

Cell content of phosphatidylinositol (4,5)bisphosphate in Ehrlich mouse ascites tumour cells in response to cell volume perturbations in anisotonic and in isosmotic media

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The labelling pattern of cellular phosphoinositides (PtdIns P_n) was studied in Ehrlich ascites cells labelled *in vivo* for 24 h with *myo*-[2- 3 H]- or *L-my*o-[1- 3 H]inositol and exposed to anisotonic or isosmotic volume perturbations. In parallel experiments the cell volume ([14 C]3-OMG space) was monitored. In hypotonic media the cells initially swelled osmotically and subsequently as expected showed a regulatory volume decrease (RVD) response. Concurrently, the cell content of PtdIns P_2 showed a marked, transient decrease and the content of PtdIns P a small, transient increase. The changes in PtdIns P_2 and PtdIns P content increased progressively with the extent of hypotonicity (in the range 1.00–0.50 relative osmolarity). No evidence was found for either hydrolysis of PtdIns P_2 or formation of PtdIns P_3 . In hypertonic medium (relative osmolarity 1.50), cells initially shrank osmotically and subsequently as expected showed a small regulatory volume increase (RVI) response. Concurrently, the cell content of PtdIns P_2 showed a marked increase and the content of PtdIns P a small decrease, i.e. changes in the opposite direction of those seen in hypotonic media. In isosmotic media with high (100 mM) or low (0.8 mM) K^+ concentration, cells slowly swelled or shrank due to uptake or loss of isosmotic KCl. Under these conditions, with largely unchanged intracellular ionic strength, the cell content of PtdIns P_2 and PtdIns P remained constant. Our results show that PtdIns P_2 is not volume sensitive *per se*, and moreover that the regulatory volume adjustments in Ehrlich ascites cells are not mediated by PtdIns P_2 hydrolysis and its subsequent production of second messengers. The simplest interpretation of the observed effects would be that PtdIns P_2 is controlled by ionic strength, probably via activation/inhibition of phosphoinositide-specific phosphatases/kinases. In Ehrlich ascites cells, as shown previously, the opposing ion channels and transporters activated during RVD and RVI, respectively, are controlled with tight negative coordination by a common cell volume ‘set-point’ that is shifted in anisotonic media, but unchanged during cell swelling in isosmotic high K^+ medium. We hypothesize that PtdIns P_2 might orchestrate this ‘set-point’ shift.

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Phosphatidylinositol (4,5)bisphosphate (PtdIns P_2) has attracted considerable interest for many years. It is the precursor for the second messengers inositol(1, 4,5)trisphosphate (Ins P_3), diacylglycerol (DAG) and phosphatidylinositol(3,4,5)trisphosphate (PtdIns P_3), and has also itself important signalling roles. It is seen as a multifunctional regulator and adaptor, modulating many cellular functions. It plays key roles in receptor-mediated signal transduction, translocation of cytoplasmic proteins containing PtdIns P_2 -binding domains, membrane

trafficking, and reorganization of the actin cytoskeleton (see, e.g. Toker, 1998; Czech, 2000; Downes *et al.* 2005; Santarius *et al.* 2006; and references therein). Moreover, it modulates directly or indirectly the function of a wide range of ion channels and transporters (see, e.g. Hilgemann *et al.* 2001; Suh & Hille, 2005), including inward rectifier K^+ channels, as studied among others by Logothetis and coworkers (see Du *et al.* 2004), and Na^+/H^+ -exchangers (see Aharonovitz *et al.* 2000).

The effect of extracellular tonicity and cell volume on cellular phosphoinositide levels has only been addressed in a limited number of studies. Hyperosmotic stress has been shown to induce increased levels of PtdIns P_2 and

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other phosphoinositides in several cell types (Van der Kaay *et al.* 1999; Nasuhoglu *et al.* 2002; Yamamoto *et al.* 2006), including plant cells (see Zonia & Munnik, 2004; and references therein). Hypotonic stress has attracted less interest, although a rapid phosphoinositide breakdown with production of cellular inositol phosphates has been demonstrated in MDCK cells (Fujii *et al.* 1999).

We have studied a conceivable role of phosphoinositides in cell volume regulation in Ehrlich ascites cells exposed to volume perturbations in anisotonic and isosmotic media. During cell swelling and shrinkage in hypotonic and hypertonic media, respectively, the cell PtdInsP₂ content is markedly changed, and changed in opposite directions under the two conditions. In striking contrast, the PtdInsP₂ content is unchanged during similar cell volume perturbations attained in isosmotic high and low K⁺ media. The results show that the PtdInsP₂ content is not volume sensitive *per se*, but rather, might be controlled by ionic strength, probably via activation/inhibition of phosphoinositide-specific phosphatases/kinases. During cell volume regulation in anisotonic media the 'set-point' for volume regulation is shifted (Harbak & Simonsen, 1999), whereas this is not the case during cell swelling in isosmotic high K⁺ medium (Harbak, Jensen & Simonsen, 2001). The 'set-point' shift in anisotonic media involves a tight coordination between the opposing volume-regulatory transport systems, and it is proposed that the multifunctional regulator PtdInsP₂ might perhaps orchestrate the setting of the volume regulatory 'set point'. Some of the present data have previously been presented in abstract form (Nielsen *et al.* 1995; Harbak *et al.* 1995).

Methods

Reagents

All reagents were of analytical grade and unless otherwise indicated obtained from Sigma-Aldrich, Denmark or Merck Eurolab Norden. *myo*-[2-³H]Inositol (with PTK-271 stabilizer, 1 mCi ml⁻¹) was from Amersham Biosciences, UK. *L-my*o-[1-³H]Inositol was obtained from New England Nuclear (NEN), Boston MA, USA on special request. 3-O-[¹⁴C]Methyl-D-glucose and ⁵¹Cr(III)EDTA complex were purchased from NEN, Boston, MA, USA.

