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MINIREVIEW

Diphosphoinositol Polyphosphates: Metabolic Messengers?

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ABSTRACT

The diphosphoinositol polyphosphates ("inositol pyrophosphates") are a specialized subgroup of the inositol phosphate signaling family. This review proposes that many of the current data concerning the metabolic turnover and biological effects of the diphosphoinositol polyphosphates are linked by a common theme: these polyphosphates act as metabolic messengers. This review will also discuss the latest proposals concerning possible molecular mechanisms of action of this intriguing class of molecules.

The discovery of cyclic AMP (Rall and Sutherland, 1958; Sutherland and Rall, 1958) introduced us to the concept of a "second messenger" (Robison et al., 1968): a diffusible molecule (or ion) that, in response to an extracellular stimulus, is rapidly generated at (or released from) a particular subcellular site and then regulates particular effector proteins within the cell so as to elicit a cellular response. Of course, evolution has a remarkable tendency to repeat a good idea, so many different second messengers are now known. The inositol phosphate family represents the convergence of several "good signaling ideas," most notably in their use of a recurring theme in the cell-signaling genre: phosphate groups. Phosphates have two especially prominent features that facilitate specificity of interactions between cell signaling entities. First, the bulky nature of the phosphate group imposes geometric constraints on ligand-protein and protein-protein interactions. Second, the phosphate's negative charge at physiological pH bestows specificity on its interactions with target proteins through multiple ionic and hydrogen bonds. The negative charges on the phosphate group also make soluble, phosphorylated molecules lipid-impermeant, so that they can be retained inside cells.

Inositol offers several additional assets for a signaling molecule. It is chemically stable, it is small (hence it diffuses through cytosol quickly), and it is only a short synthetic offshoot from the glycolytic pathway (Sherman et al., 1977). There is also a functionally significant plane of symmetry across the 2/5-axis of the inositol ring (Fig. 1). That symmetry permits one inositol phosphate to imitate another's three-dimensional phosphate recognition pattern when the orientation of the inositol ring changes in relation to the protein's ligand-binding site (Wilcox et al., 1994), although in addition the binding site itself has to be somewhat flexible (Ho et al., 2002). This phenomenon provides a molecular explanation for the metabolic promiscuity of certain inositol phosphate kinases (for review, see Shears, 2004), and it also facilitates functionally important metabolic interactions between differ-

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ABBREVIATIONS: 5-PP-InsP$_5$, 5-diphospho-D-*myo*-inositol 1,2,3,4,6-pentakisphosphate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; DIPP, diphosphoinositol polyphosphate phosphohydrolases; HGNC, Human Genome Organization Gene Nomenclature Committee; HPLC, high-performance liquid chromatography; Ins(1,3,4,5)P$_4$, D-*myo*-inositol 1,3,4,5-tetrakisphosphate; Ins(1,4,5)P$_3$, D-*myo*-inositol 1,4,5-trisphosphate; InsP$_5$, inositol pentakisphosphate; InsP$_6$, inositol hexakisphosphate; IP6K, inositol hexakisphosphate kinase; MEK, mitogen-activated protein kinase kinase; PD184352, 2-(2-chloro-4-iodophenylamino)-*N*-cyclopropylmethoxy-3,4-difluorobenzamide; PD98059, 2′-amino-3′-methoxyflavone; PH, Pleckstrin homology; PLC, phosphoinositide-specific phospholipase C; PP-InsP$_4$, diphosphoinositol tetrakisphosphate; (PP)$_2$-InsP$_4$, bis-diphosphoinositol tetrakisphosphate ("InsP$_8$"); PP-InsP$_5$, diphosphoinositol pentakisphosphate ("InsP$_7$"); PPIP5K, PP-InsP$_5$ kinase; PPP-InsP$_5$, triphosphoinositol pentakisphosphate; TNP, N^2-(*m*-trifluoromethyl)benzyl),N^6-(*P*-nitrobenzyl)purine; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene.

ent inositol phosphates (Chamberlain et al., 2007). With all of these advantageous features, it is not surprising that the inositol phosphates are a phylogenetically widespread family of second messengers.

There are also a large number of different inositol phosphates, thereby creating opportunities for substantial structural and functional diversity within this class molecules. A mere handful of these molecules is shown in Fig. 2. In fact, the inositol ring—"a six-bit signaling scaffold" (York, 2006)—can, theoretically, host 63 different combinations of one-to-six monoester phosphate groups; more than half of these possible molecules are now acknowledged to exist in nature (Irvine and Schell, 2001; Majerus et al., 2008; Michell, 2008). Yet even that understates their abundance: in the early 1990s, it became clear that the presupposed upper limit of six phosphates per inositol ring was going to be exceeded in light of reports that cells contain inositol-based molecules that are even more polar than $InsP_6$ (Europe-Finner et al., 1991; Stephens et al., 1991; Oliver et al., 1992; Wong et al., 1992). Two groups working independently (Menniti et al., 1993; Stephens et al., 1993) determined that these molecules are diphosphorylated derivatives of $InsP_5$ and $InsP_6$ (Fig. 3). More recently, evidence has arisen of diphosphorylated derivatives of $InsP_3$ and $InsP_4$ (Seeds et al., 2005). The threat that cells might also synthesize triphosphates of $InsP_6$ (Shears, 2004; Draskovic et al., 2008) foreshadows a further potential expansion of this family.

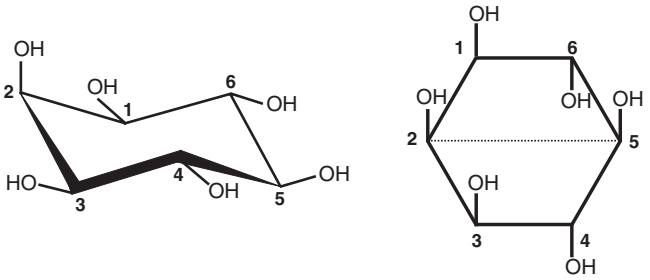

Fig. 1. The axis of symmetry across the inositol ring. The apparent simplicity of the myo-inositol molecule is deceptive. Here it is depicted both in its thermodynamically preferred "chair" form (left) and in a Haworth projection (right). The latter is not so technically discriminating, but it does have the advantage of more obviously illustrating the plane of symmetry that runs across the 2/5 axis (the broken line), thereby dividing the molecule into two chiral halves. The hydrogen atoms are omitted, for clarity. Agranoff's turtle (Agranoff, 1978) provides a popular memory aid for the carbon numbering (see also Irvine and Schell, 2001); the logic underlying the numbering system is described elsewhere (IUPAC-IUB Commission on Biochemical Nomenclature, 1969).

The initial discovery of diphosphorylated inositol phosphates immediately generated an anticipation that these molecules have some special functional significance (Menniti et al., 1993; Stephens et al., 1993). This argument goes well beyond the obvious "cells would not make them unless they are important" category. Instead, it was noted that these molecules undergo exceptionally high rates of metabolic turnover through coupled kinase/phosphatase substrate cycles (Menniti et al., 1993; Stephens et al., 1993). The investment of cellular energy in the maintenance of these cycles must be quite substantial and, presumably, biologically significant. Moreover, substrate cycles are themselves telltale signs of regulatory steps in a metabolic pathway (Hers and Hue, 1983), from which one can infer that the levels of the substrates and/or products must be worth regulating. The diphosphoinositol polyphosphates are also molecules with severe electrostatic and steric congestion. The relief of these constraints after hydrolysis of the diphosphate groups has long-been viewed as contributing to a "high-energy" reaction that ought to have biological significance. Hence, for example, the origin of the idea that diphosphoinositol polyphosphates might transfer phosphate to proteins (Stephens et al.,

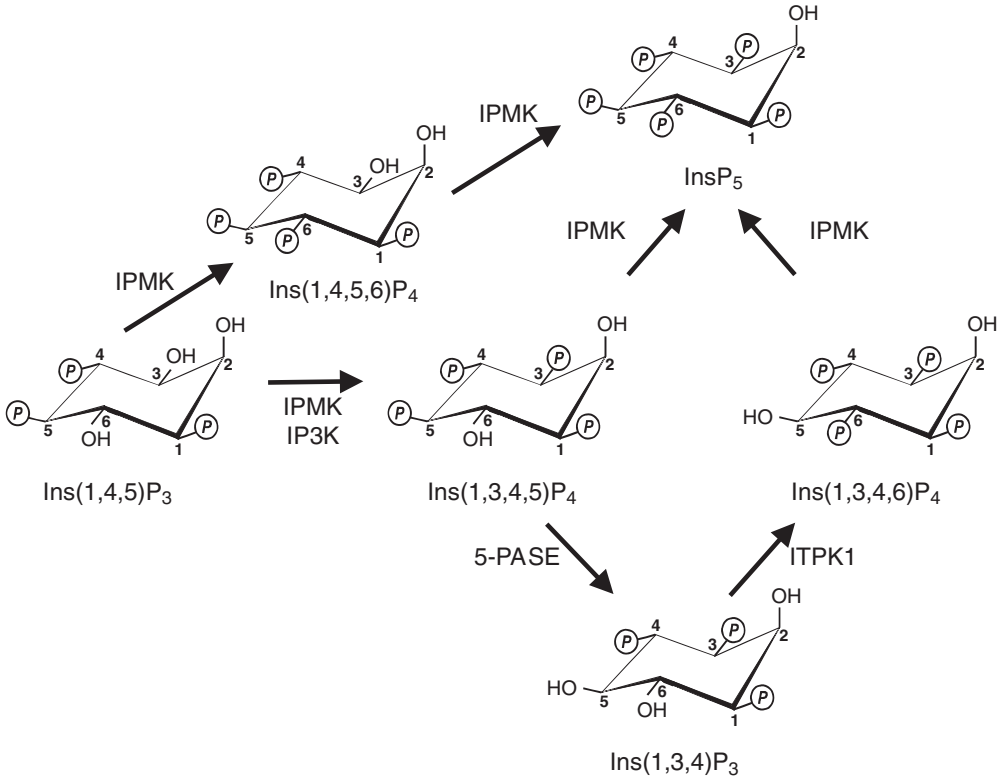

Fig. 2. Synthesis of $Ins(1,3,4,5,6)P_5$ from $Ins(1,4,5)P_3$ in mammals. In the abbreviations of the chemical structures, "Ins" indicates the myo-inositol skeleton. The number of monophosphates around the inositol ring is denoted as a suffix after the "P." The figure shows two pathways (ITPK1-dependent and -independent) by which mammalian cells convert $Ins(1,4,5)P_3$ to $Ins(1,3,4,5,6)P_5$. It is unclear which of the two routes of $Ins(1,3,4,5,6)P_5$ synthesis is quantitatively the more important. IP3K, $Ins(1,4,5)P_3$ kinase; IPMK, inositol polyphosphate multikinase (also known as Ipk2 in yeast); 5-PASE, $Ins(1,4,5)P_3$/$Ins(1,3,4,5)P_4$ 5-phosphatase; ITPK1, inositol trisphosphate kinase. There is phylogenetic variability in the isomeric nature of the $InsP_4$ produced by $Ins(1,4,5)P_3$ phosphorylation by IMPK: it is mainly $Ins(1,3,4,5)P_4$ in mammals, mainly $Ins(1,4,5,6)P_4$ in yeasts, and IPK2 from *D. melanogaster* synthesizes almost equal amounts of both $InsP_4$ isomers (Seeds et al., 2004). Note that yeasts and *D. melanogaster* do not encode ITPK1 in their genomes (Seeds et al., 2004). Finally, plants (Brearley and Hanke, 1996) and slime molds (Stephens and Irvine, 1990) can synthesize $Ins(1,3,4,5,6)P_5$ by alternate metabolic pathways that do not use $Ins(1,4,5)P_3$.

