Nutrients Induce an Increase in Inositol 1,4,5-Trisphosphate in Soybean Cells: Implication for the Involvement of Phosphoinositide-Specific Phospholipase C in DNA Synthesis

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Abstract: Phosphoinositide-specific phospholipase C (PI-PLC) hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) to generate 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (InsP_3). Both molecules serve as second messengers to carry out various cellular functions in mammals. In the present study, we demonstrate that many organic and inorganic nutrients cause the elevation of InsP_3 concentrations in cultured soybean cells. This elevation of InsP_3 content is sustained for several hours following treatment with Murashige-Skoog (MS) inorganic nutrients. Phosphate and calcium are the major components in MS salts responsible for the increase in InsP_3 levels. DNA synthesis, a measure of cell growth, was significantly suppressed by the PI-PLC-specific inhibitor 1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1H-pyrrole-2,5-dione (U-73122), whereas its near-identical analogue 1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}-hexyl)-2,5-pyrrolidinedione did not cause any suppression. Activation of PI-PLC by MS salts increased DNA synthesis and abolished the suppression of DNA synthesis caused by U-73122. Thus, we conclude that the higher cellular concentration of InsP_3 induced by MS treatment is involved in DNA synthesis.

Key words: InsP_3, phospholipase C, signal transduction, soybean.

Abbreviations:

DAG:	1,2-diacylglycerol
InsP_3:	inositol 1,4,5-trisphosphate
PtdInsP_2:	phosphatidylinositol 4,5-bisphosphate
PI-PLC:	phosphoinositide-specific phospholipase C

Introduction

Phosphoinositide-specific phospholipase C (PI-PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) on the plasma membrane generating cytosolic inositol 1,4,5-trisphosphate (InsP_3) and plasma membrane-associated 1,2-diacylglycerol (DAG), both of which serve as second messengers in many signal transduction systems (Dennis, 1983[8]; Majerus, 1992[30]; Singer et al., 1997[46]). These two molecules are known to modulate intracellular events through the regulation of intracellular free Ca^{2+} and protein kinase C isozymes, respectively (Singer et al., 1997[46]). In animal cells, hydrolysis of PtdInsP_2 by PI-PLC is a major signalling event during response to growth factors, hormones, and other extracellular signals (Berridge, 1993[3]).

Many components of the animal phosphoinositide signalling pathway are also found in plants (Coté and Crain, 1993[7]; Munnik et al., 1998[35]). Plant PI-PLCs have been cloned from *Arabidopsis thaliana,* potato *(Solanum tuberosum)* and soybean *(Glycine max)* (Hirayama et al., 1995[18]; Kopka et al., 1998[26]; Shi et al., 1995[43]), and putative cDNA or genomic clones for PI-PLC were reported from *Nicotiana rustica* and *A. thaliana* (Hartweck et al., 1997[15]; Pical et al., 1997[39]; Yamamoto et al., 1995[55]). Mammalian PI-PLC isozymes are classified into three classes, namely PI-PLCβ, γ and δ, on the basis of their primary structures. Plant PI-PLC isozymes are structurally similar to mammalian δ-type isozymes (Munnik et al., 1998[35]). Among the characterized PI-PLC genes, *Arabidopsis AtPLC1F* is transcriptionally activated during flowering (Yamamoto et al., 1995[53]), and *AtPLC1S* is activated by environmental stresses, such as dehydration, salinity and low temperature (Hirayama et al., 1995[18]). Another *Arabidopsis* PI-PLC gene, *AtPLC2,* is constitutively expressed (Hirayama et al., 1997[17]). These results suggest differential roles for PI-PLC isozymes, regulated at least in part at the transcriptional and/or post-transcriptional level.

