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Review article

Assessing the omnipotence of inositol hexakisphosphate

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Abstract

This review assesses the authenticity of inositol hexakisphosphate ($InsP_6$) being a wide-ranging regulator of many important cellular functions. Against a background in which the possible importance of localized $InsP_6$ metabolism is discussed, there is the facile explanation that $InsP_6$ is merely an ''inactive'' precursor for the diphosphorylated inositol phosphates. Indeed, many of the proposed cellular functions of $InsP_6$ cannot sustain a challenge from the implementation of a rigorous set of criteria, which are designed to avoid experimental artefacts. Published by Elsevier Science Inc.

Keywords: Inositol hexakisphosphate; phytic acid; signal transduction; inositol phosphates; calcium; magnesium

1. Introduction

> Two men say they're Jesus/One of them must be wrong
> — Dire Straits: ''Industrial Disease''

Although the first report that inositol hexakisphosphate ($InsP_6$) (Fig. 1) is present in animal cells [1] turned out to be a case of mistaken identity [2], it eventually transpired that $InsP_6$ really is widely distributed throughout the animal kingdom [3–5]. After an inauspicious inauguration as a storage molecule [6], $InsP_6$ has since become affiliated with a seemingly unconnected and wide-ranging array of important physiological activities. These include neurotransmission [4], ''priming'' of stimulus-dependent respiratory burst in neutrophils [7], activation of protein kinase C [8], inhibition of protein phosphatase activities [9], activation of L-type Ca^{2+} channels [9], a vesicle trafficking ''clamp'' [10], attenuation of agonist-induced receptor desensitization [11], an iron transporter [12], a cellular antioxidant [12,13], an activator of enzymes conducting DNA repair [14], and a regulator of mRNA export [15]. Increases in dietary $InsP_6$ have also been advocated as being of therapeutic benefit. For example, $InsP_6$ has been suggested to prevent kidney stone formation [16] and to act as an antineoplastic agent [17]. Even in plants, where $InsP_6$ has long been recognized as a phosphate storage depot [18,19], this polyphosphate's curriculum vitae was recently expanded by evidence which indicated that it modulates a K^+ channel that regulates stomatal pore closing [20]. Can $InsP_6$ really wield these multitudinous influences upon cell function?

2. The metabolic status of the mammalian $InsP_6$ pool

Some of the ideas surrounding the roles of $InsP_6$ (Section 1) provide scenarios in which changes in intracellular $InsP_6$ levels might have regulatory significance. For example, hyperosmotic stress rapidly increases $InsP_6$ synthesis in *Schizosaccharomyces pombe* [21]. In the animal kingdom, total cellular $InsP_6$ levels fluctuate at certain points in the cell cycle and during cellular differentiation [22–25]. However, shorter-term manipulations in cellular $InsP_6$ levels are rare in animal cells, and when they have been noted, they are generally of a rather subtle nature. In HL-60 cells, a 10% change in $InsP_6$ mass was observed upon stimulation with chemotactic peptide [26]. $InsP_6$ levels respond to a similarly meager extent in glucose-challenged pancreatic β-cells [9] and dopamine-activated renal epithelial cells [27].

It is possible to envisage that $InsP_6$ may regulate a cellular process independently of it undergoing rapid metabolic fluxes. One suggestion [28] is that target proteins

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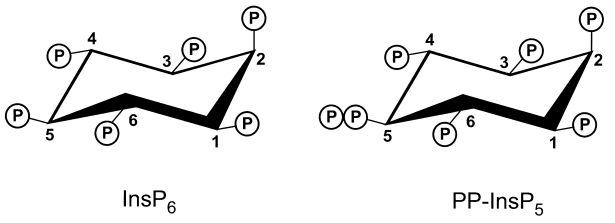

Fig. 1. Structures of $InsP_6$ and a diphosphorylated inositol phosphate. The structure of *myo*-inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$) is shown on the left. Inositol phosphate nomenclature recognizes both the number of attached phosphate ("P") groups (six in this case), and their positions around the inositol ring. Note that the 2-phosphate is axial to the plane of the inositol ring (the other five are equatorial). An example of a diphosphorylated inositolphosphate (PP-InsP_5 or "$InsP_7$") is shown on the right. See Ref. [37] for descriptions of other diphosphorylated inositol phosphates.