1993; Laussmann et al., 1996; Voglmaier et al., 1996; Hand and Honek, 2007).

Nevertheless, despite their pedigree, the diphosphoinositol polyphosphates have been slow to achieve the status of being widely accepted intracellular signals (Burton et al., 2009). One significant problem has been that their global intracellular concentrations do not change in response to the activation, by any naturally occurring extracellular agent, of a defined signal transduction pathway. This factor has been a major impediment to our attempts to place in a cell-signaling context the diversity of proposed cellular activities that have been attributed to these compounds. A major goal of this review is to assimilate evidence that points to a resolution of this problem. The key is not to consider "second messengers" as just being mediators of extracellular stimuli, as originally envisaged (Robison et al., 1968). There is little evidence that diphosphoinositol polyphosphates fulfil this requirement. Instead, I will turn to other signaling systems that respond to biochemical information that is generated from within the cell, rather than from outside of it. Examples from within this genre are the "metabolic messengers" (Rolland et al., 2001). These particular entities are biosensors that first detect variations in energy balance and subsequently communicate this information to other cellular networks, which then initiate adaptive responses. This ensures the maintenance of tight energy homeostasis, which is a fundamental necessity for cell survival (Hardie, 2004). Some recent developments (for example, Bennett et al., 2006; Lee et al., 2007; Choi et al., 2008) have led to the suggestion that diphosphoinositol polyphosphates might serve this role of coupling signaling pathways to the energetic status of the cell. This evidence is discussed here. I will also expand this new concept by arguing that it provides a common theme that rationalizes many of the apparently disparate functions of the diphosphoinositol polyphosphates. This review will also discuss the latest proposals concerning possible molecular mechanisms of action of this intriguing class of molecules.

Nomenclature

Some comments on nomenclature are warranted. This review takes an approach that might at first seem both unwieldy and unnecessarily pedantic, but it is hoped that the reader will ultimately appreciate that the field will benefit from the use of terminology that is unambiguous. Some of the current concerns with nomenclature originate from the initial discovery of these compounds (Menniti et al., 1993; Stephens et al., 1993). Stephens et al. (1993) described these molecules as "diphosphoinositol polyphosphates," which is the IUPAC-preferred terminology (IUPAC-IUB Commission on Biochemical Nomenclature, 1977). We (Menniti et al., 1993), however, classified these molecules as "inositol pyrophosphate polyphosphates"—*mea culpa*—and subsequently it has become common practice to abbreviate this to "inositol pyrophosphates" (e.g., Saiardi et al., 2002). IUPAC frowns on

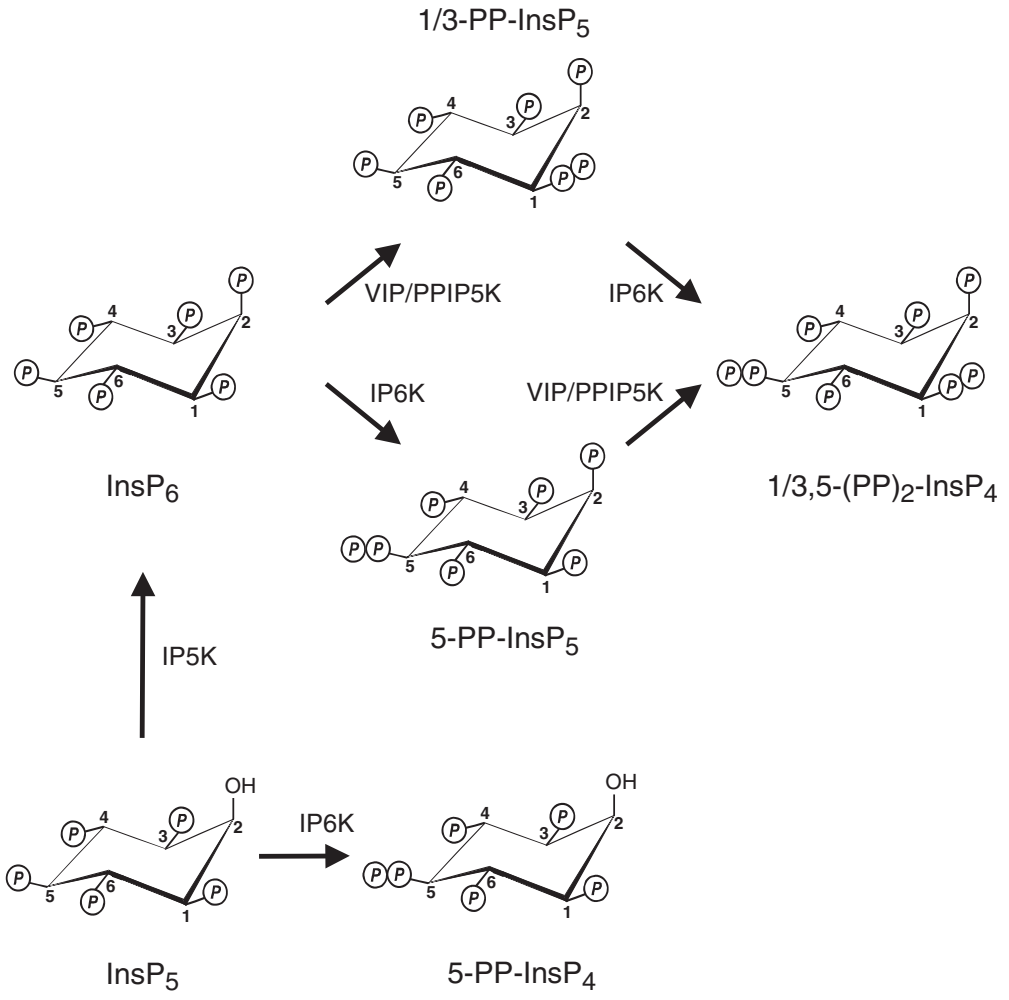

Fig. 3. Synthesis of PP-$InsP_4$, PP-$InsP_5$, and $(PP)_2$-$InsP_4$ in yeasts and mammals. This figure focuses on the best-characterized diphosphoinositol polyphosphates. Other members of this class of compounds do exist, but are less well understood (see text for details). The positions of the diphosphate groups have been determined in the following publications: Albert et al. (1997), Draskovic et al. (2008), and Lin et al. (2009). The "1/3" designation indicates that it is not yet known whether the diphosphate is present at either position 1 or 3 (or both!); the choice of position 1 in the figure is arbitrary. The 5- and 1/3-isomers of PP-$InsP_5$ are sometimes referred to as 5-IP_7 and 1/3-IP_7, respectively. The 1/3,5-$(PP)_2$-$InsP_4$ is often termed "IP_8" in the literature (see text). In addition to 5-PP-$InsP_4$, some 1/3-PP-$InsP_4$ is also formed from Ins(1,3,4,5,6)P_5 by IP6K (Draskovic et al., 2008).

this vernacular because "pyrophosphate" is a term that was originally introduced to define a diphosphate that was produced nonenzymatically, by heating (the prefix "pyro-" is derived from the Greek, meaning "fire" in this context). Another perhaps less finicky argument against using "inositol pyrophosphate" as a designation is that it is inadequate for describing a molecule that contains *both* phosphates and diphosphates. Thus, in this review, "diphosphoinositol polyphosphates" is the preferred nomenclature. However, the thesis in this review, that these molecules are metabolic messengers, applies (for now) only to the canonical members of this family: PP-$InsP_4$, PP-$InsP_5$, and $(PP)_2$-$InsP_4$ (Fig. 3). Some caution is warranted as we await further evidence of the significance of $(PP)_2$-$InsP_3$ (Caffrey et al., 2000; Ingram et al., 2003; Draskovic et al., 2008), and the diphosphorylated derivatives of $InsP_3$ and $InsP_4$ (Seeds et al., 2005). Inositol polyphosphate multikinase (see Fig. 3) can also add a diphosphate to Ins(1,3,4,5,6)P_5; the position of the diphosphate in not known, and it remains unclear whether this reaction has any biological importance (Saiardi et al., 2001a; Zhang et al., 2001).

There is also the issue of triphosphates. Some time ago, after prolonged incubation of $InsP_6$ with an $InsP_6$ kinase, we noticed that PP-$InsP_5$ was not the only product. In addition, small amounts (5–10%) of more polar material were observed (Saiardi et al., 2000). We (Saiardi et al., 2000) initially proposed that this novel molecule was an isomer of $(PP)_2$-$InsP_4$; only later (Shears, 2004) was the possibility raised that it might instead be a PPP-$InsP_5$ (i.e., a triphosphate). This alternate option was shown to be correct, after the publication of an impressive analytical study (Draskovic et al., 2008). As if this subject were not complicated enough, PPP-$InsP_5$ can be even further phosphorylated, perhaps to PPPP-$InsP_5$ (Draskovic et al., 2008). However, these are all data acquired from experiments that were performed in vitro. So, it has to be asked, do these more esoteric tri- and tetraphosphate derivatives occur in vivo, or are they merely by-products of the availability of excessive quantities of recombinant enzyme and overindulgent incubation times in vitro? This question has not yet been answered satisfactorily. In part, this may reflect technical issues; Losito et al. (2009) have found that PPP-$InsP_5$ may not be quantitatively recovered from a strong anion exchange HPLC column. It is possible, then, that some of this material is lost from HPLC assays of cell extracts. Nevertheless, some [^{3}H]inositol-labeled molecules of approximately the appropriate polarity have been observed upon HPLC analysis of a strain of *Saccharomyces cerevisiae* lacking its endogenous $InsP_6$ kinase but transformed with a human $InsP_6$ kinase gene (Draskovic et al., 2008). It still needs to be directly determined whether these compounds are actually tri- and tetraphosphates, as opposed to some of the multiply diphosphorylated derivatives that the $InsP_6$ kinase can also synthesize, from $InsP_5$ for example (Draskovic et al., 2008). More importantly, we must also be cautious in attributing biological relevance to an experiment involving supraphysiological, heterologous gene expression, and the deletion of an endogenous gene, $InsP_6$ kinase, that has many phenotypic consequences (see below).

To return to the subject of decrees from IUPAC, in this review I will agree with their contention that "Ins" is a more intuitive abbreviation for inositol than is "I" (IUPAC-IUB Commission on Biochemical Nomenclature, 1969), although both are widely deployed elsewhere (that is, inositol hexakisphosphate is written here as "$InsP_6$" rather than "IP_6"). Another point to emphasize is that the diphosphorylated derivatives of $InsP_6$ [i.e., PP-$InsP_5$ and $(PP)_2$-$InsP_4$], are frequently abbreviated in the literature to "IP_7" and "IP_8," respectively. This terminology is avoided here because it, too, can introduce confusion. For example, the aforementioned PPP-$InsP_5$ contains eight phosphates but is clearly different from $(PP)_2$-$InsP_4$ (Fig. 3). Furthermore, one might argue that the inherent inadequacies of "IP_7" as a nomenclature have led it occasionally—and incorrectly—to be defined as either a "heptaphosphate" (Mishra and Bhalla, 2002) (i.e., an entirely mythical beast endowed with a chain of seven phosphates) or a "heptakisphosphate" (Lee et al., 2007) (which would require seven phosphates to each be individually attached to a carbon—impossible, of course, because inositol has only six carbons). The usage of "IP_7/IP_8" should also be avoided because it can obscure the fact that $InsP_6$ is not the only precursor for this class of compounds; PP-$InsP_4$ is a member of this family, but it is synthesized from $InsP_5$ (Fig. 3).

