See related article, pages 132–139

Nuclear Phospholipase D1 in Vascular Smooth Muscle

Specific Activation by G Protein–Coupled Receptors

Masuko Ushio-Fukai

Activation of phospholipase D (PLD) is a major component of signal transduction cascades activated by G protein–coupled receptor (GPCR) agonists such as lysophosphatidic acid (LPA) and angiotensin II (Ang II), as well as growth factors such as PDGF and EGF that promote proliferation, migration, and inflammation of vascular smooth muscle cells (VSMCs). In mammalian cells, PLD catalyzes the hydrolysis of the principal membrane lipid phosphatidylcholine (PC), resulting in the formation of choline and bioactive lipid phosphatidic acid (PA). PA is subsequently metabolized to diacylglycerol by PA phosphohydrolase or to LPA by phospholipase A2. PLD been implicated in signal transduction, exocytosis and endocytosis, cell proliferation, cytoskeletal reorganization, and gene expression.[1–3] There are two mammalian PLD genes, PLD1 and PLD2, and two splice variants of each isoform. PLD1 has a low basal activity and is activated by the small GTP-binding proteins (Rho, Ral, and ADP ribosylation factor [Arf]) and protein kinase C (PKC). In contrast, PLD2 has a high basal activity and its in vitro activity is not or less responsive to PKC, Rho, or Arf.[1–3] Both PLD1 and PLD2 are activated by phosphatidylinositol 4,5-*bis*phosphate (PtdIns(4,5)P2), but PLD1 is inhibited whereas PLD2 is activated by oleic acid in vitro.[1,4] PLD1 and PLD2 have been proposed to mediate isoform-specific functions, based on their selective abilities and variable patterns of subcellular localization.[2] Numerous studies have used overexpression systems to show that PLD1 localizes to perinuclear vesicles,[5–7] plasma membrane,[8–10] including caveolin-enriched membrane,[11] and that PLD2 localizes to the plasma membrane,[5,12–14] and internalizes after agonist stimulation.[5,15] In contrast, endogenous PLD2 detected by a specific antibody localizes to the rim of the Golgi apparatus and the cell nucleus in rat normal rat kidney and in GH3 cells.[16] Furthermore, overexpression of HA-tagged PLD1 has been shown to cause redistribution of endogenous PLD to the significantly different location,[17] indicating that the localization obtained with overexpression systems seems to be artifactual.

During the past several years, accumulating evidence highlighted the presence of nuclear signaling pathways based on lipid hydrolysis, which are not mere duplications of those occurring at the plasma membrane. Nuclear lipids are not only concentrated in the nuclear envelope but also located further inside the nucleus.[18] Nuclear lipid second messengers are likely involved in the control of cell proliferation and gene expression.[19] PC has been associated with the nuclear envelope, the chromatin, and nuclear matrix. Previous works have reported PLD activity in cell nuclei, nuclear envelope, nuclear membranes in various cell systems.[20] However, no information has been available regarding endogenous PLD isoforms expressed inside the nucleus and their function in VSMCs.

In this issue of *Circulation Research*, Gayral et al provide evidence that endogenous active PLD1, but not PLD2, is localized in membrane-free nuclei isolated from pig aortic VSMCs.[21] Using immunoblot analysis of whole cells or purified VSMC nuclei stripped of their nuclear envelope as well as confocal microscopy, they demonstrate that both PLD1 and PLD2 are present in VSMCs but only PLD1 is expressed in inside the nuclei. The authors also demonstrate that PLD activity of purified nuclei, as measured by phosphatidylethanol (PEt) production in the presence of the PLD substrate Bodipy-PC and ethanol, is increased by PtdIns(4,5)P2 but decreased by oleic acid. Taken together, these results indicate that only PLD1 is expressed and active in VSMC nuclei. The same group previously reported that a phosphoinositide 3-kinase γ (PI3Kγ) and the two 3-phosphoinositide phosphatases SHIP-2 and PTEN are localized in VSMC membrane-free nuclei, indicating an intranuclear PtdIns(3,4,5)P3 cycle inside the nucleus.[22,23] Thus, these results suggest the existence of active nuclear phosphoinositide signaling in VSMCs.

PLD1 has been shown to be activated in vitro by small GTPases of Arf and Rho families as well as by PKC.[4] Gayral et al show that RhoA, RhoB, and Rac are present in VSMC whole cells, whereas only RhoA is expressed in the membrane-depleted nuclei using immunoblot analysis. Although data are not shown, it is stated that Arf is not found in the VSMC nucleus. They also demonstrate that treatment of nuclear fraction with C3-exoenzyme, an ADP-ribosyl transferase that blocks the activity of Rho family, decreases PEt production. Based on these observations, they conclude that RhoA regulates intranuclear PLD1 activity in VSMCs. However, because C3-exoenzyme is not specific inhibitor for RhoA, this result should be further confirmed by using dominant negative RhoA. Furthermore, Gayral et al show that a selective PKCζ pseudo-substrate (PS-PKCζ) inhibitor, but not inhibitor for classical PKCα and β, inhibits PEt production, suggesting a role of PKCζ in regulating nuclear PLD1 activity in VSMC.[21] Because PKCα is considered as a major

