


Review

Metabolism and Functions of Inositol Pyrophosphates: Insights Gained from the Application of Synthetic Analogues

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Abstract: Inositol pyrophosphates (PP-InsPs) comprise an important group of intracellular, diffusible cellular signals that a wide range of biological processes throughout the yeast, plant, and animal kingdoms. It has been difficult to gain a molecular-level mechanistic understanding of the actions of these molecules, due to their highly phosphorylated nature, their low levels, and their rapid metabolic turnover. More recently, these obstacles to success are being surmounted by the chemical synthesis of a number of insightful PP-InsP analogs. This review will describe these analogs and will indicate the important chemical and biological information gained by using them.

Keywords: metabolism; homeostasis; phosphate; cell biology; energy

1. Introduction

The inositol pyrophosphates (PP-InsPs), particularly 1-InsP₇, 5-InsP₇, and 1,5-InsP₈ (Appendix A; Figure 1) are highly ‘energetic’ and diffusible signaling molecules that play important roles in many cellular processes throughout the eukaryotic kingdoms, particularly with regards to phosphate and bioenergetic homeostasis, both of which can have profound metabolic and physiological consequences [1–8]. Indeed, it is hard to overstate the significance of dovetailing any signal transduction cascade with metabolic circuitry. All biological programs place bioenergetic demands upon an organism; its ability to adapt to environmental challenges, i.e., its long-term survival, is reliant upon signaling cascades exerting dynamic control over the uptake, storage, and utilization of various metabolic fuels [9]. Our understanding of the many roles that PP-InsPs play in these processes has been especially well-served in recent years by the contributions that organic chemists have made in synthesizing insightful PP-InsP analogs. Here, we review the nature of these tools and the knowledge gained from their use.

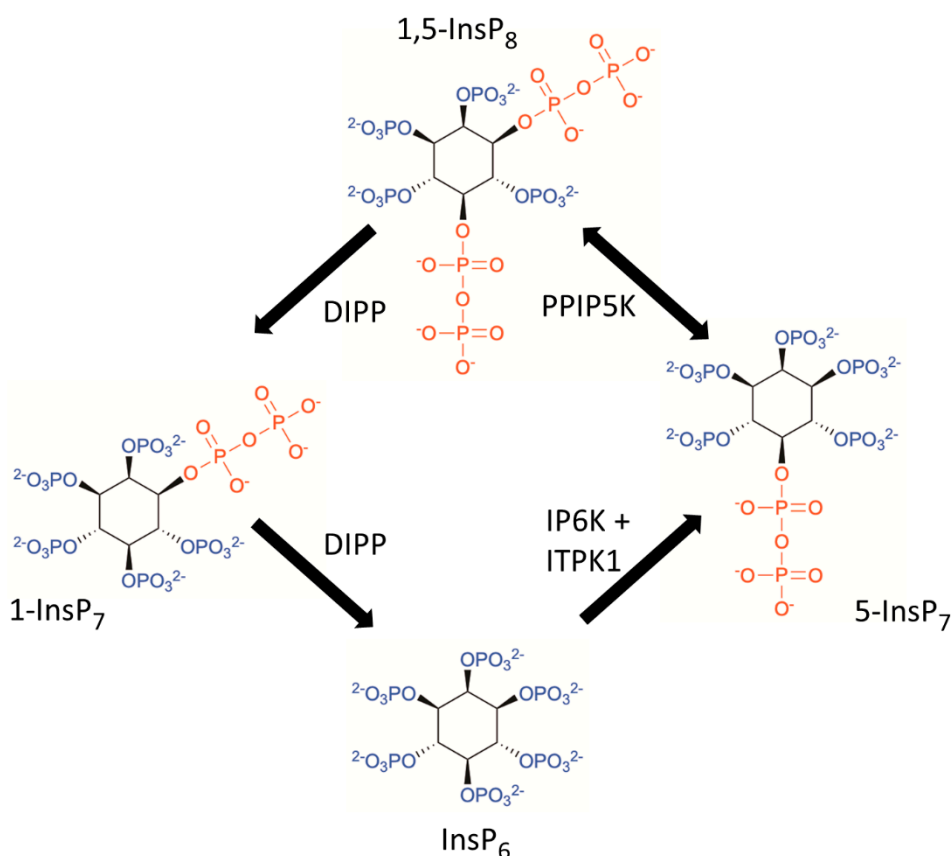


Figure 1. The inositol pyrophosphate (PP-InsP) metabolic pathway. This graphic incorporates a proposal [1,2] that the pathway for 1,5-InsP₈ turnover principally comprises a cyclical interconversion of InsP₆, 5-InsP₇, 1,5-InsP₈, and 1-InsP₇. ITPK1 = inositol 3,4,5,6-tetrakisphosphate 1-kinase; IP6K = inositol hexakisphosphate 5-kinase; PPIP5K = diphosphoinositol pentakisphosphate 1-kinase (in addition to hosting a self-contained kinase domain, this class of enzyme also contains a phosphatase domain that possesses 1,5-InsP₈ 1-phosphatase activity [2–4]); DIPP = diphosphoinositol polyphosphate phosphatase.