Another issue to be cognizant of are the different names that have been used at various times to describe the enzymes that metabolize the diphosphoinositol polyphosphates (Table 1). For example, although the phosphatases that degrade these molecules are known in the field as DIPPs (diphosphoinositol polyphosphate phosphohydrolases; Safrany et al., 1998) the Human Genome Organization Gene Nomenclature Committee (HGNC) has allocated them to the NUDT family [NUDT = nudix (nucleoside diphosphate attached moiety 'x')-type motif], in honor of their eponymous catalytic site [Gx_5Ex_5[UA]xREx_2EExGU, or similar, in which U is an aliphatic, hydrophobic residue (McLennan, 2006)]. The yeast gene is named Ddp1.

The HGNC originally proposed that "IHPK" (for inositol hexakisphosphate kinase) be used to designate the three members of the human gene family that phosphorylate $InsP_6$ and $InsP_5$ to 5-PP-$InsP_5$ and 5-PP-$InsP_4$, respectively (Fig. 3). However, in deference to more common usage, the HGNC has now adopted *IP6K* as the name for this gene family (Table 1). The orthologous yeast gene is *Kcs1* (Saiardi et al., 1999). The mammalian IP6Ks—there are three isoforms—

TABLE 1
Diphosphoinositol polyphosphate metabolism: enzyme nomenclature
There are three IP6K genes: 1, 2, and 3. There are four DIPP genes: 1, 2α, 2β, 3.

HGNC-Approved Gene Name	Catalytic Function	Other Names	*S. cerevisiae* Homolog
HISPPD2A	Kinase (and phosphatase?[a])	VIP1PPIP5K1	Vip1
HISPPD1	Kinase (and phosphatase?[a])	VIP2PPIP5K2	
IP6K#	Kinase	IHPK#	Kcs1
NUDT#	Phosphatase	DIPP#	Ddp1

[a] The protein has an "acid phosphatase"-like domain, but no catalytic activity has been reported.

have a molecular mass of 46 to 49 kDa (Saiardi et al., 1999; Schell et al., 1999; Saiardi et al., 2001b). These kinases possess a PxxxDxKxG catalytic domain, and a remote, catalytically essential SSLL (or similar) tetrapeptide. Some homologs of these kinases are rather larger. Kcs1 from *S. cerevisiae* is a 120-kDa protein (Saiardi et al., 1999). It contains leucine zippers near its C terminus that do not contribute to catalytic activity but instead are important for cell wall integrity and vacuolar biogenesis (Dubois et al., 2002). Thus, Kcs1 has other functions, and so its deletion from cells can give misleading information concerning the roles of diphosphoinositol polyphosphates.

There are separate, unrelated kinases that further phosphorylate 5-PP-$InsP_5$ to $(PP)_2$-$InsP_4$ (Shears et al., 1995). These enzymes are around 120 to 160 kDa in size, and, unlike the IP6Ks, they use an ATP-grasp catalytic domain to support kinase activity. These enzymes can also phosphorylate $InsP_6$ (Choi et al., 2007; Fridy et al., 2007; Mulugu et al., 2007), but they are not typically called "$InsP_6$ kinases," which conveniently avoids confusion with the canonical IP6K family. A recent study unequivocally determined that these particular enzymes phosphorylate the 1/3-position of the inositol ring (Lin et al., 2009). The latter work corrected an earlier tentative proposal (Mulugu et al., 2007) that the enzyme is a 4/6-kinase. Mulugu et al. (2007) was the first to describe the identify of this gene, in a study with *S. cerevisiae*, so this group retained the name (*Vip1*) that it had already been christened with, well before it was known to encode a kinase. While the latter study was in progress, our laboratory independently purified and sequenced an PP-$InsP_5$ kinase from rat brain, and we also identified the two human genes (Choi et al., 2007). York's group have also described the mammalian homologs (Fridy et al., 2007). These proteins were already listed in the genome database as HISPPD2A and HISPPD1, because they contain an acid phosphatase-like domain. The HGNC will not accept "*VIP*" as an alternative name for these genes, because that has already been allocated to a different human gene. We have proposed that they be known as PPIP5K1 and PPIP5K2 (Choi et al., 2007). This decision reflects on our conclusion that the catalytic preference of this kinase in vitro—in mammals at least—is to phosphorylate 5-PP-$InsP_5$ rather than $InsP_6$ (Choi et al., 2007). A recent pharmacological study is also consistent with the concept that 5-PP-$InsP_5$ is the main substrate in vivo (again, in mammalian cells) (Padmanabhan et al., 2009). In addition, when we overexpressed a kinase-dead mutant version of PPIP5K1 in human embryonic kidney cells, we saw no decrease in the cellular levels of any inositol phosphate (Choi et al., 2007), which argues against the proposition that the phosphatase domain has significant hydrolytic activity against inositol phosphatases in vivo.

Despite the substrate preference of the human enzyme that is explicitly implied by the PPIP5K1/2 nomenclature, it should be noted that the phosphorylation of $InsP_6$ to 1/3-PP-$InsP_5$ is in itself functionally significant, at least in yeasts and plants. For example, in *S. cerevisiae* 1/3-PP-$InsP_5$ regulates cyclin kinase activity (Lee et al., 2007; Lee et al., 2008). *Arabidopsis thaliana* appears to encode homologs of Vip1/PPIP5K in its genome, but not homologs of IP6K. Thus, Vip/PPIP5K may be the *only* source of PP-$InsP_5$ in some organisms.

Incidentally, the uncertainty over whether the diphosphate in this isomer of PP-$InsP_5$ is positioned at either the 1 or 3 position (hence the "1/3" designation; see Fig. 3) reflects an analytical impediment created by the axis of symmetry in the inositol ring (see Fig. 1). Thus, 1-PP-$InsP_5$ and 3-PP-$InsP_5$ are an enantiomeric pair, so a stereoselective technique is required to distinguish between them.

Cellular Levels, Metabolic Turnover, and the Issue of Compartmentalization

If the diphosphoinositol polyphosphates are to act as cellular signals, then the levels of one or more of this family of molecules should respond to some defined intra- or extracellular stimulus. The slime-molds, in particular *Dictyostelium discoideum* (Watts, 1984), provide a singularly dramatic example of such a phenomenon. The bacteria-munching, amoeboid form of *D. discoideum* contains approximately 10 μM concentrations of both PP-$InsP_5$ and $(PP)_2$-$InsP_4$ (Laussmann et al., 2000). When the cells are starved of their bacteria food supply, the amoeboid cells aggregate, the first of a series of morphogenetic stages that culminate in the formation of a fruiting body (Watts, 1984). During the initial aggregation process, the levels of PP-$InsP_5$ and $(PP)_2$-$InsP_4$ increase to 100 and 250 μM, respectively (Laussmann et al., 2000; Luo et al., 2003). Perhaps slime molds use these exceptionally high levels of diphosphoinositol polyphosphates as "high-energy" phosphate donors. However, these organisms are not a representative eukaryotic cell model for studying the turnover and biological functions of the diphosphoinositol polyphosphates. For a start, slime molds synthesize both 6-PP-$InsP_5$ and 5,6-$(PP)_2$-$InsP_4$ (Laussmann et al., 1996, 1997), neither of which is found in yeast and mammalian cells; these cells instead predominantly contain 1/3-PP-$InsP_5$, 5-PP-$InsP_5$, and 1/3,5-$[PP]_2$-$InsP_4$ (Albert et al., 1997; Lin et al., 2009). In addition, there are much lower concentrations of diphosphoinositol polyphosphates in yeast and mammalian cells. Levels of PP-$InsP_5$ usually lie in the 1 to 5 μM range (Fisher et al., 2002; Ingram et al., 2003; Barker et al., 2004; Bennett et al., 2006; Illies et al., 2007). The levels of PP-$InsP_4$ and $(PP)_2$-$InsP_4$ are even lower in yeast and mammalian cells, each only approximately 10 to 20% of those of PP-$InsP_5$ (Glennon and Shears, 1993; Choi et al., 2005, 2008).

The fact that most eukaryotic cells normally contain low levels of diphosphoinositol polyphosphates does not preclude these molecules from being cellular signals. Similar concentrations are found for other bioactive inositol phosphates, such as Ins(1,4,5)P_3 (Streb et al., 1983) and Ins(1,3,4,5)P_4 (Huang et al., 2007b). The issue is that such low levels add an additional layer of difficulty to the technical challenges involved in accurately measuring stimulus-dependent changes in the turnover of the diphosphoinositol polyphosphates. Currently, diphosphoinositol polyphosphates can only be quantified after their individual separation by HPLC analysis of cell extracts. There is a nonradioactive, automated, in-line HPLC assay for inositol phosphates involving a metal-dye complex (Mayr, 1988); this technique has been used to assay PP-$InsP_5$ in mammalian cells (Albert et al., 1997), but it is not quite sensitive enough to record $(PP)_2$-$InsP_4$ turnover (Lin et al., 2009). Thus, most groups assay cellular turnover of the diphosphoinositol polyphosphates after they are first radiolabeled by incubating cells with [^{3}H]inositol until isotopic equilibrium is reached (Shears, 1997). Unfortunately, this procedure takes several days (Shears, 1997). Moreover,

unless appropriate precautions are taken, the diphosphoinositol polyphosphates may not be quantitatively recovered from cell lysates, which obviously affects the data that are obtained. For example, we add 1 mM EDTA to the media used to quench and extract the cells, and also to the HPLC elution buffers, so as to chelate trace quantities of trivalent cations that otherwise can cause the diphosphoinositol polyphosphates to precipitate out of solution and be lost from the analysis. When counting the radioactivity in the HPLC eluate, some laboratories routinely use in-line scintillation counters (e.g., see Fridy et al., 2007), but our experience has been that the short count-times that are an inherent aspect of this equipment is a hindrance to the accurate assessment of the especially low cellular levels of $(PP)_2$-[^{3}H]$InsP_4$.We are also concerned that a homogeneous fluid phase may not be achieved immediately upon in-line mixing of the scintillation fluid with the high-salt HPLC buffers, which could decrease counting efficiency at approximately the point in the gradient where $(PP)_2$-[^{3}H]$InsP_4$ is normally eluted. Thus, we (Safrany and Shears, 1998) and others (Azevedo and Saiardi, 2006), believe that it is more accurate to direct the HPLC eluate to a fraction collector, manually add scintillant to each fraction, mix vigorously, and then use a traditional counter. Anyone who doubts the value of our precautions should note how difficult it was to detect changes in PP-$InsP_5$ and $(PP)_2$-$InsP_4$ levels when an in-line counter was used to assess the effects of overexpression of full-length human PP-$InsP_5$ kinase in human embryonic kidney cells (Fridy et al., 2007). In contrast, in similar experiments with the same cell line, we (Choi et al., 2007) collected and analyzed individual fractions of HPLC eluate, and we reported that $(PP)_2$-[^{3}H]$InsP_4$ levels increased 10-fold after overexpression of the PP-$InsP_5$ kinase. This point is emphasized to illustrate how varying interpretations of the biological function of an enzyme could result from practical differences in the analytical procedures that are used.

