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Yeast inositol mono- and trisphosphate levels are modulated by inositol monophosphatase activity and nutrients

Juan P. Navarro-Aviñó, José M. Bellés, and Ramón Serrano*

Instituto de Biologia Molecular y Celular de Plantas, Universidad Politécnica de Valencia-C.S.I.C., Camino de Vera s/n, Valencia 46022, Spain

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Abstract

Yeast lithium-sensitive inositol monophosphatase (IMPase) is encoded by a non-essential gene pair (*IMP1* and *IMP2*). Inhibition of IMPase with either Li^+ or Na^+ or a double null mutation *imp1 imp2* causes increased levels of inositol monophosphates and reduced level of inositol 1,4,5-trisphosphate. Overexpression of the *IMP2* gene has the opposite effects and these results suggest that IMPase activity is limiting for the inositol cycle. Addition of ammonium to cells starved for this nutrient results in a decrease of inositol monophosphates and an increase of inositol 1,4,5-triphosphate, pointing to simultaneous regulation of both inositol 1,4,5-triphosphate production and IMPase activity.

Keywords: Yeast; Inositol monophosphate; Inositol trisphosphate; Inositol monophosphatase; Nutrient starvation; Lithium; Sodium; HPLC

The inositol cycle of calcium signalling is an important regulatory pathway which transduces many external signals in animal cells [1]. This cycle starts with phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and D-*myo*-inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), the latter mediating the release of Ca^{2+} from internal stores. Several phosphatases acting in sequence (including inositolpolyphosphate 5-phosphatase, inositolpolyphosphate 1-phosphatase, and inositol monophosphatase) then convert $Ins(1,4,5)P_3$ into inositol for resynthesis of phosphoinositides. The cycle is complicated by reversible conversion of $Ins(1,4,5)P_3$ into higher phosphorylation forms with important regulatory properties [2].

The activation of the inositol cycle in yeast has been proposed to mediate some nutritional responses and, for example, ammonium addition to starved cells increases the level of $Ins(1,4,5)P_3$ [3]. However, as recently discussed [4], the mechanism of the yeast inositol cycle has only partially been characterized. Although $Ins(1,4,5)P_3$ releases calcium from yeast vacuoles [5,6] and phosphoinositide-specific phospholipase C [7,8] and inositolpolyphosphate 5-phosphatases [9,10] have been identified, the nature of the inositolpolyphosphate 1-phosphatase and of the $Ins(1,4,5)P_3$ receptor has not been clarified. Also, yeast cells contain a classical lithium-sensitive inositol monophosphatase (IMPase) but it is encoded by a non-essential gene pair [4]. As inositol monophosphate hydrolysis is required for both inositol biosynthesis [11] and recycling after phospholipase C action on phosphoinositides [1], yeast IMPase should be redundant with other phosphatases and its physiological role is unclear. On the other hand, overexpression of yeast IMPase increases intracellular calcium [4], as if the enzyme effectively modulated the inositol cycle of calcium signalling.

In the present work we have investigated the effect of gain and loss of function of yeast IMPase on the levels of inositol mono- and tris-phosphates. The results indicate that the activity of yeast IMPase modulates the inositol cycle. This cycle is regulated by nutrients such as ammonium by a mechanism involving the activation of both $Ins(1,4,5)P_3$ production and inositol monophosphate hydrolysis.

Materials and methods

Yeast strains and culture conditions. The following *Saccharomyces cerevisiae* strains were used for this study: RS16 (*MAT***a** *leu2-3112*

* Corresponding author. Fax: +34-96-387-78-59.
E-mail address: serrano@ibmcp.upv.es (R. Serrano).