2. PP-InsP Synthesis and Metabolism

Considerable attention has been given to two classes of kinases that work together to generate PP-InsPs (Figure 1): IP6Ks (see [6]), which until very recently were considered solely responsible for phosphorylating InsP₆ to 5-InsP₇, and the PPIP5Ks (see [2]), which interconvert 5-InsP₇ with 1,5-InsP₈. However, it has been enigmatic that orthologs of IP6Ks are not expressed in plants. This conundrum was recently resolved with the determination that the ‘missing’ InsP₆ kinase activity in plants is expressed by ITPK1s [10–12], a class of enzyme that had been originally characterized as active Ins(3,4,5,6)P₄ 1-kinases [13]. Human ITPK1 also expresses this InsP₆ kinase activity *in vitro* [10], but it has yet to be demonstrated if it is sufficient *in vivo* to be considered a genuine, alternate source of 5-InsP₇ synthesis. Incidentally, an ITPK1 ortholog has not been identified in any yeast or insect genome.

The major pathway for net catabolism of 1,5-InsP₈ back to InsP₆ in animal cells (see Figure 1) is proposed to occur by successive 5-β-phosphatase and then 1-β-phosphatase activities of the diphosphoinositol polyphosphate phosphatases (DIPPs), although the 1-InsP₇ (Figure 1) is only a short-lived intermediate [1,14]. In *Saccharomyces cerevisiae*, this pathway of 1,5-InsP₈ turnover is reinforced through its hydrolysis by an additional 5-phosphatase activity catalyzed by Siw14 [15,16]. No animal orthologs of Siw14 have been identified to date.

3. Metabolically Stable Analogues of 5-InsP₇

A number of studies have explored the biological activities of the PP-InsPs by pharmacological and/or genetic intervention in the metabolic pathway described by Figure 1 (see [6,8] for recent, authoritative reviews). However, the blockage of IP6K activity by itself cannot distinguish the actions of 5-InsP₇ from those of InsP₈, since the synthesis of both is reduced. The inhibition of PPIP5K activity is not a specific strategy either; both InsP₈ and 1-InsP₇ synthesis is compromised, and there is a significant elevation in 5-InsP₇ levels [17–19]. Thus, genetic and pharmacological experiments do not necessarily attribute a particular phenotype to the action (or loss of action) of one specific PP-InsP isomer. This is a situation in which metabolically stable PP-InsP analogs can be helpful, particularly with regards to biochemical and cellular assays, in which the natural PP-InsPs have a short half-life due to their inherently fast turnover rate [20,21]. Another application for such research tools is to distinguish between the two molecular mechanisms by which protein function can be modulated by the PP-InsPs: allosteric regulation upon ligand binding, versus covalent regulation by non-enzymic phosphotransfer [1,6–8,22]. The latter cannot be mimicked by metabolically stable PP-InsP analogs.

Two teams of researchers have separately pioneered the development of metabolically stable PP-InsP molecules: Barry Potter's UK group and Dorothea Fiedler's laboratory, which is now based in Germany. These reagents (Figure 2; Appendix A) were first described in 2012–2013 [23,24]. Fiedler and colleagues synthesized methylene-bisphosphonate (PCP) analogs of 5-InsP₇ in which a methylene group was substituted for the bridging oxygen of the phosphoanhydride moiety (Figure 2A and [24]). Potter's group replaced the bisphosphate of 5-InsP₇ with phosphonoacetate (PA) [23]. Subsequently, the PA- analog of 1,5-InsP₈ and PCP-analogues of 1-InsP₇ and 1,5-InsP₈ were also synthesized [25–27].

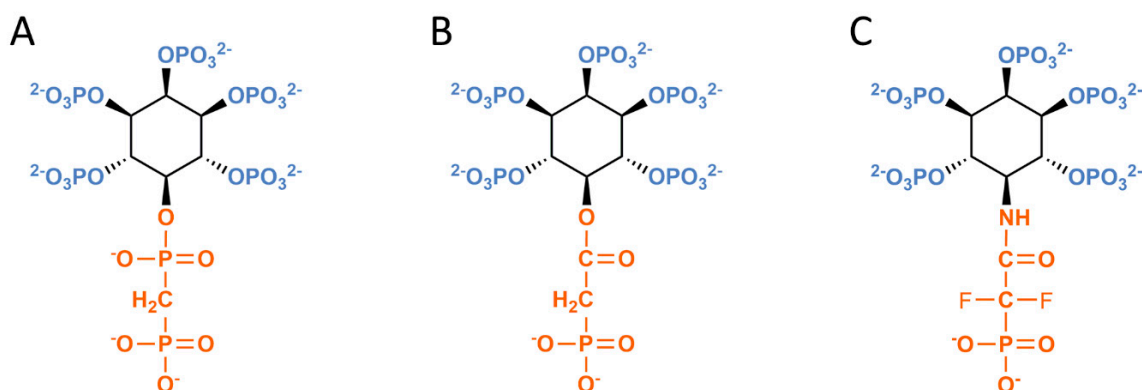


Figure 2. Metabolically stable bioisosteres of 5-InsP₇. This figure shows synthetic analogs of 5-InsP₇ in which the phosphoanhydride group is replaced with either (A) methylene-bisphosphonate (PCP), (B) phosphonoacetate (PA), (C) phosphonodifluoroacetamide.