might be tonically "clamped" into a certain state upon binding of $InsP_6$. A putative regulatory signal might then be directed at a covalent modification of the protein, which decreases its ligand affinity, thereby releasing the blockade of protein function by $InsP_6$. Alternately, if $InsP_6$ turnover is regulated, only highly localized subpools might be involved, which could be missed in global $InsP_6$ assays. This situation could be analogous to the receptor-dependent, spatiotemporal changes in cytosolic Ca^{2+}, which occur without substantial impact upon total cellular [Ca^{2+}]. Our current understanding of the significance of inositol lipid metabolism is also very similar: we no longer consider cellular physiology to be solely modulated by fluctuations in bulk cellular turnover of these molecules. Instead, various regions of the cell are believed to possess semiautonomous pools of these lipids, the turnover of which is micromanaged by a highly localized subcellular distribution of lipid kinases and phosphatases [29]. In other words, while the total $InsP_6$ pool size is relatively static, $InsP_6$ turnover can still be considerable. Evidence supporting this idea comes from the (deliberately) nonequilibrium labeled pool of [^{3}H]$InsP_6$ in thyrocytes, which declined rapidly following activation of cell-surface receptors [30]. Such rapid alteration in specific radioactivity of [^{3}H]$InsP_6$ testifies to the metabolically active nature of the polyphosphate. Stimulus-dependent increases in cellular [^{3}H]$InsP_6$ after only 2 days incorporation of [^{3}H]inositol [31,32], again probably reflects changes in specific radioactivity, as equilibrium radiolabeling of the cellular $InsP_6$ pool may take more than a week to occur [5]. Such observations provide additional evidence of the rapidity of $InsP_6$ turnover.

$InsP_6$ turnover in vivo could involve substrate cycling by $InsP_6$ phosphatases and $InsP_5$ kinases [33]. However, the only known mammalian $InsP_6$ phosphatase is closeted inside endoplasmic reticulum with restricted access to its substrates [34,35]. We suspect there are other $InsP_6$ phosphatases inside cells (Section 9) that remain to be identified. A more direct demonstration of the high rate of $InsP_6$ metabolism in vivo came from the discovery that it is actively interconverted with the diphosphorylated inositol phosphates [36] (see Fig. 1).

Naturally, the potential significance of local changes in $InsP_6$ levels depends upon whether it is $InsP_6$ or its metabolite that is the biological effector. Indeed, there is considerable, albeit indirect evidence [37], that it is the further phosphorylation of $InsP_6$ to diphosphorylated inositol phosphates that yields physiologically active metabolites. In this situation, $InsP_6$ need be no more than an "inert" metabolic reservoir. Nevertheless, in principle at least, it is a reasonable hypothesis that $InsP_6$ might induce conformational changes in a particular protein, thereby altering its inherent activity, or even influencing its interactions with other proteins. Highly polar ligands, such as $InsP_6$, could also influence the electrostatics that define the nature of protein–protein interactions [38]. For two proteins to bind, long-range delocalized electrostatic attraction is envisaged to initially promote the formation of a transient, low-affinity, complex that subsequently rearranges itself under the auspices of more specific, localized, high-affinity interactions [38,39]. Under conditions where ionic strength is physiologically relevant, inositol polyphosphates are unlikely to modify "screening" of the low-affinity, delocalized electrostatic attraction [39]. However, it is straightforward to envisage how an inositol polyphosphate might influence the subsequent formation of high-affinity complexes. Ligand binding will substantially alter the electrostatic potential in the immediate vicinity of its binding site, through the introduction of new charged moieties, neutralization of others, and alterations in the screening of electrostatic interactions within the protein. There are reports of $InsP_6$ inhibiting some protein–protein interactions [27,40]. We would expect the target protein to show highly specific recognition of the spatial arrangement of phosphate groups around the inositol ring. Thus, the impact of ligand binding could be modified by the addition or removal of phosphate groups. So, clearly, we can place $InsP_6$ metabolism in a regulatory context. Can we now justify the claimed proposals for $InsP_6$ functions?