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ura3-352, 328, 372; [12]), RS347 (RS16 with empty expression plasmid pRS699; [4]), RS1238 (RS16 with plasmid pRS699-*IMP2*; [4]), W303-1A (*MAT*a *ade2-1 can1-100 his3-11, 15 leu2-3112 trp1-1 ura3-1*; [13]), and YML89 (W303-1A with *imp1::TRP1 imp2::LEU2*). Most experiments were done with strain RS-16 and its derivatives. Strain W303-1A was used only to test the effect of the *imp1 imp2* double mutation, which is made in this genetic background. No significant differences in inositol phosphate levels were found between RS-16 and W303-1A cells. Standard methods for yeast culture and manipulation were used [14].

Cells were grown in minimal medium (SD) containing 2% glucose, 0.7% Yeast Nitrogen Base without amino acids (Difco), 50 mM succinic acid adjusted to pH 5.6 with Tris, and the amino acids, uracil and adenine, required by the different strains. LiCl (0.1 M) and NaCl (0.6 M) were added 40 min and 4 h, respectively, before harvesting the cells.

Labeling and extraction of inositol phosphates. Cultures were grown overnight in medium supplemented with 5 μCi/ml D-*myo*-[^{3}H]inositol (Amersham Pharmacia Biotech, Barcelona) up to late exponential phase (absorbance at 660 nm about 1). Soluble inositol derivatives were extracted by mixing 0.5 ml culture with 0.1 ml concentrated perchloric acid (11.6 M). After 5 min incubation on ice the cell debris was removed by centrifugation (1 min at 10,000*g*) and 0.5 ml of the supernatant was neutralized with 0.5 ml of a solution containing 1.8 M KOH and 0.4 M KCO_3H. After centrifugation of the precipitate of potassium perchlorate, 0.5 ml supernatant was used for HPLC analysis.

Analysis of inositol phosphates by HPLC. The method described by [15] was followed. Samples were injected into a 4.6 × 10-mm Guard Cartridge 10-μm SAX column linked to a 4.6 × 250-mm Partisil 10-μm SAX column (pS Phase Sep) equilibrated in Milli-Q water and maintained at 22 °C with a flow rate of 1 ml/min. Inositol phosphates were eluted with a linear gradient of ammonium phosphate (pH 3.7) as described by [16], Gradient 1. [^{3}H]Inositol phosphates were detected with a RadioFlow detector LB509 (EGG Berthold) using Optiflow scintillation mixture at a rate of 3 ml/min. Standards of D-*myo*-[2-^{3}H]Inositol 1-phosphate, D-*myo*-[2-^{3}H]Inositol 4-phosphate, and D-*myo*-[1-^{3}H]Inositol 1,4,5-trisphosphate were from DuPont NEN (ITISA, Spain).

Nitrogen starvation. Nitrogen starvation experiments were done as described by [3]. Briefly, cells were grown to late exponential phase in SD medium (supplemented with [^{3}H]inositol, see above), centrifuged, and resuspended in the same medium without ammonium sulfate. After 16 h incubation, samples were taken (starvation condition) and the cells were centrifuged and incubated in medium with ammonium sulfate for 5–30 min (ammonium readdition condition).

Results

Identification of inositol phosphates in yeast extracts

The profile of inositol phosphates present in yeast extracts is shown in Fig. 1. A conspicuous feature is that the inositol monophosphate levels are much higher (about 100-fold) than those of inositol bis- and trisphosphates. Similar results have been reported [10,17]. This situation is very different from the one of animal cerebral [18] and gland [19] tissues, where inositol mono-, bis- and tris-phosphate levels are of the same order, with only slight predominance of monophosphates.

We have characterized by co-injection with standards the two major peaks of inositol mono-phosphates present in yeast as D-*myo*-inositol 1-monophosphate (abbreviated as Ins(1)P, see [1]) and Ins(4)P (Fig. 2). It