The authors of the latter studies have referenced earlier work [28] that notes the replacement of an oxygen atom with a methylene group leads to a reduction in the acidity of the phosphonic acid when compared to the phosphoric acid. The pK_a values for the second deprotonations are such that the phosphonate will be less strongly ionized at physiological pH compared to the phosphate, potentially leading to a decreased affinity for protein receptors [28]. Despite this caveat, several significant discoveries have emerged from the application of these analogs, and these advances are described in the Sections below.

Potter group's recently synthesized a new analog in which the phosphonate CH₂ group was replaced by a more electronegative CF₂, thereby increasing the acidity of the moiety (Figure 2C) and potentially narrowing the difference in pK_a values compared to the natural diphosphate [28]. Since a difluoroacetate ester would be very labile to hydrolysis, a stable amide linkage was used (Figure 2C and [29]).

4. Bioisosteres and the Discovery of a PP-InsP Capture Site in the Kinase Domain of PPIP5K

To enforce the tight substrate specificity of the PPIP5Ks for 5-InsP₇ and, to a minor extent InsP₆, ligand entry into the catalytic pocket is guarded by substantial architectural and electrostatic constraints [30]. This situation suggests ligand is only admitted when it is presented in the appropriate orientation, i.e., a direct hit. Could that limit the enzyme's ability to attract ligand that rotates and diffuses randomly through the bulk phase? For an illustrative analogy, consider the frustratingly customary outcome of a classic carnival game: the high failure rate when attempting to throw a table tennis ball into the narrow neck of a distant goldfish bowl.

Our X-ray analysis of 5-PA-InsP₇ soaked into crystals of the kinase domain of PPIP5K (i.e., PPIP5K^{KD}) helped to uncover a near-unique mechanism by which this enzyme activity facilitates substrate access to the active site: two ligand-binding positions were observed [31]. Both ligand-binding sites cannot be occupied by substrate simultaneously, due to steric clashing. Thus, we reasoned that the crystal complexes comprised a mixture of two separate, but mutually exclusive ligand configurations. One of the binding sites for 5-PA-InsP₇ is near superimposable upon that occupied by 5-InsP₇ in the catalytic pocket (Figure 3A). A second binding site lies approximately 8 Å away, closer to the surface of the protein (Figure 3B). We propose the latter enhances substrate capture from the bulk phase, prior to its delivery into the catalytic pocket. We have described this phenomenon as a “catch-and-pass” reaction mechanism [31]. In an earlier study, we could not detect 5-InsP₇ substrate in the capture site [30]. Perhaps a slight difference in the degree of the positive charge of 5-InsP₇ vis-a-vis its synthetic analog is responsible for the different behavior. We presume that the delivery of natural substrate is more efficient compared to 5-PA-InsP₇, and hence the natural ligand occupies the capture site too transiently to yield suitable electron density.

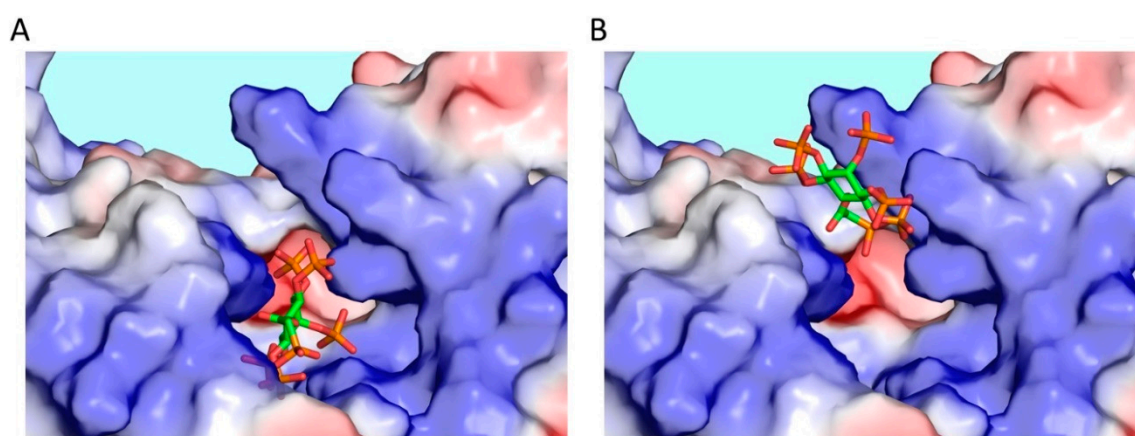


Figure 3. PPIP5K^{KD} has two substrate binding sites. X-ray crystallographic analysis of PPIP5K^{KD}/5-PA-InsP₇ crystal complexes uncovered two mutually exclusive ligand binding sites: (A) catalytic pocket; (B) substrate capture site. The protein is depicted in surface plot format, in which the intensity of the coloration (blue = positive, red = negative) corresponds to the degree of electrostatic polarization. The ligand is shown in stick form: carbon is green, phosphorous is orange, and oxygen is red.

