Emerging Functions of Nuclear Lipids

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A reader's guide to the revised STKE Review
"Nuclear lipid signaling,"
http://www.stke.org/cgi/content/full/sigtrans;2002/150/re13

Since the publication of the original review two years ago, there have been many exciting and encouraging advances in our knowledge of nuclear lipid metabolism. These advances, which are discussed in the revised version of the review, include findings that provide new hints to the functions for nuclear lipids and some of the regulatory mechanisms controlling their synthesis and metabolism. The updated review includes an entirely new section called "Other Inositol Lipids," which describes the regulation and potential functions for 3-phosphorylated lipids and other inositol lipids, including phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] and PtdIns5*P*, that have been detected in the nucleus. In addition, a new figure (Fig. 5 in the updated review) has been included that illustrates the putative functions for the various lipids and indicates some suggested points of regulation. Some highlights of the updated review (with selected reference examples) are listed below, organized by the sections that contain the most details about the topics.

PI-PLC in the Nucleus

- Nuclear phosphoinositide-specific phospholipase C β1 (PI-PLCβ1) (probably the b splice variant) may be regulated through phosphorylation by extracellular signal-regulated protein kinases (ERKs) (*1*).

Nuclear Diacylglycerol

- The nuclear localization of diacylglyerol (DAG) kinases has been found to involve several different isoforms that are regulated in complex ways.

The Cell Cycle and Signaling by Nuclear Lipids

- Complex changes in the mass levels and metabolism of nuclear inositol lipids occur during the cell cycle (*2*).

- New possible functions have been revealed for PtdIns(4,5)P_2; for example, it may be involved with premessenger RNA splicing (*3*).

Other Inositol Lipids

- New angles on the nuclear metabolism and function of the higher inositol phosphates have emerged, including a possible role for inositol hexakisphosphate in the regulation of DNA repair (*4*).

- The presence in the nucleus of 3-phosphorylated inositol lipids and the enzymes that make them has been extended. For example, a complex story is emerging involving PIKE, a nuclear guanosine triphosphatase that activates phosphatidylinositol 3-kinase (*5*). PIKE, in turn, may be regulated by the Src-homology 3 (SH3) domain of PI-PLCγ (*6*).

- Indirect evidence implies a nuclear function for PtdIns(3,4)P_2 (*7*).

Other Lipids

- A new nuclear function for phosphatidylcholine is suggested by the specific nuclear synthesis of this lipid with a saturated fatty acyl profile (*8*).

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Nuclear Lipid Signaling

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Abundant evidence now supports the existence of phospholipids in the nucleus that resist washing of nuclei with detergents. These lipids are apparently not in the nuclear envelope as part of a bilayer membrane, but are actually within the nucleus in the form of proteolipid complexes with unidentified proteins. This review discusses the experimental evidence that attempts to explain their existence. Among these nuclear lipids are the polyphosphoinositol lipids which, together with the enzymes that synthesize them, form an intranuclear phospholipase C (PI-PLC) signaling system that generates diacylglycerol (DAG) and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]. The isoforms of PI-PLC that are involved in this signaling system, and how they are regulated, are not yet entirely clear. Generation of DAG within the nucleus is believed to recruit protein kinase C (PKC) to the nucleus to phosphorylate intranuclear proteins. Generation of Ins(1,4,5)P_3 may mobilize Ca^{2+} from the space between the nuclear membranes and thus increase nucleoplasmic Ca^{2+}. Less well understood are the increasing number of variations and complications on the "simple" idea of a PI-PLC system. These include, all apparently within the nucleus, (i) two routes of synthesis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]; (ii) two sources of DAG, one from the PI-PLC pathway and the other probably from phosphatidylcholine; (iii) several isoforms of PKC translocating to nuclei; (iv) increases in activity of the PI-PLC pathway at two points in the cell cycle; (v) a pathway of phosphorylation of Ins(1,4,5)P_3, which may have several functions, including a role in the transfer of mRNA out of the nucleus; and (vi) the possible existence of other lipid signaling pathways that may include sphingolipids, phospholipase A_2, and, in particular, 3-phosphorylated inositol lipids, which are now emerging as possible major players in nuclear signaling.

Introduction

The envelope of the eukaryotic nucleus is a double membrane made of phospholipids and protein, but the intranuclear space itself also contains lipids. Some of these lipids may serve a structural function, for example as components of chromatin or the laminar layer that surrounds the nucleus [see (*1*, *2*) for review], but lipids also play a role in cellular signaling. This review will focus mostly on what we know about lipid signaling pathways within the nucleus. The discovery and establishment of these intranuclear pathways was initially accompanied by skepticism; contamination of samples with cytosolic components or traces of the nuclear envelope seemed more likely explanations. However, the evidence is now compelling that intranuclear lipid pathways exist, and perhaps the most remarkable recent development about nuclear lipid signaling is the increasing number of players and pathways. The first components identified were phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] and its precursors (Fig. 1), together with a phosphoinositide-specific phospholipase C (PI-PLC) to generate diacylglycerol (DAG), which stimulated the translocation of a protein kinase C (PKC) to the nucleus. Now other sources of DAG, multiple PKC isoforms, phospholipase A_2 (PLA_2), phosphoinositide 3-kinase (PI3K), nuclear Ca^{2+} regulation, and sphingolipids have been implicated in nuclear signaling. Some aspects of inositide signaling may have evolved first in the nucleus and "spread" to the cytoplasm later (*3*), and as we find more lipid signaling pathways in the nucleus, the possibility arises

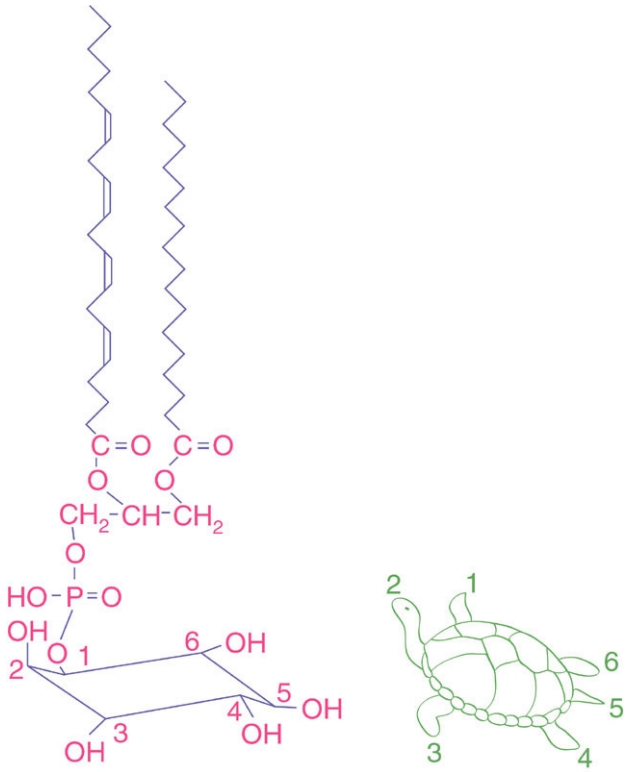

Fig. 1 Phosphatidylinositol. The figure shows (**left**) the structure of PtdIns. Also shown (**right**) is myo-inositol in the same orientation as the inositol moiety in the PtdIns, but depicted as a turtle (*21*, *155*), as an *aide memoire* to the numbering of the inositol ring. The 2-hydroxyl, the only hydroxyl that is axial in myo-inositol when it is in its chair conformation (as illustrated), is the turtle's head. In the D-numbering scheme used to designate all biological inositol lipids and inositol phosphates, the 1-hydroxyl is the turtle's front right flipper. For further explanation, see (*118*).

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that they too may have evolved first as nuclear processes.

This review is not intended to be comprehensive, but rather to be a selective discussion of what the author sees as the key issues in nuclear lipid signaling. Several comprehensive reviews on the topic are available (*1*, *2*, *4*, *5*).

A Short History

The first clue for inositides in the nucleus distinct from those in the plasma membrane came from experiments showing that in rat liver nuclei incubated with γ^{32}P-adenosine triphosphate (ATP) radioactivity was incorporated into phosphatidic acid (PtdOH), phosphatidylinositol phosphate (PtdIns*P*), and phosphatidylinositol bisphosphate (PtdInsP_2) (*6*). Removal of the nuclear envelope with detergent decreased the incorporation of phosphate, and crude fractions of membranes prepared from nuclei showed some incorporation, which led the authors to the erroneous conclusion that the kinases involved were in the nuclear envelope. A similar incorporation into polyphosphoinositides was observed in the nuclei of Friend (human erythroleukemia) cells (*7*). The latter study also showed that (i) the lipid kinases were inside the nucleus rather than in the nuclear envelope; (ii) the PtdInsP_2 was, as expected, PtdIns(4,5)P_2; (iii) the incorporation into nuclear PtdIns(4,5)P_2 increased if the cells were stimulated to differentiate (whereas parallel experiments on whole cell homogenates showed no such change); and (iv) the probable reason for the change in incorporation into PtdIns(4,5)P_2 was an increase in mass of PtdIns4*P*. Thus, the overall conclusion was that there is inositide synthesis within the nucleus distinct from that in the classic phosphoinositide (PI) cycle in the plasma membrane, and it can be controlled separately from the latter cycle (*7*).

Insulin-like growth factor 1 (IGF-1) stimulated nuclear inositide metabolism in Swiss 3T3 cells (*8*-*10*). Mass assays for DAG, PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 revealed that IGF-1 treatment of intact Swiss 3T3s caused an increase in nuclear DAG with a concomitant decrease in PtdIns4*P* and PtdIns(4,5)P_2 (*10*). No such changes occurred in parallel whole-cell experiments. However, bombesin, a "classic" PI cycle agonist, did induce changes in these lipids in whole cells, but not in the nuclei. The nuclear changes could only be detected if highly purified nuclei were made (that is, the more contaminated the nuclei, the smaller the response), which, together with the bombesin data, largely removed the concern that this was an artifact of contamination of the nuclei with other cell fractions. Treatment of cells with IGF-1 also caused translocation of PKC to nuclei (*10*, *11*). Bombesin caused no such translocation to the nucleus, whereas bombesin, but not IGF-1, caused PKC translocation to a postnuclear membrane fraction (*10*).