Media

The standard incubation medium contained (mM): 150 Na⁺, 150 Cl⁻, 5 K⁺, 1 Ca²⁺, 1 Mg²⁺, 1 HPO₄²⁻, 1 SO₄²⁻, 3.3 Mops, 3.3 Tes, and 5 Hepes (pH 7.4; 300 mosmol l⁻¹). All media were nominally bicarbonate free. Bovine serum albumin (fraction V, from Sigma; extensively dialysed against distilled water) was included in all media at 10 mg ml⁻¹. The tonicity was reduced by addition of buffered water and increased by adding extra NaCl. The

reduced Ca²⁺ concentration in the hypotonic media was not considered significant, as the RVD response in Ehrlich ascites cells is unaffected in Ca²⁺-free media (see, e.g. Hoffmann *et al.* 1993). The K⁺ concentration in isosmotic medium was varied by exchanging KCl and NaCl mole by mole.

In vivo labelling of cellular phosphoinositides and inositol phosphates

Ehrlich mouse ascites tumour cells were maintained by weekly inoculation (100 μl) into the abdominal cavity of female NMRI mice. In experiments with measurements of cellular phosphoinositides and inositol phosphates the cells were labelled *in vivo* with *myo*-[2-³H]inositol or *L-my*o-[1-³H]inositol by intraperitoneal injection of 0.3 mCi in tumour-bearing mice 24 h before harvesting the cells. The handling of the mice followed the guidelines of the institute and the procedures used in earlier work (Christensen *et al.* 1994, 2003).

Cell suspensions

The mice were killed by cervical dislocation 7 days after inoculation, and the ascites fluid collected into a large volume of standard medium. The cells were packed by gentle centrifugation, washed twice, and resuspended in standard medium. The fractional cell volume (cytocrit) of the cell suspension was measured by centrifugation of duplicate samples of the cell suspension in haematocrit tubes, and adjusted to 4–8%. The suspension of washed cells was preincubated for 20 min at 37°C in an Erlenmeyer flask on a shaking water bath in order to reach a steady state for ion content and cell volume. Aliquots of the preincubated cell suspension were transferred to an incubator with multihead magnetic stirring for ease of sampling. Silanized glass vials were used throughout. Experimental groups to be directly compared were incubated in parallel, with only a short delay between groups. An isotonic control group was included in all experiments.

Measurement of cell water volume (3-OMG space)

Cell water volume was measured using the ¹⁴C-labelled non-metabolizable glucose analogue 3-O-methyl-D-glucose (3-OMG) (0.05–0.1 μCi (ml cell suspension)⁻¹) (Kletzien *et al.* 1975). [¹⁴C]3-OMG equilibrates across the plasma membrane of Ehrlich ascites cells within seconds. Assuming that the concentration of 3-OMG in cell water is the same as the concentration in the medium, the cellular volume in which 3-OMG distributes (the 'cell 3-OMG space') is about 0.70 ml per ml original cell volume (as determined from the cytocrit

value, see above) (data not shown), consistent with 3-OMG distributing in most of the water phase of the cells. The cell content of [¹⁴C]3-OMG responds to tonicity changes as expected for a simple equilibration of 3-OMG across the cell membrane, and accordingly we have used cell 3-OMG space as a measure of cell volume. As 3-OMG equilibrates so rapidly, cell 3-OMG space reflects cell volume even during quite steep volume changes. ⁵¹Cr(III) EDTA complex (0.5–1 μC (ml cell suspension)⁻¹) was used as an extracellular marker, in order to correct for [¹⁴C]3-OMG in medium trapped in the cell pellet below the oil during the sampling procedure (see below).

Sampling from cell suspensions

From the cell suspension serial 600 μl samples were taken for determination of ¹⁴C and ⁵¹Cr radioactivity in cells and in extracellular medium. The cells were separated from the medium within seconds by centrifugation through an oil layer (phthalate ester or silicone oil mixture with appropriate density). Aliquots (200 μl) of the supernatant (the extracellular medium) above the oil were saved for analysis. The rest of the supernatant and most of the oil layer was removed by suction, the walls of the microfuge tube carefully wiped with a cotton swab, and the cell pellets suspended in distilled water by vortexing and left to lyse for an hour. The protein in cell lysate and in samples of the medium was precipitated using trichloroacetic acid (TCA; final concentration 5%, w/v). Tracer radioactivities in the TCA supernatants were measured by dual channel liquid scintillation counting with Ultima-Gold (Packard BioScience). The calculated 3-OMG spaces of the cells in any given experiment were normalized to the mean of the values of isotonic control cells, either initial values recorded in isotonic medium (see Figs. 1 and 2) or the values in a parallel control group in that same experiment (see Fig. 3).

From suspensions of *myo*-[³H]inositol labelled cells, samples were taken for extraction of phosphoinositide lipids and for analysis of cellular inositol phosphates (see below).

Extraction of phosphoinositide lipids. Samples (1000 μl) of the cell suspension were mixed with 2.5 ml ice-cold methanol containing butylated hydroxytoluene (BHT; 1.25 mg) and *trans*-1,2-diaminocyclohexano-*N,N,N',N'*-tetraacetic acid (CDTA; 5 μmol), and extracted for 30 min at 0°C. After addition of 1.25 ml chloroform the samples were extracted for 2 h at room temperature, and finally stored at -20°C until lipid extraction. The phosphoinositide lipids were extracted as previously described (Christensen *et al.* 2003). Briefly, a two-phase system was established by addition of 1.25 ml chloroform, 2.50 ml 'neutral equilibrated chloroform

phase', and 1.25 ml 2.4 M HCl. After vigorous mixing, immediately after addition of HCl, and centrifugation, the acidic aqueous phase was re-extracted with 5 ml chloroform. Both chloroform phases were re-extracted with 4 ml methanol/0.1 M HCl (1 : 1, v/v). The combined chloroform phases were finally evaporated by vacuum concentration for 2 h in preparation for deacylation (see below).