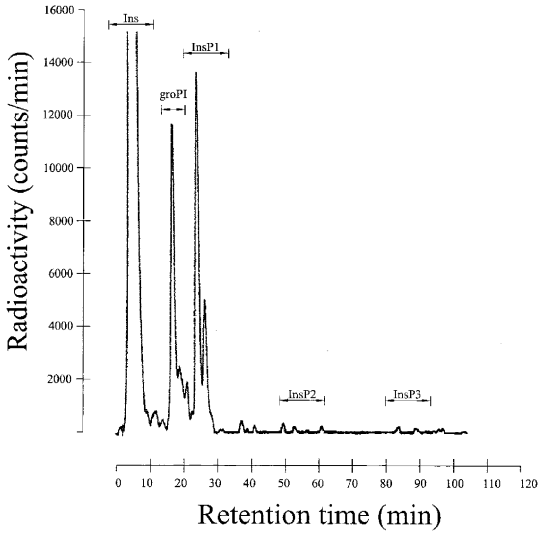

Fig. 1. Separation of [^{3}H]inositol-labeled water-soluble yeast extracts by HPLC chromatography on Partisil 10 μm SAX column. The elution positions of free inositol (Ins), glycerophosphoinositols (groPI), inositol monophosphates (InsP1), inositol bisphosphates (InsP2), and inositol trisphosphates (InsP3) are indicated.

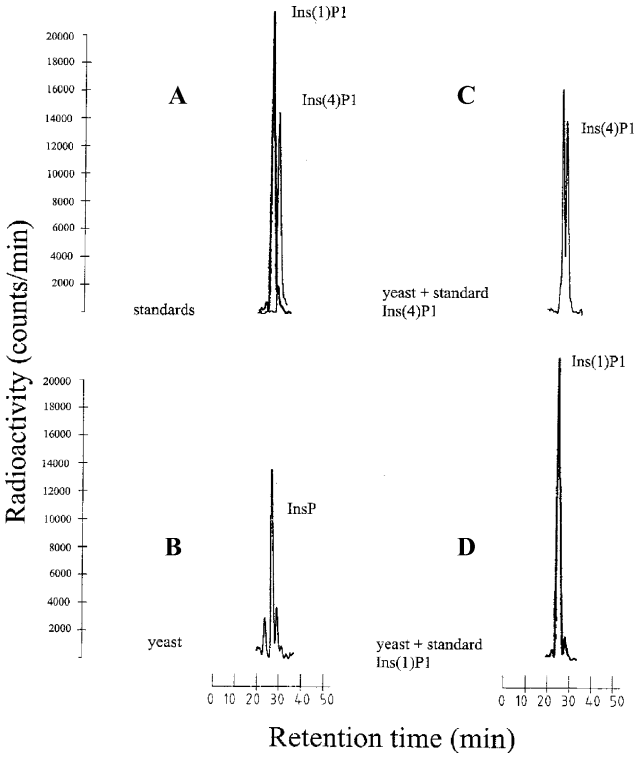

Fig. 2. Identification of Ins(1)P and Ins(4)P in yeast extracts by HPLC. (A) Ins(1)P and Ins(4)P standards; (B) major peaks in the inositol monophosphate region of a yeast extract; (C) co-injection of Ins(4)P standard (same amount as in A) with yeast extract (same amount as in B); and (D) co-injection of Ins(1)P standard (same amount as in A) with yeast extract (same amount as in B).

must be recalled that HPLC cannot resolve isomers that are enantiomers, such as Ins(1)P and Ins(3)P or Ins(4)P and Ins(6)P [1]. Ins(2)P and Ins(5)P, which exhibit

retention times intermediate between Ins(1)P and Ins(4)P, could not be resolved under our experimental conditions, but are usually present at much lower concentrations than Ins(1)P and Ins(4)P [16].

The key regulatory molecule Ins(1,4,5)P_3 was also identified by co-injection with standard (Fig. 3A).