Physiological roles of PI-PLCs in plants have been proposed in a number of systems. Auxin application generated transient changes in InsP_3 and inositol 4,5-bisphosphate (InsP_2) within minutes in *Catharanthus roseus* cells arrested in G1. The arrest was relieved following the InsP_3 and InsP_2 increase (Ettlinger and Lehle, 1988[11]). Abscisic acid treatment of guard cell protoplasts of *Vicia faba* induced a 90% increase in levels of InsP_3 within 10 s of administration, suggesting a possible role of PI-PLC in guard cell shrinking and stomatal closure (Lee et al., 1996[27]). Staxén et al. (1999[52]) demonstrated the direct involvement of PI-PLC in stomatal closure in *Commelina communis* using a PI-PLC-specific inhibitor 1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1H-pyrrole-2,5-dione (U-73122). In the leguminous plant *Samanea saman,*

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leaflet movements are driven by a circadian clock and light. A 15 – 30 s white light pulse caused an increase in $InsP_3$, $InsP_2$ and DAG in the motor organ, the pulvinus (Morse et al., 1989[33]; Morse et al., 1987[34]). $InsP_3$ is involved in Ca^{2+}-mediated pollen tube growth inhibition in *Papaver rhoeas* (Franklin-Tong et al., 1996[13]). In alfalfa, symbiosis with *Rhizobium* is initiated by lipochitooligosaccharide signals (Nod factors). It was suggested that the activity of Nod factor-responsive gene expression was mediated by PI-PLC and Ca^{2+}, based on a study with the inhibitors U-73122 and neomycin sulfate (Pingret et al., 1998[40]). A transgenic approach was recently applied in understanding the possible role of $InsP_3$ in transducing the absicisic acid (ABA) signal during seed germination and seedling growth. Transgenic plants exhibiting lower $InsP_3$ levels due to anti-sense *AtPLC1* and sense *AtP5PII* ($InsP_3$-5-phosphatase) showed no inhibition of germination and growth following ABA treatment (Sanchez and Chua, 2001[42]).

In soybean suspension cells, the G protein activator mastoparan or polygalacturonic acid elicitor activates PI-PLC, and activation of this pathway has been shown to partially regulate the oxidative burst, a process involved in plant defence (Legendre et al., 1993[28]). Glycoprotein elicitor from the phytopathogenic fungus *Verticillium albo-atrum* induced 100 – 160 % increase of $InsP_3$ in lucerne *(Medicago sativa)* suspension culture cells within 1 min of elicitation, suggesting the involvement of the phosphoinositide signalling pathway in defence responses (Walton et al., 1993[54]). Contrary to this increase in $InsP_3$ content following elicitation, Shigaki and Bhattacharyya (2000[45]) reported a reduced $InsP_3$ content in infected soybean cell suspensions for a sustainable period.

In this investigation, we have used soybean cell suspension cultures to study the possible role of PI-PLC in cell growth. We have shown that replenishment of nutrients can activate PI-PLC over an extended period of time. We have used U-73122, a compound that has been extensively used in studying possible functions of PI-PLC in mammals (for example, Bala et al., 1990[1]; Bleasdale et al., 1990[4]; Hirose et al., 1999[19]; Powis et al., 1991[41]; Smith et al., 1990 b[50]) and plants (Knight et al., 1997[24]; Koch et al., 1998[25]; Pingret et al., 1998[40]; Staxén et al., 1999[52]). Its near-identical analogue U-73343 does not inhibit PI-PLC. It has been suggested that U-73122 may be involved in uncoupling the G protein that is necessary for PI-PLC activation (Smith et al., 1990 b[50]). Staxén (1999[52]) demonstrated that the enzymatic activity of a recombinant plant PI-PLC expressed in *E. coli* was inhibited by U-73122, but not by U-73343, indicating the direct inhibitory effect of this compound on plant PI-PLC. By using this PI-PLC-specific inhibitor, we have shown that the nutrient-induced PI-PLC activity is most likely involved in increasing the DNA synthesis.