Deacylation of phosphoinositides (PtdInsP_n) and separation of deacylation products by anion-exchange chromatography. The extracted lipids were deacylated with methylamine as previously described (Christensen *et al.* 2003). Briefly, the lipids were dissolved in 300 μl *n*-butanol and mixed with 2.7 ml monomethylamine reagent (monomethylamine/methanol (5 : 4, v/v), containing CDTA (0.75 μmol), mannitol (5.5 μmol), and InsP₆-hydrolysate (600 nmol total P). The mixture was incubated for 60 min at room temperature, and subsequently evaporated by vacuum concentration overnight. The dry, deacylated product was dissolved in 1 ml 1 mM ammonium formate, and the fatty acids removed by extraction with 1.2 ml *n*-butanol/light petroleum/ethyl formate (20 : 4 : 1, v/v), followed by re-extraction with 0.75 ml of the same solvent mixture. The resulting water-soluble deacylation products of the [³H]phosphoinositides were finally separated by small-column, ambient pressure, anion-exchange chromatography, using glass columns packed with Bio-Rad AG 1-X8 formate resin, and step-wise elution with increasing concentrations of ammonium formate in formic acid (see Christensen *et al.* 2003). ³H radioactivity was recorded by liquid-scintillation counting with Hionic Fluor (Packard, BioScience) as scintillation cocktail in a Packard Tri-Carb 2300TR analyser with quench correction (tSIE/AEC). The ³H radioactivity eluted in the individual chromatographic peaks is expressed as a percentage of the total ³H radioactivity eluted from the column. The PtdInsP₂ isomers have not been resolved, but PtdInsP₂ is presumed to predominantly represent PtdIns(4,5)P₂.

Measurement of cellular inositol phosphates (InsP_n)

Samples were taken from the suspension of *myo*-[³H]inositol labelled cells and processed for chromatographic separation of inositol phosphates as previously described (Christensen *et al.* 1994). Briefly, 500 μl samples were rapidly mixed with 500 μl ice-cold 1 M perchloric acid (PCA) with 0.2% Triton X-100, dipped into an alcoholic mix of frozen carbon dioxide for a few seconds to ensure complete stoppage of enzymic activity, and finally extracted for 20 min at 0°C. After centrifugation the supernatant, still kept at 0°C, received

Table 1. Distribution of phosphoinositides in Ehrlich ascites cells (unstimulated cells)

	³ H radioactivity (% of total)	
	Series 1	Series 2
PtdIns	88.5 ± 1.22 (5)	89.9 ± 0.72 (7)
PtdInsP	2.8 ± 0.69 (5)	3.4 ± 0.66 (7)
PtdInsP ₂	8.7 ± 0.86 (5)	6.7 ± 1.09 (7)

The data are means ± s.d. and the values in parentheses are the number of independent experiments. The cells in two series of experiments were labelled *in vivo* with *myo*-[2-³H]inositol (series 1) or *L-my*o-[1-³H]inositol (series 2). ³H-labelled PtdInsP_n (PtdIns, PtdInsP, and PtdInsP₂) were measured as the water-soluble glycerophosphoinositol (GroPInsP_n) head groups resulting from methylamine-deacylation of a methanol/chloroform/HCl lipid extract of the cell suspension, and separated by small-column, ambient pressure anion-exchange chromatography. The PtdInsP_n content is given as percent of the total ³H radioactivity eluted from the column.

ethylene-diamino-*N,N,N',N'*-tetraacetic acid (EDTA; final concentration 4 mM) and was finally neutralized with tri-*n*-octylamine/freon. After centrifugation the neutralized extract (pH 6.2 approx.) was stored at -20°C until analysis. The inositol phosphates were analysed by HPLC or usually by small-column, ambient-pressure anion-exchange chromatography as previously described (Christensen *et al.* 1994). The cell content of [³H]InsP_n was calculated with correction for the content in the extracellular medium, as measured in duplicate samples of the supernatant taken before and after the hypotonic dilution (see Jørgensen *et al.* 1997).

Statistical analysis

The data are expressed as mean values ± s.d. or s.e.m. as appropriate, with *n* indicating the number of independent experiments. The figures show typical experiments, with the number of replicate experiments given in the legends. The curves were drawn by eye. In Fig. 3B two data points at 0.8 mM K⁺ have been omitted as outliers, since the deviation from the mean was 4.6 and 7.0 times s.d., respectively.

Results

In unstimulated Ehrlich ascites cells labelled *in vivo* with *myo*-[2-³H]- or *L-my*o-[1-³H]inositol and suspended in isotonic medium the labelling pattern of cellular phosphoinositides was quite reproducible between experiments (see Table 1), suggesting a dynamic balance between the phosphoinositide species. The labelling pattern was identical to that found in a previous study, demonstrating reduced aberrant ³H-labelling of

nucleotides with the use of ³H-*myo*-inositol labelled at L-C1 rather than at the C2 position (Christensen *et al.* 2003). The dominating phosphoinositide species was PtdIns, with PtdInsP₂ contributing about 7% and PtdInsP only about 3% of total PtdInsP_n. No PtdInsP₃ could be detected.

