Hypo-osmotic Stress Activates Plc1p-dependent Phosphatidylinositol 4,5-Bisphosphate Hydrolysis and Inositol Hexakisphosphate Accumulation in Yeast*

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Polyphosphoinositide-specific phospholipases (PICs) of the δ-subfamily are ubiquitous in eukaryotes, but an inability to control these enzymes physiologically has been a major obstacle to understanding their cellular function(s). Plc1p is similar to metazoan δ-PICs and is the only PIC in Saccharomyces cerevisiae. Genetic studies have implicated Plc1p in several cell functions, both nuclear and cytoplasmic. Here we show that a brief hypo-osmotic episode provokes rapid Plc1p-catalyzed hydrolysis of PtdIns(4,5)P2 in intact yeast by a mechanism independent of extracellular Ca2+. Much of this PtdIns(4,5)P2 hydrolysis occurs at the plasma membrane. The hydrolyzed PtdIns(4,5)P2 is mainly derived from PtdIns4P made by the PtdIns 4-kinase Stt4p. PtdIns(4,5)P2 hydrolysis occurs normally in mutants lacking Arg82p or Ipk1p, but they accumulate no InsP6, showing that these enzymes normally convert the liberated Ins(1,4,5)P3 rapidly and quantitatively to InsP6. We conclude that hypo-osmotic stress activates Plc1p-catalyzed PtdIns(4,5)P2 at the yeast plasma membrane and the liberated Ins(1,4,5)P3 is speedily converted to InsP6. This ability routinely to activate Plc1p-catalyzed PtdIns(4,5)P2 hydrolysis in vivo opens up new opportunities for molecular and genetic scrutiny of the regulation and functions of phosphoinositidases C of the δ-subfamily.

Phosphoinositide-based regulatory systems are ubiquitous in eukaryotes and contribute to many processes, including signaling from cell surface receptors, assembly/disassembly of the actin cytoskeleton, and vesicle trafficking. Receptor signaling through activation of PtdIns(4,5)P2 by hydrolysis of phosphoinositide-specific phospholipases C (phosphoinositidases C; PICs) is the prototype of such regulatory systems. Of the five known PIC families, PICs are ubiquitous in eukaryotes and receptor-controlled PICs of the β, γ, and δ subfamilies (1–3) are only found in metazoans. PIC has been detected only in sperm (4). A prokaryotic PIC that integrated into an emerging proto-eukaryote was probably the common ancestor of all eukaryote PICs (5), and it seems likely that this was more similar to modern PICs than the later-evolved signaling PICs (1–3). PICs might even retain some of the original functions of this ancestral PIC, so it is unfortunate that we understand so little about their regulation and functions. Improved understanding of PICs is likely to come most readily from organisms that express only one, PIC-like, PIC and that lack the PtdIns(4,5)P2-consuming Type I phosphoinositide 3-kinases. One such is Saccharomyces cerevisiae, with Plc1p (encoded by PLC1) its sole PIC.

In unstressed yeast, PLC1 deletion or mutations in the catalytic X and Y domains of Plc1p have little effect. However, the mutants display multiple problems when stressed (6, 7). They grow poorly at raised temperatures (8), stop growing at several points in the cell cycle (8), display defective cytokinesis (9), use non-fermentable carbon sources inefficiently (6), lose viability in synthetic media (10), do not grow at high osmolarity (6), show vacuole fragmentation (11) and are abnormally UV-sensitive (12). Plc1p interacts genetically and physically with Tor2p (13), with two 14-3-3 proteins (Rad24p and Rad25p) (12), with kinetochore proteins (Ndc10p and Cep3p) (14), with Sgd1p-dependent osmoregulation (15) and with stress-triggered pro teaseal degradation of the cyclin Ume3p (16). plc1Δ yeast also show faults in mRNA export from the nucleus (17), mRNA transcription (18) and chromatin organization (19, 20). Finally, mutations in two inositol polyphosphate kinases mimic some or all of the above phenotypes: Arg82p (also known as ArgRIII or Ipk2) converts Plc1p-generated Ins(1,4,5)P3 to Ins(1,3,4,5,6)P5, and Ipk1p converts Ins(1,3,4,5,6)P5 to InsP6 (17, 18, 21, 22).

The activity of Plc1p thus influences many cell activities. Some, such as chromatin maintenance, transcription, and mRNA export, are nuclear, whereas others, including vacuole homeostasis and proteasome activity, reside in the cytosol compartment. It therefore seems that the pleiotropic phenotypes of Δplc1 cells are consequences of multiple flaws in several fundamental processes, in at least two cell compartments.

Despite this substantial body of genetic evidence, there is scant information on what controls Plc1p activity in vivo. It was suggested that glucose re-admission to glucose-deprived yeast might activate Plc1p (23), but this response was later attributed mainly to polyphosphoinositide deacylation (24). It was suggested that glucose re-admission might provoke phosphoinositide turnover and activate a plasma membrane H+ pump, with Plc1p needed for both responses (25), but again deacylation may have caused much of the observed phosphoinositide loss. Nitrogen re-addition to nitrogen-starved yeast provokes rapid Ins(1,4,5)P3 formation (26), but this seems not to need Plc1p (27). Hypo-osmotic shock evokes a [Ca2+]i rise in yeast (28) and in some animal and plant cells, and hypo-osmotic shock may sometimes activate PIC (29, 30). However, the underlying phosphoinositide changes in these responses, and
Yeast Phosphoinositide C Activation in Vivo