Another approach that can assist the analysis of turnover of diphosphoinositol polyphosphates in an intact mammalian model system is to use the DDT_1-MF_2 hamster vas deferens smooth muscle cell-line (Safrany and Shears, 1998) because these cells contain approximately 10-fold higher levels of diphosphoinositol polyphosphates than are present in other mammalian cells. Others (Fridy et al., 2007; Otto et al., 2007) have successfully used genetic approaches to elevate the cellular levels of diphosphoinositol polyphosphates and thereby facilitate the analysis of their cellular turnover; this group achieved that goal by overexpression of both $InsP_5$ kinase and a G-protein ($G\alpha_q$) that activates PLC.

As noted above and in Fig. 3, two classes of enzymes (IP6K and VIP/PPIP5K) cooperate to provide two routes by which $(PP)_2$-$InsP_4$ can be synthesized from $InsP_6$ in yeast and mammalian cells. These two pathways use either 5-PP-$InsP_5$ or 1/3-PP-$InsP_5$ as an intermediate (Fig. 3). Which of these is quantitatively the most important route? This is not an easy question to answer with certainty. HPLC analyses (Albert et al., 1997) indicate that 5-PP-$InsP_5$ is the major PP-$InsP_5$ isomer to accumulate in mammalian cells. The situation is probably the same in yeast, because deletion of Kcs1, which synthesizes 5-PP-$InsP_5$ (Draskovic et al., 2008), is associated with >80% reduction in PP-$InsP_5$ levels (Saiardi et al., 2000). Therefore, even though most of the previously published assays of PP-$InsP_5$ in intact yeast and mammalian cells did not distinguish which isomer was being studied, one can safely assume it was predominantly 5-PP-$InsP_5$. However, the steady-state levels of a metabolic intermediate are not necessarily reflective of its rate of metabolism. In addition, the nature of the PP-$InsP_5$ isomers that accumulate in cells reflects upon both $(PP)_2$-$InsP_4$ synthesis *and* its metabolism. For example, when presented with 1/3,5-$(PP)_2$-$InsP_4$ as a substrate, DIPPs prefer to hydrolyze the diphosphate that is added by PPIP5K (Shears et al., 1995), which we now know to be the 1/3-diphosphate (Lin et al., 2009). A positional preference of DIPPs for one of the two diphosphate groups will clearly influence which PP-$InsP_5$ isomers are formed after $(PP)_2$-$InsP_4$ dephosphorylation (see Fig. 3). This phenomenon may help explain why the activities of the DIPPs can differentially "mask" the rates of flux through the two different PP-$InsP_5$ isomers during $(PP)_2$-$InsP_4$ synthesis (Padmanabhan et al., 2009). Nevertheless, there are two good reasons for proposing that the major pathway of $(PP)_2$-$InsP_4$ synthesis goes through 5-PP-$InsP_5$ in mammalian cells. First, kinetic data inform us that, in vitro, the human VIP/PPIP5Ks prefer to phosphorylate 5-PP-$InsP_5$ over $InsP_6$ (Choi et al., 2007). Second, there are pharmacological data that support this proposal: TNP has been used as a cell-permeant and selective inhibitor of IP6Ks without affecting the VIP/PPIP5K enzymes (Padmanabhan et al., 2009). The addition of TNP to either HeLa cells or to *S. cerevisiae* lowered the cellular levels of both $(PP)_2$-$InsP_4$ and PP-$InsP_5$ (Padmanabhan et al., 2009). These data are consistent with the idea that 5-PP-$InsP_5$ (synthesized by the TNP-sensitive IP6Ks) is the most important precursor for $(PP)_2$-$InsP_4$ (see Fig. 3), at least in "resting" cells. The situation may be different after one or more of the enzymes is either down- or up-regulated, such as occurs after hyperosmotic stress (Pesesse et al., 2004), for example.

The DIPPs are very active enzymes: four mammalian genes have been cloned, all of which encode proteins of around 20 kDa in size: type 1 (Safrany et al., 1998; Chu et al., 2004), types 2α/2β (Caffrey et al., 2000; Hua et al., 2001), and types 3α/3β (Hidaka et al., 2002; Leslie et al., 2002; Hua et al., 2003). The specificity constants (k_{cat}/K_m) for these enzymes range in value from 2×10^5 to 5×10^7 M^{-1} s^{-1}, the latter being close to the limit for diffusion-controlled encounter between enzyme and substrate (Fersht, 1985). With DIPP activities such as these, we should perhaps wonder why the cellular concentrations of diphosphoinositol polyphosphates are actually as *high* as they are! This high metabolic turnover of the diphosphoinositol polyphosphates becomes evident when cells are incubated with 1 mM fluoride, which inhibits DIPP activities (Menniti et al., 1993; Safrany and Shears, 1998). This pharmacological maneuver causes rapid, manyfold increases in the levels of diphosphoinositol polyphosphates (Menniti et al., 1993; Safrany and Shears, 1998). In the absence of fluoride, high ongoing metabolism may impede our ability to detect subtle changes in its rate, especially if metabolic regulation were to be compartmentalized to certain regions of the cell. However, fluoride is of limited practical use as a pharmacological tool, because it is so nonspecific. It is a well known protein phosphatase inhibitor; by perturbing the activities of numerous enzymes regulated by phosphorylation/dephosphorylation cycles, fluoride affects many signaling processes.

Because the DIPPs are so active, their acute spatiotempo-

ral regulation could greatly affect the turnover of the diphosphoinositol polyphosphates. However, there is no evidence that this is exploited as a short-term signaling mechanism; no covalent modification of DIPPs has been found (Safrany et al., 1998). Furthermore, studies with GFP-DIPP constructs offer no evidence of their compartmentalization; the fusion proteins were uniformly distributed throughout the cell (Leslie et al., 2002; K. Choi, S. B. Shears, unpublished data). As for the intracellular distribution of *endogenous* DIPPs, antibodies with sufficient specificity and sensitivity for such studies are not currently available.

This current lack of evidence that DIPPs might be compartmentalized affects the idea, noted later in this review and elsewhere (Burton et al., 2009), that a particular action of a diphosphoinositol polyphosphate could be facilitated if there were to be a localized elevation in its concentration. To explain the latter point, consider the example shown by another intracellular signal, cAMP. There seems little doubt that the nonuniform distribution of cAMP through the cell (i.e., its compartmentalization) is strictly dependent upon the physical separation of the "on-switch" (adenylyl cyclase) from the "off-switch" (cAMP phosphodiesterase) (Chen et al., 2008). By analogy, it is arguable that there is little possibility of there being locally elevated concentrations of diphosphoinositol polyphosphates if the highly active DIPPs are uniformly distributed throughout the cell. This last observation also makes it unclear whether a significant localized accumulation of any diphosphoinositol polyphosphates can arise from the compartmentalization of IP6K2 in the nucleus (as indicated upon overexpression of a GFP-IP6K2 fusion construct; Saiardi et al., 2001b). On the other hand, the VIP/PPIP5Ks are excluded from the nucleus (Choi et al., 2007; Fridy et al., 2007), indicating that the products of these particular kinases are probably present in lower amounts in the nucleus compared with the cytoplasm.

One possibility that has yet to be addressed is whether there might be longer-term regulation of the levels of diphosphoinositol polyphosphates through regulated changes in the degree of *DIPP* expression. For example, several DIPP2 mRNA transcripts use multiple and noncanonical polyadenylation signals (Caffrey et al., 2000). This situation is often an indicator of cells using translational control mechanisms to regulate gene expression (Edwalds-Gilbert et al., 1997). The exceptionally high G/C content of the 5′-untranslated regions of the DIPP1 and DIPP2 mRNAs (Safrany et al., 1998; Caffrey et al., 2000) may also influence mRNA stability (Kozak, 1996) and hence gene expression. Incidentally, the expression of DIPP2α and DIPP2β from a single gene relies upon an unusual mode of alternate splicing that we christened "intron boundary skidding" (Caffrey and Shears, 2001). Finally, the expression of two *DIPP3* genes (Hua et al., 2003) is of some interest because it seems to represent rare evidence in support of the concept (Force et al., 1999) of subfunctionalization after gene duplication.

Another factor that might contribute to the cellular turnover of the diphosphoinositol polyphosphates is their ability to directly phosphorylate proteins (Saiardi et al., 2004; Bhandari et al., 2007). However, current evidence indicates that there is a relatively slow-acting "off-switch" for this process: phosphatase-directed cleavage of the phosphorylated protein (Bhandari et al., 2008; Burton et al., 2009). It has been suggested that this is a manifestation of a long-lived signaling process (Burton et al., 2009). If this were the case, it would greatly limit the availability of protein substrate for phosphorylation, so that the rate of consumption of diphosphoinositol polyphosphates for this process might not be significant in relation to their ongoing high rate of metabolism by the DIPPs.

The Relationship between Cellular Bioenergetic Status and Cellular Levels of Diphosphoinositol Polyphosphates

As mentioned above, some cellular signals do not respond exclusively to extrinsic agents. Signaling activities also originate from within the cell. For example, cells use biosensors to detect variations in energy balance and subsequently communicate this information to other cellular networks, which then initiate adaptive responses. One of the most sensitive indicators of bioenergetic balance is the AMP-activated protein kinase (AMPK), which is activated by an elevated [AMP]/[ATP] ratio (Hardie, 2004). Increased [AMP] renders AMPK susceptible to activation by phosphorylation, whereupon AMPK quickly inhibits ATP-consuming anabolic processes (protein synthesis, gluconeogenesis, fatty acid synthesis) and simultaneously activates ATP-generating, catabolic pathways (glycolysis, fatty acid oxidation) (Hardie, 2004). In this way, AMPK acts to restore an appropriate cellular "energy balance." Phosphorylation and activation of AMPK are largely driven by the kinases's ultrasensitivity to just small increases in [AMP] (Hardie, 2004), which in turn are considered to represent a state of "mild energy depletion" (Inoki et al., 2003). This terminology usefully distinguishes this situation from the more severe energy stress that arises after a substantial decrease in cellular ATP levels (Browne and Proud, 2002; Inoki et al., 2003). The latter would typically only be expected to occur under rather extreme conditions such as during muscle fatigue, for example, or during an acute stress such as ischemia (Hochachka, 1999). These more significant decreases in [ATP] also prompt additional stress sentinels to become activated (Zhuo et al., 2005).