3. Criteria for testing $InsP_6$ function in vitro

It would certainly help us to evaluate the role of $InsP_6$ in vivo, if it had an effect in vitro at a concentration that we can appreciate to be physiologically relevant. Unfortunately, the latter is a rather elusive parameter. We do know that *total* cellular $InsP_6$ typically lies within the 15–100 μM range [22,41,42]. However, much of this $InsP_6$ may not be freely soluble. A number of intracellular proteins can bind $InsP_6$, at least in vitro (see below), and some of these might help buffer the free cellular concentration of this polyphosphate. There is also evidence that the surface of cellular membranes might act as an intracellular adhesive for $InsP_6$ by means of an electrostatically bonded $InsP_6$–cation–phospholipid sandwich that operates under conditions of phy-

siologically relevant ionic strength [43]. Consistent with this idea, when the cation "glue" is removed by treating isolated membranes with EDTA, $InsP_6$ is released [43,44]. Thus, we currently have little concept concerning what might be the likely concentration of available $InsP_6$ at various subcellular compartments. When $InsP_6$ is unleashed into an in vitro assay, almost any submillimolar dose can be argued as being physiologically significant. So, in this review, there are no opinions on dose–response relationships.

Just in case our limited understanding of the intracellular distribution of $InsP_6$ provided insufficient perplexity, the molecule also has some unusual chemical properties, which can create experimental responses to $InsP_6$ that may have no physiological relevance in vivo. I have therefore assembled from several sources a list of criteria, which should be considered when testing the physiological relevance of $InsP_6$-dependent phenomena that have been observed in vitro:

(i) The effect of $InsP_6$ should be observed in the presence of naturally occurring concentrations of Ca^{2+} and/or Mg^{2+} at a physiologically relevant ionic strength.
(ii) The effect of $InsP_6$ should not be imitated by ion-chelators, such as EDTA/EGTA.
(iii) Other related inositol phosphates, such as $InsP_5$ and the diphosphorylated inositol phosphates, should be shown to be less potent than $InsP_6$. PtdIns(3,4,5)P_3 should also be excluded.
(iv) It is informative if the effect of $InsP_6$ is not imitated by other conformers of $InsP_6$ or by $InsS_6$, although (Section 6) this criterion has a limited application.
(v) The target protein must show an appropriate ligand:protein stochiometry. Ideally, an $InsP_6$ binding domain needs to be characterized.
(vi) The purity of the $InsP_6$ should be verified, or at the very least, similar effects should be observed using material obtained from different sources.

Several examples can be used to justify the use of these criteria.

4. Electrostatic considerations

One approach that has been used to explore the significance of $InsP_6$ has been to screen for proteins that might bind this polyphosphate. However, these assays have frequently been performed under "ideal" ligand-binding conditions that may not extrapolate to the intracellular environment. $InsP_6$ is, of course, a molecule with a particularly high negative charge density, which gives the molecule the propensity to bind to proteins through electrostatic interactions, particularly so when the pH is lower than the isoelectric point, thereby maximizing the protein's positive charge [45]. While electrostatics can be important in contributing to some specific ligand–protein interactions (Section 2), in other cases, they may lead to the nonspecific association of $InsP_6$ with proteins in vitro. The physiologically relevant context for $InsP_6$ is almost certainly one in which at least some of its negative charge is neutralized by association with divalent cations, such as Ca^{2+} [46]. In this situation, nonspecific electrostatic interactions will be minimized, while still giving a potential target protein the opportunity for highly specific recognition of the spatial arrangement of phosphate groups around the inositol ring. Thus, interactions of $InsP_6$ with proteins in vitro have a more promising physiological context, if they can be observed in media in which both ionic strength and concentrations of cations, such as Ca^{2+} and Mg^{2+}, are similar to those of intact cells.