Materials and Methods

Plant materials

Suspension cell cultures of soybean (*Glycine max* L.) cultivar Williams 82 were maintained at 25 °C in the dark on an orbitalshaker (130 rpm) in MS medium (Murashige and Skoog, 1962[36]) supplemented with 2.22 µM 6-benzylaminopurine, 3 mg/l picloram and vitamins. pH was adjusted to 5.7 with potassium hydroxide. MS medium consisted of the following salts and a sugar: KNO_3, 1900.00 mg/l; NH_4NO_3 1650.00 mg/l; $CaCl_2 \cdot 2H_2O$, 439.80 mg/l; $MgSO_4 \cdot 7H_2O$, 370.60 mg/l; KH_2PO_4, 170.00 mg/l; FeNaEDTA, 36.70 mg/l; $MnSO_4 \cdot 4H_2O$, 22.30 mg/l; $ZnSO_4 \cdot 7H_2O$, 8.60 mg/l; H_3BO_3, 6.20 mg/l; KI, 0.83 mg/l; $NaMoO_4 \cdot 2H_2O$, 0.25 mg/l; $CoCl_2 \cdot 6H_2O$, 0.025 mg/l; $CuSO_4 \cdot 5H_2O$, 0.025 mg/l; sucrose, 30 g/l. Cultures were transferred every seven days by diluting five-fold in fresh MS medium, and experiments were performed 5 days after transfer.

Treatment of soybean cells with nutrients and inhibitors

Nine hundred microliters of soybean cell culture were incubated in 12-well tissue culture plates with shaking at 70 rpm. One hundred microliters of various nutrients, 10 times the concentration used in the regular MS medium, were added to the cell cultures (final concentrations equal to those used in the regular MS medium). When inhibitors were used along with the nutrients, a 10 µl aliquot of U-73122, U-73343, or poly-*p*-methoxyphenylmethylamine (Compound 48/80) was added to 890 µl of cell culture and 100 µl of nutrients. The cells were pre-incubated with the inhibitors for 1 h before the nutrient treatment. U-73122, its inactive analogue 1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl)-2,5-pyrrolidinedione (U-73343), and Compound 48/80 were purchased from Calbiochem-Novabiochem Corporation (San Diego, California). U-73122 and U-73343 were dissolved in dimethyl sulfoxide (DMSO). DMSO was added to water controls and MS treatments when these inhibitors were used. Neither DMSO nor water added to the samples affect cellular $InsP_3$ content. Compound 48/80 was dissolved in sterile water. Samples were collected 30 min after the treatments, unless otherwise indicated, frozen immediately in liquid nitrogen and stored at – 80 °C until use.

Radioreceptor assay of $InsP_3$

A crude extract of soybean cells was prepared according to the method described by Legendre et al. (1993[28]). In short, 500 µl of 15 % trichloroacetic acid was added to each sample and the mixture was vigorously vortexed. The samples were subsequently centrifuged at 10 000 × g for 20 min to remove insoluble material, and the supernatants were extracted four times with 5 ml of water-saturated ethyl ether. The samples were neutralized to pH 7.5 by adding appropriate amounts (5 – 8 µl) of 16 % Na_2CO_3. Radioreceptor assay was performed with a commercially available kit (TRK 1000, Amersham International plc, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturer's protocol. The binding protein used in the kit is specific to inositol 1,4,5-trisphosphate, and discriminates other isoforms of $InsP_3$, or other inositol phosphates. Cellular $InsP_3$ contents were standardized to unit protein content or dry weight. The protein concentration was determined using the Bio-Rad Protein Assay Kit.

In-vivo *labelling and separation of inositol phosphates by high performance liquid chromatography (HPLC)*

myo-[^3H]-inositol (NEN Life Science Products, Boston, Massachusetts) was added to a three-day-old cell culture at a final concentration of 50 µCi/ml, and then incubated for two days. The cell cultures were maintained in inositol-free MS medium for 10 days prior to labelling. A filter-sterilized solution of glucuronic acid (100 µg/ml) (Aldrich, Milwaukee, Wisconsin) was added to prevent incorporation of *myo*-[^3H]-inositol to glu-

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curonic acid (Loewus and Loewus, 1980[29]). Treatments were made by adding 100 µl of 10 × MS salts solution or water to an aliquot of suspension cells (900 µl). The samples were collected 30 min after the treatment and immediately frozen in liquid nitrogen, stored at – 80 °C, and a crude extract was prepared as described in the previous section.