These results were interpreted to mean that there is a PI-PLC cycle in nuclei controlled separately from the "classic" plasma membrane cycle and that its function is to generate DAG in the nucleus, which in turn causes translocation of PKC into the nucleus to phosphorylate unknown nuclear substrates (Fig. 2). The subsequent demonstration that at least one PI-PLC isoform, the β_1 isoform, is localized in the nucleus (*12*, *13*) completed what, at the time, seemed a simple story. Confusing new hints have followed in our understanding of the biological role of the nuclear PI cycle.

Boris Ephrussi is credited with saying in the early days of extranuclear (mitochondrial and chloroplast) genetics, "There are two kinds of genetics, nuclear and unclear." We have resurrected that quote for nuclear lipids (*14*); in our current state of puzzlement, it remains well justified.

Nuclear Lipid Synthesis

Nuclear lipids and the radioactivity incorporated into them largely survive detergent extraction, which suggests that some or all of the enzymes and substrates cannot be in the nuclear envelope. Vann *et al.* (*15*) titrated highly purified rat liver nuclei with increasing concentrations of the detergent Triton X-100, and quantified the lipids and kinases remaining. As little as 0.04% (w/v) Triton X-100 removed all of the nuclear envelope as judged by electron microscopy. (It is interesting to note that 1% Triton X-100 is routinely used to remove the nuclear envelope in many standard protocols.) After extraction with 0.04% Triton X-100, almost all of the PtdIns kinase, PtdIns4*P* 5-kinase, and DAG kinase remained, implying that these are truly intranuclear enzymes. In this context, the enzymes were assayed by the addition of exogenous lipid substrates. If the nuclei were incubated with only radiolabeled ATP and no added substrates, as a way of assessing the ability of the kinases to phosphorylate endogenous substrates, then 25 to 50% of the three kinases' activities were still present after nuclear envelope removal, despite the removal of >90% of the PtdIns and DAG. This result, together with other data (*16*), implies that PtdIns and DAG may exist as "compartmentalized" pools, which can be "channeled" as substrates for the PtdIns and DAG kinases.

In nuclear fractionation studies, PtdIns4*P* 5-kinase, DAG ki-

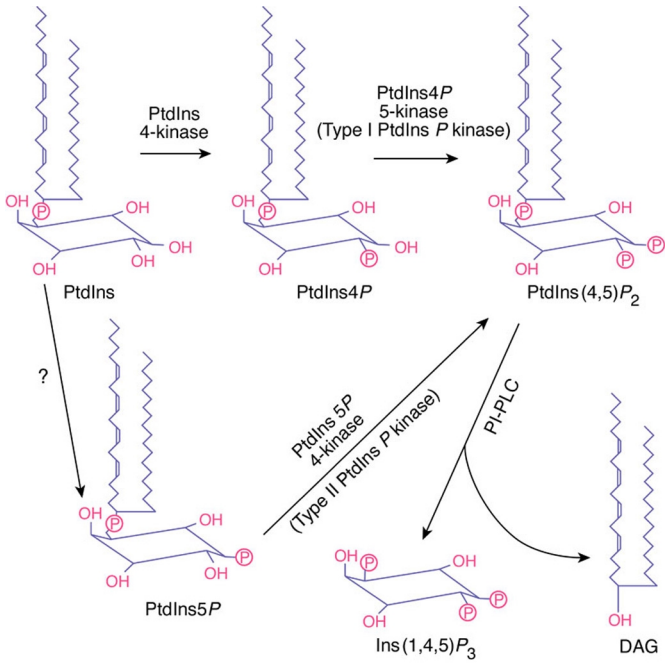

Fig. 2. The PI-PLC pathway. This figure depicts the "classic" pathway by which PtdIns(4,5)P_2 and Ins(1,4,5)P_3 are generated from PtdIns (*156*). Also shown is PtdIns5*P*, the alternative precursor for PtdIns(4,5)P_2. It is not yet certain how this lipid is synthesized, but it is the favored substrate for the Type II PtdIns*P* kinases (*18*).

nase, and PI-PLC were associated with "internal matrix" structures, whereas PtdIns kinase was found exclusively in the "peripheral matrix" (*17*), consistent with the observation that PtdIns kinase is more easily extracted with Triton X-100 (*15*). The differential localizations of these enzymes suggests that the PtdIns4*P* made in one place in the nucleus has to get to another place to be further phosphorylated to PtdIns(4,5)P_2. This idea is reinforced by in vitro experiments that quantified the radioactivity incorporated into the 4 and 5 phosphate moieties of nuclear PtdIns(4,5)P_2 in Friend cells (*7*) and rat liver (*15*). The results showed that, initially, a majority of the [^{32}P] radiolabel is in the 5 phosphate, which suggests that most of the initial PtdIns4*P* substrate for PtdIns4*P* 5-kinase is unlabeled and has not been synthesized during the in vitro incubation of the isolated nuclei (Fig. 2). However, with increasing time of incubation, radiolabel becomes nearly equal in the 4 and 5 phosphates of PtdIns(4,5)P_2 (*15*), so if PtdIns kinase and PtdIns4*P* 5-kinase are in different intranuclear locations, the PtdIns4*P* can move from one place to another in few minutes in vitro. In these particular experiments, removal of the ATP caused disappearance of radioactivity from PtdIns4*P*, PtdIns(4,5)P_2, and PtdOH, suggesting that some nuclear enzymes can dephosphorylate these phospholipids. However, the radioactivity only decreased to a limited degree and then remained constant, implying that the lipids exist in pools with differing access to these phosphatases (*15*). Clearly, we have much more to learn about the topography of these pathways.

A further complication in the interpretation of experiments on PtdIns(4,5)P_2 synthesis lies in the identification of Type II PtdIns*P* kinases as PtdIns5*P* 4-kinases (*18*), rather than as PtdIns4*P* 5-kinases as had previously been assumed (Fig. 2). Type II PtdIns*P* kinases are certainly present in the nucleus (*13*, *19*, *20*), yet so are Type I enzymes ["true" PtdIns4*P* 5-kinases (*19*)]. The radiolabeling data from rat liver nuclei discussed above (*15*) are consistent with the predominant route of nuclear PtdIns(4,5)P_2 synthesis being the same as that in whole cells, by 5-phosphorylation of PtdIns4*P*. The physiological function of the Type II enzymes is currently unclear, but is presumed to be one of three possibilities (*18*, *21*): (i) to catalyze a minor, but compartmentalized and specialized, route of PtdIns(4,5)P_2 synthesis, a possibility consistent with the increasing number of functions suggested for PtdIns(4,5)P_2 (*22*); (ii) to remove PtdIns5*P*, which might have a specific intracellular function; and (iii) to serve as an alternate route for PtdIns(3,4)P_2 synthesis, a function suggested by the ability of Type II PtdIns*P* kinases to use PtdIns3*P* as substrate, although PtdIns5*P* is preferred. The presence of Type II PtdIns*P* in the nucleus suggests that any one of these three possible functions may apply inside the nucleus. Because the genome of *Saccharomyces cerevisiae* reveals no obvious Type II PtdIns*P* kinase homologs, it seems likely that, as with the functions of these enzymes in the cytoplasm, their intranuclear function may be an evolutionarily recent aspect of nuclear physiology.

The Type II PtdIns*P* kinase isoform(s) found in the nucleus are not yet established clearly. One set of data (*19*) suggests that both α and β isoforms are nuclear, whereas another study shows that the α is cytosolic and the β is nuclear (*20*). The nuclear localization of the Type IIβ PtdIns*P* kinase is caused principally by the presence in its structure of a 16 amino acid length of α-helix (*20*). One proposed resolution to this contradiction concerning the α isoform is that because these Type II PtdIns*P* kinases form dimers (*23*, *24*), they may also form heterodimers; in this way some Type IIα PtdIns*P* kinase may be "carried" into the nucleus as a heterodimer with the β isoform. However, we have done extensive cotransfection experiments with Type IIα and IIβ PtdIns*P* kinases, and found no evidence that they heterodimerize (*25*).

Many other lipids have been identified in the nucleus, and, as with the inositol lipids, the evidence suggests that they are within the nucleus rather than in the nuclear membrane. Phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtan), sphingomyelin, and phosphatidylserine (PtdSer) are present in nuclei (*1*, *26*, *27*), and nuclear phosphatidylglygerol (PtdGro) may have an important role in translocating and activating PKC (*28*). All of this prompts the question of where these lipids are synthesized. Extensive studies on nuclei isolated from brain (*29*) have documented complete de novo phospholipid synthesis pathways, but these fractions may have been contaminated with endoplasmic reticulum (ER). More importantly, the nuclei in these experiments (*29*) had intact envelopes, and the studies we have outlined so far draw a clear line between what is in the nuclear envelope and what is not. The nuclear envelope could be regarded in this context as a specialized part of the ER, and it seems very likely that it would have the capability to synthesize most phospholipids. Consistent with this, Baker and Chang (*30*) have suggested that in intact nuclei the major source of DAG substrate for the (predominantly intranuclear) nuclear DAG kinase is indeed the nuclear envelope. However, evidence has been increased for the presence of the enzymes necessary for PtdCho synthesis in membrane-free nuclei [see (*31*) and references therein], and indeed this might represent a pool of PtdCho with a specific fatty acid profile (*31*) (see below under "Other Lipids").

How lipids might enter the nucleus from the envelope is not clear, but it is interesting that immunocytochemical and Western blot analysis of nuclei (with and without envelopes) showed that the α isoform of the PtdIns exchange protein is partly localized in nuclei (*32*, *33*). Several functions for the PtdIns exchange protein have been proposed, and these may all be relevant within the nucleus: (i) The exchange protein brings PtdIns into the nucleus from the envelope, (ii) it transports PtdIns to different locations within the nucleus, or (iii) it serves to present more substrate to PtdIns kinase, and thus regulates the activity of that enzyme (*34*).