There is a pharmacological tool for studying the actions of cellular sentinels that react to increases in [AMP]: 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). Upon its uptake into cells, AICAR is phosphorylated to 5-amino-4-imidazolecarboxamide riboside monophosphate, an AMP mimetic that, for example, activates AMPK (Sabina et al., 1985; Merrill et al., 1997). It should be emphasized that this experimental approach is designed to invoke relatively mild energy stress (that is, an increase in [AMP]) without there being a significant effect upon [ATP]. We (Choi et al., 2008) have recently reported that the treatment of cells with AICAR causes a reduction in the cellular levels of $(PP)_2$-$InsP_4$. This effect was most easily detected in experiments with the DDT_1-MF_2 smooth muscle cell line (Choi et al., 2008), in which "resting" levels of $(PP)_2$-$InsP_4$ are unusually high (Safrany and Shears, 1998). In other cell types, the levels of $(PP)_2$-$InsP_4$ are often too low to accurately assess inhibition of its synthesis. In these other cell types, this inhibitory effect of AICAR was most readily observed when the drug was used to antagonize the severalfold elevation in $(PP)_2$-$InsP_4$ levels that normally accompanies hyperosmotic stress (Pesesse et al., 2004; Choi et al., 2008). Incidentally, this osmotic response is an area of study that could benefit from further research, because we do not yet understand the mechanisms that are involved or the biological significance.

We have previously argued that the degree of osmotic stress that activates $(PP)_2$-$InsP_4$ synthesis lies within the biologically relevant range of conditions to which cells are normally exposed (Yang et al., 2008).

The mechanism by which increases in [AMP] inhibit $(PP)_2$-$InsP_4$ synthesis is still unknown, although Choi et al. (2008) unexpectedly found that the inhibition of $(PP)_2$-$InsP_4$ synthesis after the incubation of cells with AICAR is independent of its canonical target, AMPK (Choi et al., 2008). So, clearly, there must be other proteins that can respond to the changes in [AMP] that AICAR—through its conversion to 5-amino-4-imidazolecarboxamide riboside monophosphate—is considered to mimic. If we are to look for candidates, we should consider that the AMP-binding cystathionine-β-synthase module present in AMPK also occurs in a large range of diverse proteins, including ATP-binding cassette transporters, voltage-gated chloride channels and transporters, a variety of other transporter families, and a number of enzymes (Biemans-Oldehinkel et al., 2006). We (Choi et al., 2008) have also perturbed [AMP] by incubating cells with the mitochondrial poison oligomycin, but in the presence of glucose to sustain glycolytic ATP synthesis. Again, the relatively mild energy-stress that ensued was associated with a decline in $(PP)_2$-$InsP_4$ synthesis (Choi et al., 2008). These observations led us (Choi et al., 2008) to propose that the levels and hence the signaling strength of $(PP)_2$-$InsP_4$ might provide a novel link between signaling and bioenergetic networks. In other words, $(PP)_2$-$InsP_4$ was hypothesized to be a metabolic messenger.

It seems likely that the activities of the VIP/PPIP5K proteins may be regulated, and now that these enzymes have been cloned (Choi et al., 2007; Fridy et al., 2007; Mulugu et al., 2007), we hope that mechanistic information will shortly be forthcoming. The regulation of VIP/PPIP5K activity may not only involve the kinase domain by itself, which only accounts for the N-terminal one-third of the entire protein (Choi et al., 2007; Fridy et al., 2007; Mulugu et al., 2007). In addition, the VIP/PPIP5Ks also possess an apparently catalytically inactive phosphatase domain followed by long C termini (Choi et al., 2007; Fridy et al., 2007; Mulugu et al., 2007). Perhaps these are regulatory domains? Because regulation of protein function by phosphorylation is such a widespread regulatory process, recent advances in phosphoproteomic methods (Blackburn and Goshe, 2009) may help us gain insight into the regulation of VIP/PPIP5K activity. For example, such techniques have already demonstrated that EGF treatment of HeLa cells increases the degree of phosphorylation of Ser475 in the phosphatase domain of PPIP5K1 (Olsen et al., 2006).

The cellular levels of $(PP)_2$-$InsP_4$ are reduced after cell treatment with either of three MEK inhibitors: U0126, PD98059, or PD184352 (Pesesse et al., 2004; Choi et al., 2005, 2008). It is important to note that this inhibition of $(PP)_2$-$InsP_4$ synthesis is not a result of blockade of the MEK/extracellular signal-regulated kinase cascade. We can make this assertion because the efficient "knock-down" of either MEK or extracellular signal-regulated kinase using RNA interference had no impact upon $(PP)_2$-$InsP_4$ levels (Choi et al., 2008). Instead, it has turned out that all three of the MEK inhibitors have a puzzling "off-target" effect of somehow impairing cellular energy homeostasis (Dokladda et al., 2005; Choi et al., 2008). Again, these effects upon energetic status are relatively mild, with the MEK inhibitors primarily elevating [AMP] and not altering [ATP]. Although we cannot yet explain this phenomenon, it does provide additional pharmacological evidence of the close association between $(PP)_2$-$InsP_4$ levels and cellular energy status (Choi et al., 2008). This is also a remarkable turn of events for three such structurally dissimilar drugs that, until recently, were considered to be quite specific MEK inhibitors (Dokladda et al., 2005; Bain et al., 2007).

This idea that the cellular levels of the diphosphoinositol polyphosphates might be dependent upon the metabolic health of the cell can help us view in a new light an otherwise puzzling observation: the inhibition of cellular synthesis of $(PP)_2$-$InsP_4$ after elevations in intracellular [cAMP] (Safrany and Shears, 1998). By using several commercially available cAMP analogs as pharmacological tools, we have excluded both protein kinase A (Safrany and Shears, 1998) and exchange protein directly activated by cyclic AMP (K. Hidaka, K. Choi, and S. B. Shears, unpublished data) from mediating the inhibition of $(PP)_2$-$InsP_4$ synthesis by cAMP. We are still searching for another mechanistic explanation. Perhaps $(PP)_2$-$InsP_4$ metabolism is controlled by a metabolic sensor that poorly discriminates between cAMP and AMP? This sensor, if it were to exist, could not be AMPK, because as noted above, this particular kinase does not affect $(PP)_2$-$InsP_4$ synthesis. In any case, it is known that cAMP cannot activate AMPK directly (Carling et al., 1989; Henin et al., 1996). Despite that, increases in cellular cAMP do lead to an activation of AMPK in adipocytes (Yin et al., 2003; Daval et al., 2005). A similar effect occurs in DDT_1-MF_2 cells (K. Choi and S. B. Shears, unpublished data). This puzzling but reproducible phenomenon has apparently received little subsequent attention. Is it possible that a sustained elevation in cAMP might somehow stress the energy status of the cell? For example, others (Epperson et al., 2005) have hypothesized that, in those cells that have high levels of cAMP generation and metabolism, this might generate sufficient AMP to activate a sensor of energy-stress; Epperson et al. (2005) of course thought that this sensor would be AMPK, but we could speculate further that maybe $(PP)_2$-$InsP_4$ synthesis might also be regulated by some other entity that reacts to the AMP that is inevitably produced from cAMP. In any case, theses are reasons to suggest that the response of $(PP)_2$-$InsP_4$ levels to cAMP reflects the roles of diphosphoinositol polyphosphates as metabolic signals.

While an increased [AMP] may be considered symptomatic of a relatively mild bioenergetic challenge, a decline in [ATP] places a considerably greater metabolic stress upon cells. A number of energy-homeostasis processes respond to changes in cellular ATP levels (for example, see Ruiz-Stewart et al., 2004; Zhuo et al., 2005). Recent work in our laboratory suggests that this list should now include diphosphoinositol polyphosphates: we (K. Choi and S. B. Shears, unpublished data) have found that an oligomycin-induced, 15% decrease in cellular [ATP] elicits a 50% drop in $(PP)_2$-$InsP_4$ levels in the DDT_1 MF-2 smooth muscle cell-line (we do not yet understand the mechanism underlying this effect). In addition, in the same experiments, there was also a 15% decrease in PP-$InsP_5$ levels (K. Choi and S. B. Shears, unpublished data; as discussed above, almost all of the PP-$InsP_5$ that accumulates in mammalian cells is the 5-PP-$InsP_5$ isomer). In earlier experiments in which [ATP] levels were perturbed by cell

treatment with antimycin, the concentration of PP-$InsP_5$ decreased much faster ($t_{1/2}$ = 5 min) than did the levels of all of the other inositol phosphates (Oliver et al., 1992). The explanation for these rapid responses of PP-$InsP_5$ to a drop in cellular [ATP] may lie in the IP6Ks exhibiting a relatively high K_m value of approximately 1 mM for ATP (Voglmaier et al., 1996; Saiardi et al., 1999), which lies within the range of values (1–5 mM) usually accredited to the cytosolic [ATP] in mammalian cells (Soboll et al., 1978). Just to emphasize how unusual this situation is, it should be noted that the $InsP_4$ and $InsP_5$ kinases display a K_m for ATP that is less than 100 μM (Tan et al., 1997; Verbsky et al., 2002). In other words, IP6Ks have an atypically low affinity for ATP that could make 5-PP-$InsP_5$ synthesis especially sensitive to fluctuations in cytosolic [ATP].

The high K_m of the IP6Ks for ATP might also explain an otherwise puzzling observation that the synthesis of both PP-$InsP_4$ and PP-$InsP_5$ synthesis is inhibited by 20 to 50% after short-term (<2 h) treatment of liver cells with thapsigargin (Glennon and Shears, 1993; Padmanabhan et al., 2009). Perhaps IP6K activity decreases in direct response to the decline in [ATP] levels that others have shown can accompany cell-treatment with thapsigargin (Waring and Beaver, 1996). Furthermore, others (Tamás et al., 2006) have shown thapsigargin to activate the AMPK cascade. This phenomenon was suggested to represent a homeostatic adjustment to an increased energy-demand after activation of Ca^{2+}-dependent signaling pathways (Tamás et al., 2006). In any case, the latter study provides a context for understanding why the levels of a metabolic messenger might respond to thapsigargin. Yet again, a previously inexplicable behavior of a diphosphoinositol polyphosphate can be rationalized by considering the molecule to be a metabolic messenger.

What might be the biological consequences of this decrease in the levels of 5-PP-$InsP_5$ and $(PP)_2$-$InsP_4$ that seems to accompany a decline in the bioenergetic health of the cell? It should first be noted that a reduction in the rate of synthesis of diphosphoinositol polyphosphates will, just by itself, reduce cellular ATP consumption. This in itself might help defend cellular energy balance. It is also known from genetic work with yeasts that when the synthesis of the diphosphoinositol polyphosphates is blocked, the cells grow more slowly (Saiardi et al., 2000), and there are defects in endocytic trafficking (Saiardi et al., 2002) and DNA repair (Luo et al., 2002). These are biological processes that normally consume significant quantities of cellular energy. If these cellular activities are constrained in response to reduced levels of diphosphoinositol polyphosphates, that might provide a mechanism for energy conservation when the cell is bioenergetically challenged. Clearly, these are speculative ideas at present. We need to obtain more precise information on the biological actions of diphosphoinositol polyphosphates, particularly in mammalian cells. This is essential to future progress in this field of research.

