Minireview

# Biochemistry and genetics of inositol phosphate metabolism in *Dictyostelium*

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**Abstract Biochemical and genetic data on the metabolism of inositol phosphates in the microorganism *Dictyostelium* are combined in a scheme composed of in five subroutes. The first subroute is the inositol cycle as found in other organisms: inositol is incorporated into phospholipids that are hydrolysed by PLC producing Ins(1,4,5)$P_3$ which is dephosphorylated to inositol. The second subroute is the sequential phosphorylation of inositol to Ins$P_6$; the Ins(3,4,6)$P_3$ intermediate does not release $Ca^{2+}$. The third subroute is the sequential phosphorylation of Ins(1,4,5)$P_3$ to Ins$P_6$ in a nucleus associated fraction, whereas the fourth subroute is the dephosphorylation of Ins(1,3,4,5,6)$P_5$ to Ins(1,4,5)$P_3$ at the plasma membrane. This last route mediates Ins(1,4,5)$P_3$ formation in cells with a disruption of the single *PLC* gene. Finally, we recognize the formation of Ins$P_7$ and Ins$P_8$ as the fifth subroute.**



*Key words:* Ins(1,4,5)$P_3$; Phospholipase C; Cloning; Chemotaxis; Signal transduction

## 1. Introduction

*Dictyostelium* is a fungus that diverted from the evolutionary mainstream about 1 billion years ago, after the diversion of yeast but before the splitting between animals and plants [1]. The amoeboid cells live in the soil where they feed on bacteria. When the bacteria become scarce, starvation induces the expression of a cAMP sensory system; some cells start to secrete cAMP to which cells in the surrounding respond with a chemotactic reaction. A cell aggregate is formed that can be composed of up to 100 000 cells. Finally, a fruiting body is formed consisting of spores embedded in a slime droplet on top of highly vacuolized cells arranged in a stalk. When food is available, spores germinate and amoebae will search for food by chemotaxis.

Extracellular cAMP stimulates several second messengers pathways, including adenylyl cyclase, guanylyl cyclase, phospholipase C, and the opening of plasma membrane $Ca^{2+}$ channels. Directed cell locomotion and specific gene expression are the two main cellular responses induced by extracellular cAMP. Cells possess surface receptors that bind cAMP. Four genes encoding cAMP receptors have been identified (see [2]). The deduced amino acid sequences predict that they pass the membrane seven times, typical for receptors that interact with G-proteins. *Dictyostelium* contains only one gene encoding a Gβ subunit. Cells with a deletion of Gβ are strongly defective in many responses, including the activation of adenylyl cyclase, guanylyl cyclase, phospholipase C, chemotaxis and gene expression [3]. Interestingly, the cAMP-mediated opening of $Ca^{2+}$ channels still occurs in cells lacking Gβ, suggesting that this response is G-protein-independent [4]. The *Dictyostelium* genome uncovers eight genes encoding Gα subunits [5]; their function in regulating PLC is described below.

The metabolism of inositol phosphates is very complex, and *Dictyostelium* is not an exception. Mass detection has identified at least 25 compounds in this organism, with relatively large concentrations of Ins$P_6$, Ins$P_7$ and Ins$P_8$ [6]. In this review inositol phosphate metabolism will be subdivided in eight parts, and some attention will be paid to their localization in the cell and their regulation. A division is made into (1) de novo synthesis of inositol, (2) conversion of inositol to phospholipids in membranes, (3) formation of Ins(1,4,5)$P_3$ from PtdIns(4,5)$P_2$ by phospholipase C, (4) degradation of Ins(1,4,5)$P_3$ to inositol in the cytosol, (5) sequential phosphorylation of inositol to Ins$P_6$ in the cytosol, (6) formation of Ins(1,4,5)$P_3$ from Ins(1,3,4,5,6)$P_5$ by a new enzyme at the plasma membrane, (7) phosphorylation of Ins(1,4,5)$P_3$ to Ins$P_6$ in the nucleus, and finally the formation of higher inositol phosphates such as Ins$P_7$ and Ins$P_8$ (see Fig. 1).