The apparent $InsP_6$-dependent stimulation of protein kinase C activity in crude cell extracts prepared from insulinoma HIT T15 cells was shown to rely upon Ca^{2+} being absent [8], so, based on the above criterion, the significance of this effect is doubtful. Actually, even in the absence of Ca^{2+}, this was a rather small effect of $InsP_6$ — about a 20% change in kinase activity was recorded. Furthermore, the specificity of action of $InsP_6$ (Section 6) was not studied. Divalent cations were also absent from media in which $InsP_6$ was shown to bind with nanomolar affinity to some pleckstrin homology (PH) domains [47]. Therefore, their affinity for $InsP_6$ may be overestimated. A similar criticism can be made of demonstrations of high-affinity $InsP_6$ binding to (i) vinculin, a cytoskeletal protein regulating cell adhesion, spreading and motility [48]; (ii) myelin proteolipid protein, which participates in myelin deposition [49]; (iii) coatomer, which regulates vesicle traffic between the ER and the Golgi [50,51]; and (iv) phosphotyrosine-binding domains [52]. Besides, there is no evidence that $InsP_6$ alters the recognized physiological function of any of these particular target proteins. Divalent cations were also absent when $InsP_6$ was shown to inhibit three different species of serine/threonine protein phosphatases [9]. In any case, it is difficult to envisage how a specific signalling consequence could result from a global effect of $InsP_6$ upon the activities of several different types of protein phosphatases.

As well as performing in vitro assays with $InsP_6$ in appropriate assay media, the physiological relevance of $InsP_6$-binding studies could be highlighted by demonstrations of an appropriate, saturable ligand:protein stoichiometry, and also by identification of a conserved $InsP_6$-binding domain. However, a more widespread utilization of domain structure should also be considered; structure is typically more conserved than is sequence [53].

5. More cation-binding issues

The negative charge density of $InsP_6$ provides this molecule with considerable prowess as a chelator of divalent and trivalent cations [12,46]. The idea that $InsP_6$

was a neurotransmitter [4] has met a somewhat dilatory demise with its proponents [54], following the recognition that nonspecific ion chelation by $InsP_6$ was responsible [55]. We should be particularly careful to avoid this pitfall again. For example, control experiments should be performed to demonstrate that EGTA and/or EDTA do not mimic the effects of $InsP_6$. It should also be noted that neurotransmission, and other proposed extracellular roles for $InsP_6$ (e.g., neutrophil "priming" [7]), face the challenge that mammalian cells have never been shown to deliver this polyphosphate to the extracellular environment (e.g., Ref. [56]).

Although a potential scourge for in vitro experiments with $InsP_6$, its ion-binding properties have occasionally been invoked when considering physiological roles. For example, the remarkable affinity of $InsP_6$ for iron inhibits this metal's ability to catalyze the formation of hydroxyl radicals in vitro [12]. Thus, it has been suggested that $InsP_6$ might transport iron within the cytosol in a form that protects against the potentially lethal consequences of free radical formation [12]. This proposal extends an earlier observation that $InsP_6$ is a powerful antioxidant in vitro [13]. The apparent affinity constant for Fe^{3+} and $InsP_6$ ($>10^{18}$ [12]) appears to provide ample opportunity for this interaction to occur in vivo. Yet, most of the cell's iron pool is safely bound to proteins, such as ferritin [57]. Admittedly, cells still retain a small, cytosolic pool of free Fe^{2+}, about 1 μM in size, but the fact that this remains redox-active [57] argues $InsP_6$ has only a limited role in preventing iron-dependent oxidative processes in vivo.

The chelation of Ca^{2+} by $InsP_6$ has been suggested to be physiologically important in blocking the formation of crystals of calcium oxalate and calcium phosphate [16]. $InsP_6$ was further proposed to be excreted in urine (in amounts that are in direct proportion to its dietary intake), thereby, it was suggested, preventing kidney stone formation [16]. However, the published descriptions of $InsP_6$ being a constituent of urine are indirect, and far from being conclusive. The samples of urine were simply batch-chromatographed using ion-exchange resin [58], and the "$InsP_6$ fraction" could have contained many other phosphorylated compounds.

It remains to be seen if ion chelation can account for the putative antineoplastic effect of $InsP_6$, when it is administered in heroically high (mM) concentrations in a number of isolated cell systems [17,59]. These results largely represent the persistent efforts of a single laboratory, and, moreover, there is no compelling epidemiological evidence that links dietary $InsP_6$ to cancer prevention in humans [60].