TABLE I Yeast strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| YPH499 | MatA ura3–52 lys2–801 ade2–101 trpl–Δ63 his3–Δ200 leu2–Δ1 | ATCC 76625 |
| BY4742 | MatA his3Δ1 leu2–3,122 lys2Δ280 ura3Δ0 | Euroscarf strain Y10000 |
| YPH410 | plc1::HIS3 | Dr. C. Flick |
| YPH42 | plc1::kanMX4 | Euroscarf strain Y17135 |
| YPH42 | arg82::kanMX4 | Euroscarf strain Y13531 |
| YPH42 | ipk1::kanMX4 | Euroscarf strain Y13674 |
| YPH42 | kest1::kanMX4 | Euroscarf strain Y13956 |
| YPH42 | leb6::kanMX4 | Euroscarf strain Y13923 |
| YPH42 | sttd::kanMX4 | This study |
| BY4742 + pUG36-PLC1 | MatA his3Δ1 leu2–3,122 ura3Δ0::URA3 PLC1 | This study |
| YPH410 | plc1::kanMX4 + pUG36-PLC1 | This study |
| YES32 (PIK1::TRP1) | MatA ade2–101 his3::2000 leu2–Δ1 lys2–801 trpl–Δ1 ura3::Pik1::TRP1 | Dr. D. DeWald |
| YES95 (pik1–63::TRP1) | MatA ade2–101 his3::2000 leu2–Δ1 lys2–801 trpl–Δ1 ura3::pik1–63::TRP1 | Dr. D. DeWald |

how these are linked to other cellular events, remain uncertain.

In this study, we present evidence that hypo-osmotic stress speedily activates Plc1p-catalyzed PtdIns(4,5)P₂ hydrolysis at

the plasma membrane in S. cerevisiae, and we define the source of the hydrolyzed PtdIns(4,5)P₂ and the metabolic fate of the liberated Ins(1,4,5)P₃.

MATERIALS AND METHODS

Yeast Strains—Table I lists the yeast strains used.

Growth of ³²H]-inositol-labeled Yeast and Procedure for Hypo-osmotic Shock—Cells were grown exponentially with [³²H]-inositol (5 μCi per ml), and maintained in the presence of this label throughout all manipulations until they were killed. They were acclimatized to hypertonic saline during 5–10 min, 0% B; 5–10 min, ramp to 75% B; 5–10 min, to 100% B; and then isocratic to 120 min. Radioactivity was measured using a flow detector (see above). A different gradient, using the same column and Solutions A and B, was used to confirm that the major inositol phosphates observed in the present chapter were: (a) either iso-osmotic (retaining 0.9 ml NaCl) or hypo-osmotic (lacking the 0.9 ml NaCl); and (b) either Ca²⁺-free or supplemented with Ca²⁺ (Ca²⁺–reconstituted): the final Ca²⁺/calretor combinations were 1.65 ml Ca²⁺/0.5 ml BAPTA/0.25 mm EGTA (Ca²⁺–reconstituted) or 0.5 ml BAPTA/0.25 mm EGTA (Ca²⁺–free). After 2 min, cells were killed, and inositol lipids and phosphates extracted and analyzed. The high ionic strength (10–100 mM NaCl) greatly decreases chelation by EGTA (and slightly by BAPTA) (31–33), so there will have been slightly more [Ca²⁺]free in the assays than the nominal value of 0.9 ml given by simple subtraction.

Extraction and Analysis of [³²H]-inositol-labeled Phosphoinositides and Inositol Polysphosphates—Methods were adapted from those described previously (34, 35). Cells were killed with 2 volumes of ice-cold CH₃OH, 0.12 ml of HCl solution (usually 5% 0.5 ml cell suspension), vortexed (15 s), and sedimented (3000 × g, 5 min, 4 °C). The pellet was suspended in 0.3 ml of 2.1 ml methanol/water containing 0.08 ml HCl, 6.7 ml 1% saturated NaHPO₄, 0.5 ml EGTA, and 0.5 ml MgCl₂; and vortexed (30 s). Glass beads (0.8 g, diameter 425–600 μm, washed with 2 ml methyl di-potassium InsP₃ in 0.1 ml HCl) were added. The slurry was alternately vortexed (30 s) and cooled for on ice (30 s) for 12 cycles, despite >90% of the yeast. The 0.47 ml of methanol (containing 0.12 ml HCl and 1.33 ml of chloroform were added, the slurry was vortexed (30 s), and the monophosphate mixture was then added on ice (15 min). Bovine brain phosphoinositides (10 μg, Type I, Folch fraction V) were added in 2:1 (v/v) chloroform/methanol, and samples were vortexed (30 s) and cooled on ice. After adding 0.4 ml of a mixture of 0.1 ml HCl, 100 ml NaHPO₄, 5 ml tetra-n-butyl-ammonium hydrogen sulfate (TEBAS), 2.5 mm EDTA, and 2.5 mM EGTA, mixing yielded a two-phase system (separated at 3000 × g, 5 min, 4 °C). 500 mM chloroform-rich lower phase was transferred to a glass vial and dried in vacuo (unheated Speedvap), and excess HCl was removed by repeated drying in vacuo with methanol. Lipids were deacylated (monomethylamine reagent, 53 °C, 40 min) and glycerophosphoesters recovered (35). They were resolved by anion-exchange HPLC on a 250 × 4.6 mm Partisphere 5-SAX column, eluted at 1 ml/min with a complex gradient: Solution A was H₂O, and Solution B was 1.25 mM (NH₄)₂HPO₄, pH 3.8. The gradient was: 0–5 min, 0% B; 5–45 min, ramp to 12%; 45–52 min, ramp to 20%; 52–64 min, ramp to 100%; then isocratic to 80 min. The eluate was continuously mixed with 3 ml/min UltimaFlo scintillant and fed through an on-line detector (averaging period 0.1 min).