## 2. De novo synthesis of inositol and formation of inositol phospholipids

*Dictyostelium* cells can grow on a semi-synthetic defined medium that does not include inositol [7], indicating that cells can make this sugar. The precursor is most likely glucose 6-phosphate that is converted to Ins3P and subsequently dephosphorylated to inositol, as in other organisms [8]. No experimental evidence for the presence of an Ins3P synthase is available, except that introduction of [$^{14}$C]glucose into cells by electroporation results in the formation of small amounts of [$^{14}$C]Ins$P_6$ (Van Haastert, unpublished observations); it is known that Ins$P_6$ is formed mainly from inositol via Ins3P (see below).

When [$^3$H]inositol is introduced into *Dictyostelium* cells by electroporation, the label is rapidly converted to PtdIns reaching a maximum after 10 min [9,10]. It then takes a relatively long time to form PtdIns4P (maximal after 45 min), which is converted within 15 min to PtdIns(4,5)$P_2$. Except for developmentally regulated PtdIns 4-kinase [11], little is known about the enzymes involved in inositol phospholipid metabolism.

A family of five phosphatidylinositol kinases (PIK) have been cloned in *Dictyostelium* by Zhou et al. [12]. PIK5 shows high homology with the family of yeast Vsp34p and presum-

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ably phosphorylate PtdIns at the 3-position. Such an enzyme was suggested by the detection of small amounts of PtdIns3P in *Dictyostelium* [13]. Reduced expression of PIK5 leads to slow growth and abnormal development. PIK4 encodes a putative PtdIns 4-kinase; attempts to disrupt the gene were unsuccessful, suggesting that it might be essential for growth. PIK1, PIK2 and PIK3 are most closely related to p110 PtdIns 3-kinase that use PtdIns4P and PtdIns(4,5)$P_2$ as substrates. Disruption of a single *PIK1*, *PIK2*, or *PIK3* gene has no effect, while double knockouts *PIK1/PIK3* or *PIK2/PIK3* appear to be lethal. The double knockout *PIK1/PIK2* grows slowly and shows abnormal development. When these cells are labelled with $^{32}PO_4$, reduced levels of PtdIns$P_3$ are observed without alterations of PtdIns(3,4)$P_3$ levels [14]. A PtdIns4P 5-kinase has not been identified yet.

## 3. Phospholipase C

### 3.1. Regulation of phospholipase C

PLC is present mainly in the plasma membrane and to some extend in the cytosol and in a nuclear fraction. The enzyme is strictly $Ca^{2+}$-dependent with half-maximal stimulation at about 0.5 μM $Ca^{2+}$ [15,16]. In cells, cAMP stimulates PLC enzyme activity about 2-fold. Regulation of PLC activity was studied in detail using mutants with deletions of genes encoding specific cAMP receptors (cAR) and Gα subunits. Activation of PLC by cAMP does not require cAR1, and probably also not cAR3 [17]. Some cAMP analogues that previously were shown to be chemotactic antagonists, inhibit PLC activity; this inhibition requires cAR1 expression. In a cell-free extract GTPγS stimulates PLC activity. Stimulation of PLC by cAMP in cells or by GTPγS in lysates is lost in mutants with a deletion of the *Gα2* gene, suggesting that activation of PLC is mediated by an unknown cAMP receptor and the G-protein G2. Inhibition of PLC by cAMP antagonists is lost in cells with a deletion of the *Gα1* gene. Thus, it is suggested that inhibition of PLC by cAMP occurs via cAR1 and Gα1 [17].