6. Judging the specificity of $InsP_6$ action

The demonstration of specificity of action of $InsP_6$ should be a crucial goal. For example, $InsP_6$, when microinjected into *Xenopus* oocytes, appeared to attenuate the desensitization of heterologously expressed substance P receptors [11]. However, Ins(1,3,4,5,6)P_5, and several other $InsP_5$ isomers, all closely imitated this effect of $InsP_6$ [11]. In the latter example, and in several other cases where Ins(1,3,4,5,6)P_5 and $InsP_6$ have elicited similar effects in vitro, it has been noted that the total cellular levels of both polyphosphates are very similar, and so the idea that they are functionally redundant has been promulgated [4,11,40,54]. I will argue here that this is not likely to be correct. Metabolic fluxes through the cellular pools of Ins(1,3,4,5,6)P_5 and $InsP_6$ are largely independently regulated [56] by sophisticated control processes [10]. This seems both inefficient and nonspecific if the two polyphosphates were to duplicate each other's functions. Thus, when $InsP_6$ has an experimental action that Ins(1,3,4,5,6)P_5 can closely imitate, then we should seriously consider that this is either a consequence of inadequate assay conditions that overemphasize the efficacy of one of the polyphosphates, or worse, that the effect of both compounds has little physiological significance.

The diphosphorylated inositol phosphates are also close metabolic relatives of $InsP_6$ [36,61]. These diphosphates should be excluded when examining specificity of $InsP_6$, but when doing so, the possibility of differential metabolism should be accounted for. There is widespead, active phosphatase activity towards the diphosphorylated inositol phosphate [62]. To preserve their half-life in cell-free assays, phosphatase inhibitors, such as fluoride [36], should be added. Differential metabolism may also have been an issue in experiments where several minutes exposure to Ins(1,4,5)P_3 was shown to be much less effective than $InsP_6$ at inhibiting a plant cell K^+ conductance [20]. In part at least, this lower apparent efficacy of Ins(1,4,5)P_3 may reflect it being rapidly depleted by phosphatases [63]. Alternately, since the effects of $InsP_5$ and $InsP_4$ isomers were not examined, it is possible that these products of $InsP_6$ hydrolysis might be the more active modulators of K^+ conductance.

Another group of compounds that might not immediately be obvious for specificity testing are the inositol lipids, but in fact they can compete effectively for some "$InsP_6$-binding" sites. For example, $InsP_6$ binds to vinculin [48], but this protein commands more attention as a target for inositol lipids [64]. Other examples include synaptotagmin, arrestin, AP-2, and AP-180 (Section 7). Determination of a protein's affinity for inositol lipid frequently utilizes soluble analogues with short, fatty acyl chains. Unfortunately, this can unnaturally decrease apparent ligand affinity by reducing any direct contacts that the protein may have with the natural and longer fatty acyl chains. Another factor that may contributed to the overall affinity of protein for a phospholipid surface in vivo is partitioning of the protein into the membrane. Ligand avidity [65] is yet another issue. A clustered array of inositol lipids on a membrane surface can act as a multivalent ligand with a collective functional

affinity that is higher than that of individual (and soluble) lipid molecules. Thus, when determining whether a protein might have a preference for binding $InsP_6$ or inositol lipids, there are considerable opportunities to underestimate functional lipid affinity.

Ligand avidity may also be relevant to a curious problem that my laboratory first noted some time ago [34,66]. Although the molecular size of $InsP_6$ is only 660 Da (Fig. 1), the polyphosphate can take on the properties of a much larger molecule, eluting from size exclusion columns with an apparent size of 9 kDa [34]. The polyphosphate is also not readily dialyzable [14,66,67]. An acceptable explanation for this phenomenon is still lacking, but it seems $InsP_6$ can self-aggregate into a multivalent, macromolecular complex. The latter [65] can have a higher *apparent* binding affinity to a target protein than will individual $InsP_6$ molecules. To avoid overestimating a protein's affinity for $InsP_6$, binding assays should be performed in media with physiologically relevant ionic strength, in which $InsP_6$ behaves more like a molecule of 660 Da [14,67]. Appropriate levels of divalent cations should also be present (Section 5).

There is another issue relating to specificity that might also threaten the validity of some experiments with $InsP_6$ in vitro. There may be unexpected cross-reactivity between inositol phosphates and other cellular polyphosphates that do not even contain inositol, such as the competitive dephosphorylation of diphosphorylated inositol phosphate and diadenosine polyphosphates by some Nudt hydrolases [62]. Thus, an effect of $InsP_6$ that is observed in vitro may be satisfied by a completely unrelated polyphosphate in vivo. This potential problem, by its very nature, is always difficult to exclude.