Another poorly understood issue is the physicochemical form of intranuclear lipids. They are obviously not a "classic" lipid bilayer, because they are resistant to detergents. The only likely alternative is that the intranuclear lipids are in some form of proteolipid complex similar to, for example, PtdIns(4,5)P_2 in association with the cytoskeleton (*35*). Evidence from immunocytochemical studies with antibodies that recognize PtdIns(4,5)P_2 are contradictory with regard to what part of the nucleus is associated with the PtdIns(4,5)P_2 (*2*, *36*). The resistance of PtdIns(4,5)P_2 to detergent extraction (*15*) implies a strong interaction with proteins, and some possible partners for PtdIns(4,5)P_2 include the laminar layer, a nuclear skeleton, a component of an intranuclear cytoskeleton (*37*), structures associated with spliceosomes (*36*), or invaginations of the laminar layer into the nucleus similar to those that have been suggested for the nuclear envelope (*38*). We have a lot to learn about subnuclear structures (*39*) that will clarify the associations of phospholipids with them.

PI-PLC in the Nucleus

There is a huge literature on nuclear PI-PLC (*2*, *4*, *5*, *40*). Some PI-PLCγ isoforms may be nuclear, but these reports are not unequivocal, because cytoplasmic contamination was not always eliminated or immunohistochemistry was performed with only one antibody. The two main players to be discussed here are PI-PLC β_1 and PI-PLC δ (probably δ_4).

Although it is generally accepted that there is some PI-PLC β_1 within the nucleus, it is still not certain how much is there and how it is controlled. Experiments on Swiss 3T3 nuclei originally suggested that all of the cell's PI-PLCβ_1 is nuclear (*12*). This conclusion was based on immunocytochemistry data from a single mixture of antibodies, which suggested an entirely nuclear location, and subcellular fractionation, which confirmed the nuclear localization. However, the subcellular fractionation started with different protocols depending on whether nuclei or cytoplasm were to be prepared (that is, the two fractions were not derived from the same starting homogenate). Conversely, in rat liver, although there is some PI-PLC β_1 in the nucleus, most of it is cytoplasmic (*13*), although the problem with this study is that the enzyme may be released from the nuclei during their preparation. PI-PLC β_1 has been extensively studied as a heterotrimeric guanine nucleotide-binding protein (G protein)-controlled enzyme in the cytoplasm (*41*); thus, it seems very unlikely that it is all within the nucleus. Further studies on the difference between nuclear and cytosolic PI-PLC β_1 (*42*) have now led to a general agreement that some, but not all, PI-PLC β_1 is nuclear (*13*). The difference between complete nuclear and partial nuclear localization is important, because if all of the PI-PLC β_1 were nuclear, it would have to be regulated in situ, and this begs the question of how it is regulated. If it is only partially nuclear, an obvious means of regulation is to translocate it in and out of the nucleus.

One observation from C6Bu-1rat glioma cells may explain some of the controversies in PI-PLC β_1 localization. In C6Bu-1 cells, of the two splice variants of PI-PLC β1 (*43*), one, PI-PLC β_1b, shows a much more pronounced nuclear localization than the other, PI-PLC β_1a, which is predominantly cytosolic. However, in transfected Rat-2 cells, PI-PLC β_1a is mostly nuclear unless some critical lysine residues are mutated to remove a putative lysine-rich localization sequence that is common to both splice variants (*44*). Transfection experiments are subject to their own caveats because abnormally high levels of protein are achieved; it may be that endogenous proteins show a stronger contrast between the two variants (*43*). Faenza *et al*. (*45*) have shown that transfection of Friend cells with the two splice variants demonstrates that both proteins are in the nucleus. But the PI-PLC β_1b is entirely nuclear, whereas the PI-PLC β_1a is in the cytosol and nucleus. For the ensuing discussion, I will assume that it is probably the b splice variant that is physiologically relevant in nuclear signaling. The most interesting question of all remains to be answered: If PI-PLC β_1b is truly a (or the) major nuclear player, how is it regulated—by its production (for example, by splicing control), by its translocation, or by some uncharacterized mechanism of regulation within the nucleus?

PI-PLCβ_1 is classically a G protein-controlled enzyme. Although there are reports that some G proteins, for example α_i (*46*, *47*), and some regulators of G proteins (*48*) can translocate to the nucleus, there is as yet no consistent data that suggest G protein regulation of nuclear PI-PLCβ_1. Some recent studies from Lucio Cocco and his colleagues may give us the first insights into the regulation of PI-PLC in the nucleus. Their data have pointed toward the possibility that extracellular signal-regulated and mitogen-activated protein kinase (ERK) ERK-1 or ERK-2, which can translocate into the nucleus, may phosphorylate PI-PLCβ_1b (*49-51*). PI-PLCβ_1 immunoprecipitated from nuclear extracts of IGF-1-stimulated cells is phosphorylated predominantly on Ser^{982}, a phosphorylation that is blocked by the ERK kinase (MEK) inhibitor PD 98059 in the intact cells, and mimicked by recombinant ERK-2 in vitro (*51*). Similar data have been reported from interleukin-2 activated natural killer (NK) cells (*52*). The functional consequence of PI-PLCβ_1 phosphorylation by ERKs is not yet clear. However, a PI-PLBβ_1 construct that has an alanine at residue 982 has a dominant-negative effect on the mitogenic action of IGF-1 (*51*). A similar Ser^{982} to Ala mutant with its nuclear localization sequence removed had no effect, highlighting the likelihood that a nuclear inositide event is contributing to IGF-1's effects (*51*). Removal of PI-PLC β_1 by antisense DNA (*53*) also attenuated IGF-1-stimulated cell growth in Swiss 3T3 cells, an observation consistent with data above and the stimulation by IGF-1 of a nuclear PI-PLC activity in these cells (*8-10*).

Nuclear targeting of PI-PLC β_1 is probably also involved in the differentiation of Friend cells, because the differentiation process induced by dimethylsulfoxide (DMSO) can be inhibited by transfection with a PI-PLC β_1a construct that shows some nuclear localization, but not by mutant constructs (with the nuclear localization sequence deleted) that are predominantly cytosolic (*42*). Avazeri *et al*. (*54*) have documented a translocation of PI-PLCβ_1 (which they believed to be predominantly the β_1a splice form) to the nucleus, initially to perichromatin and interchromatin granules, and later to the nucleoplasm, during the resumption of meiosis in the mouse oocyte. Moreover, injection of a monoclonal antibody to the enzyme into the germinal vesicle (nucleus) inhibited vesicle breakdown (*54*). There may be a function for the nuclear PI cycle in aspects of nuclear function other than proliferation, such as differentiation and meiosis, that are yet to be uncovered.

Phosphorylation of nuclear PI-PLCβ_1 by PKC (probably PKCα) inhibits its activity (*55*). The activation of nuclear PI-PLC by IGF-1 stimulation of Swiss 3T3 cells has long been known to be transient, returning to low levels within an hour (*8*, *10*). This transient activation is accompanied by a translocation of PKC to the nucleus (*10*, *11*), and Xu *et al*. (*55*) have suggested that phosphorylation of PI-PLCβ_1 on Ser^{887} causes this transience by inhibiting the enzyme. This negative feedback loop is an interesting complication in the unfolding story of nuclear PI-PLCβ_1 regulation.

Thus, some PI-PLC β_1, probably mostly PI-PLCβ_1b, can be confidently localized in the nucleus, and both its regulation and function are beginning to be understood. However, data regarding the presence of other isoforms in the nucleus are not so clear. The other proposed major player is PI-PLCδ_4, which was cloned from a cDNA library from regenerating rat liver and which was suggested to be primarily nuclear, especially during the S phase in the cell cycle (*56*). Another study failed to show any nuclear localization of PI-PLCδ_4 [(*57*) and see (*2*) for detailed discussion of the issues], and at present, we do not know the contribution that this isoform makes to nuclear inositide metabolism.

However, it does seem likely that there is more than one isoform of PI-PLC in nuclei. Two pieces of evidence suggest some functional redundancy either of different PI-PLC isoforms, or

of nuclear inositide signaling in general, at least in the context of cell proliferation. First, removing PI-PLCβ_1 from Swiss 3T3 cells with antisense DNA (*53*) or inhibition with a dominant-negative construct (*51*) largely prevents the mitogenic response to IGF-1, yet the cells still grow well in culture. Second, although knockout mice lacking the *PI-PLCβ_1* gene exhibit neurological problems, they can grow to adulthood (*58*). So, either nuclear PI-PLCβ_1 has an nonessential role in nuclear function, or there is another isoform that can substitute or compensate. Note that many eukaryotes, such as yeasts and plants, do not have a PI-PLCβ_1. Indeed, they contain only δ isoforms, so if there is an intranuclear PI-PLC cycle in these organisms, it must use a PI-PLCδ.

A final point that is relevant in considering the whole issue of nuclear PI-PLCs emerges from a study on the subcellular localization of PI-PLCδ_1 (*59*). This isoform is generally regarded as having a cytosolic localization, but this may be a result of a steady-state cycling in and out of the nucleus, with the equilibrium being maintained in favor of the cytosol by a nuclear export signal contained within the EF-hand domain of PI-PLCδ_1 (*59*). This has important implications for how the localization of PI-PLCs is viewed, because any technique used to determine a protein's localization is only visualizing a moment in time, which reflects a steady state. Other isoforms of PI-PLC may spend enough time inside the nucleus to have an effect, but not long enough for us to detect them there.

Nuclear Protein Kinase C

Several isoforms of PKC can be found in the nucleus or can translocate there (*1*, *2*). In the context of this review, the key issue is whether PKC translocates to or becomes activated in the nucleus as a consequence of nuclear lipid signaling. This is not always easy to assess. For example, one of the first reports of a translocation of PKC to the nucleus was in B lymphocytes, and it was caused by activation of Ia-binding ligands (antibodies to protein Ia, a cell surface major histocompatibility (MHC)-encoded protein in B cells), or by increasing adenosine 3′-5′ monophosphate (cAMP) (*60*). But there are no data addressing the question of whether or not that translocation of PKC was caused by an increase in DAG in the nucleus.