The 8 Å journey that 5-InsP₇ takes when moving between its two binding sites has been modeled by the application of molecular dynamics simulations [32]. This was how we established the role of Glu192 as a molecular ratchet that moves towards the space vacated by the substrate. That is, the Glu192 electrostatically enforces one-way substrate delivery [32]. The concept that this residue is catalytically important has been verified experimentally—a Glu192Gln mutation dramatically reduces kinase activity, presumably by compromising substrate transfer into the active site [31].

The new phosphonodifluoroacetamide analog (Figure 2C) has also been soaked into crystals of PPIP5K^{KD} and found to closely mimic the occupation of the catalytic pocket by 5-InsP₇ (Figure 2C

and [29]); the capture site was not occupied. Interestingly, there is a Mg^{2+} ion that makes contact with 5-InsP₇ in its crystal complex with PPIP5K^{KD}, but the metal is missing from the complex with the phosphonodifluoroacetamide analog. Therefore, it was suggested that this new analog might be particularly useful for studying those PP-InsP/protein interactions that are Mg^{2+} -independent [29]. As it happens, there may be many such interactions: it has been noted by others that, to date, Mg^{2+} is missing from all of the published non-catalytic protein/PP-InsP complexes that have been captured by x-ray crystallographic analyses [33].

The analysis of a crystal complex of PPIP5K^{KD}/1,5-PA-InsP₈ showed that this particular analog of the PPIP5K enzymatic product also occupies both ligand binding sites [25]. This observation raises the possibility that the product escapes from the catalytic pocket via the same capture site. In contrast, in corresponding crystal complexes that contain either 1,5-PCP-InsP₈ or 5-PCP-InsP₇, these ligands were only observed in the catalytic pocket [25,27], just as is the case with the natural substrates [30,34]. Nevertheless, the PA-version was more informative because it identified the capture site. This comparison underscores the scientific value of probing biological systems with a variety of different analogs.

5. The Positional Specificity of DIPP: Information Gained from PCP-InsPs

The DIPP shows a high rate of hydrolytic activity towards all PP-InsPs [14], but there is a puzzling aspect to the enzymes' positional specificity. Biochemical assays have indicated that the 5- β -phosphate is preferentially cleaved from 1,5-InsP₇ [14]. Despite that, these enzymes show a greater preference for the 1- β -phosphate of 1-InsP₇ compared to the 5- β -phosphate of 5-InsP₇ [14,35]. Moreover, PCP analogs have been useful in validating the enzyme's preference for 1-InsP₇ over 5-InsP₇. PCP analogs are not hydrolyzed by DIPP, but by competing with the active site, they act as inhibitors of PP-InsP hydrolysis [25,26,35]. Fiedler and colleagues showed that 1-PCP-InsP₇ is an 80-fold more potent inhibitor of DIPP activity than is 5-PCP-InsP₇ [26]. Such data are consistent with the aforementioned observation that DIPP prefers to hydrolyze 1-InsP₇ over 5-InsP₇. It is noteworthy that these biochemical assays were performed in media containing the Mg^{2+} that is necessary for activity. In contrast, Mg^{2+} was absent when Potter and colleagues [35] assessed apparent ligand affinities for DIPP by using differential scanning fluorimetry. Under such conditions, the apparent K_d values for 1-InsP₇ and 5-InsP₇ are very similar, while the apparent K_d value for 5-PCP-InsP₇ is 3 to 4-fold lower than that for 1-PCP-InsP₇ [35]. The fact that PCP versions of PP-InsPs recapitulate this Mg^{2+} -dependence for DIPP's preferential apparent affinity for 1-InsP₇ over 5-InsP₇ is testimony to their value as bioisosteres.

6. Application of Metabolically-Stable PP-InsP Analogues in Studies of Protein Pyrophosphorylation

By definition, metabolically-stable analogs of PP-InsPs cannot participate in phosphoryl transfer reactions; in fact, the PCP-analogues inhibit protein pyrophosphorylation, which presumably reflects their competitively inhibiting the natural PP-InsP from binding to the protein [26]. Such a phenomenon speaks to the pyrophosphorylation event requiring a specific binding event that could enforce PP-InsP specificity [26]. That is another valuable conclusion to be drawn from the use of these analogs.

Metabolically-stabilized derivatives of PP-InsPs (like PP-InsPs themselves) cannot penetrate into intact cells. Thus, to date, the use of these analogs to probe the possibility of protein pyrophosphorylation has been restricted to cell-free, *in vitro* assays. For example, there has been an investigation into the roles of PP-InsPs in regulating phosphorylation of interferon regulatory factor 3 (IRF3) [36]. This is a key step in inducing cellular interferon- β 1 expression in response to viral infection. It was found that IRF3 phosphorylation *in vitro* is stimulated by 1-InsP₇ and/or InsP₈ [36]. Since the corresponding PA-analogues of 1-InsP₇ and InsP₈ proved to be inactive in this assay, it was concluded that the underlying mechanism for IRF3 activation likely involved protein pyrophosphorylation.

Another example has been a study of PP-InsP-mediated regulation of vesicle trafficking driven by cytoplasmic dynein 1 [37]. The latter is an important microtubule-based motor in many eukaryotic

cells. In vitro, the dynein intermediate chain (IC), a non-catalytic dynein subunit, was shown to be pyrophosphorylated by 5-InsP₇ on its N-terminus, which in turn promotes dynein-IC binding to the p150Glued subunit of dynactin [37]. Notably, 5-PCP-InsP₇ was considerably less effective at enabling these protein-protein interactions [37], consistent with the idea that protein pyrophosphorylation is required.