The upper phase plus interface, containing water-soluble inositol polyphosphates, were incubated at 30 °C for 30 min, centrifuged (105,000 × g, 30 min, 30 °C) and the interfacial pellet discarded. The volume was made 2 ml with H₂O, and acid was neutralized with an appropriate volume of 1 ml NaOH containing 2.5 mM EDTA, 2.5 mM EGTA, and 37.5 mM HEPES, and samples were stored at −20 °C. They were analyzed by HPLC on a 250 × 4.6 mm Partisphere 5-SAX column, eluted at 1 ml/min with a complex gradient: Solution A was H₂O, and Solution B was 1.25 mM (NH₄)₂HPO₄, pH 3.8. The gradient was: 0–5 min, 0% B; 5–10 min, ramp to 7%; B; 10–40 min, ramp to 9% B; 40–110 min, ramp to 100% B; then isocratic to 120 min. Radioactivity was measured with a flow detector (see above). A different gradient, using the same column and Solutions A and B, was used to confirm that the major inositol polyphosphate that accumulated during hypo-osmotic shock was InsP₃ (Fig. 1): 0–5 min, 0% B; 5–10 min, ramp to 7%; 10–25 min, ramp to 70% B; 25–95 min, ramp to 78% B; 95–110 min, ramp to 100% B; then isocratic to 120 min. Fractions were collected for static scintillation counting.

Recombinant GST-Arg82p and GST-Ipk1p—ARG82 and IPK1 ORFs were PCR-amplified from yeast genomic DNA using sense and antisense primers (0.3 μm for ARG82: 5'-ATACCGGGAATCTGGATACGCTGAAATGTAATTACC-3' and 5'-CAGCCCTCGAGTTATTTATTTGAAGTATG-3' for IPK1: 5'-ATAGGAAATCCGATTCACTGACCGAGTGT-3' and 5'-CCGCGCTGATTTATTTAAGTTATG-3') and Expand proofreading DNA polymerase (25 cycles, denaturing at 94 °C (2 min initially, then 45 s reaction), annealing at 56 °C (1 min) and extension at 72 °C (3 min 6 min in final reaction). Products were purified on Qia-ex II resin. An ~0.9 kb fragment covering the ARG82 ORF was digested with BamHI and Xhol and inserted into pGEX4T1 to make pGEX4T1-ARG82. An ~1.1 kb PCR fragment covering the IPK1 ORF was digested with EcoRI and Xhol and inserted into pGEX4T1 to make pGEX4T1-IPK1. pGEX4T1-ARG82 and pGEX4T1-IPK1 were transformed into BL21 competent Escherichia coli. To induce expression of GST-Arg82p and GST-Ipk1p, suspensions were diluted to an OD₆₀₀ of 0.1–0.2 in 1 liter of LB + 100 μg/ml ampicillin, grown to an OD₆₀₀ of 0.5–0.6 (~1 h) and treated with 2.0 ml isopropyl-1-thio-β-galactopyranoside. After 4 h at 37 °C, cells were harvested (3000 × g, 10 min, 4 °C), washed thrice in polyphosphate-buffered saline (PBS: 140 mNaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 10 mM dithiothreitol, 550 μM phenylmethylsulfonyl fluoride, and 2 mM EDTA) and suspended in 40 ml of PBS buffer supplemented with 5 mg/ml aprotinin, 5 mg/ml leupeptin, 5 mg/ml bestatin, 3 mg/ml pepstatin, and 1 mg/ml E64 (also present in subsequent PBS buffers). E. coli were disrupted
resulting glycerophosphoesters separated by an-
diluted 4-fold, and after various periods lipids were extracted, de-
precursor mixture of [3H]inositol and inositol made
precipitation were therefore independent events. When cells and
expression of the pTL336 plasmid in a
This region of the chromatogram was devoid of clear-cut peaks in most experiments, but small but distinct peaks were seen in others, whether because of better HPLC or slight variations in metabolism we do not know.
To confirm the identity of the putative InsP6, an aqueous extract from [14C]inositol-labeled and stressed cells was co-
the PtdIns(4,5)P2 complement of the extracted cells would have been about 50% greater than the figures reported.
When aqueous phases from unstressed [3H]inositol-labeled yeast were analyzed, there were substantial labeled peaks co-
when multicopy overexpression of Ptc1p was achieved in wild-type cells, it caused no substantial modification either of basal or stimulated
hypersensitive to inositol phosphoinositides than wild-type cells (Fig. 1B). They showed no significant changes in PtdIns(4,5)P2 complement during hypo-osmotic stress (Fig. 1, A and B), but the rapid and transient PtdIns4P accumulation still occurred (not shown).
Expression of Ptc1p from a multicopy plasmid in a ptc1 mutant strain restored [3H]PtdIns(4,5)P2 breakdown in response to hypo-osmotic stress (not shown). When multicopy overexpression of Ptc1p was achieved in wild-type cells, it caused no substantial modification either of basal or stimulated
hypotonic stress (not shown). When multicopy overexpression of Ptc1p was achieved in wild-type cells, it caused no substantial modification either of basal or stimulated
hypotonic stress (not shown). When multicopy overexpression of Ptc1p was achieved in wild-type cells, it caused no substantial modification either of basal or stimulated
The kinase activities of GST-
Hypo-osmotic Shock Did Not Stimulate PtdIns(4,5)P2 Hydrolysis in Δptc1 Yeast—Δptc1 yeast incorporated slightly less
[3H]inositol into their phosphoinositides than wild-type cells (Fig. 1B). They showed no significant changes in PtdIns(4,5)P2 complement during hypo-osmotic stress (Fig. 1, A and B), but the rapid and transient PtdIns4P accumulation still occurred (not shown).
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hypo-osmotic stress quickly stimulates Ptc1p-catalyzed PtdIns-(4,5)P2 hydrolysis; Ptc1p-generated Ins(1,4,5)P3 is the precursor of all S. cerevisiae inositol polyphosphates; and Ins(1,4,5)P3 is converted to InsP6 so fast that no detectable intermediates accumulate.