It can be informative if an in vitro effect of $InsP_6$ is only weakly imitated by $InsS_6$, since the latter is a nonphysiological analogue, which also has high negative charge density. Conformers of $InsP_6$ may also be useful; in the naturally occurring *myo*-$InsP_6$, only the 2-phosphate is axial to the plane of the inositol ring (the other five are equatorial, see Fig. 1). In contrast, *scyllo*-$InsP_6$ has no axial phosphates, whereas *neo*-$InsP_6$ has two (the 2- and 5-phosphates). It should be noted that these particular negative controls are most useful when they yield just such a result. If an action of, say, $InsS_6$ in vitro is nearly as equipotent as $InsP_6$ (e.g., activation of L-type Ca^{2+} channels, Ref. [9]), this may be evident that the effect has no physiological significance, but such a result may instead reflect the particular pharmacology of an $InsP_6$-binding site. $InsS_6$, *scyllo*-$InsP_6$, and *neo*-$InsP_6$ are not present in animal cells, so there has been no evolutionary pressure to exclude these compounds from being effective ligands.

Recently, *neo*-$InsP_6$ and *scyllo*-$InsP_6$ were found not to imitate the apparent osmoregulatory ability of *myo*-$InsP_6$ to inhibit an inwardly rectifying K^+ current in guard cell protoplasts in two plant species [20]. Unfortunately, this otherwise promising result is somewhat tempered by the use of *myo*-$InsP_6$ from only one source. Some commercial preparations of $InsP_6$ have repeatedly been shown to be seriously contaminated [68,69]. At the very least, the experimenter should verify that different batches of $InsP_6$, obtained from more than one source, all act in a similar manner; further purification of $InsP_6$ by HPLC [69] is strongly recommended.

7. $InsP_6$ and the regulation of protein traffic

A fundamental problem in cell biology is to understand the spatial and temporal control of protein traffic in macromolecular, vesicular structures. This complex transport process is necessary for the biogenesis of plasma membranes, lysosomes, and endosomes, the secretion of proteins and other materials from the cell, and the accumulation of molecules from the extracellular environment. The specificity of such processes is also harnessed to generate structurally and functionally polarized apical and basolateral surfaces to many cell types. Some years ago, an increasing number of proteins that regulate vesicle traffic were discovered to bind $InsP_6$, so the polyphosphate has drawn some attention as a potential modulator of these processes (see Ref. [28] for a review). Has this idea withstood the test of time?

Type I synaptotagmin, which is part of the synaptic vesicle complex, promotes exocytosis by "sensing" local changes in $[Ca^{2+}]$ and by interacting with several other proteins [70]. Recombinant synaptotagmin binds certain inositol phosphates, with $InsP_6$ being a particularly potent ligand [71]. Speculation that such ligand binding might affect vesicle traffic has been further fueled by two reports: first, microinjection of (an uncertain quantity of) $InsP_6$ into the presynaptic terminal of the giant squid synapse inhibits neurotransmitter release from synaptic vesicles [72]. Second, $InsP_6$ inhibits catecholamine release from permeabilized bovine adrenal chromaffin cells [73]. However, neither of these two responses were specific for $InsP_6$, as Ins(1,3,4,5,6)P_5 and Ins(1,3,4,5)P_4 were also effective [72]. The effect of $InsP_6$ upon catecholamine release was also attenuated by Ca^{2+} [73] (see Section 5). Also worrying is the finding that, compared to recombinant synaptotagmin, the native protein has a lower affinity for $InsP_6$, and instead prefers to bind Ins(1,3,4,5)P_4 and Ins(1,3,4,5,6)P_5 [74,75]. In any case, it appears that synaptotagmin — especially the native protein — binds inositol lipids with even higher affinity than inositol phosphates [70,75,76]. Thus, it is the binding of synaptotagmin to PtdIns(4,5)P_2 in the plasma membrane that is now more generally envisaged to promote docking and fusion of exocytic vesicles [70,75,76]. It is an exaggeration to assert, as was the case in one recent review [77], that $InsP_6$ "...is *known* to modulate the trafficking of synaptic vesicles in mammalian cells through a direct interaction with synaptotagmin" (*my emphasis*).