PKCα translocates to the nucleus and perinuclear region of NIH 3T3 cells after treatment with the phorbol ester PMA (*61*). In cardiac myocytes, after PMA treatment PKCα, PKCβII, and PKCζ translocate predominantly to the perinuclear region, whereas the δ and ε isoforms start in the nucleus and move to the cytosol (*62*). Surprisingly, in these cardiac cells, similar patterns for translocation of PKC isoforms were observed when the cells were exposed to norepinephrine, which would be expected to stimulate the plasma membrane PI cycle. Moreover, none of the PKC isoforms showed any major shift to the nucleus in response to serum, which would be expected to stimulate the nuclear PI cycle, as it does in Swiss 3T3 cells (*10*). Thus, it is evident that most PKC isoforms can be found at one time or another in the nucleus, either in "resting" or in stimulated cells, and presumably one or more of the wide choices of potential PKC substrates (*1*, *2*) act as substrates for each PKC isoform. But relating nuclear PKC to nuclear DAG generation, and thus making it relevant to this review, is currently almost impossible—with two exceptions.

The first exception was described above in the section on "history." An antibody raised to the COOH-terminus of the α isoform showed PKC translocating to the nucleus when nuclear DAG was increased (*10*, *11*), but not when DAG was generated in the plasma membrane by stimulation of the cells with bombesin (*10*). A caveat of these experiments is that the specificity of the antibody has not been systematically explored, so the translocation event may not only involve PKCα. A similar coincidence of nuclear DAG generation and PKC translocation [probably the α isoform, because the antibody used was the same as that in the previously described experiments [N.B.] (*10*, *11*)] was also found in regenerating rat liver (*63*). These two examples represent the closest correlation between the apparent generation of DAG within the nucleus and a translocation of PKC to the nucleus. How the DAG in the nucleus is "detected" by the PKC is a complicated question that remains unanswered (*1*). In the preceding section, the idea was discussed briefly that proteins such as PI-PLCδ_1 may be cycling in and out of the nucleus (*59*). If that idea is applied to a PKC, it prompts the suggestion that the formation of DAG within the nucleus could simply "trap" the PKC during a transient visit to the nucleus.

The second convincing link between PKC and nuclear lipids, and currently the only clear link with physiological nuclear function, comes from the extensive and elegant studies by Alan Field's group on the nuclear translocation of PKCβII in HL-60 cells. The early work showed that PKCβII is a major physiological player in the phosphorylation of lamins (especially lamin B) that accompanies nuclear breakdown at the G2 to M transition (*64*). Bryostatin was used as a specific pharmacological tool to activate βII translocation (*65*), and a physiologically relevant stimulus, platelet-derived growth factor (PDGF), causes a similar translocation and lamin B phosphorylation (*66*). The specificity of the translocation of PKCβII and of the lamin B phosphorylation can be largely attributed to the presence of nuclear PtdGro interacting with the extreme COOH-terminus of the PKCβII (*28*, *67*). This is a remarkable observation that leads to another player in nuclear lipid signaling. Where the nuclear PtdGro comes from, and whether its levels change as part of a regulatory mechanism, are both unanswered questions.

A potential physiological (indeed, pathological) significance of this PKCβII story has been suggested by the ability of elevated PKCβII to induce cancer in vivo in a tissue (the colon) in which it is overexpressed transgenically (*68*) and by the specific increase in this PKC isoform in chemically-induced colon cancers (*69*). A link with this carcinogenic action of PKCβII and the anti-carcinogenic effects of ω-3 fatty acids has also recently been suggested (*70*). Although these data provide a circumstantial case for PKCβII being involved in carcinogenesis in vivo, they do not show whether its ability to enter the nucleus is the crucial factor in this action.

Nuclear Diacylglycerol

The DAG part of the nuclear lipid story has become very complicated over the last few years. Because of the close temporal correlation between a decrease in nuclear polyphosphoinositides and an increase in nuclear DAG with PKC translocation (*10*, *11*), it was assumed that DAG was being produced largely from PtdIns(4,5)P_2. However PtdCho is another obvious source of DAG in the cell (*71*, *72*), and DAG produced in the nuclei of II-C 9 fibroblasts was , judged by its fatty acid profile, derived from PtdCho and not PtdIns (*73*). The cells were stimulated with thrombin, which activates a G protein-coupled receptor, and the nuclei were isolated from them without the use of detergents and would therefore have intact envelopes; thus, most of

the DAG in these nuclear preparations is probably in the envelope (*15*). However, our own data (*16*) on DAG species in nuclei free of envelopes has confirmed the interpretation (*73*) that intranuclear DAG has a fatty acyl saturation profile, as assayed by argentation chromatography, consistent with PtdCho, and not PtdIns, as a source.

The generation of DAG from PtdCho, as well as from inositol lipids in nuclei, provides an explanation for an apparent lack of correlation between nuclear DAG concentrations and PI-PLC activity in nuclei prepared from differentiating MEL cells. Nuclei isolated from differentiated cells generated less DAG in vitro than nuclei from undifferentiated cells, and less DAG can be extracted from nuclei of differentiated cells compared to those from undifferentiated cells-(*16*). If PI-PLC activity in these nuclei from differentiated and nondifferentiated cells is assayed with an exogenous PtdIns(4,5)P_2 substrate, there is no change in activity seen in the differentiated cells. However, there is undoubtedly a PI-PLC activity in these MEL cell nuclei. So what is happening to PI-PLC-generated DAG? The answer is that in the nuclei of these cells PI-PLC-derived DAG appears to be preferentially phosphorylated by a DAG kinase, because radiolabeled PtdOH generated by incubating nuclei with [γ^{32}P]-ATP in vitro has a fatty acyl profile much more like PtdIns than PtdCho (*16*). The DAG kinase preference for PI-PLC-derived DAG is apparently related to access of the DAG substrate to the enzyme rather than the actual DAG specificity of the kinase. This conclusion is suggested by the observation that destroying the structure of the nucleus with deoxycholate removes any specificity of nuclear DAG kinase with respect to DAG species. Only with endogenous substrate in an intact, but membrane-free, nucleus does the PtdOH labeled have a highly unsaturated profile that resembles PtdIns (*16*).

Thus, MEL cells appear to have at least two distinct pools of DAG in nuclei, generated from different phospholipids in different subnuclear locations. The one derived from inositides is rapidly phosphorylated to PtdOH. However, treatment with specific inhibitors of PI-PLC showed that most DAG generated in vitro in nuclei isolated from HL-60 cells or Swiss 3T3 cells is derived from PtdIns and not PtdCho (*74*, *75*). Moreover, treatment of whole cells with these PI-PLC inhibitors suggests that this PtdIns-derived DAG may be the physiologically relevant nuclear PKC pool with regard to PKC translocation (*74*, *75*). This suggestion is also supported by the data of Deacon *et al*. (*76*) in U937 cells (discussed in more detail in the next section). In MEL cells (*16*), PI-PLC inhibitors did not inhibit DAG generated in nuclei in vitro, but did inhibit the [^{32}P]-labeled PtdOH formation, as might be expected because it appears to be generated from PtdIns-derived DAG. This suggests that the relative contributions of PtdCho and PtdIns to overall nuclear DAG levels may be different between different cell types. A final point is that neither inhibition of PtdCho-PLC with a reportedly selective PtdCho-PLC inhibitor (though its selectivity in mammalian systems is not clear), nor inhibition of phospholipase D (PLD) with ethanol had any effect on nuclear DAG generation in MEL cells (*16*). Thus, in these cells the enzyme responsible for generating nuclear DAG from PtdCho remains unknown. It is possible that reversal of nuclear synthesis of PtdCho (*31*) is responsible.

To return to the subject of nuclear DAG kinase, the important role of this enzyme in regulating nuclear DAG levels has recently been emphasized (*77*). DAG kinase-ζ is a nuclear-localized isoform (*78*), and this nuclear localization is apparently in part regulated by PKC phosphorylating the nuclear localization sequence in the myristoylated alanine-rich C kinase substrate (MARCKS) domain of the DAG kinase (*77*). The consequence of phosphorylation of this domain is to keep the DAG kinase-ζ in the cytosol, presumably resulting in increased intranuclear DAG levels (*77*). Whether it is a nuclear or cytosolic PKC that phosphorylates this domain is not yet clear. Only α and γ PKC appeared capable of effecting this phosphorylation, and it is interesting that, as discussed in the preceding section, PKCα is one of the PKCs for which nuclear translocation in response to nuclear DAG generation is best documented. The physiological significance of this nuclear DAG kinase localization was shown by its inhibitory effect on cell growth (*77*). Martelli *et al*. (*79*) reported an increase in nuclear DAG kinase activity in IGF-1-stimulated Swiss 3T3 cells, which may reflect a translocation of DAG kinase-ζ. Two DAG kinase inhibitors caused increased PKC activity in the nuclei (*79*), again supporting the suggested role (*77*) of nuclear DAG kinase in regulating nuclear PKC (by removing the DAG, which stimulates the latter). Hogan *et al*. (*80*) suggested that interaction of DAG kinase-ζ with syntrophins may influence the degree to which it localizes in the nucleus, and the physiological significance of this interaction and its relationship with the PKC-regulated localization discussed above remains to be explored.

Other isoforms of DAG kinase have also been reported to translocate to the nucleus. For example, in T cells DAG kinase α translocates to the nuclear matrix in response to concanavalin A or activation of the T cell receptor (*81*). In IIC9 cells stimulated by thrombin (see above), a translocation of the θ isoform of DAG kinase occurs (*82*). The story of nuclear DAG kinases, and how their localization and nuclear functions are regulated, are still emerging.

The previous section concluded that more than one PKC may be translocated to the nucleus, perhaps at different times for more than one intranuclear function. Now that we know that there is probably more than one pool of nuclear DAG, perhaps regulated by more than one process, the cell has a choice of DAG kinases that can translocate to the nucleus might seem reasonable.