Following anisotonic perturbation, however, the cell content of PtdInsP₂ and PtdInsP was markedly changed. In hypotonic medium there was a marked decrease in the cellular PtdInsP₂ content that reached a minimum after about 2 min and partially recovered after 5 min (see Fig. 1B). Concurrently, there was a small, transient increase in PtdInsP (see Fig. 1C). A similar rapid, transient decrease in PtdInsP₂ content was observed in a much earlier study using ³²P-labelled cells (Christensen *et al.* 1988).

The sum of the ³H radioactivity eluted from the columns remained constant (not illustrated), and no evidence of the presence of PtdInsP₃ could be detected in any experiment, not even at the time of maximal decrease in PtdInsP₂ (no ³H radioactivity eluted at higher eluent concentrations than InsP₃, see column labelled 'Rest' in Fig. 1C). In parallel control groups with isotonic dilution, as expected no changes in PtdInsP₂ and PtdInsP could be detected (not illustrated). Of note, following the hypotonic dilution (relative osmolarity 0.50) no changes could be detected in cytosolic inositol phosphates (InsP_n), when analysed by small-column chromatographic separation of neutralized PCA extracts of serial aliquots of the cell suspension, and with due correction for the content in the extracellular medium (single experiment, data not shown). In particular, no increase in cellular InsP₃, InsP₄, or InsP₂ could be demonstrated. In separate experiments with HPLC analysis of the PCA extracts, the cell content of both Ins(1,4,5)P₃ and Ins(1,3,4)P₃ remained constant, consistent with previous results (see Jørgensen *et al.* 1997).

The time-dependent changes in phosphoinositide pattern were concurrent with the changes in cell water (3-OMG space, see Fig. 1A). Cells showed the well-known RVD response, studied in detail in this cell type among many others (see, e.g. Hoffmann *et al.* 1993). After the initial, nearly osmometric cell swelling the cells partially recover their initial, isotonic cell volume by KCl loss during the RVD response. The volume recovery is only partial, because the 'set-point' for the RVD response is elevated in hypotonic media (see Harbak & Simonsen, 1999).

The changes in cellular PtdInsP₂ and PtdInsP content increased with the degree of hypotonicity, as summarized in Table 2 (see also Fig. 1C). Concurrently, the steady-state 3-OMG space attained after completed RVD response and reflecting the elevation of the volume regulatory 'set-point' also increased with the degree of hypotonicity (see Table 2).

In hypertonic medium the cell content of PtdInsP₂ and PtdInsP was also shifted, and in a direction opposite of that observed in hypotonic medium (see Fig. 2B and

Table 2). Again, the sum of the ³H radioactivities eluted from the columns remained constant (not illustrated).

The changes in phosphoinositide pattern were, as in hypotonic medium, concurrent with changes in 3-OMG space (see Fig. 2A). After the initial, nearly osmometric cell shrinkage, the cells showed a small RVI response that was only just detectable, reflecting the marked depression of

the cell volume 'set-point' in the hypertonic medium (see Harbak & Simonsen, 1999).

Addition of glucose to a suspension of Ehrlich ascites cells leads to a marked transient fall in cytosolic ATP concentration, due to the 'Crabtree effect' as described by Overgaard-Hansen (1965). Addition of glucose (10 mM, at 4–5% cytocrit), with a nearly 50% reduction in ATP concentration, was, however, without any effect on the