Because the levels of diphosphoinositol polyphosphates seem to be so closely tied to the bioenergetic status of the cell (see above), we should also consider what might be the biological impact if the levels of the polyphosphates were elevated after an *increase* in the cellular [ATP]. For one possible explanation, consider pancreatic β-cells. These cells are metabolically specialized so that cellular [ATP]/[ADP] ratios increase dramatically in response to elevations in serum glucose levels (Detimary et al., 1998). The unusual sensitivity of IP6Ks to fluctuations in cellular [ATP] would seem to be an appropriate mechanism by which cellular 5-PP-$InsP_5$ concentration in β-cells could be tied to serum glucose levels. This could be an important topic to pursue because 5-PP-$InsP_5$ has been shown to stimulate insulin release, in an impressive series of electrophysiological experiments (Illies et al., 2007). More recently, mice in which the *IP6K1* was deleted were found to have reduced levels of circulating insulin (Bhandari et al., 2008), and TNP-mediated inhibition of IP6K was shown to reduce insulin secretion from the Min6 pancreatoma cell line (Padmanabhan et al., 2009). The 5-PP-$InsP_5$ enhances secretion of the hormone by increasing the size of the rapidly releasable pool of insulin vesicles (Illies et al., 2007). This subset of granules is near the plasma membrane, where they are poised to undergo exocytosis as soon as the cell receives the appropriate stimulus (e.g., elevated glucose). This process, which drives the initial phase of insulin secretion, was enhanced either by overexpression of IP6K or by direct application of PP-$InsP_5$ in a dose-dependent manner over the physiologically relevant concentration range of 1 to 10 μM (Illies et al., 2007). Any isomer of PP-$InsP_5$ was able to elicit an increase in insulin secretion (Illies et al., 2007), which has led to speculation that PP-$InsP_5$ acts by phosphorylating some (as-yet undefined) target protein, because, as discussed below, such a molecular action also promises not to be restricted to any particular isomer of PP-$InsP_5$. The lack of any specificity for PP-$InsP_5$ suggests that $(PP)_2$-$InsP_4$ might also stimulate insulin secretion, but this has yet to be tested. In any case, we can conclude that organismal energy metabolism is regulated by the diphosphoinositol polyphosphates.

The idea that cellular levels of the diphosphoinositol polyphosphates might reflect cellular bioenergetic status adds a new context to recent observations that PP-$InsP_4$ levels influence telomere length, in yeast at least (Saiardi et al., 2005; York et al., 2005). The shortening of telomeres is a topic of interest because it accompanies the inevitable process of cellular senescence in somatic cells (Herbig et al., 2006; Feldser and Greider, 2007). This quite normal biological process is beneficial in an animal's early life because it has tumor-suppressive properties (Sedivy, 2007). However, cellular senescence is detrimental in older animals because it contributes to the functional decline of various organ systems (i.e., the ageing process) by inexorably depleting tissues of the functional cells that are required to maintain organ homeostasis. The timing of this telomere-dependent phenomenon of "good things going bad" (Campisi and d'Adda di Fagagna, 2007), or, more technically, "antagonistic pleiotropy" (Shayman and Kirkwood, 1987), is increasingly recognized to be influenced by environmental stress (Rando, 2006; Sedivy, 2007) and, notably, cellular energy status (Narala et al., 2008); cellular senescence is associated with a decline in bioenergetic health (Wang et al., 2003; Zwerschke et al., 2003). The observation that telomere length in yeast is also correlated with the synthesis of very long-chain fatty acids (Ponnusamy et al., 2008) makes this subject even more intriguing because, in mammals, systemic energy balance is influenced by circulating long-chain fatty acids (Lam et al., 2005). It could be productive to study whether there is a connection between PP-$InsP_4$ levels and telomere length in animal cells that reflects interplay between cellular bioenergetic homeostasis and the diphosphoinositol polyphosphates.

It is also worth noting that some of the proteins that participate in telomere homeostasis [for example the highly conserved REM (MRE11/RAD50/NBN) complex] also function to regulate DNA repair (Czornak et al., 2008). This observation suggests that it is not coincidental that both telomere maintenance (Saiardi et al., 2005; York et al., 2005) and DNA repair (Luo et al., 2002) require input from the diphosphoinositol polyphosphates; perhaps the polyphosphates act upon both processes through a common mechanism. This could be an important topic, especially as DNA repair is an energy-intensive process that has to be coordinated with the mechanisms that maintain cellular energy homeostasis (Olovnikov et al., 2009).

So far, we have discussed circumstances in which the levels of diphosphoinositol polyphosphates positively correlate with the metabolic health of the cell, as described by the levels of ATP and AMP. It is noteworthy that apoptosis is an important circumstance in which the relationship between adenine nucleotides and diphosphoinositol polyphosphates can sometimes be reversed. This observation comes in part from experiments in which an ovarian carcinoma cell line was treated with cisplatin (Nagata et al., 2005), a platinum-based chemotherapeutic agent that not only cross-links DNA but also impairs cellular bioenergetic health (Rodríguez-Enríquez et al., 2009). Yet, after cisplatin treatment, the rate of synthesis of 5-PP-$InsP_5$ initially increased, apparently as a result of persistent activation of IP6K2 (Nagata et al., 2005). If an increased synthesis of PP-$InsP_5$ is normally a signal of bioenergetic health (see above), is it possible that the cisplatin-mediated activation of IP6K2 "misleads" the cell, preventing it from countering the bioenergetic stress that cisplatin has induced? In such an event, it can be appreciated that the metabolic crisis could be exacerbated, a sort of "double-whammy" for cellular energy-balance. Apoptosis is frequently the ultimate outcome of a sustained failure to adapt to metabolic stress (Jin et al., 2007). Is this why cisplatin-treated cells in which IP6K is overexpressed then succumb to apoptosis? A recent study (Morrison et al., 2009) indicated that apoptosis could even be induced by microinjection into a cell of 25 μM 5-PP-$InsP_5$ (the product of IP6K activity; Fig. 3).

Because apoptosis is recognized to be a means by which emerging cancer cells can be purged (Jin et al., 2007), perhaps IP6K activity can be exploited as an anticancer defense mechanism. Indeed, the overexpression of IP6K augments the proapoptotic actions of not only cisplatin (Nagata et al., 2005) but also other cellular stressors—etoposide, hydrogen peroxide, hypoxia (Nagata et al., 2005)—which, again, compromise bioenergetic homeostasis (Lambotte, 1977; Hara and Abiko, 1995; Rodríguez-Enríquez et al., 2009). IP6K2 also enhances the pro-apoptotic actions of interferon-β (Morrison et al., 2001; Morrison et al., 2002). It might be worth studying whether the latter effect is related to the decrease in cellular [ATP] reportedly induced by interferon-β (Lewis et al., 1996).

Type 2 IP6K might have been selected for this pro-apoptotic process because of its unique, stress-dependent relocation from the nucleus to Bax-positive (i.e., damaged) mitochondria; the type 1 and 3 IP6Ks do not show this effect (Nagata et al., 2005). However, a subsequent study failed to detect increased mitochondrial IP6K2 during apoptosis (Morrison et al., 2005). Perhaps instead the activity of the type 2 enzyme is unique among the IP6K family in being activated by certain pro-apoptotic stimuli, through covalent modification, for example (Nagata et al., 2005), or by impeding the association of IP6K2 with the 90-kDa heat shock protein, which normally inhibits the kinase activity (Chakraborty et al., 2008). IP6K2 also has the ability to bind to tumor necrosis factor receptor-associated factor-2, thereby attenuating the latter's influence over the antiapoptotic nuclear factor-κB signaling pathway (Morrison et al., 2007).

In the discussion of PP-$InsP_5$ turnover in the preceding paragraphs, we have largely discussed the relationship that the 5-PP-$InsP_5$ isomer has with cellular energy balance. In yeast, at least, the quantitatively minor 1/3-PP-$InsP_5$ isomer also has a biologically important role that is directly relevant to metabolic well being: 1/3-PP-$InsP_5$ participates in phosphate homeostasis (Lee et al., 2007). Inorganic phosphate is, of course, an essential nutrient that is required in large amounts for nucleic acid and phospholipid biosynthesis, but it is also essential for cellular energy metabolism. Lee et al. (2007) have shown that total PP-$InsP_5$ levels are upregulated in yeast cells grown for 1 to 2 h in low-phosphate media. Genetic evidence indicates that it is the 1/3-isomer of PP-$InsP_5$ that must have accumulated (Lee et al., 2007), although this was not directly confirmed. In any case (see above), the levels of the other PP-$InsP_5$ isomer, 5-PP-$InsP_5$, would not be expected to rise when cells are bioenergetically stressed, which is the expected outcome of phosphate starvation (Martinez et al., 1998). As discussed in more detail below, elevated 1/3-PP-$InsP_5$ levels inhibit a cyclin kinase activity that regulates the expression of phosphate responsive (*Pho*) gene products that are important for extracellular phosphate generation and assimilation, such as a phosphate transporter and a secreted acid phosphatase (Springer et al., 2003). Thus, the data published by Lee et al. (2007) can be considered to have provided us with a classic demonstration of the actions of a metabolic messenger: a biosensor (1/3-PP-$InsP_5$) that helps the cell avoid perturbations in energy balance when extracellular inorganic phosphate is limiting. Because the turnover of diphosphoinositol polyphosphates is quite high, the yeast cells must invest a significant amount of energy to sustain the 10-fold increase in the levels of 1/3-PP-$InsP_5$ observed during phosphate deprivation (Lee et al., 2007). However, yeasts have significant energy reserves in the form of inorganic polyphosphate, which is mobilized during the initial stages of phosphate starvation (Martinez et al., 1998). Thus, a short-term investment of cellular energy in the increased synthesis of 1/3-PP-$InsP_5$ might be justified when, hopefully, it can subsequently be repaid by improved scavenging of extracellular organic phosphates. Nevertheless, the window of opportunity for this adaptation will likely be open only for a limited time; both ATP and inorganic polyphosphate levels decline substantially after a few hours of phosphate starvation (Martinez et al., 1998), which would be expected (see above) to put severe strain upon the ability of yeasts to sustain elevated levels of diphosphoinositol polyphosphates.

As noted by others (Bennett et al., 2006), there are earlier observations (albeit less well characterized) that also speak to there being lines of communication between cellular phosphate status and diphosphoinositol polyphosphates. For example, one group uncovered the molecular identity of IP6K1 while pursuing an explanation for its ability to stimulate cellular uptake of inorganic phosphate (Schell et al., 1999).

Consistent with this observation, the *kcs1*Δ strain of *S. cerevisiae* was found to exhibit reduced inorganic phosphate uptake (Saiardi et al., 2004). Deletion of *Kcs1* has also been shown to activate the PHO system (Auesukaree et al., 2005), although in retrospect, one might now suggest that this reflects compensatory up-regulation of *Vip1* expression (for example, see York et al., 2005) and elevation of 1/3-PP-$InsP_5$ levels.

Protein Phosphorylation by Diphosphoinositol Polyphosphates?

Only relatively recently has solid information emerged that offers possible mechanisms of action of the diphosphoinositol polyphosphates. One proposal has drawn from a long-standing recognition (Stephens et al., 1993; Laussmann et al., 1996; Hand and Honek, 2007) that the hydrolysis of one of their phosphate groups must be associated with a significant free-energy change. It was consideration of this point that led Stephens et al. (1993) to hypothesize that the diphosphoinositol polyphosphates might act as phosphate donors in phosphotransferase reactions. Saiardi et al. (2004) and Bhandari et al. (2007) have actively pursued this idea. These groups have shown, at least in vitro, that all of the diphosphoinositol polyphosphates can phosphorylate certain proteins. The consensus phosphorylation site is a serine that is surrounded by acidic residues (Saiardi et al., 2004). The appropriate target sequence is especially well represented in proteins such as Nsr1 (yeast nucleolin), NOPP140, and TCOF1, which are all nucleolar residents (Saiardi et al., 2004). The primary function of the nucleolus is to synthesize ribosomes (Boisvert et al., 2007). It is therefore of interest that there is evidence of genetic interactions between ribosomal biogenesis and the synthesis of diphosphoinositol polyphosphates (Horigome et al., 2009). Moreover, ribosomal biogenesis consumes 80% of the energy expenditure of a proliferating cell (Thomas, 2000). Thus, any regulation of the rate of ribosome synthesis that might occur after changes in the phosphorylation status of nucleolar proteins could have a significant impact upon cellular bioenergetic homeostasis. This may be one means by which the actions of diphosphoinositol polyphosphates can be linked to cellular energy conservation, which, the reader may recall, I have speculated to be the *raison d'être* for their roles as metabolic messengers (see above).