The Cell Cycle and Signaling by Nuclear Lipids

What are the functions of nuclear lipids? A large number of modulatory or structural functions may be fulfilled by nuclear lipids, but further understanding of these roles must wait until we know more about nuclear structure. For the present context, this section will assume that PI-PLC hydrolysis of PtdIns(4,5)P_2 is the major signaling output of nuclear inositides, so it will focus on two questions: (i) What are the substrates for nuclear PKC? and (ii) How do they contribute to nuclear function? Unfortunately, there is a multitude of suggested substrates for PKC, arguably so many that only some can be physiologically relevant (*1*). Most of these substrates relate to events in the cell cycle in one way or another, so pinpointing the place in the cell cycle at which there is evidence for nuclear DAG generation, PI-PLC activation, and PKC translocation should be informative. There are a number of other reports of growth factors altering nuclear inositide metabolism or DAG levels, such as interferon (*83*, *84*), interleukin-1 (*85*), interleukin-2 (*52*), or interleukins 12 and 15 (*86*), but we do not yet have the information to fit them into the physiology of cell cy-

cle regulation in vivo.

Current circumstantial evidence points to a role for nuclear DAG generation or PKC activation at two points in the cell cycle. Perhaps the best-documented "pulse" of nuclear DAG is the 60-min transient production in IGF-stimulated Swiss 3T3 cells (*10*). However, these were quiescent cells (serum-starved), and it is not entirely obvious to what stage of a normal cell cycle these are equivalent. Experiments in which PI-PLCβ_1 was removed from Swiss 3T3 cells by antisense DNA (*53*) showed that the response of the cells to IGF-1 was compromised, but the cells grew well in culture. So, at least in this particular cell line, it may be that the IGF-1 signaling pathway has an especially strong link with nuclear PI-PLC. Transfection of Friend cells with PI-PLCβ_1 (either the a or b splice variant, both of which were at least partly nuclear) stimulated cell cycle progression. This was explained partly by an increase in constitutive levels of the cyclin D3-cdk4 complex, and Rb phosphorylation plus its dissociation from E2F. All of these events are consistent with a general increase in cell cycle progression, but do not tell us where the increased nuclear PI-PLC is having its effect. As mentioned above, a PI-PLCβ_1 construct lacking its nuclear localization sequence was also tested and it caused no changes in cell cycle progression, reinforcing the likelihood that a nuclear inositol lipid story is involved. One intriguing observation that may lead to clarification of a mechanism is that the increase in Rb phosphorylation in the PI-PLCβ_1-transfected cells was only detectable on Ser^{795}, even though in serum-stimulated cells the expected phosphorylation of Ser^{780}, Ser^{807}, and Ser^{811} was also detected (*45*). Thus, some specific aspect of cell cycle progression may be altered by increasing PI-PLCβ_1 in the nucleus.

When nuclear PI metabolism is followed through the cell cycle, the timing of nuclear DAG increases or changes in inositide turnover often occur close to the beginning of S phase. In regenerating rat liver (*63*), an increase in nuclear DAG and PKC has been detected at the beginning of S phase, and in synchronized HeLa cells (*87*), S phase is accompanied by a decrease in nuclear PtdIns, although in neither case could the timing be very precise.

Following MEL cells through a cycle after release from nocodazole block showed no increase in nuclear DAG at any point in the cell cycle, but, as discussed above, most of this DAG is derived from PtdCho (*16*). As discussed in the previous section, the PtdIns-derived DAG in MEL cell nuclei is phosphorylated by DAG kinase, so an indirect measure of PtdIns-derived DAG can be made by incubating isolated nuclei with [γ^{32}P]-ATP and quantifying the incorporation into PtdOH. When PtdIns-derived DAG was assayed in this way, it increased roughly in concert with the number of cells in G_1. This increase was blocked by adding PI-PLC inhibitors to the nuclei during the incorporation with [γ^{32}P]-ATP, consistent with activation of nuclear PI-PLC at this time (*16*). Because of the uncertainty of precisely timing the cell cycle, it may be possible to unite all the observations discussed in this paragraph by suggesting that a pulse of PI-PLC activation takes place somewhere at the G_1 to S transition.

Even if the last statement is true, the changes in nuclear inositide metabolism during this part of the cell cycle are not as simple as that. As MEL cells enter S phase, there is a marked increase in incorporation into PtdIns4*P* and PtdIns(4,5)P_2 if their nuclei are isolated and incubated in vitro with radiolabeled ATP (*88*). This increase is consistent with an indirect effect of the increase in PtdIns(4,5)P_2 hydrolysis by PI-PLC and is presumably similar to the increased incorporation of radiolabel into inositides if the classic plasma membrane PI-PLC pathway is stimulated. However, if mass levels of PtdIns(4,5)P_2 are measured they hardly change, implying a tight regulation of PtdIns 4-kinase and PtdIns4*P* 5-kinases. Although mass levels of PtdIns4*P* levels increase, as might be expected from the implied stimulation of PtdIns 4-kinase as the cells enter S phase, the timing of this mass increase happens in G_1 and is already decreasing as the cells enter S phase (*88*). Thus, either PtdIns 4-kinase and PtdIns4*P* 5-kinase are subject to regulations separate in mechanism and timing, or there are multiple pools of inositol lipids and kinases, as discussed above [see (*15*, *17*)]. The behavior of PtdIns5*P* (*18*, *87*, *89*) is also intriguing, because, like PtdIns4*P*, it increases as the cells go through G_1 and decreases as they enter S phase. Compared to the changes in PtdIns4*P*, the changes in PtdIns5*P* are much more pronounced, with PtdIns5*P* disappearing to undetectable levels by the time the cells are fully in S phase (*88*).

The other point in the cell cycle affected by nuclear inositide signaling is the G_2 to M boundary. If PKCβII plays a role in nuclear disassembly, as the evidence for its role in lamin phosphorylation (*64*) would circumstantially suggest, then some generation of nuclear DAG at the G_2 to M transition might be expected. Nuclear DAG levels increase at the G_2 to M transition in HL-60 cells, and this increase was blocked by a PI-PLC inhibitor (*74*). The fatty acid profile of this DAG remains to be determined (see below). In the same cells, PKC inhibition leads to complete cell cycle arrest in G_2 (*90*), presumably because the nuclei cannot disassemble. The data from HL-60 cells, therefore, present a strong case for a rise in PI-generated DAG leading to PKC-phosphorylation of lamin B, in turn leading to nuclear disassembly.

Deacon *et al*. (*76*) have reinforced this picture in another promonocytotic cell line, U937s. They used elutriation of cells from a proliferating suspension to isolate cells at G_1, S, and at the G_2 to M boundary, which has the advantage of avoiding any potentially cytotoxic cell cycle inhibitors, such as nocodazole [for example, compare (*16*, *88*)]. In the nuclei of U937 cells at the G_2 to M boundary, there was a specific increase in stearoyl-arachidonoyl DAG levels (probably PtdIns-derived), and a concomitant translocation to the nucleus of PKCβII. The interesting difference between these cells and MEL cells (*16*) is reinforced by Deacon *et al*. (*76*). If isolated U937 nuclei are incubated with radiolabeled ATP, there is virtually no incorporation into PtdOH (only with exogenous DAG substrate was DAG kinase activity detected), whereas in MEL cells, there was robust incorporation specifically into PtdOH with a fatty acyl profile resembling inositol lipids (*16*). Overall the picture of PtdIns-derived DAG recruiting PKCβII to the nucleus at the G_2 to M boundary is a compelling one. This cannot be the same as the putative G_1 to S boundary nuclear PI-PLC response discussed above, so there appear to be two distinct points in the cell cycle at which PI-derived DAG is formed. The timing and the function of PtdCho-generated DAG remain unknown.

However, the original data for nuclear localization of DAG kinase-ζ (*78*) were gathered from nonproliferating cells, that is, neurons in culture or in situ. One possible explanation for high levels of DAG kinase-ζ in these cells would be permanent suppression of nuclear DAG levels in nonproliferating cells. That interpretation requires in turn that fully differentiated cells,

which have entirely resigned from the proliferation business, still have active nuclear lipid metabolism, so it is likely that nuclear DAG must fulfill functions not associated with the cell cycle. A number of possible links between inositol lipids or PKC and transcriptional events have been suggested [reviewed in (*2*)]. For example, PtdIns(4,5)P_2 can influence chromatin structure by facilitating the interaction between chromatin and *Brahma*-related gene associated factors (BAF) (*91*). Another intriguing link to transcriptional events may be the association between histone H1 and PtdIns(4,5)P_2, which suppresses the inhibitory effect of HI on basal transcription in a *Drosophila* system (*92*). PtdIns(4,5)P_2 binds with high specificity to a PKC phosphorylation site on the histone, and both PtdIns(3,4,5)P_3 and PtdIns4*P* exhibit lower affinity for histone phosphorylated at this site. These are promising candidate functions of PtdIns(4,5)P_2 in the nucleus, but their physiological relevance remains to be explored.

Another exciting possibility has emerged for an intranuclear function of PtdIns(4,5)P_2—it might be involved in premessenger RNA (pre-mRNA) splicing (*36*). This idea started with the detection of detergent-resistant nuclear PtdIns(4,5)P_2, which constitutes at least half of the total nuclear PtdIns(4,5)P_2 (*15*), in electron-dense intranuclear particles using a specific antibody to PtdIns(4,5)P_2. During the cell cycle, the PtdIns(4,5)P_2 detected by this antibody was intranuclear during interphase, but during mitosis it dispersed to the cytoplasm, only re-entering the nucleus when it was fully reformed (*36*). When in the nucleus, the PtdIns(4,5)P_2 showed a close colocalization with SC-35, a classic marker for interchromatin granular structures (IGCs), which are believed to act as storage compartments for splicing factors. There was also partial colocalization with other markers associated with IGCs, such as hyperphosphorylated RNA Pol IIo. The colocalization changed through the cell cycle, leading to the overall conclusion that PtdIns(4,5)P_2 might be associated with a dynamic structure connected with the process of pre-mRNA processing. This association apparently requires the presence of RNA, but not DNA.