7. Protein Stability

Potter and colleagues have used differential scanning calorimetry to show that the binding to DIPPs of either 1-PCP-InsP₇ or 5-PCP-InsP₇ elevates the protein's thermal transitions temperature—which reflects increased protein stability—to almost the same degree as the corresponding PP-InsPs [35]. The authors posited that any protein stabilization by metabolically-stable PCP-InsPs might facilitate the formation of tractable crystal/ligand complexes for X-ray structural analysis.

8. Interrogation of the 5-InsP₇ Interactomes

Fiedler's group has prepared affinity resins to which 5-PCP-InsP₇ has been immobilized by attachment of a linker to the 2-phosphate. This metabolically-resistant ligand has been deployed to search for 5-InsP₇ binding partners in lysates of *S. cerevisiae* [38]. This particular application for the PCP-analogue yielded several interesting conclusions. Firstly, gene ontology analysis of the 'hits' showed a significant over-representation of proteins with bioenergetically important roles, such as nucleotide metabolism, glucose metabolism, and the energetically-intensive process of ribosome biogenesis. The nature of this cohort dovetails with the overarching concept that PP-InsPs play multiple roles at the interface of signaling and metabolic homeostasis [1].

This work was also informative because of the two separate screening conditions that were used: One contained 1 mM exogenous Mg²⁺, and in a second screening, endogenous Mg²⁺ was chelated by the addition of EDTA [38]. Similar numbers of proteins were identified in each screen (98 and 84, respectively), but only 21 overlapped both datasets. This is a singular observation that speaks to the existence of two structurally- and operationally-distinct PP-InsP binding sites. The authors speculated that Mg²⁺-independent binding could largely depend upon basic, positively charged amino-acid residues, whereas Mg²⁺-dependence may reflect the previously-demonstrated absolute dependence upon the cation for non-enzymic pyrophosphorylation of phosphor-Ser within an acidic context [39,40].

The 5-PCP-InsP₇ affinity resin has also been used to screen for binding partners in cell extracts of the parasite, *Trypanosoma cruzi* [41]. As is the case for yeast extracts (see above), proteins involved in ribosome biogenesis and nucleotide turnover were both over-represented in the list of captured proteins. One of the novel targets, an uncharacterized protein, which is a putative phosphoribosyl pyrophosphate synthase, was found to contain a candidate SPX domain, which was recently shown to be a widespread PP-InsP binding module [42]. A novel pyrophosphorylation target was also identified and validated: a hypothetical choline/carnitine *o*-acetyltransferase domain containing protein. Further work is needed to illuminate the biological significance of 5-InsP₇ interactions with these proteins.

Fiedler and colleagues found that only 22 of the 5-PCP-InsP₇ binding proteins in their yeast cell extracts (approximately 20% of the total) were *not* enriched in parallel screens performed with bead-immobilized InsP₆ [1,38]. In discussing this observation, the authors asked how the other 80% of their 'hits' could perform as a specific regulatory molecule for 5-InsP₇ in vivo, given that this PP-InsP has a total cellular concentration that is only 2–5% that of InsP₆. This is a conundrum that permeates the field; other studies have also described binding proteins that show similar affinities towards PP-InsPs and InsP₆ (e.g., [42]). Perhaps in part, the answer is provided by several separate lines of evidence—albeit indirect in nature (summarized in ref [2])—that indicate much of the cell's complement of InsP₆ is not free in the cytoplasm but instead may be accumulated into a number of cellular compartments, in which it cannot compete for PP-InsP binding sites.

In addition, Fiedler's group speculated that there was another factor that contributed to their identification of only a small number of binding proteins with specificity for 5-PCP-InsP₇ over

InsP₆: if a protein requires a 2-phosphate monoester for efficient binding, then its selectivity may be compromised by linker attachment at that position. This consideration prompted the group to subsequently synthesize triplexed affinity reagents – a mixture of linker attachments at either the 1-, 2- or 3-positions [43]. By using these more advanced reagents to screen lysates from HEK293 and HCT116 cells, the laboratory identified a 3-fold higher number of proteins exhibiting specificity for 5-PCP-InsP₇ over InsP₆, compared to their earlier study with yeast lysates. Among new potential PP-InsP binding proteins that should prove interesting to pursue are the kinase LKB1, which phosphorylates and regulates AMPK, and several enzymes that participate in purine metabolism; these are interesting targets in the context that PP-InsPs act as metabolic regulators.

9. Delivery of PP-InsPs and PP-InsP Analogues into Live Cells Using Nanocarriers

The ability to deliver an intracellular signal into cells can directly facilitate studies into its biological actions. However, the anionic nature of polyphosphates prohibits them from crossing the plasma membrane [44]. There are workarounds to this barrier, but several are unsatisfactory because they are physically or biochemically damaging, such as microinjection, electroporation, or chemical transfection (in any case, Lipofectamine 2000 does not efficiently transfer PP-InsPs into cells [45]). To circumvent these restrictions, the negative charges can be ‘hidden’ by incorporating the polyphosphates into biocompatible nanoparticles that can be accumulated into cells by endocytosis. For example, liposomes have been used to deliver a number of polar molecules into cells: microRNAs [46], siRNA [47], antisense DNA [48], and ATP [17,49]. We have recently adapted this technique in order to transfer PP-InsPs into cells; the cargo was spontaneously released during a 3–4 h loading protocol [50,51]. The mechanism of intracellular release appears to involve the fusion of the endocytosed liposomes with the endosomal and/or lysosomal membranes, causing the cargo to be ejected into the cytoplasm [52].