### 3.2. Phospholipase C gene and protein

One gene encoding phosphatidylinositol-specific phospholipase C (*Dd-PLC*) has been identified in *Dictyostelium* [18]. Mammalian cells possess three classes of PLC isozymes that all require $Ca^{2+}$ ions for full activity [19,20]. PLCβ is regulated by G-proteins; PLCγ contains SH2 and SH3 domains and is activated by tyrosine kinase receptors. Regulation of enzymes of the PLCδ class is largely unknown; it has been suggested that only $Ca^{2+}$ ions regulate enzyme activity. The deduced primary sequence of Dd-PLC places it in the class of PLCδ isozymes. Cells with an inactivated *Dd-PLCδ* gene have lost all detectable PLC activity [6]. Also extended searches by PCR or low-stringency hybridization does not give any indication for another *PLC* gene.

Using the three-dimensional structure of mammalian PLC [21], the deduced primary sequence of Dd-PLCδ can now be interpreted. The N-terminal segment of about 300 amino acids is composed of a PH-domain and four EF-hands domains. This is followed by a split catalytic domain; all eukaryotic PLC isozymes have an insertion in the catalytic domain of variable length and function. At the C-terminus of Dd-PLCδ, a conserved C2 domain is recognized that may help in binding of the enzyme to the membrane [22]. In rat PLCδ 135 amino acids can be deleted from the N-terminus without loss of activity [23], but this is probably not the case in *Dictyostelium* where deletion of 44 amino acids leads to inactivation of the enzyme (Drayer et al., unpublished observations). The insertion in the catalytic domain of Dd-PLCδ is predicted to be a strong EF-hand $Ca^{2+}$-binding domain. Mutations of those amino acids that would chelate the $Ca^{2+}$ ion have no effect on the $Ca^{2+}$-sensitivity of the enzyme, but lead to reduced maximal PLC activity; apparently this $Ca^{2+}$ fulfills a structural role and is not the $Ca^{2+}$ site of regulating the activity of PLC [16]. Deletion of small parts of the C-terminal segment of Dd-PLCδ destroys enzyme activity; a similar observation was made for rat PLCδ [23].

### 3.3. Deletion of phospholipase C gene

Unexpectedly, cells with a disruption of the *Dd-PLCδ* gene grow with the same rate as wild-type cells, they show chemotaxis and differentiate without observable defects. Even more unexpectedly, cells contain nearly normal Ins(1,4,5)$P_3$ levels as measured using mass assays, although they have no detectable PLC activity [6]. On further analysis, we observed in [$^3$H]inositol-labelled cells that [$^3$H]Ins(1,4,5)$P_3$ has very low specific activity in the *plc*-null mutant and thus was formed from a source that is not rapidly labelled with [$^3$H]inositol. In wild-type and *plc*-null cells [$^3$H]inositol is rapidly converted to [$^3$H]PtdIns(4,5)$P_2$ (see above in Section 2) and only slowly to [$^3$H]Ins$P_6$ via the de novo synthesis route (see below in Section 5). In wild-type cells [$^3$H]Ins(1,4,5)$P_3$ has approximately the same specific activity as its presumed precursor [$^3$H]PtdIns(4,5)$P_2$. The relatively cold [$^3$H]Ins(1,4,5)$P_3$ in *plc*-null cells and the reduced mass of Ins(1,3,4,5,6)$P_5$ in this mutant [6] led to the speculation that a phosphatase acting on higher inositol phosphates may mediate Ins(1,4,5)$P_3$ synthesis in cells lacking PLC activity (see below in Section 6).

## 4. Degradation of Ins(1,4,5)$P_3$

Ins(1,4,5)$P_3$ is degraded in the cytosol of *Dictyostelium* cells by two phosphatases at the 5- and 1-position, respectively [24]. Mammalian cells only possess a 5-phosphatase, whereas plants have only the 1-phosphatase [25]. In growing *Dictyostelium* cells, both enzymes are present, but the 5-phosphatase disappears during multicellular differentiation [26]. As the 5-phosphatase does not degrade Ins(1,3,4,5)$P_4$, the enzyme may be a type-II 5-phosphatase as classified in mammalian cells [27]. The products of Ins(1,4,5)$P_3$ degradation, Ins(1,4,)$P_2$ and Ins(4,5)$P_2$, are both dephosphorylated to Ins4P, which is further degraded to inositol.