$InsP_6$ binds with nanomolar affinity to the α subunit of the tetrameric AP-2 complex [78,79] and to monomeric AP-180 [80,81]. Both of these "assembly" proteins pro-

mote an early event during endocytosis, namely, the polymerization of clathrin into a lattice that coats the surface of the endocytic vesicle, so as to provide structural integrity. AP-2 is present in many tissues, whereas AP-180 is synaptosomal-specific. One consequence of $InsP_6$ binding to these assembly proteins, in vitro at least, is inhibition of clathrin lattice formation [78,80,81]. These assembly assays were performed in the presence of divalent cations (see Section 4), and both $Ins(1,3,4,5,6)P_5$ and $InsS_6$ were considerably much less active than $InsP_6$. Thus, $InsP_6$ emerged as a potential inhibitor of endocytosis [78,80,81]. The phosphorylation of $InsP_6$ to PP-$InsP_5$ yields an even more potent inhibitor [81]. However, more recent studies with both AP-180 [82] and AP-2 [79] suggest that inositol lipids are more important ligands than $InsP_6$. Moreover, the postulated role of this inositide-binding domain now points elsewhere, not in a down-regulatory context, but as a *positive* membrane-recruitment motif [83]. For example, endocytosis is inhibited when the inositide-binding domain is deleted from the α subunit of AP-2, which then does not get incorporated into clathrin-coated vesicles [83].

The binding of $InsP_6$ to arrestins has also drawn attention in the vesicle trafficking field [84]. Arrestins are recruited to G protein-coupled receptors following their receptor-dependent phosphorylation. One of the consequences of arrestin binding is receptor desensitization. In the case of the so-called "nonvisual" arrestins (e.g., β-arrestin and β-arrestin2), desensitization is in part due to internalization of the receptor. It has been argued that arrestin-dependent receptor trafficking can be inhibited by $InsP_6$ [11]. This proposal emerged after microinjection into *Xenopus* oocytes of $InsP_6$ (and $InsP_5$, see Section 6), appeared to attenuate the desensitization of heterologously expressed substance P receptors [11]. However, other evidence points to inositol lipids being the functional ligand for β-arrestin2, by recruiting the protein to coated pits; this targeting, and the concomitant stimulation of receptor-endocytosis, is strongly impaired upon deletion of the inositide-binding domain from β-arrestin2 [84].

8. $InsP_6$ and DNA repair

Genomic stability relies on an efficient means of repairing radiation- and chemical-induced double-stranded breaks in DNA. Nonhomologous end-joining represents one such mechanism. This end-joining reaction, as catalyzed in vitro by a partly purified protein fraction eluted from a phosphocellulose column (denoted PC-C), was recently found to be activated by $InsP_6$ [14]. This effect of $InsP_6$ tolerates several of the criteria described in Section 3. For example, the authors confirmed by NMR and mass spectroscopy that $InsP_6$ was an endogenous activator of DNA repair, and then they recapitulated the effect using commercial $InsP_6$ obtained from two independent sources [14]. This effect of $InsP_6$ was observed in the presence of 0.5 mM Mg^{2+} (the significance of which is described in Section 4). In specificity tests, $InsS_6$, $Ins(1,3,4,5,6)P_5$, $Ins(1,3,4,5)P_4$, and $Ins(1,4,5)P_3$ were all less effective than $InsP_6$ [14]. The authors do note, however, that diphosphorylated inositol phosphates should also be tested for specificity. Otherwise, these data are quite alluring.

The protein(s) in fraction PC-C through which $InsP_6$ acts were not directly characterized; presumably there are many proteins in PC-C, in addition to the small number already known to promote the end-joining reaction. A DNA-dependent protein kinase (DNA-PK) emerged as a promising candidate for an $InsP_6$ target, since it bound this polyphosphate, even in the presence of divalent cations [14]. However, stoichiometry, specificity, and the affinity of ligand binding are important factors that remain to be determined. The authors of this study [14] propose that $InsP_6$ might influence the interaction of DNA-PK with other proteins in the multimeric end-joining complex. Possible mechanisms by which this may occur were discussed in Section 2.