For example, these methods supported the discovery that 1,5-InsP₈ regulates a cellular pathway for Pi efflux that is dependent upon Xenotropic and Polytopic Retrovirus Receptor 1 (XPR1). It was experiments with PPIP5K^{-/-} cells that initially led us to this idea that the Pi efflux pathway is licensed by 1,5-InsP₈ [50]. To interrogate this genetically-based conclusion with an independent approach, we set out to complement the phenotype using our liposome delivery method. We delivered metabolically-stable 1,5-PCP-InsP₈ into cells, and this rescued Pi efflux in a dose-dependent manner; delivery of either 1-PCP-InsP₇ or 5-PCP-InsP₇ was ineffective [50].

The relatively slow (3–4 h) liposomal delivery protocol described above has the disadvantage that it is not so useful for the delivery of the natural PP-InsPs; due to their rapid metabolism, it is hard to distinguish the functions of the original cargo from those of its metabolites. To ameliorate this restriction, we recently described the loading into populations of cultured cells of thermosensitive liposomes that only become permeable when the temperature exceeds 37 °C [53]. These liposomes are also doped with a photothermal, near infra-red carbocyanine dye, and can be induced to ‘melt’ and release their cargo within 5 min of illumination of the cultures by a high-power, near infra-red light-emitting diode. We used this methodology to deliver either 1,5-InsP₈ or 1,5-PCP-InsP₈ into HCT116 cells; both enhanced the rate of cellular Pi uptake to the same extent [53]. These quantitatively equivalent effects of 1,5-InsP₈ and 1,5-PCP-InsP₈ in a cell-based bioassay testify to the value of the PCP analog as a functional bioisostere when studying rapid signaling events that do not involve protein pyrophosphorylation.

In a more recent study, we pursued the discovery that DIPP1 not only hydrolyzes PP-InsPs such as 5-InsP₇, but also cleaves the 5′-end 7-methylguanosine cap of a specific subset of mRNAs, under the guise of its NUDT3 pseudonym [54]. Decapping exposes the mRNA 5′-end to exonuclease-mediated degradation; this decay pathway plays critical roles in early animal development, cell growth and proliferation, immune response, and mRNA quality control [55]. We posited that 5-InsP₇ might competitively inhibit mRNA decapping, and thereby stabilize the DIPP1-sensitive transcripts. This proposal was confirmed by liposome-mediated delivery of 5-PCP-InsP₇ into HCT116 cells; this procedure elevated the levels of several DIPP1 mRNA substrates [51].

In this context, it is notable that 5-InsP₇ levels fluctuate in response to the bioenergetic status of the cell (i.e., ATP levels) [56]; this homeostatic process underlies the role of 5-InsP₇ in mediating nutrient-stimulation of insulin secretion from pancreatic β -cells [56]. These observations rationalize 5-InsP₇ as being a rheostatic signaling molecule that protects bioenergetic health in the face of environmental challenges [1,6]. Thus, our delivery of PCP-InsP₇ into cells using liposomes has unveiled an epitranscriptomic control process; we have shown how 5-InsP₇ acts in a molecular pathway by which mRNA structure and stability can be modified in response to fluctuations in the extracellular environment.

Inhibition of 5'-decapping is associated with P-body accumulation [57,58]; the latter are highly dynamic, membrane-less biomolecular condensates that sequester certain mRNAs [59]. While the original concept that P-bodies regulate mRNA decay continues to be pursued [58,60], these organelles are now recognized as important sites for sequestration and storage of mRNAs away from the translating pool [61]. We have shown that liposome delivery of 5-PCP-InsP₇ into cells elicits a >3-fold increase in the numbers of P-bodies per cell [51]. We are currently investigating if this effect on P-body dynamics is either a secondary consequence of the elevated levels of stabilized transcripts or a separate function for 5-InsP₇ (e.g., perhaps through electrostatic interactions with mRNA, just as polyvalent interactions between arginine and RNA assist the phase separation process [62]). As for the ultimate fate of P-body enriched mRNAs, it has been proposed that these transcripts can be rapidly reintroduced into the translating pool, for example, in response to certain environmental cues [58,63,64].

Alternative nanocarriers for PP-InsPs have been described, such as guanidinium-rich oligocarbonate transporters, which, like the liposomes described above, enter cells by the endocytic pathway and spontaneously release their cargo within approximately 4 h [45]. The latter study by Jessen and colleagues is also significant because it described the first synthesis and application of a 'caged' PP-InsP (Figure 4A and see Section 10 for further discussion).