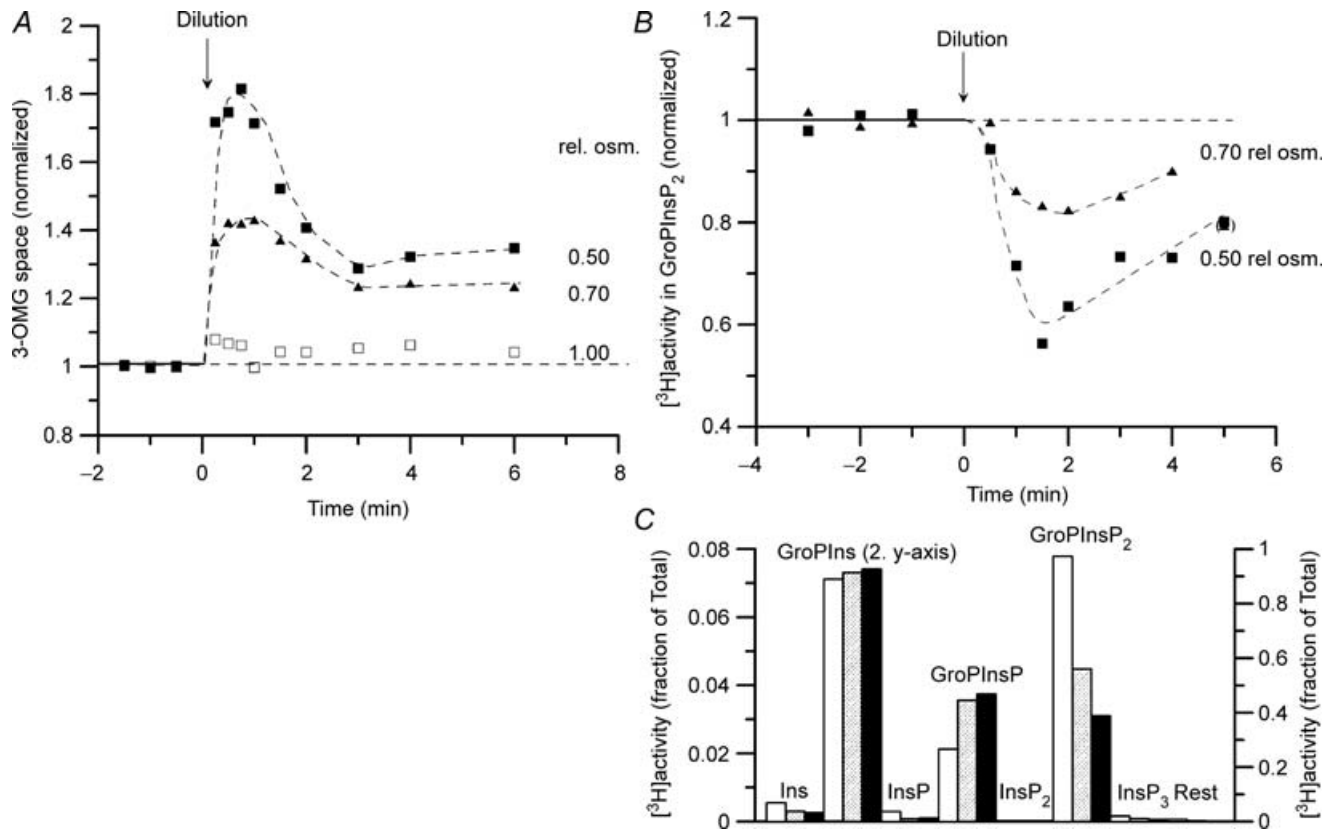


Figure 1. Effect of hypotonic exposure on cell water (3-OMG space) and on PtdInsP₂ content in Ehrlich ascites cells

At zero time (arrow) the cell suspensions were diluted with an equal volume of 2/5 strength incubation medium (diluted with buffered water) or of buffered water, to give final, relative osmolarity at 0.70 and 0.50, respectively. A parallel group (control, not illustrated) was diluted with the same volume of the standard incubation medium. A, the cell suspension was preincubated with [¹⁴C]3-O-methylglucose, and with ⁵¹Cr(III)EDTA as a marker of trapped volume in the cell pellet. For the serial aliquots of the cell suspension taken after the dilution, the radioactivity recorded was corrected such that all samples nominally represent the same number of cells. The calculated 3-OMG spaces of the cells are given normalized to the mean of the initial values in isotonic medium. Typical results from 4 and 3 replicate experiments, respectively. The small increase in 3-OMG space in the control group receiving isotonic dilution was found consistently (see Table 2 and Fig. 2A) and probably represents a small systematic error in the correction applied to compensate for the dilution of the cell suspension (see above). The 3-OMG data presented have not been corrected for this error. B, the cells were labelled *in vivo* with L-myco-[1-³H]inositol, and the water-soluble glycerophosphoinositols (GroPInsP_n, derived from PtdInsP_n) and inositol phosphates (InsP_n) in the deacylated lipid extracts of the cell suspension separated by small-column anion-exchange chromatography. The cell content of [³H]GroPInsP₂, representing PtdInsP₂, is calculated as the fraction of the total ³H radioactivity eluted from the column and given normalized to the mean of the initial values in isotonic medium. Single experiment. Consistent results were obtained for cells labelled with myco-[2-³H]inositol in 4 and 3 experiments at 0.70 and 0.50 relative osmolarity, respectively. C, peak areas representing InsP_n and GroPInsP_n in elution profiles of deacylated lipid extracts for samples taken 1.5 min after exposure to isotonic medium (open columns) and to hypotonic medium at 0.70 (hatched columns) and 0.50 (filled columns) relative osmolarity, respectively. Note that the column for GroPIns refers to the second y-axis (right). Typical experiment.

Table 2. 3-OMG space, PtdInsP₂ and PtdInsP in Ehrlich ascites cells following anisotonic exposure

Rel. osmolarity	3-OMG space ^a	PtdInsP ₂ ^a	PtdInsP ^b
1.50	0.752 ± 0.018 (2)	1.237 ± 0.002 (2)	0.802 ± 0.008 (2)
1.00 ^c	1.056 ± 0.011 (5)	0.986 ± 0.003 (3)	1.030 ± 0.017 (4)
0.90	1.087	0.899	1.042
0.80	1.181 ± 0.005 (2)	0.848 ± 0.015 (3)	1.104 ± 0.048 (3)
0.70	1.244 ± 0.007 (4)	0.840 ± 0.012 (4)	1.120 ± 0.033 (3)
0.50	1.283 ± 0.014 (3)	0.787 ± 0.038 (3)	1.180 ± 0.053 (2)

The data are normalized means ± S.E.M., with the number of independent experiments given in parentheses. ^aMean of data points 3, 4 and 5 min after anisotonic exposure, normalized to mean of data points taken before anisotonic perturbation. ^b'Peak' value 1 min after anisotonic exposure, normalized to mean of data points taken before anisotonic perturbation. ^cParallel control groups receiving isotonic dilution. The small increase in 3-OMG space probably represents a small systematic error (see Legend to Fig. 1).

cellular PtdInsP₂ and PtdInsP content (two experiments, not illustrated).

In isosmotic medium cells swelled slowly at high K⁺ concentration (100 mM) and conversely shrank slowly at low K⁺ concentration (0.8 mM), reflecting net uptake and loss, respectively, of KCl and osmotically obliged cell water (see Fig. 3A). Interestingly, the cell PtdInsP₂ content remained constant during cell swelling and cell shrinkage

in isosmotic media with high and low K⁺ concentration, respectively (see Fig. 3B).