At least three InsP isomers are produced in *Dictyostelium* via different routes. Ins1P is formed from the phospholipid PtdIns by a phospholipase C reaction, Ins4P is formed by degradation of Ins(1,4,5)$P_3$, whereas Ins3P is formed by de novo synthesis from glucose 6-phosphate or by phosphorylation of inositol. Specific enzymes and HPLC were used to discriminate between these isomers [28]. Pulse-labeling of cells with [$^3$H]inositol reveals that Ins1P is the first labelled InsP isomer, followed by Ins4P, whereas almost no Ins3P is formed; this kinetics of InsP formation is consistent with the extremely rapid formation of [$^3$H]PtdIns, the slower formation of [$^3$H]PtdIns(4,5)$P_2$ and [$^3$H]Ins(1,4,5)$P_3$, and the still slower phosphorylation of inositol to [$^3$H]Ins3P.

Inositol monophosphatases are generally inhibited by lith-

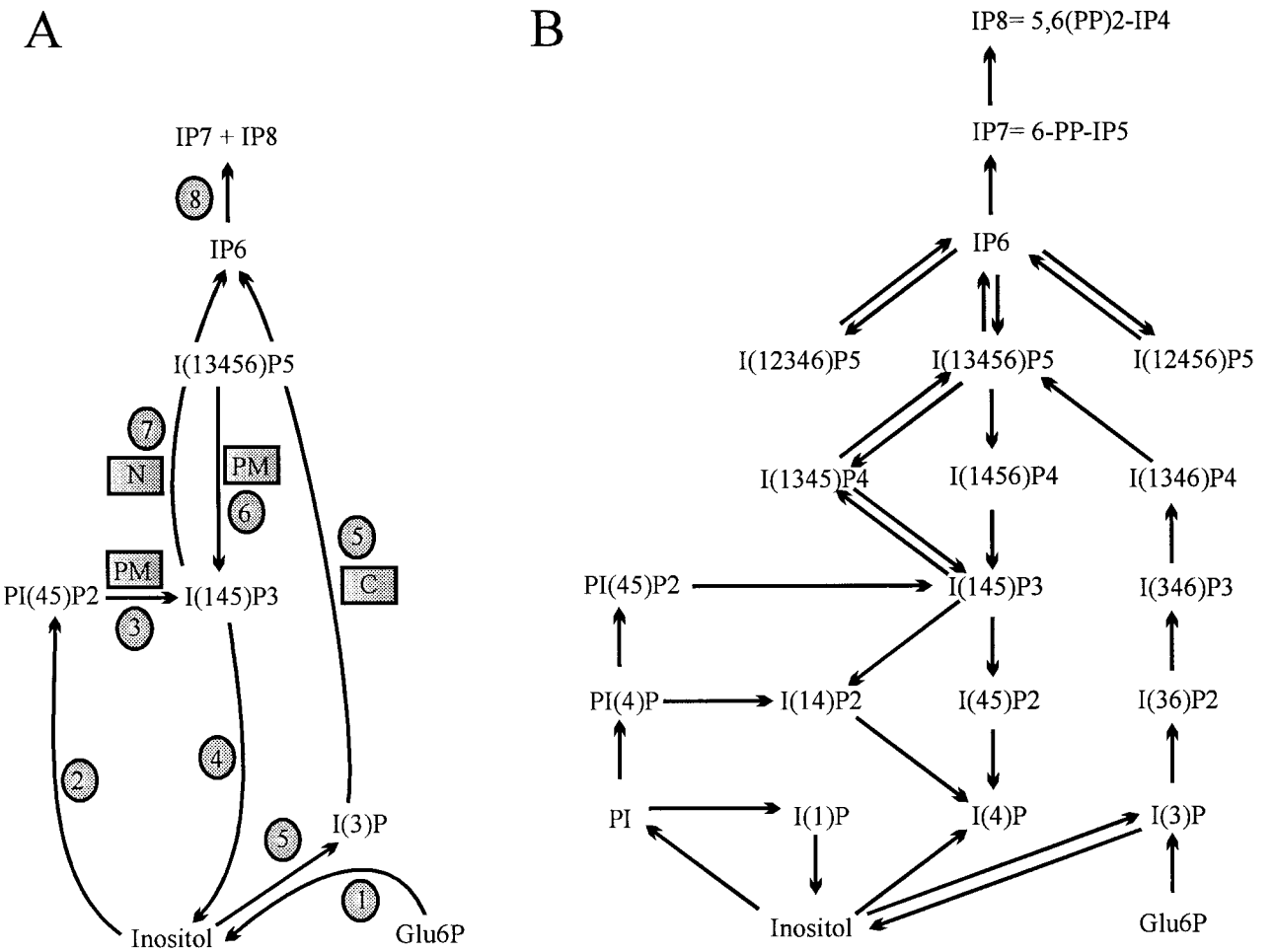


Fig. 1. Scheme of inositol phosphate metabolism in *Dictyostelium*. A: The overall scheme, depicting the numbered subroutes, their location (c = cytosol; n = nucleus; m = plasma membrane), and the main compounds. B: All reaction steps and intermediates.

ium in an uncompetitive mechanism, which has been used to explain the therapeutic effects of lithium [29]. In mammalian cells one lithium-sensitive monophosphatase degrades all InsP isomers [30]. *Dictyostelium* contains three enzymes that degrade InsP isomers with very different substrate specificity and sensitivity to lithium [28]. The first