Perhaps the most intriguing observation in addition to indirect guilt-by-association, was that removing PtdIns(4,5)P_2 (or proteins associated with it) by immunoprecipitation from HeLa cell nuclear extracts caused a specific inhibition of pre-mRNA splicing in that extract (*36*). It remains to be determined exactly what PtdIns(4,5)P_2 is interacting with in this context, and thus whether its involvement with pre-mRNA processing, if any, is direct or indirect by virtue of its interaction with some part of the nuclear skeleton or intranuclear cytoskeleton, which is a structural requirement for processing. Also, we don't know whether the PtdIns(4,5)P_2 here is acting as itself or as a substrate for PI-PLC, although the immunoprecipitation data (*36*) imply the former. PtdIns(4,5)P_2 certainly has a wide range of functions in the cytoplasm [for examples, see (*22*)] so, given the complexities of nuclear inositide metabolism discussed in this section, more than one intranuclear function also seems likely.

Nuclear Ca2+

Despite some controversy about nuclear lipid signaling, there is considerable agreement about the principles. Nuclear Ca^{2+} signaling remains among the most controversial topics; at issue is the question of whether Ca^{2+} signals are generated within the nucleus separately from those in the cytoplasm (*93*, *94*). Hydrolysis of PtdIns(4,5)P_2 by a PI-PLC can take place in the nucleus, so some InsP_3 is generated there. InsP_3 receptors may be present in the inner nuclear membrane (*93-95*), with their InsP_3-binding domains facing the nucleoplasm and their intrinsic channels spanning the inner nuclear membrane (*95*, *96*). Therefore, potential exists for Ca^{2+} to be released into the nucleoplasm from the compartment between the outer and inner nuclear membrane (Fig. 3). This compartment has a high Ca^{2+} concentration as a result of the activity of Ca^{2+} pumps in the outer nuclear membrane. Finally, ryanodine receptors, or another protein responsive to cyclic adenosine diphosphate (ADP)-ribose (cADPR), may also exist in an orientation similar to that of the InsP_3 receptors (*95-97*).

Thus, it is possible that by generating InsP_3 within the nucleus, the nuclear inositide cycle could create nuclear Ca^{2+} signals that are controlled separately from those Ca^{2+} signals that are well known in the cytoplasm. Is this only a possibility or does it actually happen? There is currently no unequivocal answer to this question. Problems with Ca^{2+}-sensing dye calibration and other artifacts have made proving the existence of distinct nuclear Ca^{2+} signals almost impossible (*93*, *98*). Some examples will be discussed to illustrate these problems.

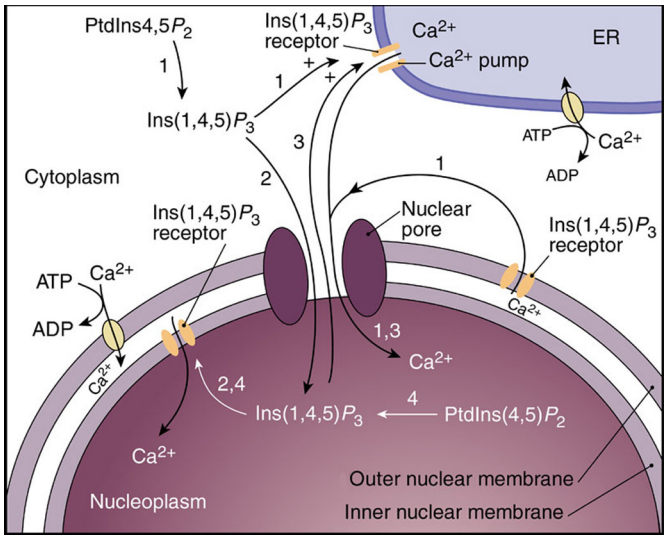

Fig. 3. Regulation of Ca^{2+} in the nucleus. Shown here are the various ways in which Ins(1,4,5)P_3 can increase Ca^{2+} in the nucleus. These are numbered in the figure as follows: (**1**) InsP_3 mobilizes Ca^{2+} from the ER or nuclear envelope, and then the Ca^{2+} enters the nucleus through the nuclear pore. (**2**) Ins(1,4,5)P_3 generated in the cytoplasm enters the nucleus and mobilizes Ca^{2+} from the nuclear envelope by activating Ins(1,4,5)P_3 receptors facing the nucleoplasm. (**3**) Ins(1,4,5)P_3 generated in the nucleus exits into the cytoplasm through the nuclear pores and mobilizes Ca^{2+} in the cytoplasm, which then enters the nucleus. (**4**) Ins(1,4,5)P_3 is generated in the nucleus, and activates Ins(1,4,5)P_3 receptors as in (2).

In *Xenopus laevis* oocytes, nuclear InsP_3 receptors were activated by InsP_3 micro-injected into the nucleus even when heparin, an InsP_3 receptor-blocker, was present in the cytoplasm. This cytosolic heparin did prevent cytosolic InsP_3 from generating a Ca^{2+} signal, suggesting that InsP_3 couldn't get through the nuclear pore complex from the cytoplasm (*99*). A similar relationship between nuclear and cytosolic Ca^{2+} is suggested by ex-

periments on starfish oocytes. In these cells, injection of heparin or antibodies against the InsP_3 receptor into the nucleus prevented the increase in nuclear Ca^{2+} that could be generated by intranuclear photorelease of InsP_3. If the heparin or antibodies were injected into the cytoplasm, they had no such inhibitory effect on nuclear Ca^{2+} signals (*100*, *101*). Thus, overall these data show clearly that InsP_3 in the nucleus can release intranuclear Ca^{2+}. A similar relationship with cADPR and nuclear Ca^{2+} was also shown, because antagonists to cADPR receptors micro-injected into nuclei prevented Ca^{2+} signals generated by the intranuclear photorelease of cADPR (*100*), but if the inhibitors were injected into the cytoplasm they had less effect.

In further experiments, injection into the nucleus of antagonists to InsP_3 or cADPR receptors had a partial inhibitory effect on nuclear envelope breakdown induced by treatment of the oocytes with 1-methyladenine, a natural initiator of meiosis (*101*). This suggests that intranuclear mobilization of Ca^{2+} by InsP_3 may be a significant component of the physiological process of meiosis. Even so, the physiological source of that InsP_3, nucleus or cytoplasm, is still unclear. Moreover, in hamster oocytes, InsP_3 photoreleased inside the nucleus produced a nuclear Ca^{2+} signal that did not arise from the nucleoplasmic space, but instead appeared to be initiated from a cortical region beside the nucleus (*102*). This suggests that in hamster oocytes, to generate an increase in nuclear Ca^{2+} concentration, the InsP_3 must exit from the nucleus through the nuclear pores and act on cytoplasmic InsP_3 receptors. This mechanism cannot exist in the *Xenopus* or starfish oocyte experiments described above, because the data *(99-101)* directly contradict it. The difference between these systems, oocytes from mammals versus amphibians or echinoderms, makes drawing common conclusions very difficult.

A particularly clear view on the issue of differentiating between primary (generated within the nucleoplasm) and secondary (generated in the cytoplasm and passing into the nucleus) Ca^{2+} signals comes from experiments on HeLa cells (*103*). In these experiments, rapid confocal scanning was used to look at elementary Ca^{2+} events. A small puff of Ca^{2+} released from the endoplasmic reticulum near the nucleus is "magnified" by virtue of the lower Ca^{2+} buffering power of the nucleoplasm versus the cytoplasm. Were the same cells examined by methods other than fast confocal scanning, the nuclear Ca^{2+} signal would appear to be entirely independent of any cytosolic Ca^{2+} increase, which is what has been claimed for a number of tissues [for example, see (*104*) and (*93*) for review]. Finally, invaginations of the nuclear envelope (*38*, *105*) also create problems in determining exactly where a "nuclear" Ca^{2+} signal started.

In conclusion, unequivocal evidence for a primary nuclear Ca^{2+} signal that initiates from nuclear InsP_3 generation is lacking at present. Existing data suggest that most nuclear Ca^{2+} signals actually arise in the cytoplasm. But the evidence discussed above also provides a strong argument that nuclear InsP_3 receptors exist that can be made to respond to injected or photoreleased intranuclear InsP_3. However, the large body of evidence discussed throughout this review shows that hydrolysis of PtdIns(4,5)P_2 by a PI-PLC can happen within the nucleus, and that this generates InsP_3; thus, there is surely a possibility that Ca^{2+} signals can be initiated in the nucleus.

Another Ca^{2+}-mobilizing second messenger, cADPR, may also play an important role in calcium signaling in the nucleus (*96*, *101*). Two papers have provided evidence that CD38, an isoform of the enzyme ADP-ribosyl cyclase (which synthesizes cADPR), is localized in the inner nuclear membrane with its catalytic site facing the nucleoplasm (*97*, *106*). The enzyme is active, and adding cADPR or its precursor nicotinamide adenine dinucleotide (NAD^+) to nuclei increased intranuclear Ca^{2+} concentration (*95*, *97*) or stimulated release of calcium from the isolated nuclei (*106*). It may be that cADPR is another second messenger that could, like InsP_3, be synthesized within the nucleus to increase nuclear Ca^{2+} concentration.

Other Inositol Phosphates

Inositol phosphates are not lipids, but it is appropriate to discuss their nuclear functions here, not least because their common precursor is probably PtdIns(4,5)P_2. A highly specific Ins(1,3,4,5)P_4-binding site in rat liver nuclei has been identified, and a stimulatory effect of this inositol phosphate on nuclear Ca^{2+} uptake has been reported (*107*). The same group has also partially purified a putative nuclear Ins(1,3,4,5)P_4 receptor (*108*), which, based on a partial sequence, is clearly not $GAP1^{IP4BP}$, currently the most promising putative cytoplasmic InsP_4 receptor (*109*). The significance of these findings for nuclear Ca^{2+} homeostasis awaits clarification. We sought to identify similar InsP_4-specific binding sites in highly purified rat liver nuclei (*15*) using carrier-free [^{32}P]-Ins(1,3,4,5)P_4 (*110*), which should have been able to detect even low amounts of high affinity specific binding sites. However, we could detect only low affinity sites that did not discriminate between InsP_4 and InsP_6 (*111*).