The separation of inositol phosphates by HPLC was based on the method of Irvine et al. (1985[20]). A Partisil 10 SAX anion exchange column (Phenomenex, Torrance, California) was initially washed with water for 8 min, and then the eluant (1.7 M ammonium formate adjusted to pH 3.7 with phosphoric acid) was increased linearly to 100% over 24 min, and the buffer held at this concentration for 10 min. After elution, the buffer concentration was decreased linearly to water over 2 min. Ninety-five fractions were collected over the elution period, and analyzed by scintillation counting. Peaks were identified by comparing with authentic standards.

Labelling of DNA in vivo

Cell cultures (15 ml) with an appropriate treatment were incubated with shaking at 25 °C for 15 h. The cells were then pulse-labelled for 1 h by incubating with 50 µCi [^{3}H]-thymidine (NEN Life Science Products, Boston, Massachusetts). DNA was extracted with a QIAGEN DNeasy Plant Maxi Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. The amount of total DNA was determined spectrophotometrically, and the incorporation of [^{3}H]-thymidine was quantified by scintillation counting. DNA synthesis rate was determined by calculating the ratio of tritium-labelled DNA to total DNA.

In vitro *phosphatase activities on InsP$_3$*

Two milliliters of suspension cells were treated with either MS salts at the final concentration prescribed for the standard MS medium, or water as a control. Cells were sedimented by centrifugation at 1000 g for 10 min and resuspended in Buffer A (120 mM KCl, 20 mM Tres/Hepes, 5 mM EGTA and 1 mM dithiothreitol, pH 7.2). Samples were ground with a glass homogenizer in Buffer A containing 1 µg/ml each of aprotinin, pepstatin, leupeptin and antipain (Sigma, St. Louis, Missouri). The crude extract was centrifuged at 755 g for 5 min, and the supernatant was centrifuged at 60000 g for 60 min. The supernatant was desalted on a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated with 35 ml of Buffer A, and was loaded with 2.5 ml of the crude extract. The sample was eluted with 3.5 ml Buffer A.

Dephosphorylation was assayed in a buffer consisting of 120 mM KCl, 20 mM Tris/Hepes and 0.3 mM MgCl$_2$ (Buffer B). The reaction was carried out at 30 °C by adding 450 µl of crude extract in a 1 ml reaction mixture containing 0.3 µCi of tritium-labelled IP$_3$ and 15 µM unlabelled InsP$_3$. The reaction was stopped after 15 min by adding 500 µl of 15% trichloroacetic acid, followed by extraction with 5 ml water-saturated ethyl ether four times. The samples were neutralized to pH 7.0 by adding appropriate volumes of 16% Na$_2$CO$_3$. Phosphatase products were analyzed by HPLC using the same method as for InsP$_3$ analysis. Injection volume was 200 µl.

Results

Cellular InsP$_3$ levels are increased by various nutrients

We investigated the association of PI-PLC activity with cell growth, using cell cultures of soybean cultivar Williams 82. We estimated the activity of PI-PLC by measuring one of its hydrolysis products, InsP$_3$, by a radioreceptor assay. The other product of PtdInsP$_2$ hydrolysis, DAG, was not measured in our study because of a high background resulting from phospholipase D activation, and biosynthesis of phospholipids in the ER and plastids (Coté and Crain, 1993[7]; Munnik et al., 1998[35]).

Following replenishment of 5-day-old cell cultures with MS medium, the InsP$_3$ content started to increase within 20 min of treatment and remained at significantly higher levels for at least 60 min, as compared to that in water-treated control cells (data not shown). Samples were collected 30 min after the nutrient treatment, based on the result of a time course experiment (data not shown). The InsP$_3$ content in cells increased approximately eight-fold following treatment with complete MS medium (Fig. **1 A**). When components of MS medium were tested individually, inorganic MS salts caused a significantly greater increase in InsP$_3$ than did sucrose (Fig. **1 A**). Glucose showed a similar effect to sucrose. Since MS salts appeared to have a greater effect than sugars on cellular IP$_3$ levels, this treatment was used in further studies.