Effect of altering IMPase activity on inositol phosphate levels

We have altered IMPase activity by three types of manipulations: (a) overexpression of the *IMP2* gene; (b) double gene disruption *imp1 imp2*; and (c) addition of the inhibitors lithium or sodium. As indicated in Table 1, gain of function of IMPase (YEpIMP2) results in decreased levels of inositol monophosphates (40–50%) and increased level of Ins(1,4,5)P_3 (about 2-fold). Loss of function of IMPase in the double mutant had the opposite effect: increased levels of inositol monophosphates (4- to 5-fold) and decreased level of Ins(1,4,5)P_3 (40%). Inhibition of IMPase with lithium or sodium produced changes in the same direction as in the *imp1 imp2* double mutation, although the magnitude of the effect was lower as if only partial inhibition of the IMPase were achieved. As expected, lithium had no significant effects on the inositol phosphate levels of the *imp1 imp2* double mutant.

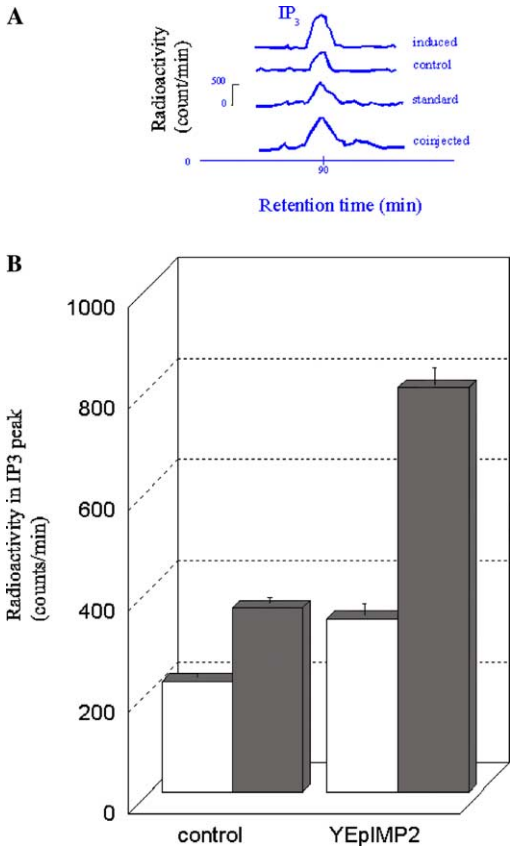

Fig. 3. Ins(1,4,5)P_3 in yeast extracts. (A) Identification of Ins(1,4,5)P_3 in yeast extracts (control) by coinjection with standard (coinjected). Standard alone (same amount as coinjected) is also shown (standard) and the trace labeled as "induced" corresponds to the increased level of Ins(1,4,5)P_3 after addition of ammonium to starved cells. (B) Effect of overexpression of IMPase (YEpIMP2) and of addition of ammonium (filled bars) to starved cells (empty bars) on Ins(1,4,5)P_3 levels.

Table 1
Effect of altering IMPase activity on inositol phosphate levels

Conditions	Ins(1)P	Ins(4)P	Ins(1,4,5)P_3
Control	"100"	"100"	"100"
YEp*IMP2*	52 ± 9	41 ± 8	204 ± 10
imp1 imp2	535 ± 17	450 ± 23	42 ± 6
LiCl	326 ± 16	270 ± 19	75 ± 7
imp1 imp2 + LiCl	550 ± 21	425 ± 18	57 ± 12
NaCl	260 ± 15	210 ± 23	91 ± 2

Yeast cells with either overexpression (YEp*IMP2*), gene disruption (*imp1 imp2*) or with inhibitors of IMPases (LiCl or NaCl) were extracted and the levels of Ins(1)P, Ins(4)P, and Ins(1,4,5)P_3 quantified as described in Materials and methods. Results are the average of three experiments (± standard deviation) and are presented as per cent of control values for the wild type strain without inhibitors. These corresponded to 1–3 × 10^4, 2–5 × 10^3, and 1–1.5 × 10^2 counts/min mg cells for Ins(1)P, Ins(4)P, and Ins(1,4,5)P_3, respectively, for the different experiments.