The information in Fig. 2, D and E, reinforces these deductions. First, the concurrent time courses of PtdIns(4,5)P2 depletion and InsP6 accumulation (Fig. 2D) suggest a direct relationship between PtdIns(4,5)P2 loss and InsP6 synthesis. Secondly, titration of cells with the PIC inhibitor U73122 (37, 38) caused a progressive inhibition of PtdIns(4,5)P2 depletion that was matched by similar inhibition of InsP6 accumulation (Fig. 2E).

Rapid Conversion of Ins(1,4,5)P3 to InsP6 in Vivo—Early work indicated that S. cerevisiae extracts exhibit a kinase activity that converts Ins(1,4,5)P3 to Ins(1,4,5,6)P4 (39) and that soluble enzyme(s) from Schizosaccharomyces pombe can quickly convert Ins(1,4,5)P3 to InsP6, mainly via Ins(1,4,5,6)P4.
and Ins(1,3,4,5,6)P₆ (40, 41). In *S. cerevisiae*, Arg82p catalyzes the first two steps (18, 42) and Ipklp the final step (17, 22), and Kcs1p can further phosphorylate InsP₆ to a pyrophosphate derivative (abbreviated as PP-InsP₆) (21, 43).

We determined which inositol polyphosphates accumulate during hypo-osmotic stress in mutants lacking Arg82p, Ipklp or Kcs1p, with the results in Fig. 3 and Table II. Hypo-osmotic stress provoked an essentially normal loss of PtdIns(4,5)P₂ in all of these mutants (Table II).

Approximately 9004 arg82 cells contained a small peak of Ins(1,4,5)P₃ (Fig. 3B, inset; note the expanded scale) but the main feature of the inositol phosphate complement in these cells was the presence

![Image of Fig. 2](https://example.com/fig2.png)

**Fig. 2.** InsP₆ accumulates rapidly in hypo-osmotically shocked *S. cerevisiae*. A, the inositol phosphate complements of unstressed [³H]inositol-labeled wild-type (BY4742) and Δplc1 (BY4742 plc1::kanMX4) are compared with cells that were hypo-osmotically shocked for 2 min.

B, authentic [³H]-labeled InsP₆ (solid line) precisely co-migrated during anion-exchange HPLC with the [¹⁴C]-labeled InsP₆ formed in [¹⁴C]inositol-labeled yeast during 2 min of hypo-osmotic shock (for details, see "Materials and Methods"). C, inositol polyphosphates other than InsP₆ changed little during a 2-min hypo-osmotic shock. D, reciprocal decrease in PtdIns(4,5)P₂ and increase in InsP₆ when an hypo-osmotic shock was applied to cells that overexpressed Plc1p (BY4742 plc1::kanMX4 + pUG36–PLC1): wild-type cells behaved similarly. E, the phosphoinositidase C inhibitor U73122 inhibited both PtdIns(4,5)P₂ depletion and InsP₆ accumulation during hypo-osmotic stress. The bar at the left represents unstressed cells and that at the right cells that were hypo-osmotically stressed (2 min) in the absence of U73122. For each panel, the results are representative of 2–4 experiments that yielded similar results.

|                  | Iso-osmotic | Hypo-osmotic (2 min) | Change |
|------------------|-------------|----------------------|--------|
|                   | 3H (cpm × 10⁻³) | 3H (cpm × 10⁻³) |        |
| **C**            |             |                      |        |
| PtdIns(4,5)P₂    | 41.2 ± 1.6  | 27.3 ± 1.1           | -13.9  |
| InsP₆            | 12.9 ± 0.72 | 33.1 ± 1.5           | +20.2  |
| PP-InsP₆         | 2.2 ± 0.1   | 4.9 ± 0.23           | +2.7   |

and Ins(1,3,4,5,6)P₆ (40, 41). In *S. cerevisiae*, Arg82p catalyzes the first two steps (18, 42) and Ipklp the final step (17, 22), and Kcs1p can further phosphorylate InsP₆ to a pyrophosphate derivative (abbreviated as PP-InsP₆) (21, 43).
TABLE II

Comparison of the inositol polyphosphate complements of control and hypo-osmotically stressed wild-type yeast with the inositol phosphate complements of similarly treated cells lacking the various inositol polyphosphate kinases

For each strain, the information is representative of that gathered from 2–4 experiments. Where no figure is recorded, the quantity of 3H detected in the relevant compound was near or below the detection limit for the experiment.