Discussion

Like nearly all other animal cells, Ehrlich ascites cells regulate their volume in anisotonic media, as studied in detail in this cell model before (see, e.g. Hoffmann *et al.* 1993). In hypotonic media the initial osmometric cell swelling is followed by an RVD (regulatory volume decrease) response with KCl loss via swelling-activated K⁺ and Cl⁻ channels (see Niemeyer *et al.* 2000; Pedersen *et al.* 1998), and in hypertonic media the initial cell shrinkage is followed by a small and barely detectable RVI (regulatory volume increase) response with activation of the Na⁺,K⁺,2Cl⁻-cotransporter, as illustrated in Figs. 1A and 2A. In both cases the volume recovery in Ehrlich cells is clearly incomplete, because the 'set-point' for volume regulation, as discussed further below, is shifted in anisotonic media, being elevated in hypotonic media and depressed in hypertonic media (see Harbak & Simonsen, 1999). In the anisotonic media the cell volume changes are concurrent with changes in the cell PtdInsP₂ and PtdInsP content. In hypotonic media PtdInsP₂ decreases and PtdInsP increases, with changes in the opposite direction in hypertonic media (see Figs. 1B and 2B and Table 2). The decrease in the cell PtdInsP₂ content in hypotonic medium cannot be caused by phosphoinositide kinase-mediated phosphorylation of PtdInsP₂, since no

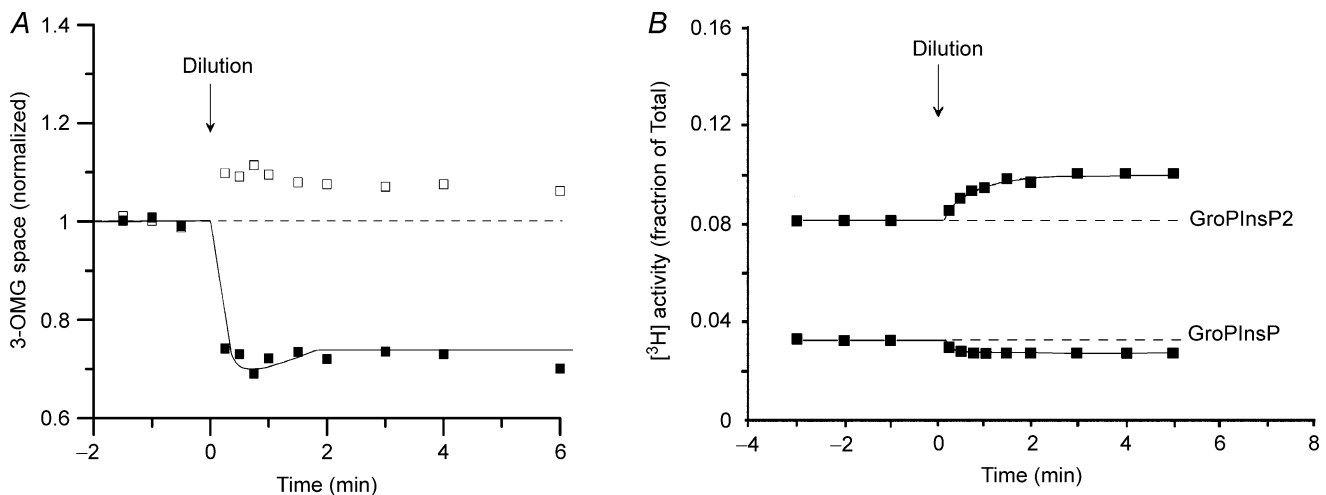
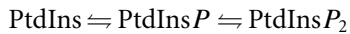


Figure 2. Effect of hypertonic exposure on cell water (3-OMG space) and on PtdInsP₂ and PtdInsP content in Ehrlich ascites cells

At zero time (arrow) the cell suspensions were diluted with an equal volume of double-osmolarity incubation medium, to give final, relative osmolarity at 1.50. Protocol otherwise as in Fig. 1. A, typical results from 5 and 2 replicate experiment at 1.0 (□) and 1.50 (■) relative osmolarity, respectively. The small increase in 3-OMG space in the control group receiving isotonic dilution probably represents a small systematic error (see legend to Fig. 1). In the hypertonic medium the small RVI response indicated was genuine, as evidenced from a number of other experiments in hypertonic media, and is small in size, because the volume regulatory 'set-point' is markedly depressed in hypertonic media (see Harbak & Simonsen, 1999). B, typical experiment. One other experiment gave essentially identical results.

PtdInsP₃ was detectable, not even at the time of maximal decrease in PtdInsP₂ (see Fig. 1C). Also, the decrease in PtdInsP₂ cannot be due to a swelling-induced activation of phosphoinositidase C with hydrolysis of PtdInsP₂, since no increase in cytosolic inositol phosphates, including both Ins(1,4,5)P₃ and Ins(1,3,4)P₃, could be detected (see Results).

The reciprocal changes in cellular PtdInsP₂ and PtdInsP content both in hypotonic and in hypertonic medium would suggest a shift in the 'PtdInsP_n shuttle':



In hypotonic medium the shift would be to the left and in hypertonic medium to the right, perhaps being caused by activation/inhibition of phosphoinositide-specific phosphatases/kinases. As a simple interpretation the shifts could conceivably be due to the changes in cytosolic ATP concentration concomitant to the cell volume changes. This interpretation seems unlikely, however, since no changes in PtdInsP₂ and PtdInsP content could be detected during a marked transient fall of nearly 50% in cytosolic ATP concentration induced by addition of glucose (see Results). In support of the proposed shift in the 'PtdInsP_n shuttle' in anisotonic media, hypertonicity has recently been shown to induce a rapid

increase in PtdInsP₂ in HeLa cells, due to membrane recruitment and activation of PtdIns(4)5-kinase by a volume-dependent, calyculin A-sensitive protein phosphatase-mediated dephosphorylation of the 5-kinase (Yamamoto *et al.* 2006).