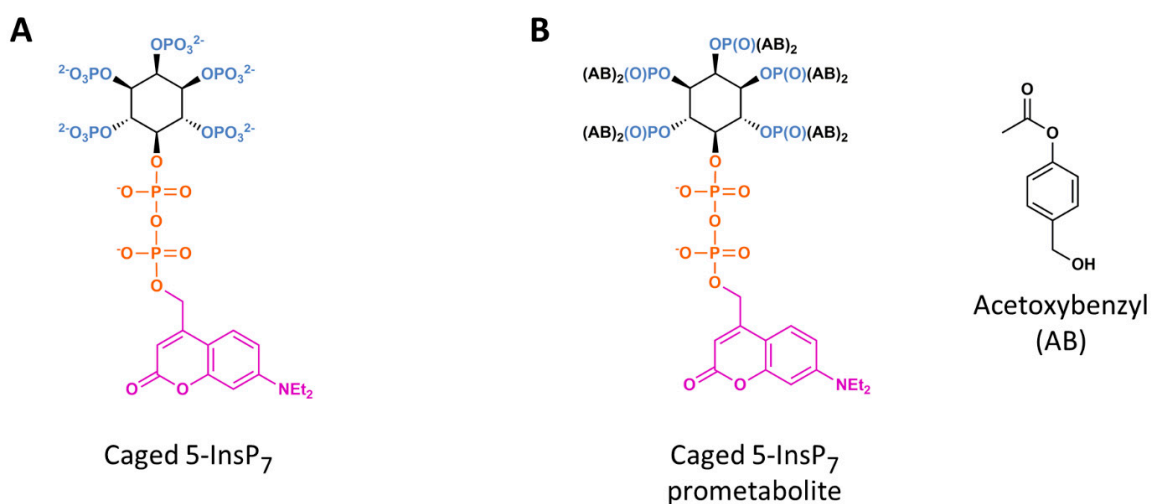


Figure 4. The structure of a (A) caged 5-InsP₇ and (B) its 'prometabolite'.

10. Caged and Cell-Permeant PP-InsPs

Jessen and colleagues produced caged 5-InsP₇ by adding [7-(diethylamino)coumarin-4-yl]methyl (DEACM) to the 5- β -phosphate (Figure 4A). The molecule was delivered into cells using guanidinium-rich oligocarbonate transporters [45]. This method offers a dramatic improvement to the temporal and spatial resolution of PP-InsP signaling. Uncaging was accomplished by cell exposure to a 30-s pulse from a 375 nm UV laser—the first example of controlled 5-InsP₇ augmentation inside a living cell. Thus, it was possible to interrogate the hypothesis [65,66] that 5-InsP₇ inhibits growth-factor mediated stimulation of AKT partly by competing with its PH domain (AKT^{PH}) binding site for the signaling phospholipid, PtdIns(3,4,5)P₃, which normally drives AKT translocation from the

cytoplasm to plasma membrane, where it encounters upstream activating protein kinases. In their experiments, Jessen and colleagues uncaged 5-InsP₇ after their AKT^{PH} construct had already been relocated to the plasma membrane by prior addition of growth factors [45]. A release of AKT^{PH} was observed after 5 min, and nearly completed by 15 min. The length of this time frame could indicate that it depends upon 5-InsP₇ metabolism to 1,5-InsP₈, but that seems unlikely because the latter is a significantly weaker AKT^{PH} ligand [67]. Perhaps instead there are physical constraints that slow down access of 5-InsP₇ to AKT^{PH} that is already associated with PtdIns(3,4,5)P₃. Indeed, even in vitro, 5-InsP₇ is less potent at competing for the AKT^{PH} – bound PtdIns(3,4,5)P₃ if the lipid is prebound to the protein [66].

Another approach to delivering PP-InsPs into cells is to use analogues that are inherently cell-permeant. This can be accomplished by masking the negative charges with lipophilic groups that are naturally removed once the compound has entered the cell; the application of this method to an inositol phosphate (InsP) was first described in 1994 [68]. Acetoxymethyl esters were deployed to mask the phosphate groups, while butyrate was added to the hydroxyls. The resultant, uncharged molecule can penetrate the plasma membrane, and once it is inside cells, intracellular esterases cleave the masking groups and yielded the native inositol phosphate. This technique has been adapted by Jessen and colleagues [69], such that caged 5-InsP₇ was made cell-permeable by attaching acetoxybenzyl groups to the monophosphates and DEACM was added to the 5-β-phosphate (Figure 4B). Cell loading and removal of the acetoxybenzyl moieties was completed within 4 hr. Subsequently, UV-mediated uncaging of 5-InsP₇ was found to block Ca²⁺ oscillations in the mouse pancreatic insulinoma (MIN6) cell-line, after a delay of 15 min [70]. In a subsequent study using caged 1,5-InsP₈, its photorelease instantly stopped Ca²⁺ oscillations [71]. Thus, in the earlier study, it seems likely that the delay was due to 5-InsP₇ phosphorylation to 1,5-InsP₈. It can be anticipated that inhibition of Ca²⁺ signaling in β-cells by 1,5-InsP₈ would inhibit exocytic insulin secretion [72], but this remains to be demonstrated.