| Strain          | PtdIns(4,5)P2 | Ins(1,4)P2 | Ins(1,4,5)P3 | Ins(1,4,5,6)P4 | Ins(1,3,4,6)P4 | InsP5 | PP-InsP4 | InsP6 | PP-InsP5 |
|-----------------|---------------|------------|--------------|----------------|----------------|-------|----------|-------|----------|
| **Wild-type**   |               |            |              |                |                |       |          |       |          |
| Control         | 41.2 ± 1.6    | 2.8 ± 0.12 | 0.11 ± 0.01  | 0.4 ± 0.015    | 0.150 ± 0.01   | 0.58 ± 0.03 | 0.18 ± 0.01 | 12.9 ± 0.72 | 2.2 ± 0.01 |
| Hypo shock      | 27.3 ± 1.1    | 3.2 ± 0.15 | 0.17 ± 0.01  | 0.43 ± 0.02    | 0.17 ± 0.012   | 0.79 ± 0.04 | 0.21 ± 0.01 | 33.1 ± 1.5  | 4.9 ± 0.23  |
| Change          | −13.9         | +0.4       | +0.06        | +0.03          | +0.02          | +0.21  | +0.03    | +20.2  | +2.7     |
| **Δarg82**      |               |            |              |                |                |       |          |       |          |
| Control         | 40.4 ± 1.8    | 24.5 ± 0.98| 1.2 ± 0.06   |                |                |       |          |       |          |
| Hypo shock      | 28.6 ± 1.1    | 42.7 ± 1.8 | 2.2 ± 0.06   |                |                |       |          |       |          |
| Change          | −11.8         | +18.2      | +1.0         |                |                |       |          |       |          |
| **Δipk1**       |               |            |              |                |                |       |          |       |          |
| Control         | 42.4 ± 1.9    | 2.1 ± 0.09 | 11.5 ± 0.5   | 10.0 ± 0.45    |                |       |          |       |          |
| Hypo shock      | 27.7 ± 1.3    | 4.6 ± 0.19 | 32.9 ± 1.4   | 23.5 ± 0.84    |                |       |          |       |          |
| Change          | −14.7         | +2.5       | +21.4        | +13.5          |                |       |          |       |          |
| **Δacs1**       |               |            |              |                |                |       |          |       |          |
| Control         | 43.4 ± 2.1    | 2.1 ± 0.08 | 1.6 ± 0.08   | 23.9 ± 0.72    | 0.04 ± 0.005   |       |          |       |          |
| Hypo shock      | 26.4 ± 1.2    | 4.6 ± 0.21 | 4.5 ± 0.22   | 57.2 ± 1.5     | 0.045 ± 0.005  |       |          |       |          |
| Change          | −17.0         | +2.5       | +7.4         | +2.9           | +33.3          | +0.005|          |       |          |

Fig. 3. The inositol phosphates formed during hypo-osmotic stress in yeast lacking Arg82p or Ipk1p. A, the inositol polyphosphates in [3H]inositol-labeled Δarg82 (BY4742 arg82::kanMX4) cells, both unstressed and following a 2 min hypo-osmotic stress. The stressed trace has been displaced upwards to facilitate comparison, the scale is expanded for the InsP3 region of the trace, and an elution profile of standard inositol polyphosphates is shown for reference. B, the inositol polyphosphates in [3H]inositol-labeled Δipk1 (BY4742 ipk1::kanMX4) cells, both unstressed and following a 2-min hypo-osmotic stress. Compare the traces in A and B with the wild-type trace in Fig. 2A.
of greatly increased amounts of multiple isomers of InsP2, notably Ins(1,4,5)P2, which is likely to be a direct dephosphorylation product of Ins(1,4,5,6)P4 (44, 45) (Fig. 3B). The Δarg82 cells were devoid of other inositol phosphates with 3 or more phosphate groups (Fig. 3B and Table II). The Ins(1,4,5)P3 peak became larger during hypo-osmotic stress, but none of the Plc1p-generated Ins(1,4,5)P3 was converted to more highly phosphorylated products. Instead, much of the PtdIns(4,5)P2-derived radioactivity accumulated as the Ins(1,4,5)P3 metabolite Ins(1,4)P2 (Table II and Fig. 3B).

Δipk1 cells also accumulated no InsP6, but they did show major accumulations of InsP4, and its pyrophosphorylated derivative PP-InsP4, together with small amounts of InsP4 isomers. All of these were more abundant after hypo-osmotic shock (Fig. 3C and Table II).

In most respects, Δkes1 cells behaved like wild-type cells (Table II). However, they had higher basal and stimulated InsP4 complements and, as expected, they made no PP-InsP4. They accumulated more InsP4, and PP-InsP4, than wild-type cells, both with and without hypo-osmotic stress (Table II).

Conversion of Ins(1,4,5)P3 to InsP6 by a Mixture of GST-Arg82p and GST-Ipk1p—Being surprised by how fast cells metabolized Ins(1,4,5)P3 to InsP6, with no intermediates accumulating in more than trace amounts, we used recombinant enzymes to reproduce this pathway in vitro. Ins(1,4,5)P3 that was incubated with a mixture of GST-Arg82p and GST-Ipk1p was quickly and quantitatively converted to InsP6. Easily detected intermediate InsP5 was not the expected reaction product of Arg82/Ipk1-catalyzed InsP6 synthesis, appeared transiently during the early phases of the Ins(1,4,5)P3 to InsP6 conversion catalyzed by GST-Arg82p plus GST-Ipk1p (not shown).

Stimulated PtdIns(4,5)P2 Hydrolysis Occurs at the Plasma Membrane—To determine where PtdIns(4,5)P2 hydrolysis occurs in hypo-osmotically stressed cells, we expressed a dimeric GFP-PH domain construct based on the PtdIns(4,5)P2-selective PH domain of PIC61 in the cells (36). Much of this construct localized around the cell periphery, and there were no obvious concentrations of fluorescence on any intracellular organelles (Fig. 4, inset). This suggests that much of the PtdIns(4,5)P2 in S. cerevisiae is at the inner face of the plasma membrane.