enzyme is similar to the mammalian enzyme as it is inhibited by lithium and hydrolyses both Ins1P, Ins4P and to a lesser extend Ins3P. The second enzyme only hydrolyses Ins4P and is not inhibited by lithium, whereas the third enzyme is also lithium-insensitive and degrades especially Ins3P and also Ins1P and Ins4P. Lithium has diverging effects on the relative levels of InsP isomers: inositol levels are hardly affected, Ins1P is increased to 600%, Ins4P to 190%, whereas Ins3P levels were reduced to 72%. These data are consistent with the specificity and relative sensitivity to lithium of the three monophosphatases in *Dictyostelium* [28]. Clearly, the monophosphatase activity is not the target of lithium for its pronounced effects during development of this organism [31].

## 5. Sequential phosphorylation of inositol to $InsP_6$

Incubation of the cytosol with inositol and ATP results in the formation of large amounts of $InsP_6$ [32]. The first step is the synthesis of Ins3P by a *myo*-inositol D-3 hydroxykinase [13]. The phosphorylation of Ins3P to $InsP_6$ is strictly sequential, with the intermediates $Ins(3,6)P_2$, $Ins(3,4,6)P_3$, $Ins(1,3,4,6)P_4$, and $Ins(1,3,4,5,6)P_5$. Thus, the $InsP_3$ isomer is different from the important $Ins(1,4,5)P_3$ that is produced by phospholipase C and liberates $Ca^{2+}$ from internal stores. The $InsP_5$ isomer appears to be an important compound because it intersects with several other metabolic routes (see Fig. 1 and below).