The transfer of the phosphate group from the diphosphoinositol polyphosphate to a protein substrate occurs independently of protein kinase activity (Saiardi et al., 2004). This phenomenon is particularly remarkable because in the absence of enzymatic assistance, phosphoric anhydrides are considered to be chemically stable, protected by their negative charges from rapid attack by water and other nucleophiles (Westheimer, 1987). The coordination of the negative charge by Mg^{2+} seems to enable the phosphate transfer to occur (Saiardi et al., 2004; Bhandari et al., 2007). There is also a requirement that the target proteins must first be "primed" by an initial casein kinase 2-dependent phosphorylation event (Bennett et al., 2006; Bhandari et al., 2007). Furthermore, the evidence now points to the diphosphoinositol polyphosphates actually further phosphorylating the serine that is initially phosphorylated by casein kinase 2 (Bhandari et al., 2007). These data provide a provocative and novel idea concerning the molecular action of diphosphoinositol polyphosphates and unveil evidence of a novel mechanism of covalent modification: nonenzymic diphosphorylation of serine. But does this occur in vivo?

The last question is difficult to answer directly, in no small part because of considerable technical challenges. Despite ongoing improvements in the sensitivity of mass spectrometry, it remains a daunting task to identify changes in phosphorylation status of a particular protein in cell lysates (Blackburn and Goshe, 2009), let alone to distinguish between serine diphosphates and serine monophosphates in a peptide fragment. Perhaps in the future it might be possible to develop antibodies against diphosphoserine that can achieve the same goal. Until that time, only indirect approaches have been possible. For example, Saiardi et al. (2004) used yeast cells in which the $InsP_6$ kinase (Kcs1) that makes 5-PP-$InsP_5$ was genetically eliminated. They found that the degree of phosphorylation of endogenous Nsr1 was substantially reduced in these cells. This is a promising observation, but it should be noted that the deletion of Kcs1 impairs cell-wall integrity (Dubois et al., 2002) and compromises a number of yeast's normal biological processes: vacuolar biogenesis (Saiardi et al., 2000, 2002), endocytosis (Saiardi et al., 2002), stress responses (Dubois et al., 2002), and DNA recombination (Luo et al., 2002). Moreover, the expression of at least 20 yeast genes changes in response to the elimination of Kcs1 (El Alami et al., 2003). In such circumstances, a change in the phosphorylation status of Nsr1 could be argued to arise independently of PP-$InsP_5$ synthesis per se and might instead reflect the cell's adjustments to the many molecular responses to the *kcs1*Δ genotype.

Another point to discuss is that the deletion of the *Nsr1* gene in *S. cerevisiae* has been observed to be associated with a doubling of intracellular levels of PP-$InsP_5$ and $(PP)_2$-$InsP_4$ (Saiardi et al., 2004). These increases were proposed to reflect a reduced demand for diphosphoinositol polyphosphate turnover, once this proposed target of phosphorylation was eliminated (Saiardi et al., 2004). However, the validity of this proposal might now be questioned by the expansion of the number of proteins now put forward as substrates for protein phosphorylation by the diphosphoinositol polyphosphates (Bhandari et al., 2007). If there really are such a large number of protein substrates, removing just one of them would not be expected to significantly affect the cellular levels of diphosphoinositol polyphosphates, especially if the putative serine-diphosphate is long-lived (Burton et al., 2009), because this also limits the impact of the phosphorylation process upon the turnover of the phosphate donors. Once again, we should perhaps consider that the changes in levels of PP-$InsP_5$ and $(PP)_2$-$InsP_4$ in the *nsr1*Δ strain might be an indirect effect, perhaps a consequence of the accompanying growth-impaired phenotype.

Another issue to consider is that $InsP_6$ is quite an effective inhibitor of protein phosphorylation by diphosphoinositol polyphosphates (Saiardi et al., 2004). This might not always be an obstacle in the slime-molds, particularly the aggregated form of *D. discoideum*, in which levels of $(PP)_2$-$InsP_4$ match those of $InsP_6$ (Laussmann et al., 2000). Moreover, *D. discoideum* is apparently unique in its synthesis of the 5,6-diphosphate isomer of $(PP)_2$-$InsP_4$ (Laussmann et al., 2000; Lin et al., 2009); the hydrolysis of one of these vicinal diphosphates is an especially "high-energy" reaction that could facilitate phosphotransfer to proteins (Hand and Honek, 2007).

Thus, slime molds might be a singularly appropriate model in which to study the biological relevance of protein phosphorylation by diphosphoinositol polyphosphates. In contrast, because in all other eukaryotic cells the cellular levels of $InsP_6$ are at least 25-fold higher than the diphosphoinositol polyphosphates, the latter will likely only be capable of phosphorylating proteins in an a microenvironment from which $InsP_6$ is relatively excluded. This scenario is plausible. There is certainly evidence that some $InsP_6$ is divided into metabolically separated "pools" (Otto et al., 2007). Other data showing a punctate distribution of the $InsP_5$ 2-kinase within certain cellular structures such as nucleoli and stress granules also indicates that intracellular $InsP_6$ synthesis is compartmentalized (Brehm et al., 2007). On the other hand, the apparently tight metabolic equilibrium between [^{3}H]inositol-labeled pools of $InsP_6$ and PP-$InsP_5$ (Menniti et al., 1993) argues strongly that $InsP_6$ is not actually physically separated from the diphosphoinositol polyphosphates in vivo.

Using the human homolog of Nsr1—nucleolin—as a model, we (Yang et al., 2008) have searched for evidence that its phosphorylation by diphosphoinositol polyphosphates might be physiologically relevant. We made the assumption that, if Saiardi et al. (2004) and Bhandari et al. (2007) are correct, the degree of nucleolin phosphorylation should increase as the cellular levels of $(PP)_2$-$InsP_4$ and/or PP-$InsP_5$ are elevated. We also noted previous experiments demonstrating that the phosphorylation of nucleolin is associated with its transfer from the nucleolus into the nucleoplasm (Kim et al., 2005). Thus, the extent to which nucleolin accumulates in the nucleoplasm can be anticipated to provide a measure of its degree of phosphorylation. We therefore manipulated cellular levels of diphosphoinositol polyphosphates in an osteosarcoma cell line using a combination of hyperosmotic stress and some pharmacological tricks (Yang et al., 2008). We found that a hyperosmotic challenge indeed caused nucleolin to accumulate in the nucleoplasm—suggesting its degree of phosphorylation was increased—but this response occurred independently of changes in levels of diphosphoinositol polyphosphates (Yang et al., 2008). Nevertheless, our experiments with nucleolin can only be considered an indirect test of the hypothesis put forward by Saiardi et al. (2004) and Bhandari et al. (2007). Further progress in this area requires new methods to be developed that can directly detect protein diphosphorylation by diphosphoinositol polyphosphates in vivo.

One might anticipate that if diphosphoinositol polyphosphates were indeed to phosphorylate proteins in vivo, then the reverse reaction—dephosphorylation of the protein—might also be a regulated event. So far, however, no such phosphatase activity has been observed; in fact, the diphosphorylated proteins are notably resistant to dephosphorylation when added to cell lysates (Bhandari et al., 2007). This metabolic stability has been argued to be biologically significant by ensuring that signaling through this process is long-lived (Burton et al., 2009). Nevertheless, the identification of the requisite phosphatase, even if it is not very active, is essential to bolstering the credentials of this hypothesis.

Finally, it is also a little puzzling from a signaling perspective that each of the individual diphosphoinositol polyphosphates have similar abilities to phosphorylate proteins in vitro (Bhandari et al., 2007). Why should the cell invest resources in synthesizing several highly phosphorylated molecules that all have an identical mechanism of action? And, if all of the different diphosphoinositol polyphosphates act in the same way, why does the cell sometimes independently regulate their turnover (for example, see Pesesse et al., 2004; Choi et al., 2005, 2007, 2008; Lee et al., 2007)? These questions suggest that there must be other mechanisms of action of the diphosphoinositol polyphosphates. In fact, in the case of regulation by 1/3-PP-$InsP_5$ of yeast cyclin kinase activity (see below), it is almost certain that 1/3-PP-$InsP_5$ does not act by directly phosphorylating a protein. One can make that statement with some confidence because any of the diphosphoinositol polyphosphates can donate a phosphate to a protein, at least in vitro (Bhandari et al., 2007). In contrast, Lee et al. (2007) described how the kinase inhibition was relatively specific for the 1/3-isomer of PP-$InsP_5$, whereas 5-PP-$InsP_5$ was considerably less effective. Such specificity suggests the participation of a more traditional receptor-based signaling mechanism, as discussed below.

Do Cells Contain "Receptors" for Diphosphoinositol Polyphosphates?

A traditional role for an intracellular signal is for it to selectively bind to an intracellular "receptor" and thereby alter some inherent property of the protein. In a group of mid-1990 publications, we identified several PP-$InsP_5$-binding proteins; at that time, it seemed of potential interest that they all have in common a role in regulating vesicular traffic: Coatomer, AP2, and AP180 (previously sometimes called "AP3") (Fleischer et al., 1994; Shears et al., 1995; Ye et al., 1995). The affinity of AP180 for PP-$InsP_5$ was determined to be 3- to 5-fold greater than it is for $InsP_6$ (Ye et al., 1995; Saiardi et al., 2002), and these observations were considered to support the idea that PP-$InsP_5$ plays a role in endocytosis (Ye et al., 1995; Saiardi et al., 2002). However, these ligand-binding assays were performed before there was a full appreciation of the biological importance of Mg^{2+} coordinating the negative charge of these highly electronegative polyphosphates (Torres et al., 2005). Because our earlier binding assays (Fleischer et al., 1994; Shears et al., 1995; Ye et al., 1995) did not include divalent cations, the values of the binding affinities that were obtained must now be interpreted cautiously, as discussed previously (Shears, 2001; Bennett et al., 2006). Moreover, we subsequently demonstrated that the domain in AP180 that binds PP-$InsP_5$ also binds inositol lipids (Ye et al., 1995). The current consensus in the field is that the lipids are the physiologically relevant ligands, and most notably for phosphatidylinositol 4,5-bisphosphate, they are viewed as *promoting* endocytosis (Legendre-Guillemin et al., 2004). This has rather muddied the waters with regard to understanding the significance of our in vitro clathrin assembly assays, which indicated that endocytosis should be *inhibited* when AP180 binds either the lipids (Hao et al., 1997) or the diphosphoinositol polyphosphates (Ye et al., 1995).