We then analyzed, in parallel, the time-courses of three hypo-osmotically induced events in the cells expressing GFP-PIC61: changes in plasma membrane fluorescence, PtdIns(4,5)P2 depletion and InsP6 accumulation (Fig. 4). [PtdIns(4,5)P2] declined to a nadir of ~60% of the starting value at 2 min, before gradually rising again. As before, the relationship between PtdIns(4,5)P2 depletion and InsP6 accumulation was approximately reciprocal (Fig. 4). In the experiment shown, [PtdIns(4,5)P2] rose briefly after imposition of the hypo-osmotic stress, maybe as a secondary effect of the early increase in its precursor PtdIns(4,5)P2 (see above).

Plasma membrane GFP fluorescence tracked the PtdIns(4,5)P2 changes remarkably closely (Fig. 4), indicating that much of the Plc1p-accessible PtdIns(4,5)P2 was at the plasma membrane. There was no detectable labeling with GFP-PIC51-PH of other cellular organelles whose membranes would contain an unknown proportion of cell PtdIns(4,5)P2 (e.g. nucleus, Golgi), so we have no indication whether Plc1p-catalyzed PtdIns(4,5)P2 occurred at any of those sites.

Ca2+ Entry Is Not the Immediate Activator of Plc1p—There is evidence from animal cells that hypo-osmotic shock sometimes triggers Ca2+ entry, and that in such situations the consequent rise in [Ca2+]cyt may sometimes activate PIC8 (for references, see the Introduction). We therefore determined whether changing the availability of extracellular Ca2+ would influence the S. cerevisiae response to hypo-osmotic stress.
Hypo-osmotic stress provoked normal PtdIns(4,5)P_2 hydrol-
ysis and InsP_6 accumulation in the /H9004
lsb6 cells and in the
temperature-sensitive
pik1-63
cells at their non-permissive
temperature (Table III). However, it provoked no change in the
already low PtdIns(4,5)P_2 complement of
/H9004
stt4 cells, and no
additional InsP_6 accumulated in those cells during iso-osmotic
stress (Table III).

These results suggest that Stt4p makes most of the
PtdIns(4,5)P_2 in
S. cerevisiae
, and that knocking out
STT4
eliminates the synthesis of PtdIns(4,5)P_2 at the plasma mem-
brane. It also seems likely that that Stt4p makes all of the
PtdIns(4,5)P_2 that is susceptible to hydrolysis by hypo-osmot-
ically activated Plc1p.

**DISCUSSION**

There is still little understanding of the biological function(s)
of PICs or of how they are controlled in vivo (2, 3). However,
mouse sperm need PIC4 to initiate the acrosome reaction and
achieve efficient fertilization (56), and spore germination is
aberrant in
Dictyostelium
that lack this organism’s only PIC
(57). Moreover, PLC
1 accumulates abnormally in the neuro-
fibrillary tangles of Alzheimer’s disease (58, 59) and in brain
subjected to hyperoxic stress (60) or aluminum toxicity (61),
and Alzheimer’s patients and spontaneously hypertensive rats
harbor unusual PLC61 alleles (62–64).

There has been a common hope that PICs might transduce
receptor signals, and studies of mammalian PICs have suggested some possible activating G-proteins (2). Two G proteins regulate Dictostelium PICs (65). In particular, a receptor for Conditioned Medium Factor liberates G$_{q}$Y from association with G$_{s1}$ and stimulates PIC (66). However, an alternate model has implicated elevated intracellular [Ca$^{2+}$] as a possible PICs activator, maybe as a result of Ca$^{2+}$ entry through capacitative channels (67).

Plc1p, yeast’s only PIC, is PICs-like and has been genetically implicated in numerous cell functions (see the Introduction). The traditional view of PICs is that their primary function is to make the second messengers InsP$_{6}$ and the related inositol polyphosphates, and some of these have previously unsuspected functions in the nucleus (Refs. 17–20, 22, 68, and see the Introduction). Our results confirm the earlier observation that _S. cerevisiae_ must contain Plc1p if they are to make InsP$_{6}$ (68).

Previous work has not provided clear information on how Plc1p is supplied with PtdIns(4,5)P$_{2}$ or how its activity is regulated. The experiments reported in this paper newly establish or reinforce several important features of the Plc1p pathway. First, Stt4p and Mss4p convert PtdIns to the Plc1p-sensitive PtdIns(4,5)P$_{2}$. Second, hypo-osmotic stress rapidly activates Plc1p-catalyzed PtdIns(4,5)P$_{2}$ hydrolysis. Third, much of the stress-induced PtdIns(4,5)P$_{2}$ hydrolysis occurs at the plasma membrane. Fourth, the burst of Ins(1,4,5)P$_{3}$ that is liberated following Plc1p activation is immediately converted to InsP$_{6}$ by Arg82p and Ipk1p. Finally, Kcs1p pyrophosphorylates some of this InsP$_{6}$ to PP-InsP$_{5}$.

Not only do these results confirm the previously reported roles of Arg-82, Ipk1p, and Kcs1p in converting Plc1p-derived Ins(1,4,5)P$_{3}$ to InsP$_{6}$ and its pyrophosphorylated derivatives, but they also demonstrate that the normal cell complement of these enzymes is capable of keeping pace with the explosive production of Ins(1,4,5)P$_{3}$ that is triggered by hypo-osmotic challenge.