On the other hand, Jessen and colleagues also described an apparently separate effect of 1,5-InsP₈, which, theoretically at least, could *promote* exocytosis, through PP-InsP's interaction with synaptotagmin-like protein 4 (Slp-4)/granuphilin [71]. The latter is required for insulin granules to dock to the plasma membrane, but such vesicles are not fusion-competent until granuphilin is subsequently detached [73]. A mechanism for granuphilin release has previously been proposed: competition from cytosolic InsP₆ for plasma-membrane associated PtdIns(4,5)P₂ binding to granuphilin's C2A and C2B binding domains [74]. However, 1,5-InsP₈ was shown in Jessen's study to also bind to each of these two domains in vitro ($K_d = 0.6$ and $5 \mu\text{M}$, respectively [71], as compared to 1.8 and $21 \mu\text{M}$ for InsP₆ [74]). Moreover, the caged 1,5-InsP₈ proved useful in validating PP-InsP's interaction with granuphilin in intact cells; a transfected and membrane-associated eGFP-tagged C2AB domain from granuphilin was found to redistribute into the cytoplasm following uncaging of the 1,5-InsP₈ [71]. That is, 1,5-InsP₈ was shown to be functionally active even in the presence of steady-state levels of InsP₆. Nevertheless, further work is required to determine how the cell might coordinate the seemingly opposing effects upon insulin secretion that could arise from 1,5-InsP₈ inhibiting Ca²⁺ signaling while also causing granuphilin relocalization.

11. FAM-5-InsP₇

Recently, Jessen's group synthesized a fluorescein-tagged analog of 5-InsP₇ (FAM-5-InsP₇) [51,53]. This, in itself, is notable for being the first description of the application of click chemistry to the synthesis of a PP-InsP analog, and the synthetic pathways that were involved [53] illuminate a gateway to the preparation of other novel molecules to facilitate further PP-InsP research. The value of this particular molecule is enhanced by the placement of the fluorescent tag onto the 5-β-phosphate, thereby imparting metabolic stability upon the normally labile diester phosphate [51].

We have used this new analog to validate our liposome delivery protocols [51,53] by exploiting the phenomenon of self-quenching that occurs when a fluorescent probe is present at high concentrations inside vesicles [75,76]. Thus, the gain of signal due to unquenching was used to follow the intracellular

release of the FAM-5-InsP₇ from the vesicles, predominantly into the cytoplasm, by using the tools of confocal microscopy and flow cytometry [51,53].

12. Conclusions

Based on the successes described above, we can confidently predict that further, dramatic developments in our understanding of PP-InsP biology will result as additional synthetic analogs provide information from inside intact cells and eventually in vivo. Two developments in particular appear close to fruition. First, photocage technology has improved to the point that the toxic effects of UV-mediated uncaging can be avoided by using photosensitive moieties that respond to visible light while retaining good water solubility [77]. Second, it has been proposed that one oxygen atom of the β -phosphoryl group of a PP-InsP can be replaced with a sulfur atom, so as to create an analog that can transfer a thiophosphoryl group to protein substrates for pyrophosphorylation [78]. Once transferred, the thiophosphate group can be derivatized and identified by appropriate antibodies. An InsP₇- β S reagent could be delivered into cells using the nanoparticle delivery systems described above. These and other technical innovations will increase the opportunities available to those working in this unique field of signal transduction.

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Appendix A

A Note Concerning Inositol Pyrophosphate (PP-InsP) Nomenclature: The literature in this field has a divergent usage of abbreviations to denote individual inositol pyrophosphates and their analogues, accompanied by dueling opinions as to which system is the more accessible to a multidisciplinary audience; one approach to nomenclature is systematic in nature, whereas the other is more colloquial. The latter is the option taken in the text of the current study. Nevertheless, each of the corresponding systematic abbreviations are given here in parentheses, along with full definitions: 1-InsP₇ (1-PP-InsP₅) = 1-diphospho-*myo*-inositol 2,3,4,5,6-pentakisphosphate; 5-InsP₇ (5-PP-InsP₅) = 5-diphospho-*myo*-inositol 1,2,3,4,6-pentakisphosphate; 1,5-InsP₈ (1,5-[PP]₂-InsP₄) = 1,5-bis-diphosphoinositol 2,3,4,6-tetrakisphosphate; 5-PP-InsP₄ (also 5-PP-InsP₄) = 5-diphospho-*myo*-inositol 1,3,4,6-pentakisphosphate; 1-PCP-InsP₇ (1-PCP-InsP₅) = *myo*-inositol 1-methylenediphosphonate-2,3,4,5,6-pentakisphosphate; 5-PCP-InsP₇ (5-PCP-InsP₅) = *myo*-inositol 5-methylenediphosphonate-1,2,3,4,6-pentakisphosphate; 1,5-PCP-InsP₈ (1,5-PCP-InsP₄) = *myo*-inositol 1,5-bis(methylenediphosphonate)-2,3,4,6-tetrakisphosphate; 1-PA-InsP₇ (1-PA-InsP₅) = *myo*-inositol 2,3,4,5,6-pentakisphosphate-1-phosphonoacetate; 5-PA-InsP₇ (5-PA-InsP₅) = *myo*-inositol 1,2,3,4,6-pentakisphosphate-5-phosphonoacetate; 1,5-PA-InsP₇ (1,5-PA-InsP₅) = *myo*-inositol 2,3,4,6-tetrakisphosphate-1,5-bis(phosphonoacetate).

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