The effect of MS salts on cellular InsP$_3$ content was followed in a time course experiment. A rapid increase in InsP$_3$ content was observed following MS salts treatment. High InsP$_3$ levels, that were four times those of water controls, were sustained for approximately 1 h following the treatment (Fig. **1 B**). The InsP$_3$ levels then gradually decreased with time. However, higher levels than those of the control were still evident 8 h after the MS salts treatment (Fig. **1 B**).

We also measured the InsP$_3$ levels in cells treated with MS salts by HPLC. We detected a significant increase in the amount of InsP$_3$ when cells were treated with MS salts (Fig. **2**, Table **1**), confirming the radioreceptor assay results (Fig. **1**). In this HPLC analysis, an increase in inositol 1,4-bisphosphate content was also observed in cells treated with MS salts, as compared to that in water control cells (Fig. **2 A**, Table **1**). In subsequent experiments only the radioreceptor assay was carried out, considering its ease, sensitivity and reliability in measuring InsP$_3$ content.

Identification of components in MS salts responsible for the cellular IP$_3$ increase

MS salts are a mixture of 13 different salts (Murashige and Skoog, 1962[36]). Therefore, we proceeded to identify the components in MS salts that are responsible for the increase in cellular InsP$_3$ content. Because of the possibility that a combination of two or more components is required for the increase in InsP$_3$, we made treatments with the omission of one component at a time from MS salts, rather than testing each single component individually. Complete MS salts (all 13 salts combined) increased the InsP$_3$ content approximately five-fold compared to the water control. When one component of MS salts was omitted at a time, cellular InsP$_3$ content was reduced as compared to that induced by the total MS salts in many

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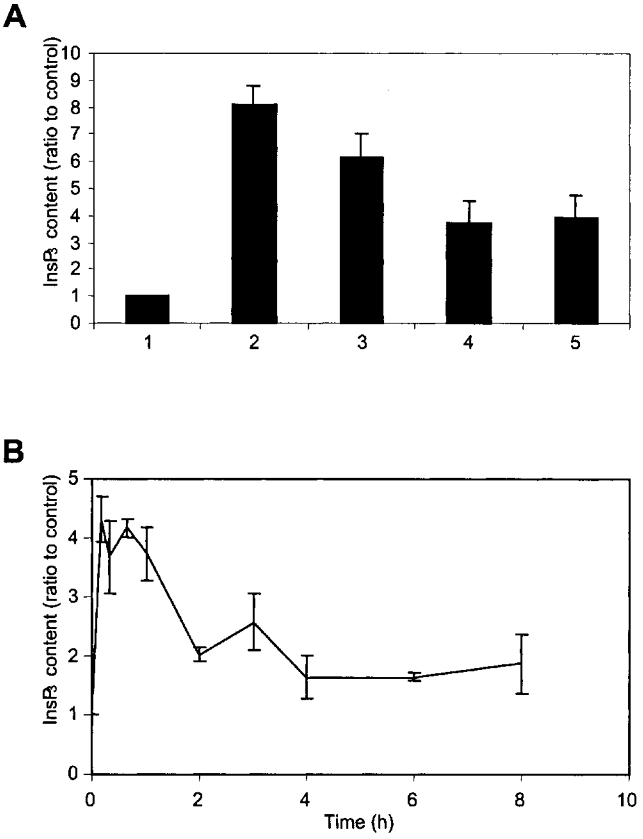

Fig. 1 Cellular $InsP_3$ increase by various nutrients. All the data represent means of three replications and are expressed as ratios to the control. Error bars indicate standard error of the mean. (**A**) Various nutrients were added to soybean suspension cultures, and incubated for 30 min prior to measurement of cellular $InsP_3$ content. $InsP_3$ content is expressed as a ratio to the water control. 1, water control; 2, MS salts and sucrose (87.6 mM); 3, MS salts; 4, sucrose (87.6 mM); 5, glucose (87.6 mM). The concentration of $InsP_3$ in the water control was 88.0 pmol/g fresh weight. (**B**) Time course of cellular $InsP_3$ content following the treatment of soybean cell suspensions with MS salts.