_S. cerevisiae_ has three PtdIns 4-kinases: Plk1p (49, 51), Stt4p (50, 52, 69–71), and Lsb6p (54, 72). Our results suggest that Stt4p makes most of a yeast cell’s PtdIns4P and, in particular, that this includes all of the PtdIns4P that is precursor to the plasma membrane PtdIns(4,5)P$_{2}$ hydrolyzed by stress-activated Plc1p (Table III). This tallies with a recent demonstration that much of cellular Stt4p is at the plasma membrane (71). Earlier genetic studies ascribed Stt4p a function upstream of Mss4p (50) and offered evidence that Stt4p, Mss4p, and Plc1p all lie on a single pathway (73). Our study vindicates these genetic deductions.

Our conclusion that Stt4p makes the bulk of yeast PtdIns4P is in apparent conflict with a previous study that assigned approximately equal roles in PtdIns4P (and PtdIns(4,5)P$_{2}$) synthesis to Stt4p and Pik1p (52). How are these studies to be reconciled? Readily, since the previous study used a brief period of pulse-chase [3H]inositol labeling to label yeast phospholipids. Although this is a convenient technique for labeling cells it does not label lipid pools to close to equilibrium with added inositol, which means that it cannot validly be used to determine the relative rates of synthesis of one product by multiple enzymes in _vivo_.

It has long been apparent that much of the PtdIns(4,5)P$_{2}$ in animal cells is at the plasma membrane (74): the best available estimate puts that proportion at 60–70% (75). There is no equivalent information for yeast, but our evidence that most of its Plc1p-sensitive PtdIns(4,5)P$_{2}$ is at the plasma membrane tallies with indications from other work. The first pointer was that Mss4p-generated PtdIns(4,5)P$_{2}$ is needed for the integrity of the subplasmalemmal cytoskeleton (46). While this manuscript was under consideration, it also became apparent that Mss4p only makes PtdIns(4,5)P$_{2}$ efficiently when it is at the plasma membrane (76); Mss4p in the nucleus makes little PtdIns(4,5)P$_{2}$.

The pathway to Plc1p-responsive PtdIns(4,5)P$_{2}$ and the onward metabolism of Ins(1,4,5)P$_{3}$ in hypo-osmotically stimulated cells can be summarized thus in Scheme 1.

![Scheme 1](image)

Growing and unstressed cells contain about half as much InsP$_{6}$ as cells that have been acutely hypo-osmotically stressed, so cells that are receiving no overt stimulus must tonically support a slow but continuous rate of PIC-catalyzed PtdIns(4,5)P$_{2}$ hydrolysis. How this slow and sustained Plc1p activity is regulated and where in the cell this basal PtdIns(4,5)P$_{2}$ hydrolysis occurs remain to be determined.

The GFP-PICs1-PH construct reported that the plasma membrane PtdIns(4,5)P$_{2}$ complement never decreased by more than about one-half during hypo-osmotic stress, even when the stressed cells were overexpressing Plc1p. This suggests that cells maintain close control of this pathway even when they contain Plc1p in abundance. It also suggested that some form of feedback control must restrain further stress-stimulated PtdIns(4,5)P$_{2}$ hydrolysis after a couple of minutes, at a time when the PtdIns(4,5)P$_{2}$ level reaches its nadir and [InsP$_{6}$] stabilizes at a new, and roughly doubled, plateau concentration.

How does hypo-osmotic stress activate Plc1p sufficiently for about half of the PtdIns(4,5)P$_{2}$ in a cell to be hydrolyzed within a couple of minutes? One view is that PICs activation is a simple response to elevation of cytosolic [Ca$^{2+}$] (67). MDCK cells seem to provide the only precedent for translocation of PICs to the plasma membrane and activation in response to hypo-osmotic shock, but this apparently occurs without a need for Ca$^{2+}$ entry (29), and our results suggest that a rise in cytosolic [Ca$^{2+}$] does not trigger hypo-osmotic Plc1p activation in yeast (see “Results”). How Plc1p is activated remains to be determined. One possibility is that the primary sensor is a still-to-be-identified membrane stretch receptor protein, in which case the key question would be how its activation signal is transmitted onwards to Plc1p.

Intriguingly, Plc1p activation seems not to be reversed immediately if the stress is removed. When isotonicity was quickly restored midway through the most rapid phase of...
hypo-osmotically driven PtdIns(4,5)P₂ hydrolysis, ongoing PtdIns(4,5)P₂ hydrolysis continued normally for at least the next minute or so.²

Given the remarkable speed of PtdIns(4,5)P₂ hydrolysis and InsP₆ synthesis in the stressed yeast, without any substantial accumulation of intermediates, we wondered where in the cells the responsible enzymes and the inositol polyphosphate products were located. Similar events seem to occur in S. pombe, though in this case in response to hyper-osmotic challenge (41). Direct information on how inositol phosphates are distributed within eukaryotic cells is scant. The clearest data, from HL60 promyeloid cells, place most of the inositol polyphosphates, including InsP₆ and Ins₁,3,4,5,6,2P₆, either in the cytosol or in a pool that is in free and rapid exchange with that compartment (77). Moreover, the PP-InsP₆ that is made from InsP₆ seems to influence vacuolar morphology in the yeast cytoplasm (21). By contrast, much of the recent work on InsP₆ and its close metabolic relatives in yeast has pointed to important actions in the nucleus (17–20, 68).