In cell lysates $InsP_6$ is rapidly dephosphorylated to $Ins(1,2,4,5,6)P_5$ and $Ins(1,2,3,4,6)P_5$, and rephosphorylated to $InsP_6$ [32]. It has been calculated that these in vitro enzyme activities are sufficiently high to give half-times of conversions to $InsP_6$ for $Ins(1,2,4,5,6)P_5$ and $Ins(1,2,3,4,6)P_5$ in the order of 0.8 and 6.4 s, respectively. As the concentration of these $InsP_5$ isomers are 20–40 μM, these dephosphorylation/phosphorylation shunts would consume relevant amounts of energy [32]. Van der Kaay et al. [33] have tried to find evidence for the presence of these shunts in vivo by incubating cells with $^{32}P$-labeled inorganic phosphate, isolating [$^{32}P$]$InsP_6$, and determining which position of $InsP_6$ is labelled. It was observed that after a short incubation of cells with $^{32}P$, $InsP_6$ was predominantly labelled at the 6-position, whereas the 4- and 5-positions were relatively cold. Thus, no indications for the proposed futile $InsP_5$/$InsP_6$ cycles were found in vivo [33].

## 6. Formation of Ins(1,4,5)$P_3$ from Ins(1,3,4,5,6)$P_5$ at the plasma membrane

Cells with a deletion of the single *PLC* gene have nearly normal Ins(1,4,5)$P_3$ levels, suggesting that another route for the formation of Ins(1,4,5)$P_3$ must exist, at least in *Dictyostelium*. Several higher inositol phosphates with phosphates at the 1-, 4-, and 5-positions were tested as potential substrates for the formation of Ins(1,4,5)$P_3$ in cell lysates [34]. Small amounts of Ins(1,4,5)$P_3$ are produced from Ins$P_6$; Ins(1,2,3,4,5)$P_5$ and Ins(1,2,4,5,6)$P_5$ are not converted to Ins(1,4,5)$P_3$. The only Ins$P_5$ isomer that is a very good substrate for Ins(1,4,5)$P_3$ production in lysates from both *plc*-null and wild-type cells is Ins(1,3,4,5,6)$P_5$, which is the same Ins$P_5$ isomer with reduced mass in *plc*-null cells. The intermediate products of the reaction are Ins(1,3,4,5)$P_4$ and Ins(1,4,5,6)$P_4$, both compounds are degraded to Ins(1,4,5)$P_3$ [26]. This degradation of Ins(1,3,4,5,6)$P_5$ at the 3- and 6-positions is most likely mediated by one enzyme [35].

The enzyme is strictly $Ca^{2+}$-dependent with half-maximal activity at 0.9 μM $Ca^{2+}$ for all substrates. Cell fractionation studies demonstrate the enzyme to be localized at the inner face of the plasma membrane. Interestingly, the Ins(1,3,4,5,6)$P_5$ 3/6-bisphosphatase is activated by the surface cAMP receptor; activation probably occurs via the elevation of intracellular $Ca^{2+}$ mediated by opening of a plasma membrane $Ca^{2+}$ channel, since activation is absent when extracellular $Ca^{2+}$ is removed [35].

Mammalian cells contain an enzyme called multiple inositol polyphosphate phosphatase (MIPP) that shows some resemblance with the *Dictyostelium* enzyme [34,36]. Both enzymes use Ins(1,3,4,5,6)$P_5$ as substrate and produce Ins(1,4,5)$P_3$. However, the main mammalian enzyme from rat liver preferentially dephosphorylates at the 3-position. Furthermore, the mammalian enzyme resides in the endoplasmatic reticulum where it has no excess to its substrate [36]. An Ins(1,3,4,5)$P_4$ 3-phosphatase has been detected in human erythrocytes that is present at the plasma membrane and dependent on $Ca^{2+}$ ions as the *Dictyostelium* enzyme [37]. It is possible that mammalian cells may contain two MIPP isozymes, a $Ca^{2+}$-independent enzyme localized in the ER, and a $Ca^{2+}$-dependent enzyme at the plasma membrane.