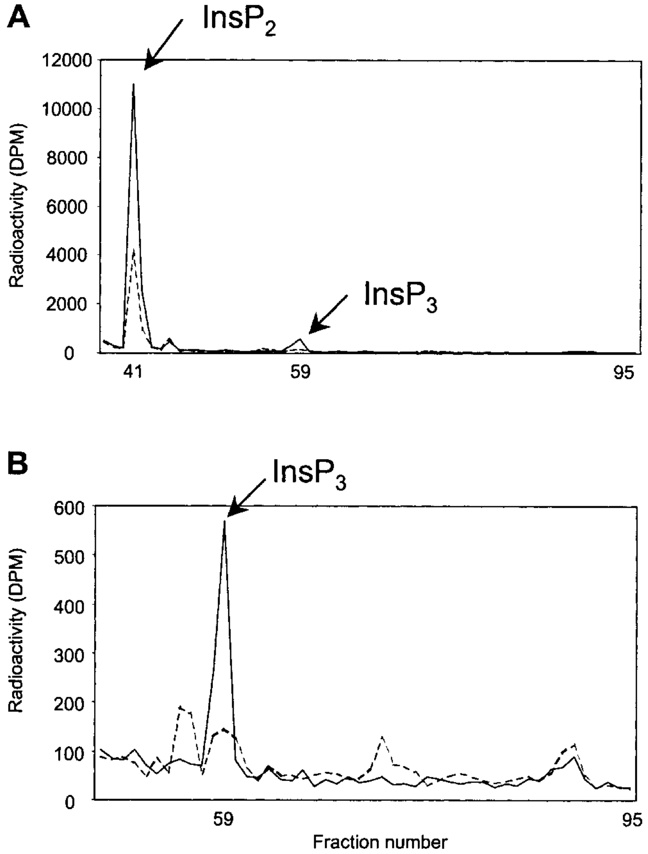

Fig. 2 Representative HPLC profiles of [^{3}H]-inositol labelled crude soluble extracts from soybean cells. (**A**) Profiles of a representative experiment showing fractions 38 – 95. (**B**) Profiles showing fractions 48 – 95; solid line, MS salts-treated cells; broken line, water control.

Table 1 Production of cellular $InsP_2$ and $InsP_3$ following MS salts treatment

	$InsP_2$	$InsP_3$
MS salts treated	10730 ± 325	931 ± 94
Water control	5047 ± 342	308 ± 10

Samples were collected 30 min after the MS salts treatment. Figures are radioactivity in DPM for $InsP_2$ and $InsP_3$ peaks as means of three replications ± standard error. $InsP_2$ counts are for fraction 41. $InsP_3$ counts are for fractions 58 and 59 combined.

treatments, indicating that more than one component contributed toward the increase in cellular $InsP_3$ content (Fig. **3**). The omission of calcium and phosphate from MS salts showed the greatest effects, while omission of certain other salts, such as magnesium and manganese, also showed significant but lesser effects. When both calcium and phosphate were omitted from MS salts, the increase in cellular $InsP_3$ content was completely abolished.

Effect of hyperosmosis on the increase in cellular IP_3 content

Sugars change the osmotic status of cell suspensions significantly. In order to separate the contribution of hyperosmosis from possible nutritional effects, we used mannitol and 2-deoxyglucose as nutrient analogues. Mannitol is not readily utilized by plants, and 2-deoxyglucose is an analogue of glucose. In our time course experiment, glucose treatment resulted in significantly higher levels of $InsP_3$ increase compared to the treatments with mannitol or 2-deoxyglucose (Fig. **4**). All the sugar treatments caused a significant increase in cellular $InsP_3$ content over the basal levels of the water control, with a similar temporal change (Fig. **4**). These results indicate that a part of the glucose-induced PI-PLC activation was caused by the nutritional effects of glucose.