We attempted to explore this further by comparing the intracellular distributions of biologically functional GFP-Plc1p, GFP-Arg82p, and GFP-Ipk1p constructs with the distribution of an over-expressed nuclear-targeted construct (the nuclear localization signal of SV40 large T antigen coupled to DsRed (78)). The DsRed construct was only in the nucleus, but the over-expressed GFP-Plc1p, GFP-Arg82p, and GFP-Ipk1p were present in both cytoplasm and nucleus, in each case at a higher concentration in the latter (not shown). This leaves the situation unresolved, and an important question for the future will be to determine whether Plc1p and the various inositol phosphate kinases really do carry out multiple functions in more than one cell compartment.

Here we have only discussed a relatively straightforward series of events that have at their center Plc1p activation in cells subjected to osmotic perturbation. We have not addressed how a lack of Plc1p and its products, the inositol polyphosphates discussed here and sn-1,2-diacylglycerol, cause dysregulation of multiple cell functions and thus the many plc1 phenotypes. Our observations make a method for physiologically controlling the catalytic activity of Plc1p available for the first time, and this should facilitate detailed examination of these other questions. We also have evidence, to be reported elsewhere, that activation of Plc1p-catalyzed PtdIns(4,5)P₂ hydrolysis participates in the responses of S. cerevisiae to high temperature, glucose readmission and nitrogen readmission, in surprisingly complex ways.

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REFERENCES
1. Rhee, S. G. (2001) Annu. Rev. Biochem. 70, 281–312
2. Rebecchi, M. J., and Ponteley, S. N. (2000) Physiol. Rev. 80, 1291–1335
3. Katan, M. (1998) Biochim. Biophys. Acta 1436, 5–17
4. Cox, I. J., Larman, M. G., Saunders, C. M., Hashimoto, K., Swann, K., and Lai, F. A. (2002) Receptor 192, 611–623
5. Michel, R. H. (1987) in Perspectives on Receptor Classification (Gerschkowitch, V. P., ed) pp. 95–101, Alan R. Liss Inc, New York
6. Flick, J. S., and Thorner, J. (1995) Mol. Cell. Biol. 15, 5861–5876
7. Yokos, O., Kato, H., Matsui, T., Yagisawa, H., Nogami, M., Ueno, I., and Toh-e, A. (1993) J. Biochem. 113, 145–148
8. Payne, W. R., and Fitzgerald-Hayes, M. (1993) Mol. Cell. Biol. 13, 4351–4364
9. Flick, J. S., and Thorner, J. (1998) Genetics 148, 33–47
10. Seeley, E. S., Kato, M., Margolis, N., Wickner, W., and Elzten, G. (2002) Mol. Biol. Cell 13, 792–794

² N. M. Perera, unpublished data.
60. Tanino, H., Kusuda, T., Nagasawa, K., Shimohama, S., and Fujimoto, S. (2001) *Biol. Pharm. Bull.* **24**, 1241–1245.
61. Tanino, H., Shimohama, S., Sasaki, Y., Sumida, Y., and Fujimoto, S. (2000) *Biochem. Biophys. Res. Commun.* **271**, 620–625.
62. Katsuya, T., Higaki, J., Miki, T., Kohara, K., Yagisawa, H., Tanase, H., Mikami, H., Serikawa, T., Nojima, H., and Ogihara, T. (1992) *Biochem. Biophys. Res. Commun.* **187**, 1359–1366.
63. Yagisawa, H., Fuji, M., and Hirata, M. (1999) *Biochem. Soc. Trans.* **27**, 652–657.
64. Yagisawa, H., Tanase, H., and Nojima, H. (1991) *J. Hypertens.* **9**, 997–1004.
65. Bominaar, A. A., and Van Haastert, P. J. (1994) *Biochem. J.* **297**, 189–193.
66. Brazill, D. T., Lindsey, D. F., Bishop, J. D., and Gomer, R. H. (1998) *J. Biol. Chem.* **273**, 8161–8168.
67. Allen, V., Swigart, P., Cheung, R., Cockcroft, S., and Katan, M. (1997) *Biochem. J.* **327**, 545–552.
68. York, J. D., Guo, S., Odom, A. R., Spiegelberg, B. D., and Stolz, L. E. (2001) *Adv. Enzyme Regul.* **41**, 57–71.
69. Yoshida, S., Ohya, Y., Nakano, A., and Anraku, Y. (1994) *Mol. Gen. Genet.* **242**, 631–640.
70. Trotter, P. J., Wu, W. I., Pedretti, J., Yates, R., and Voelker, D. R. (1998) *J. Biol. Chem.* **273**, 13189–13196.
71. Audhya, A., and Emr, S. D. (2002) *Dev. Cell* **2**, 593–605.
72. Minogue, S., Anderson, J. S., Waugh, M. G., dos Santos, M., Corless, S., Cramer, R., and Hsuan, J. J. (2001) *J. Biol. Chem.* **276**, 16635–16640.
73. Cutler, N. S., Heitman, J., and Cardenas, M. E. (1997) *J. Biol. Chem.* **272**, 27671–27677.
74. Downes, P., and Mitchell, R. H. (1982) *Cell Calcium* **3**, 467–502.
75. Watt, S. A., Kular, G., Fleming, I. N., Downes, C. P., and Luceog, J. M. (2002) *Biochem. J.* **363**, 657–666.
76. Audhya, A., and Emr, S. D. (2003) *EMBO J.* **22**, 4223–4236.
77. Stuart, J. A., Anderson, K. L., French, P. J., Kirk, C. J., and Michell, R. H. (1994) *Biochem. J.* **303**, 517–525.
78. Rodrigues, F., van Hemert, M., Steensma, H. Y., Corte-Real, M., and Leao, C. (2001) *J. Bacteriol.* **183**, 3791–3794.