*Dictyostelium* cells have two routes to make Ins(1,4,5)$P_3$, via PLC and via a Ins(1,3,4,5,6)$P_5$ 3/6-bisphosphatase. In cells with a blockade of the PLC route Ins(1,4,5)$P_3$ levels are maintained using the second route. The observation that out of 20 inositol phosphates only Ins(1,3,4,5,6)$P_5$ levels are reduced in *plc*-null cells suggest that this phosphatase route of Ins(1,4,5)$P_3$ formation is recruited in the mutant to maintain Ins(1,4,5)$P_3$ levels. Does this redundancy mean that Ins(1,4,5)$P_3$ (and thus PLC) is very important, or is Ins(1,4,5)$P_3$ one of the many inositol phosphate metabolites that has no role in signal transduction? Inactivation of the 3/6-bisphosphatase gene may answer this question.

## 7. Phosphorylation of Ins(1,4,5)$P_3$ to Ins$P_6$ in the nucleus

When investigating the phosphorylation of inositol phosphates, we observed that both inositol and Ins(1,4,5)$P_3$ can be converted to Ins$P_6$ in cell lysates [33]. Careful cell lysis and fractionation reveals phosphorylation of inositol to take place in the cytoplasm as described by Stephens and Irvine [13,32], whereas phosphorylation of Ins(1,4,5)$P_3$ occurs only in a nucleus-associated fraction of the cell lysate. In order to observe enzyme activity, the nuclei should be broken, suggesting that the enzymes are localized inside the nucleus or the nuclear envelope [33]. Ins(1,4,5)$P_3$ is phosphorylated first at the 3-position, then at the 6-position and finally at the 2-position. It is not known how Ins(1,4,5)$P_3$ and its products enter or leave the nuclear compartment. One may suggest via the nucleopore, but incubation of isolated nuclei with [$^3$H]$H_2O$ and [$^{32}$P]Ins(1,4,5)$P_3$ reveals the rapid equilibration of $^3$H-label whereas the $^{32}$P-label is excluded from the nucleus (Van Dijken, Van der Kaay and Van Haastert, unpublished observations).

Using the same nuclear preparations, we have detected several enzyme activities in broken nuclei, including PtdIns kinases, PLC and Ins(1,4,5)$P_3$ phosphatases. This suggest that the nucleus or a nucleus-associated fraction contains nearly all enzyme activities.

## 8. Ins$P_7$ and Ins$P_8$

*Dictyostelium* cells contain relatively large amounts of two inositol phosphates that elute from ion-exchange columns after Ins$P_6$ [38,39]. Their identification was performed using NMR and specific enzymes; Ins$P_7$ is D-6-PP-Ins$P_5$, whereas Ins$P_8$ was identified as D-5,6-bis-PP-Ins$P_4$ [40]. The route of Ins$P_7$ formation is not known; Ins$P_8$ is derived from Ins$P_7$ by a 5-kinase. The pyrophosphates in Ins$P_7$ and Ins$P_8$ probably have a high energy potential. Vogelmaier et al. demonstrated that the phosphorylation of Ins$P_6$ to Ins$P_7$ by a purified rat kinase can run in reverse, transferring a phosphate group to ADP to form ATP [41].

## 9. Conclusions

The metabolism of inositol phosphate in *Dictyostelium* is slowly emerging. This organism contains a single *PLC* gene belonging to the PLCδ class. Yeast and probably plants also possess only a PLCδ; apparently PLCβ and PLCγ isozymes and expansion of the three subtypes to a very large family took place after the diversion of plants and animals. Two routes for the formation of Ins(1,4,5)$P_3$ have been identified, PtdIns(4,5)$P_2$-PLC and Ins(1,3,4,5,6)$P_5$-3/6-bisphosphatase. These two routes are likely to be present in animals, where the Ins$P_5$-phosphatase may contain subtypes that are localized in different cellular compartments. The function of higher inositol phosphates are still poorly understood. Isolation of mutants with altered metabolism may uncover the function of these compounds. The identification of the main routes of formation and degradation of Ins$P_6$, Ins$P_7$ and Ins$P_8$ in *Dictyostelium* is an important first step towards understanding the function of these compounds that attain such high concentrations in many cells.

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