It has been described that ammonium addition to starved yeast cells results in increased levels of Ins(1,4,5)P_3 (measured by a radioligand assay system) as if this nutrient activated the inositol cycle [3]. We have corroborated these results by using an HPLC assay (Fig. 3) and in addition we have found that ammonium addition induces a decrease of inositol monophosphate levels (Table 2). Similar levels of inositol phosphates were measured at 5, 15, and 30 min after ammonium addition (data not shown).

As indicated in Fig. 3B, overexpression of IMPase not only increases basal Ins(1,4,5)P_3 levels but also potentiates the ammonium response.

Effect of ammonium addition to starved cells on inositol phosphate levels

Table 2
Effect of starvation and readdition of ammonium on inositol phosphate levels

Conditions	Ins-P	Ins(1,4,5)P_3
Control	"100"	"100"
$NH4^+$ Starvation	240 ± 10	168 ± 7
$NH4^+$ Addition	17 ± 2	334 ± 15

Yeast cells (control) were starved for ammonium ($NH4^+$ starvation), supplemented with the nutrient ($NH4^+$ addition) and the inositol phosphates determined as described in Materials and methods. Ins-P: inositol monophosphates (Ins(1)P+Ins(4)P). Results are the average of three experiments (± standard deviation) and are presented as per cent of control values without starvation. These corresponded to 1.5–3 × 10^4 and 1–1.5 × 10^2 counts/min mg cells for Ins-P and Ins-P_3, respectively, for the different experiments.

Discussion

The present work was motivated by the observation that the yeast lithium-sensitive IMPase was non-essential for growth and therefore its participation in the inositol cycle was unclear [4]. Our present results indicate for the first time that increased activity of yeast IMPase results in decreased levels of inositol monophosphates and increased levels of Ins(1,4,5)P_3, while decreased activity of the enzyme has opposite effects. Therefore, although other yeast phosphatases may act on inositol monophosphates during the essential metabolic pathways of inositol biosynthesis and recycling, the activity of the lithium-sensitive IMPase modulates the levels of inositol phosphates. Also, the fact that overexpression of IMPase increases the level of Ins(1,4,5)P_3 explains the observed increase in intracellular free calcium of IMPase-overexpressing yeast cells [4].

One novel aspect of the yeast inositol cycle is deduced from the observation that yeast inositol monophosphates and Ins(1,4,5)P_3 change in opposite directions upon manipulation of IMPase activity (Table 1). This supports the hypothesis [4] of feed-back inhibition of Ins(1,4,5)P_3 production caused by inositol monophosphates. Related to this fact is the observation (Table 2) that activation of the inositol cycle by ammonium addition to starved cells [3] not only increases Ins(1,4,5)P_3 levels but also decreases inositol monophosphate levels. And overexpression of IMPase increases the induction of Ins(1,4,5)P_3 by ammonium (Fig. 3B). These results can be explained by simultaneous activation by ammonium of Ins(1,4,5)P_3 production and inositol monophosphate hydrolysis. Therefore the operation of the yeast inositol cycle is at variance with the situation in animal systems, where all inositol phosphates are increased upon activation of the cycle and lithium is used to foster their accumulation [18,19].

The yeast inositol cycle needs to be further investigated because it seems different from the well characterized inositol cycle of animal cells [1]. The mechanism of the proposed feed-back inhibition of Ins(1,4,5)P_3 production by inositol monophosphates and of the activation of yeast IMPase by ammonium deserves further investigation. Also, the pathway(s) generating Ins(1,4,5)P_3 needs to be investigated. Recent results indicate that the relative low levels of inositol bis- and trisphosphates in yeast are caused by high activity of inositol polyphosphate kinase [20]. This pathway generates the regulatory metabolites inositol tetrakis-, pentakis-, and hexakis-phosphates [2], the latter being required for efficient messenger RNA export from the yeast nucleus [21]. This inositol polyphosphate kinase pathway also generates an alternative source of Ins(1,4,5)P_3 as it has been shown in *Dictyostelium* that, in addition to the classical phospholipase C pathway, Ins(1,4,5)P_3 production can occur by inositol pentakisphosphate hydrolysis [22].

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