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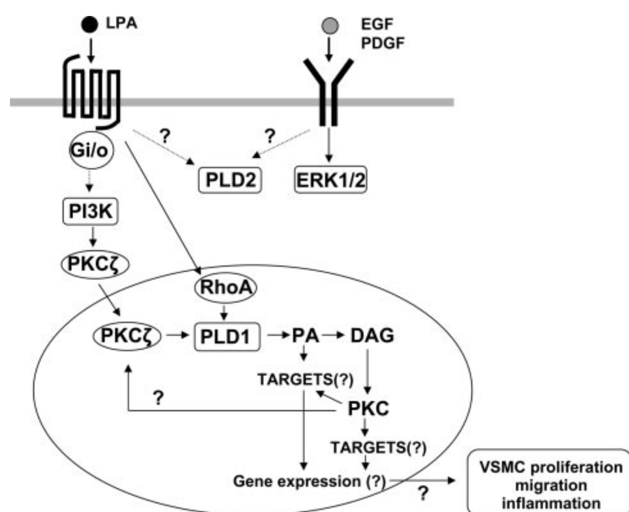

Proposed mechanisms for nuclear PLD1 activation by LPA in VSMCs.

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regulator of PLD1,[3] it is possible that cytosolic PLD1 may be regulated by PKCα in VSMCs. Furthermore, the relationship between RhoA and PKCζ has not been determined in their study. These points should be clarified in future studies using dominant negative, siRNA, and/or antisense knockdown approaches.

The most novel aspect of the report by Gayral et al is the finding that treatment of intact VSMCs but not nuclei with PI3K inhibitors partially prevented nuclear PLD1 activation, indicating an important role of "cytosolic" PI3K in turning on nuclear PLD1. This result also suggests that active PI3K identified in VSMC nuclei[21] does not contribute to nuclear PLD1 activity, whereas previous report shows that PtdIns(3,4,5)P3 can stimulate PLD activity in vitro.[24] Moreover, another important issue is that nuclear PLD1 is activated specifically by GPCR but not by receptor tyrosine kinases (RTK; Figure). Gayral et al measured PLD activity in purified nuclei from VSMCs stimulated with GPCR agonists LPA and Ang II or RTK agonists PDGF and EGF and show that nuclear PLD1 is selectively activated by GPCRs. They also show that PS-PKCζ and PI3K inhibitors block LPA-induced nuclear PLD1 activity to the same extent, and that LPA-induced nuclear translocation of PKCζ is dependent on cytosolic PI3K. Moreover, both C3-exoenzyme and pertussis toxin inhibit LPA-induced nuclear PEt production. Thus, these results suggest an involvement of heterotrimeric Gi/G0 protein, RhoA and PI3K/PKCζ pathway in LPA-stimulated intranuclear PLD1 activity in vivo (Figure). However, their relationships remain unclear in the current study. It is unknown whether Ang II–induced nuclear PLD1 activity is regulated by the same mechanisms. It should be noted that in rat VSMCs Ang II activates PLD through cSrc and RhoA as well as pertussis toxin-insensitive Gα12 and G$\beta\gamma$, but not Gαq11, subunits[25] and that in rabbit aortic VSMCs Ang II selectively stimulates PLD2 through PKCζ activation.[26,27] Additional experiments will be necessary to reconcile these observations.

At present there is no information regarding a role of nuclear PLD1 in VSMCs. Given that LPA and Ang II play an important role in hypertension and atherosclerosis, it is likely that nuclear PLD1 is involved in vascular proliferating disorders. Candidate targets for nuclear PLD-generated PA include PtdIns[4] P 5-kinases which synthesize PIP2[28] and are present in the speckle domains of the nucleus.[29] Speckles are subnuclear structures that are enriched in pre-messenger RNA splicing factors. Thus, it is tempting to speculate that PA generated from nuclear PLD1 may be involved in pre-mRNA splicing or mRNA metabolism and export, thereby regulating gene expression. More detailed studies are required to understand the role of nuclear PLD1 in VSMCs. There are many unanswered questions. What are the downstream targets of nuclear PLD1-derived PA in the nucleus? What is the functional role of nuclear PLD1 in LPA- and Ang II–stimulated VSMCs? Accumulating evidence suggest that GPCR agonists can transactivate RTKs to mediate growth-promoting effects in VSMCs. How do GPCR agonists only, but not RTK agonists, selectively stimulate nuclear PLD1 activity? Does PKCζ translocate into nucleus and bind to PLD1 after GPCR stimulation? Are results obtained by Gayral et al cell type– or species-specific? What are relationships between RhoA and PI3K-PKC in GPCR-stimulated nuclear PLD1 activation? Addressing these questions will be essential to our understanding the precise signaling pathways regulating PLD1 in VSMC nuclei as well as the role of nuclear PLD1 in vascular cells under normal and pathological conditions.

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Disclosures

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