC, and PLD in non-nuclear membranes, indicating that autonomous signaling mediated by PLA_2, PLC, and PLD operates within the nucleus. Its modulation is independent of similar events taking place in other subcellular organelles including plasma membranes. In the nucleus, PLA_2, PLC, and PLD use nuclear membrane phospholipids as substrates for the generation of arachidonic acid and its metabolites and DAG. Under normal conditions these metabolites are involved in cell proliferation and differentiation processes. However, excessive production of arachidonic acid metabolites under pathological situations results in oxidative stress, inflammation, and neurodegeneration [29, 30].

Many questions remain to be answered regarding the involvement of signaling in the nucleus induced by PLA_2, PLC, and PLD. For example, how many isoforms of PLA_2, PLC, and PLD are present in the nucleus and what are their proportions and characteristics? Two forms of calcium-independent phospholipase A_2 (PtdEtn-PLA_2 and PlsEtn-PLA_2) are present in LA-N-1 cell nuclei [23, 24]. The kinetic properties of these enzymes are similar to the corresponding enzymes in the cytosol. However, others have reported differences between nuclear and cytosolic enzymes in response to PLA_2 inhibitors [16, 31]. At present nothing is known about the regulation of nuclear PLA_2, PLC, and PLD. Detailed investigations are required on the extent of cross talk between nuclear and cytosolic PLA_2, PLC, and PLD activities. Nuclear PLA_2, PLC, and PLD activities should be characterized and antibodies should be prepared against the isoforms. The availability of antibodies against nuclear PLA_2, PLC, and PLD isoforms would not only aid in their localization in the nucleus, but would be useful in determining their role in lipid transport and trafficking. Another important question that must be addressed in future studies is what factors determine the specificity of cellular responses at cellular and subcellular levels and how much of this specificity is dictated by the interplay among second messengers generated by PLA_2, PLC, and PLD? Thus, more studies are required to understand the metabolic significance of the interplay among PLA_2, PLC, and PLD at nuclear and non-nuclear levels in cultured cells of neuronal and glial origin and in nuclear preparations obtained from normal brain.

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