In isosmotic medium increasing or reducing the external K⁺ concentration results in slow changes in the cell volume (see Fig. 3A). Under these conditions, however, the cell PtdInsP₂ content is unaffected by the cell volume changes (see Fig. 3B), in contrast to the findings during cell volume changes in anisotonic media. The cell swelling and shrinkage attained after 30 min in high and low K⁺ medium correspond roughly to those that are attained in anisotonic media at 0.70 and 1.50 relative osmolarity, respectively, and that showed marked concurrent changes in PtdInsP₂ content. The absence of a PtdInsP₂ response during the cell volume changes in isosmotic media shows that PtdInsP₂ is not volume sensitive *per se* and is not simply acting as a volume 'sensor' or indicator. The absence of a PtdInsP₂ response in isosmotic media shows, moreover, that the response observed in anisotonic media cannot be caused by volume-dependent changes in cytosolic enzyme concentrations or in 'macromolecular crowding' (Minton *et al.* 1992), or be dependent on membrane tension *per se* or on mechanical transduction by the actin cytoskeleton (see Czech, 2000).

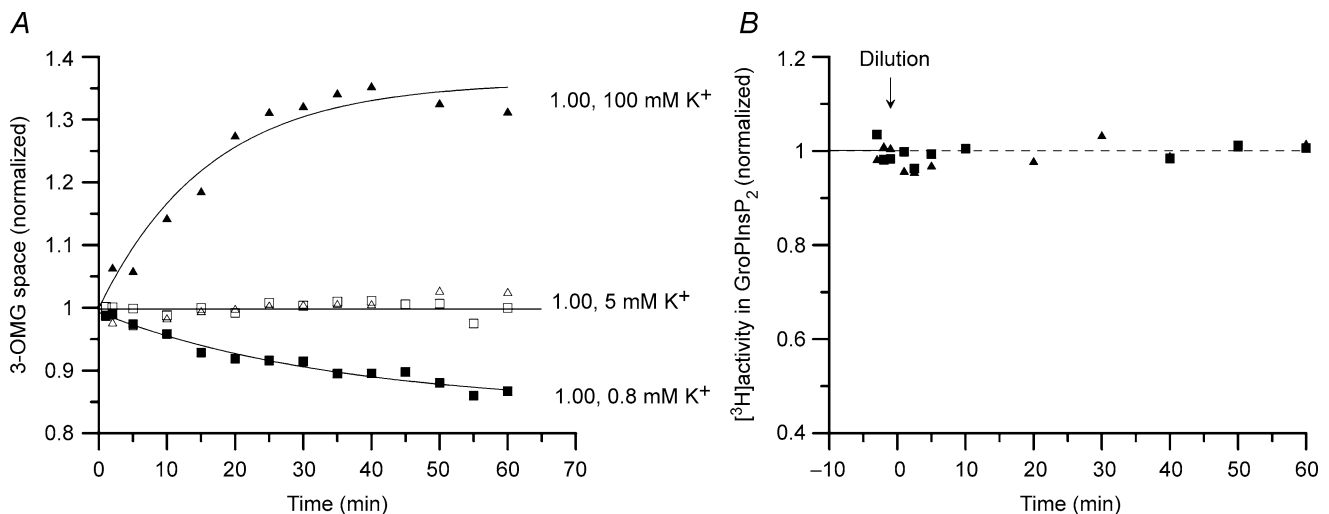


Figure 3. Cell water (3-OMG space) and PtdInsP₂ content in Ehrlich ascites cells during incubation in isosmotic media with increased or decreased K⁺ concentration

At zero time (arrow) the cell suspensions were diluted 3- or 6-fold with isosmotic incubation medium with either high or low K⁺ concentration (obtained by replacing NaCl and KCl mole by mole), to give a final K⁺ concentration at 100 mM and 0.8 mM, respectively (marked with ▲ and ■). Parallel groups (control, marked with open symbols) were diluted with the same volumes of standard incubation medium (5 mM K⁺). Protocol otherwise as in Fig. 1. A, typical experiment. The calculated 3-OMG spaces of the cells are normalized to the mean for the control group in isosmotic medium at 5 mM K⁺. One other experiment gave similar results. B, typical experiment. Other experiments with similar protocol gave consistent results. In 2, 3, and 3 further experiments at 5 mM, 0.8 mM, and 100 mM K⁺, respectively, the PtdInsP₂ content was followed with 5 min intervals for 35 min, and normalized to the mean value within the group. The normalized data were pooled at each K⁺ concentration, and were at all K⁺ concentrations adequately fitted by a horizontal line. s.d. was calculated at s.d. = 0.045 (*n* = 14), 0.027 (*n* = 23), and 0.027 (*n* = 18), respectively, *n* being the number of pooled data points at 5, 0.8, and 100 mM K⁺, respectively.

PtdIns P_2 has been implicated in both direct and indirect modulation of the function of ion channels and transporters (see recent review by Hilgemann *et al.* 2001; Zeng *et al.* 2003). However, it seems in the present case unlikely that the depletion of cell membrane PtdIns P_2 is directly involved in the activation of the K⁺ and Cl⁻ channels mediating the RVD response in hypotonic medium. The K⁺ and Cl⁻ channels are activated also during the isosmotic cell swelling, since a rapid KCl efflux can be induced by restoring a gradient for K⁺ and Cl⁻ efflux by a sudden reduction of the external K⁺ concentration (Harbak, Jensen & Simonsen, 2001), and this channel activation is seen in the absence of changes in cell membrane PtdIns P_2 .

An important difference between the cell volume perturbations in isosmotic and in anisotonic media is that the cytoplasmic ionic strength is largely unchanged during cell volume changes in isosmotic media, but is markedly reduced and increased, respectively, during cell swelling and shrinkage in anisotonic media. In parallel with the ionic strength, the cell PtdIns P_2 content remains constant during isosmotic cell volume perturbations, but markedly changes, and in opposite directions, during hypotonic and hypertonic cell swelling and shrinkage.