In later ligand-binding experiments that also did not contain divalent cations, Luo et al. (2003) reported that 5-PP-$InsP_5$ bound to the PH domain of *D. discoideum* CRAC (cytosolic regulator of adenylyl cyclase) (Luo et al., 2003). The affinity of PP-$InsP_5$ was reportedly similar to that of $Ins(1,3,4,5)P_4$, which is the "headgroup" of phosphatidylinositol (3,4,5)-trisphosphate (Luo et al., 2003). This and other evidence suggested that competition between 5-PP-$InsP_5$ and

phosphatidylinositol (3,4,5)-trisphosphate for binding to this PH domain regulated the intracellular distribution of the CRAC, which is a key factor for determining the directionality of chemotaxis of *D. discoideum* (Luo et al., 2003). The fact that *D. discoideum* contains such exceptionally high levels of diphosphoinositol polyphosphates (see above) makes this hypothesis of special interest. However, it is harder to justify the biological relevance of the apparent binding of PP-$InsP_5$ to PH domains in several mammalian proteins: PKB, PIKE, and TIAM (Luo et al., 2003). The latter argument is made because, in mammalian cells, the levels of PP-$InsP_5$ are 100-fold lower than they are in *D. discoideum*. In any case, others (Downes et al., 2005) have been unable to reproduce the observation that PP-$InsP_5$ binds to PKB, at least. A separate, detailed study of the PH domain in PDK1 detected no binding of PP-$InsP_5$ (Komander et al., 2004).

Arguably the most promising—and selective—intracellular "receptor" for a diphosphoinositol polyphosphate was recently identified by Lee et al. (2007, 2008) in experiments with *S. cerevisiae*. We should note at the outset that these binding assays were not only performed in the presence of Mg^{2+}, thereby making the data more reliable (see above), but in addition the ligand binding was even found to depend upon divalent cations being present (Lee et al., 2007). In this work, 1/3-PP-$InsP_5$ was shown to bind to the Pho80/Pho85/Pho81 cyclin-dependent kinase/cyclin kinase inhibitor complex. [In an effort to spare the reader some confusion, we should note that in Lee et al. (2007, 2008), the isomer in question is said to be 4/6-PP-$InsP_5$. Only subsequently (Lin et al., 2009) was it determined to actually be the 1/3-isomer; also see above for an explanation].

The biological significance of 1/3-PP-$InsP_5$ binding to the Pho80/Pho85/Pho81 complex is as follows: when *S. cerevisiae* has limited access to inorganic phosphate, Pho81 inhibits cyclin kinase activity, so that it no longer hyperphosphorylates the transcription factor Pho4 (Kaffman et al., 1994), which then enters the nucleus to drive the transcription of genes important for phosphate generation and assimilation, such as a phosphate transporter and a secreted acid phosphatase (Springer et al., 2003). Lee et al. (2007, 2008) have demonstrated that 1/3-PP-$InsP_5$ augments the inhibitory activity of Pho81. This is a relatively specific effect, because 5-PP-$InsP_5$ is much less effective (Lee et al., 2007). In addition, from the key perspective of physiological relevance, the inhibitory effect of 1/3-PP-$InsP_5$ was unaffected by a 50-fold excess of $InsP_6$ (Lee et al., 2008). Thus, $InsP_6$ is unlikely to interfere with this action of 1/3-PP-$InsP_5$ in vivo, even though cellular $InsP_6$ levels are 25-fold greater than those of PP-$InsP_5$ (Lee et al., 2007). 1/3-PP-$InsP_5$ seems to either interact with both Pho81 and Pho80/Pho85 or to stabilize the inhibitory association of Pho81 with Pho80/Pho85 (Lee et al., 2008). There are also strong indications of a more complex relationship between Vip1 and the Pho80/Pho85/Pho81 system that deserves further attention. For example, the expression of *Vip1* is regulated by Pho4 (Maerkl and Quake, 2007). Of further interest is the observation that the Vip1 protein is phosphorylated by Pho80-Pho85, at least in vitro (Dephoure et al., 2005). Pho85—and its human homolog, Cdk5—are also important for cell cycle progression, polarized cell growth, and cytoskeletal dynamics (Huang et al., 1999, 2007a), thus raising the possibility that such activities might also be regulated by the products of Vip/PPIP5K activity.

The1/3-PP-$InsP_5$ inhibits cyclin kinase activity in vitro with an IC_{50} value of 55 μM (Lee et al., 2007). Lee et al. (2007) conclude that this is physiologically relevant in phosphate-restricted yeast, in which they estimated that total PP-$InsP_5$ levels can rise to 10 to 30 μM (a 10-fold increase over basal levels). It is useful to look closely at how this estimate was obtained. To arrive at this value, total intracellular PP-$InsP_5$ concentration in [^{3}H]inositol-labeled cells was estimated from the ratio, PP-[^{3}H]$InsP_5$/[^{3}H]$InsP_6$, and the value of the denominator was *assumed* to be 100 μM (Lee et al., 2007). Is this a realistic assumption? It does have some precedents in eukaryotic cells: *D. discoideum* synthesizes 300 μM $InsP_6$ (Laussmann et al., 2000), but this organism seems uniquely adapted in this respect. Some transformed hemopoietic cell lines have been reported to contain 90 to 100 μM $InsP_6$ (Bunce et al., 1993). However, another species of yeast, *Schizosaccharomyces pombe* contains only 36 μM $InsP_6$ (Ingram et al., 2003). All of the other published estimates of cellular $InsP_6$ levels—including some direct mass assays—are in the range of 15 to 60 μM (Szwergold et al., 1987; Pittet et al., 1989; Irvine and Schell, 2001; Barker et al., 2004; Letcher et al., 2008). So 100 μM $InsP_6$, although not an implausible concentration, would certainly be an unusually high value, which means that the levels of PP-$InsP_5$ in *S. cerevisiae* may also have been overestimated.

In addition, we should note that Lee et al. (2007) calculated the concentration of 1/3-PP-$InsP_5$ from two more or less equally sized [^{3}H]inositol-labeled peaks that eluted after $InsP_6$ during HPLC analysis. If these two peaks are actually two different isomers of PP-$InsP_5$ (Fig. 3), only half of this "total PP-[^{3}H]$InsP_5$" can represent the one specific isomer, 1/3-PP-$InsP_5$, that inhibits cyclin kinase activity. In this scenario, cellular levels of 1/3-PP-$InsP_5$ are overestimated by a factor of 2. If instead one of these two "post-$InsP_6$" peaks were to comprise $(PP)_2$-$InsP_4$, then "total PP-[^{3}H]$InsP_5$" would actually include $(PP)_2$-$InsP_4$ and 5-PP-$InsP_5$, as well as genuine 1/3-PP-$InsP_5$, leading to an even larger overestimation of the actual 1/3-PP-$InsP_5$ levels. At the very least, therefore, we should reduce by half the concentration of 1/3-PP-$InsP_5$ that should be calculated from the two "post-$InsP_6$" peaks. Once this correction is made, and after also taking a more conservative value for the intracellular levels of $InsP_6$ (15–60 μM instead of 100 μM), the actual levels of 1/3-PP-$InsP_5$ after phosphate starvation could lie in the range of 3 to 9 μM, well below the 55 μM required to half-maximally inhibit cyclin kinase activity in vitro.

On the other hand, we should not forget that very persuasive genetic data indicate that the cyclin kinase activity of Pho80/Pho85 is inhibited by the catalytic activity of Vip1, which synthesizes 1/3-PP-$InsP_5$ (Lee et al., 2007; Mulugu et al., 2007). One of the key experiments involved a *vip1*Δ strain of *S. cerevisiae*. In these cells, Pho4 was constitutively cytoplasmic and therefore incompetent to activate transcription, even in phosphate-starved yeast. Remember that Pho4 is inactive when it is hyperphosphorylated by the Pho80-Pho85 cyclin/cyclin-dependent kinase (Kaffman et al., 1994). Thus, in the *vip1*Δ strain, and therefore in the absence of 1/3-PP-$InsP_5$, the cyclin kinase could not be inactivated by low phosphate conditions (Lee et al., 2007). Data as telling as these indicate that we should offer suggestions as to how the genetic results might be reconciled with the biochemical observations. For example, it may be productive to determine

the effects of 1/3,5-$(PP)_2$-$InsP_4$ upon cyclin kinase activity. Alternately, perhaps other regulatory factors remain to be discovered that sensitize the cyclin kinase so that 1/3-PP-$InsP_5$ acts with greater potency in vivo. These and other possible explanations are especially worth pursuing, because the isomer-specific association of 1/3-PP-$InsP_5$ with Pho81 and/or Pho80/Pho85 in vitro is, to date, the best characterized example of a specific "receptor" for a diphosphoinositol polyphosphate. In addition to further confirming that this interaction is biologically significant, it will also be important to explicitly define the amino acid residues that mediate these interactions and to search for similar motifs in other proteins, such as Cdk5, the mammalian homolog of Pho85 (Huang et al., 1999). In this way, we might uncover additional actions of 1/3-PP-$InsP_5$.

Having said all that, however, a couple of additional comments that relate to the aforementioned "post-$InsP_6$" peaks are warranted. Recall (see above) that the data from Lee et al. (2007) can be interpreted to indicate that, in addition to 1/3-PP-$InsP_5$, the cellular levels of 5-PP-$InsP_5$ and/or 1/3,5-$[PP]_2$-$InsP_4$ may also be elevated in *S. cerevisiae* during phosphate starvation. If this interpretation is correct, then clearly the initial response of the yeast cells to impending bioenergetic stress is different from that which would be expected in mammalian cells. As discussed above, in mammalian cells, the levels of 5-PP-$InsP_5$ and/or 1/3,5-$[PP]_2$-$InsP_4$ often seem to *decrease* when bioenergetic health is compromised. Burton et al. (2009) has even indicated that they have unpublished data showing that phosphate-starved *S. cerevisiae* do indeed exhibit a decrease in PP-$InsP_5$ levels. Presumably, these authors are referring to the 5-PP-$InsP_5$ isomer, because this is the predominant isomer in *S. cerevisiae* (see above). Thus, it would be useful to clarify the responses of each of the individual diphosphoinositol polyphosphates to phosphate starvation in yeast. It seems that much more information is required before we can fully appreciate the molecular actions of diphosphoinositol polyphosphates in vivo.

Conclusion

The idea that diphosphoinositol polyphosphates are second messengers—as originally defined (Robison et al., 1968)—is hard to justify when their cellular levels do not respond to extracellular agents. This situation has prompted the proposal in this review that these molecules respond to an intrinsic change in cellular circumstances: the cellular bioenergetic status. This hypothesis does have the advantage of providing a context for the conditions in which changes in the levels of these molecules has actually been observed. However, even if the idea that diphosphoinositol polyphosphates are metabolic messengers makes a convincing treatise, this review has shown that many questions remain unanswered. Finding the answers is a task of considerable importance. Adaptation to energy-stress is essential to the very survival of the cell. A fuller understanding of cellular energy-sensing machinery is potentially also of use as an exploitable target for cancer therapy (Sofer et al., 2005; Swinnen et al., 2005). Finally, our data raise the possibility of a new phenomenon associated with aging: attenuation of inositol pyrophosphate signaling, because of its hypersensitivity to the slight but progressive decline in cellular energy homeostasis that others have noted during the approach to cellular senescence (Wang et al., 2003; Miyoshi et al., 2006). Pharmacological or genetic intervention in the pathways of inositol pyrophosphate signaling may therefore ultimately prove to be of benefit to human health.

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