Other discoveries point to roles for higher inositol phosphates, those containing more than three phosphate moieties, in other aspects of nuclear function. In preparations of cytosol from the slime mold *Dictyostelium*, there is no detectable Ins(1,4,5)P_3 3-kinase activity (*112*). Furthermore, in these cytosolic extracts, the formation of InsP_6 by sequential phosphorylations of inositol occurs through a route that does not include Ins(1,4,5)P_3 (*112*). However, nuclear fractions from the same species can phosphorylate Ins(1,4,5)P_3 all the way to InsP_6 by a different route, implying a separate inositol phosphate metabolism in nuclei compared to the cytosol (*113*).

Data from experiments on yeast point to functions for higher inositol phosphates within the nucleus. Three yeast genes apparently involved in mRNA transport from the nucleus were identified as components of the major inositol phosphate-generating pathway in yeast (*114*). One is the yeast PI-PLC, and another is an Ins(1,3,4,5,6)P_5 2-kinase (Fig. 4). The third gene was suggested from indirect evidence to encode an enzyme that converts Ins(1,4,5)P_3 to Ins(1,3,4,5,6)P_5, and subsequently has been cloned and shown to be an Ins(1,4,5)P_3 multi-inositol phosphate kinase [Ins(1,4,5)P_3 multikinase] (*115*, *116*). The enzyme can phosphorylate Ins(1,4,5)P_3 to produce Ins(1,4,5,6)P_4, and then further phosphorylate the Ins(1,4,5,6)P_4 to Ins(1,3,4,5,6)P_5 (*115*, *116*). Yeast strains lacking any one of these three proteins—PI-PLC, Ins(1,4,5)P_3 multikinase, or Ins(1,3,4,5,6)P_5 2-kinase—show marked defects in their ability to export mRNA from the nucleus (*114*, *116*, *117*). The simplest interpretation of these experiments is that InsP_6 is required for mRNA transport out of the nucleus. There may be some complications in this interpretation [see (*118*) for review], such as the intriguing demonstration by York's group (*119*) that the correlation between enzymatic activity in vivo of the Ins(1,3,4,5,6)P_5 2-kinase and mRNA export is not absolute. Despite this, a role for inositol polyphosphates in RNA transport represents a remarkable new angle on inositol phosphate func-

tion in the nucleus.

As if one intranuclear function for InsP_6 were not enough, Hanakahi *et al*. (*120*) have proposed a role for it in double-stranded DNA repair. They assayed DNA end-joining in vitro and found that the nuclear extract had to be supplemented with cytosol for full activity. They purified the active agent in cytosol and found it to be InsP_6. The InsP_6 was probably exerting its effect by interacting with DNA-dependent protein kinase, and a putative binding domain was identified (*120*). However, more recently Ku, a partner protein to DNA-dependent protein kinase, was identified as the more likely InsP_6-binding protein (*121*). The full significance of both nuclear InsP_6 stories remains an enticing prospect for further investigation.

Several papers on the Ins(1,4,5)P_3 multikinase (*116*, *117*) have expanded the story of intranuclear inositol phosphates into transcriptional regulation, and here things are more complicated [see refs (*118*, *122*) for further discussions]. The gene for the Ins(1,4,5)P_3 multikinase was originally discovered to encode a protein that is a part of the transcriptional complex that regulating the expression of genes involved in the metabolism of arginine. The phenotype is usually studied by testing the ability of yeast to grow with arginine or ornithine as a sole nitrogen source. Mutants lacking the Ins(1,4,5)P_3 multikinase, also known as ArgIII or Arg82, can neither grow on such a medium, nor can they form the protein complex that associates with the *ARG5,6* promoter (*116*). However, if the yeast express a catalytically inactive mutant of the protein, they can still form the protein complex that associates with the *ARG5,6* promoter. However, this complex is probably not active, because these yeast cannot grow with arginine as a sole nitrogen source (*116*). Dissociation of the kinase activity of the Ins(1,4,5)P_3 multikinase from the regulation of arginine metabolism in yeast has been shown by Dubois *et al*. (*123*). Mutation of an acidic stretch of amino acids in Arg82, through which it interacts with two transcription factors containing MADS (McM1 Agamous Deficiens Serum Response Factor) boxes, resulted in a phenotype resembling the null *Arg82* mutant, despite the fact that this construct was still catalytically active. The simple (probably too simple) conclusion is that the enzyme is mediating protein-protein interactions in the nucleus, and that inositol phosphates are not involved, but much remains to be clarified [see (*118*) for further discussion]. One last point worth noting is that the mammalian Ins(1,4,5)P_3 multikinases, the orthologs of Arg82, do not actually contain the acidic stretch of amino acids suggested to be crucial for protein-protein interactions, so it is possible that this transcriptional regulation story is confined to only a few species.

Another piece of data suggesting that the functions of inositol polyphosphates are going to become more complicated lies in the profiles of inositol phosphates made in vivo by the yeast strains lacking the Ins(1,4,5)P_3 multikinase. These yeast are, as expected, low in Ins(1,3,4,5,6)P_5 and InsP_6 (*116*). A more detailed analysis of what they actually can synthesize (*117*) shows that they compensate for lack of Ins(1,3,4,5,6)P_5 by making other InsP_5 isomers, Ins(1,2,3,4,5)P_5 or Ins(1,2,3,5,6)P_5 [these two inositol phosphates are an enantiomeric pair and are indistinguishable by the high pressure liquid chromatography techniques used to identify them (*118*)]. The Ins(1,4,5)P_3 multikinase-deficient yeast were still able to synthesize some InsP_6, and perhaps most significantly, to maintain measurable levels of the pyrophosphate-containing inositol phosphates InsP_5(*PP*) and InsP_4(*PP*)$_2$, more commonly known as InsP_7 and InsP_8, which are synthesized (Fig. 4) by phosphorylation of InsP_6 (*117*). These observations leave wide open the question of which inositol phosphate is doing what. It seems likely that, as has happened with inositol lipids and phosphates in the cytoplasm, the players and functions will multiply and become more complicated as more experiments are performed.

Other Inositol Lipids

The discovery of 3-phosphorylated lipids has impinged on almost all aspects of cell biology, including nuclear lipid signaling. A member of the PI3K family that is associated with a p85 regulatory subunit has been reported in rat liver nuclei (*124*). Metjian *et al*. (*125*) have demonstrated in HepG2 cells a serum-induced translocation of the G protein βγ-regulated isoform of Type Iγ PI3K, and have suggested that this translocation may be driven by an association with Gβγ through the p101 regulatory subunit that confers Gβγ sensitivity on this isoform. Bacqueville *et al*. (*126*) have found similar results in smooth muscle cells, where a G_i- and G_o-regulated PI3K activity [compare with (*46*)] in nuclei was reported. This nuclear PI3K in smooth muscle cells could also possibly be the Type Iγ PI3K. PTEN, a PtdIns(3,4,5)P_3 3-phosphatase, is also partly nuclear, and some correlative studies have implied a role for nuclear localization in its tumor-suppressive function (*127*, *128*).

Increases in nuclear PI3K activity occur upon stimulation with nerve growth factor (NGF) of the rat pheochromocytoma cell line PC12 (*129*), and this activation may involve PIKE, a nuclear guanosine triphosphatase (GTPase) that enhances nu-

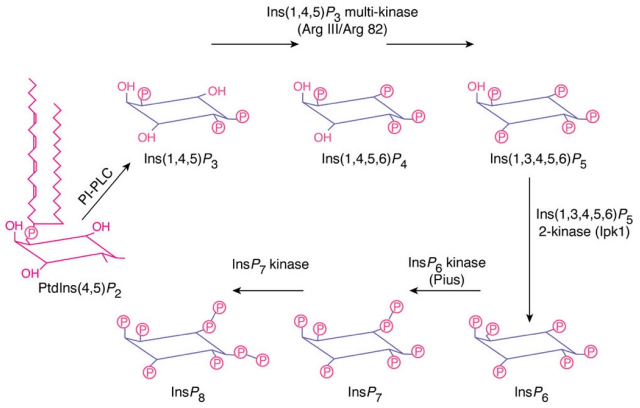

Fig. 4. Higher inositol phosphate metabolism. The probable pathway of Ins(1,4,5)P_3 metabolism in yeast is shown. Some of this pathway is likely to be localized within the nucleus. Ins(1,4,5)P_3 is generated from PtdIns(4,5)P_2 by a PI-PLC, and is then phosphorylated twice by an Ins(1,4,5)P_3 multikinase to Ins(1,3,4,5,6)P_5. The multikinase is also known as Ipk2, ARGIII, or ARG 82 (*116*, *117*). Ins(1,3,4,5,6)P_5 is phosphorylated to InsP_6 by Ins(1,3,4,5,6)P_5 2-kinase, also known as Ipk1 (*114*). The InsP_6 can be further phosphorylated to the pyrophosphate-containing inositol phosphate InsP_7, by an InsP_6 kinase also known as PiUS (*114*, *157*). The InsP_7 is finally phosphorylated to InsP_8 by an InsP_7 kinase that has not yet been cloned. The isomeric configuration of InsP_7 and InsP_8 is unknown in mammalian cells or yeast, so they are depicted here as Ins(1,2,3,4,5)P_5(6)*PP* and Ins(1,2,3,4)P_4(5,6)PP_2, the predominant isomers found in *Dictyostelium* (*158*).

clear PI3K activity (*130*). This activation by PIKE requires the p85 regulatory subunit of the PI3K and is, thus, distinct from the Ras-activation of these enzymes that occurs through interactions with the catalytic subunit. A fascinating connection with the nuclear localization of PI-PLCγ has emerged. The SH3 domain of PI-PLCγ_1 can act as a guanine nucleotide exchange factor (GEF) of PIKE (*131*), an observation that accounts for the surprising earlier discovery that PI-PLCγ_1 does not have to be catalytically active to have mitogenic activity, but needs only its SH3 domain (*132*). The nuclear function of PIKE remains an exciting new area for exploration.