9. A different approach: genetic studies

Clearly, we face substantial difficulties in interpreting the effects of $InsP_6$ in cell-free assays. However, alternative strategies, using in vivo models, are beginning to emerge. For example, as we begin to understand the molecular nature of the enzymes that synthesize and metabolize $InsP_6$, and then manipulate their levels of expression, we will be provided with new opportunities to alter $InsP_6$ levels in its physiologically relevant milieux. Which are the enzymes that are involved? The de novo pathway of $InsP_6$ synthesis has been most carefully studied in model systems outside the animal kingdom: in the slime-mould, *Dictyostelium* [85], in yeasts [15,21], and in the duckweed *Spirodela polyrhiza* [86]. In all cases, $Ins(1,3,4,5,6)P_5$ was identified as the immediate precursor of $InsP_6$. Deletion of the $Ins(1,3,4,5,6)P_5$ 2-kinase gene from *Saccharomyces cerevisiae* has yielded new ideas on $InsP_6$ function (Section 10). This new approach preempts many of the difficulties that have plagued in vitro studies with this polyphosphate.

Unfortunately, in animal cells, the molecular identity of an $Ins(1,3,4,5,6)P_5$ 2-kinase currently eludes us. A possible alternative tool has been cloned [87,88], namely, the mammalian multiple inositol polyphosphate phosphatase (MIPP) that hydrolyzes $InsP_6$. This is an intriguing protein that is predominantly located inside the endoplasmic reticulum, where its access to $InsP_6$ is restricted [34,35]. Cells obtained from mice in which the MIPP gene was deleted have only slightly higher levels of $InsP_6$ than wild-type cells, and the "knockout" animals show no obvious phenotypic defects [35]. This result suggests that there are other $InsP_6$ phosphatases that can compensate for the loss of MIPP, but,

otherwise, these experiments provide no fresh insight into the cellular roles of $InsP_6$.

We [89] have recently discovered that cellular $InsP_6$ levels can be acutely reduced by the SopB protein from *Salmonella*. However, this approach is not recommended for the specific study of $InsP_6$ action, since SopB has so many other gross effects upon cells. For example, SopB activates Akt [90], dephosphorylates $InsP_5$ [89,91], activates PLC [89], and stimulates the Rho-based GTPase family that promotes cytoskeletal rearrangements and gene transcription [89].

10. $InsP_6$ and the regulation of nuclear mRNA export

Nuclear export of mRNAs requires a series of events: pre-mRNA processing (splicing, polyadenylation, and capping), ribonucleoprotein targeting to the nuclear pore complex, and energy-dependent translocation through the pore. Three yeast gene mutations have been identified, which share the common phenotypes of impaired mRNA export and near-complete loss of $InsP_6$ synthesis [15]. One of the defective genes (named *ipk1*) was found to encode Ins(1,3,4,5,6)P_5 2-kinase activity, an enzyme that immunofluorescence microscopy revealed to be concentrated at the nuclear periphery [15]. This localization suggests a strategic targeting of $InsP_6$ synthesis to a potential site of action — the export machinery of the nuclear pores. Further genetic studies also address the issue of specificity; there is no evidence for participation in this process of the diphosphorylated derivatives of $InsP_6$ [92,93]. It was proposed that $InsP_6$ might either regulate the conformation of the nuclear pore complex, or facilitate removal of an export inhibitor [15]. These options could be interesting possibilities to pursue. However, more information is required concerning a mutant form of *ipk1* that lacks its 45 residue carboxy terminus (*ipk1–5*; Ref. [92]): the original synthetic lethal screen that led to the identification of mutant alleles of *ipk1* was a temperature-sensitive *gle* mutant defective for another, essential mRNA export factor [15]. The *ipk1–5* mutant is also synthetically lethal, yet, it supports a substantial proportion of the $InsP_6$ synthesis seen in the wild-type yeast [92]. It will be important to fit this observation into the overall hypothesis.

11. Summary

I have outlined in this review several, rigorous criteria that I believe should be implemented for the analysis of the relevance of the purported effects of $InsP_6$. In fact, many of the published experimental observations do not yet stand up to such close scrutiny. It is my hope that a closer examination of the proposed roles of $InsP_6$ in these cellular events, and the other ideas I have put forward, will now prompt a more careful consideration of the significance of $InsP_6$ and other higher inositol phosphates.

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