There has been considerable interest in the intracellular Cl⁻ concentration as a regulated parameter, and the existence of a Cl⁻ sensor has been suggested (see, e.g. O'Neill, 1999; Lytle & McManus, 2002; and references therein). In our experimental set-up with changes in the external K⁺ concentration in isosmotic media, the cells swell or shrink by taking up or releasing isosmotic KCl, that is, KCl at a concentration of about 160 mM. This is about the same as the normal K⁺ concentration in unperturbed cells in isosmotic media, so the intracellular K⁺ concentration will not change much during these isosmotic cell volume changes. The intracellular Cl⁻ concentration, on the other hand, is about 50 mM in unperturbed Ehrlich ascites cells. This means that when the cells swell by taking up isosmotic KCl, the intracellular Cl⁻ concentration will increase. Conversely, when the cells shrink by releasing isosmotic KCl, the intracellular Cl⁻ concentration will decrease. (For a discussion of the changes in intracellular Cl⁻ concentration due to isosmotic KCl fluxes, see Fig. 2 in O'Neill, 1999.) Despite these changes in the intracellular Cl⁻ concentration, the PtdIns P_n content is unchanged during isosmotic volume perturbations.

The simplest interpretation of the present results would be an effect of ionic strength on the cellular level of PtdIns P_2 . On the other hand it cannot be excluded that the cells could perhaps have both a volume and a Cl⁻ sensor, with the effect of increasing cell volume and decreasing Cl⁻ concentration augmenting each other in hypotonic media, whereas in isosmotic high K⁺ medium the effect of increasing cell volume and increasing Cl⁻ concentration

following the isosmotic KCl uptake would counteract each other.

During cell volume regulation opposing ion channels and transporters are activated during the RVD and RVI response, respectively. In Ehrlich ascites cells the activities of these opposing transporters are controlled with tight negative coordination by a common cell volume 'set-point' (Harbak & Simonsen, 1999). Thus, a cell volume above the 'set point' elicits an RVD response with silent RVI transporters, and conversely a cell volume below the 'set-point' elicits an RVI response with silent RVD transporters. It is intriguing that this volume-regulatory 'set-point' is elevated in hypotonic medium and depressed in hypertonic medium (Harbak & Simonsen, 1999), but unchanged during cell swelling in isosmotic high K⁺ medium (Harbak *et al.* 2001). It would appear that in anisotonic media there is a connection between the changes in ionic strength, the changes in PtdIns P_2 , and the shift in cell volume 'set-point'. The intracellular signalling mechanism involved remains, however, to be identified.

Cell volume plays an essential role in the regulation of a multitude of cellular functions (see, e.g. Lang *et al.* 1998; and references therein), and the signal transduction involved in cell volume regulation has been studied intensively (see, e.g. review by Wehner *et al.* 2003). The 'sensing' mechanism(s) for the regulation of volume regulatory transporters remain(s), however, enigmatic. The present study implies a putative role of phosphoinositides in intracellular signalling in cell volume regulation. PtdIns P_2 is found to change concurrent with the 'set-point' shift during cell volume regulation in anisotonic media, but to remain unchanged during isosmotic cell volume regulation where the cell volume 'set-point' is also unchanged. The results show that PtdIns P_2 is not volume sensitive *per se*, and moreover, that the regulatory volume adjustments in Ehrlich ascites cells are not mediated by PtdIns P_2 hydrolysis and its subsequent production of the second messengers DAG and Ins P_3 , corroborating previous findings demonstrating the absence of Ca²⁺ signalling during RVD in Ehrlich ascites cells (Jørgensen *et al.* 1997). The simplest interpretation of the observed effects is that PtdIns P_2 is controlled by the changes in ionic strength in anisotonic media, probably via activation/inhibition of phosphoinositide-specific phosphatases/kinases in the 'PtdIns P_n shuttle'. PtdIns P_2 itself is a multifunctional regulator that is known to modulate a number of ion channels and transporters. We hypothesize, however, that PtdIns P_2 , at least in Ehrlich ascites cells, does not interact directly with the volume regulatory transporters, but rather acts 'upstream' to control the volume-regulatory 'set-point' setting. It would be interesting to study if a similar mechanism might operate in other cell types.

The activity of volume regulatory transporters is often affected by protein phosphatase/kinase activators/

inhibitors, indicating a role for phosphorylation in the regulation of transporter activity. The hypothesis proposed above that changes in PtdInsP₂ under anisotonic conditions are controlled by the activity of phosphatases/kinases in the 'PtdInsP_n shuttle', and itself controls the volume-regulatory 'set-point' setting, implies that an effect of a phosphatase/kinase activator/inhibitor on the activity of a volume regulatory transporter does not necessarily reflect a direct effect on the specific transporter, but might perhaps rather reflect a 'set-point' shift. In support of this notion it may be noted that the protein phosphatase 1 (PP1) inhibitor calyculin A raises the volume regulatory 'set-point' in Ehrlich ascites cells in isotonic medium and activates the Na⁺,K⁺,2Cl⁻-cotransporter (Harbak, 1999). Clearly, in this case the elevation of the 'set-point' would account for the cotransporter activation. The effect of calyculin A on PtdInsP₂ was not recorded in the study by Harbak (1999), but the PtdIns(4)5-kinase is, at least in HeLa cells, activated by a calyculin A-sensitive protein phosphatase-mediated dephosphorylation (Yamamoto *et al.* 2006). Hence, PtdInsP₂ would be expected to decrease in the presence of calyculin A, with an elevated 'set-point' as the predicted result, by analogy with the effects of hypotonicity.

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