The effectors of PtdIns(3,4,5)P_3 in the nucleus are not yet certain, but a PtdIns(3,4,5)P_3-binding protein called PIP_3BP can translocate to the nucleus under certain conditions (*133*). However, it may not be only PtdIns(3,4,5)P_3 that should be considered for a nuclear function. Using a monoclonal antibody apparently specific for PtdIns(3,4)P_2, Yokogawa *et al*. have detected this lipid, now known to have its own independent role as a second messenger, on the cytoplasmic surface of nuclei in vivo (*134*). Also, the Type II PI3K, PI3K C2α, contains a nuclear localization sequence and associates with nuclear speckles in HeLa cells (*135*). Its close relative, PI3K C2β has been shown to increase in nuclei 20 hours after partial hepatectomy, with a possible calpain-mediated modification of the enzyme (*136*).

It may be that PtdIns3*P* should be considered for an intranuclear role too, because in higher plants the PI3K, which is believed to be a Type III isoform capable of phosphorylating only PtdIns, is found to have a punctate intranuclear distribution consistent with nuclear transcription sites (*137*). Using a PtdIns3*P*-specific probe, Gillooly *et al*. (*138*) found PtdIns3*P* in nuclei (especially nucleoli) of yeast and BHK (mammalian) cells, although it could be a breakdown product from other 3-phosphorylated inositol lipids.

Finally, in this context of inositol lipid function, it is worth reiterating that, as reviewed above in the Cell Cycle section, PtdIns(4,5)P_2 itself may have functions in the nucleus, such as participation in RNA splicing (*36*). Also, the presence of a Type II PtdIns*P* kinase in nuclei (*20*), plus the detection of its probable substrate, PtdIns5*P*, which changes markedly during the cell cycle (*88*), might indicate that PtdIns5*P* also has a role to play. PtdIns itself has been implicated in nuclear envelope assembly (*139*), and we may have seen only the tip of the iceberg with regard to nuclear inositol lipid function.

Other Lipids

In several cellular systems PLA_2 can translocate to a nuclear fraction or the nuclear envelope (*140*-*142*). Prostanoid receptors have also been reported to show a perinuclear distribution (*143*), so there may be a regulatory mechanism involving the liberation and use of arachidonic acid near the nucleus. In the context of this review, it is more relevant to ask whether any such PLA_2-involved signaling occurs inside the nucleus. A nuclear matrix-associated PLA_2 of unknown isoform has been reported, along with its possible control by polyphosphoinositides (*144*). One of the low molecular weight (Type I or II) PLA_2s may be in the nucleus of proliferating, but not nonproliferating, U_{III} cells (*145*). There is abundant evidence for a potential PLA_2 substrate, PtdCho, in the nucleus, so it is possible that fatty acids are generated within the nucleus. However, although there is no obvious function at the moment, in the context of fatty acid release it is worth noting the intriguing change in the fatty acyl profile of nuclear PtdEthan in IIC-9 cells that follows stimulation of the cells with thrombin (*27*). This may be some form of remodeling similar to that documented for ethanolamine-containing species of phospholipid in other tissues (*146*). Such a process could take place in the nuclear envelope, or possibly even in the nucleus.

An additional possible aspect of nuclear PtdCho function has been suggested by Hunt *et al*. (*31*). The fatty acid profile of intranuclear PtdCho in IMR-32 neuroblastoma cells is markedly different from the whole cell profile, in that there is a much higher degree of saturation [this is consistent with the highly saturated PtdCho found by D'Santos *et al*. in MEL cell nuclei (*16*)]. Through the elegant use of mass spectroscopy and deuterated choline, Hunt *et al*. (*31*) showed that nuclear PtdCho is not synthesized de novo in this saturated state, but is probably synthesized with a more typical fatty acid profile and subsequently remodeled within the nucleus. This remodeling may be similar to the way that PtdIns is remodeled in cells after synthesis to acquire its stearoyl-arachidonoyl rich profile (*147*). Hunt *et al*. (*31*) made some provocative calculations that suggested that PtdCho may occupy 10% of the nuclear volume. There is a tempting parallel here with the saturated (predominantly dipalmitoyl) PtdCho found in lung surfactant, a material whose unusual physical properties is attributed to this dipalmitoyl PtdCho (*148*). Could it be that this lipid forms an important part of chromatin structure by some kind of liquid crystalline phase?

Sphingomyelin is probably present in the nucleus as part of the nuclear matrix (*149*), and a sphingomyelinase has been purified from nuclei (*150*). At least in AH7974 hepatoma cells, a neutral sphingomyelinase 1 is reportedly almost entirely in the nuclear matrix (*151*). Originally, sphingomyelin was believed to have a structural role (*152*). However, over the last decade, sphingolipids and their metabolites (most notably sphingosine-phosphate and ceramide) have emerged as a set of signaling molecules of considerable significance in cell biology. Tsugane *et al*. (*153*) suggested that a ceramidase is also present in rat liver nuclei, and Simbulan *et al*. (*154*) reported a very specific inhibitory effect of sphingosine on the synthesis of RNA primers by primase in vitro. A whole new range of possibilities for sphingomyelin metabolism and signaling in the nucleus remains to be explored.

Conclusion

Despite the very large literature on nuclear lipid signaling (*1*, *2*, *4*, *5*), we are still in the beginning of understanding it. Even a comparatively "simple" topic, such as the PI-PLC pathway, which in the plasma membrane is now so fully mapped out, is still confusing in the nucleus. We are faced with the most basic questions: Where in the nucleus, and in what physical form, is the PtdIns(4,5)P_2 substrate for these enzymes? What are the relevant signals generated by PtdIns(4,5)P_2 hydrolysis, Ins(1,4,5)P_3 or DAG, or both, and what are the molecular events, and in turn the physiological endpoints, that follow their production (Fig. 5)?

Considering our profound ignorance about the nuclear counterpart of the well-characterized cytoplasmic PI-PLC system, it is not surprising that other aspects of nuclear lipid signaling that are still baffling in the cytoplasm or plasma membrane elude our comprehension completely. How is the DAG from PtdCho generated and what is it doing? Why are there two pathways of PtdIns(4,5)P_2 synthesis in nuclei? What is the signifi-

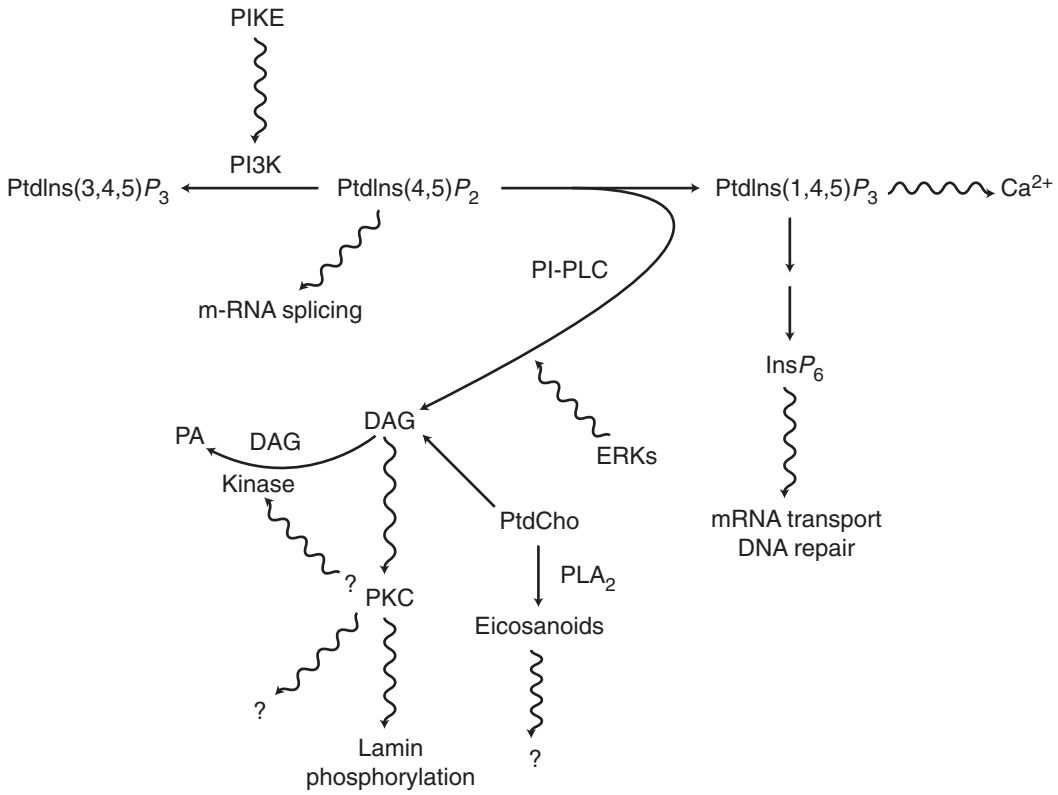

Fig. 5. Potential functions and points of regulation in the nuclear lipid cycle. The straight lines are reactions in the biosynthetic and metabolic pathway. The wavy lines represent regulatory interactions.

cance of the nuclear Ins(1,4,5)P_3 phosphorylation pathway, and, in particular, what is its relationship to mRNA export? How many ways of regulating intranuclear Ca^{2+} are physiologically significant, and what is their role in cell function? And the biggest question of all, is nuclear lipid signaling a central and indispensable part of eukaryotic nuclear function, or is a late evolutionary refinement? If the latter, to what end is it directed?

Multiple questions can be disheartening, because they emphasize how little we understand. But they can also be a call to arms. We know from all the evidence documented above and described elsewhere (*1*, *2*, *4*) that nuclear lipid signaling does exist. We also know that the main reason that we know so little about it is that it is very difficult to study, as anyone who has tried to do so will testify. Since the early days of nuclear lipid signaling, two major changes have occurred: We have many new techniques and tools to study molecular cell physiology (*5*), and we understand much more about the pathways and processes outside the nucleus that must be similar to those inside it. The clearest message from all the data described above is that the nucleus is a compartmentalized organelle that in many respects resembles a miniature cell, with a level of complexity and refinement of lipid signaling contained within it similar to that found in the cytoplasm. So, if nothing else, this review might serve as an encouragement and enticement to other investigators to enter the fray and find out just what is going on within this microcosm of cellular signaling.

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