Increased $InsP_3$ content results from PI-PLC activation

Brearley et al. demonstrated that, *in vivo*, increased $InsP_3$ levels in *Commelina communis* resulted from the cleavage of $PtdInsP_2$ by activated PI-PLC (Brearley et al., 1997[5]). However, the increase in cellular $InsP_3$ content in different plants or under different conditions could also be attributed to decreased degradation of $InsP_3$ to other inositol phosphate molecules, or to an unknown $InsP_3$ biosynthetic pathway. To examine whether

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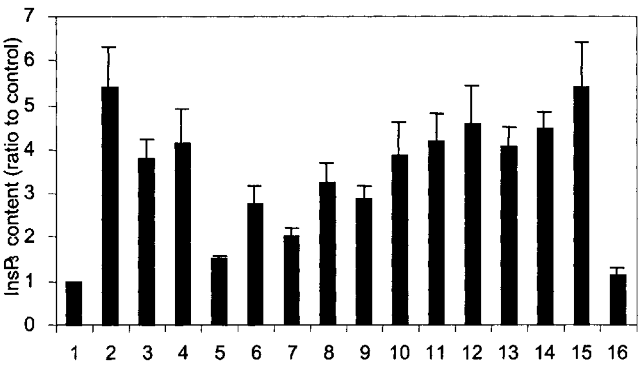

Fig. 3 Identification of components of MS salts responsible for the cellular $InsP_3$ increase. 1, water control; 2, complete MS; 3–16 represent the omissions of one component of MS at a time. e.g.: 3, KNO_3; 4, NH_4NO_3; 5, $CaCl_2 \cdot 2H_2O$; 6, $MgSO_4 \cdot 7H_2O$; 7, KH_2PO_4; 8, FeNaEDTA; 9, $MnSO_4 \cdot 4H_2O$; 10, $ZnSO_4 \cdot 7H_2O$; 11, H_3BO_3; 12, KI; 13, $NaMoO_4 \cdot 2H_2O$; 14, $CoCl_2 \cdot 6H_2O$; 15, $CuSO_4 \cdot 5H_2O$; 16, $CaCl_2 \cdot 2H_2O$ and KH_2PO_4. Samples were collected 30 min after the treatments.

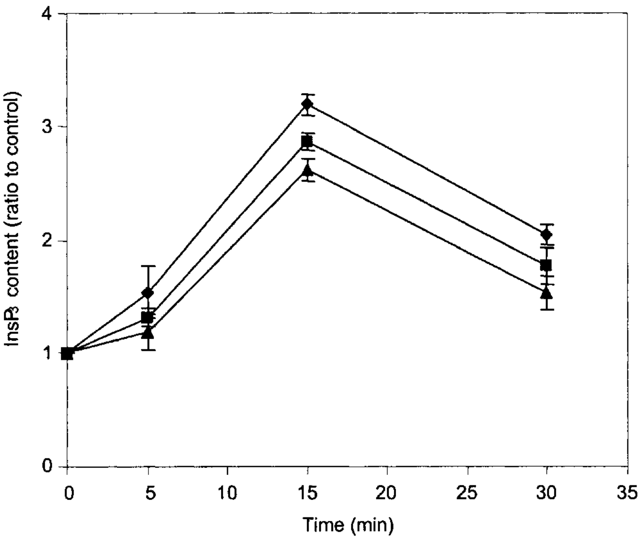

Fig. 4 Osmotic effects of sugars on cellular $InsP_3$ content. Glucose (◆), mannitol (■) and a glucose analogue 2-deoxyglucose (▲) were added to soybean suspension cultures, and incubated for 5, 15, or 30 min to measure cellular $InsP_3$ content. The molar concentration of each sugar was the same as of sucrose in the MS medium (i.e., 87.6 mM). $InsP_3$ content is expressed as a ratio to the control. All the data represent means of three replications and error bars indicate standard error of the mean.

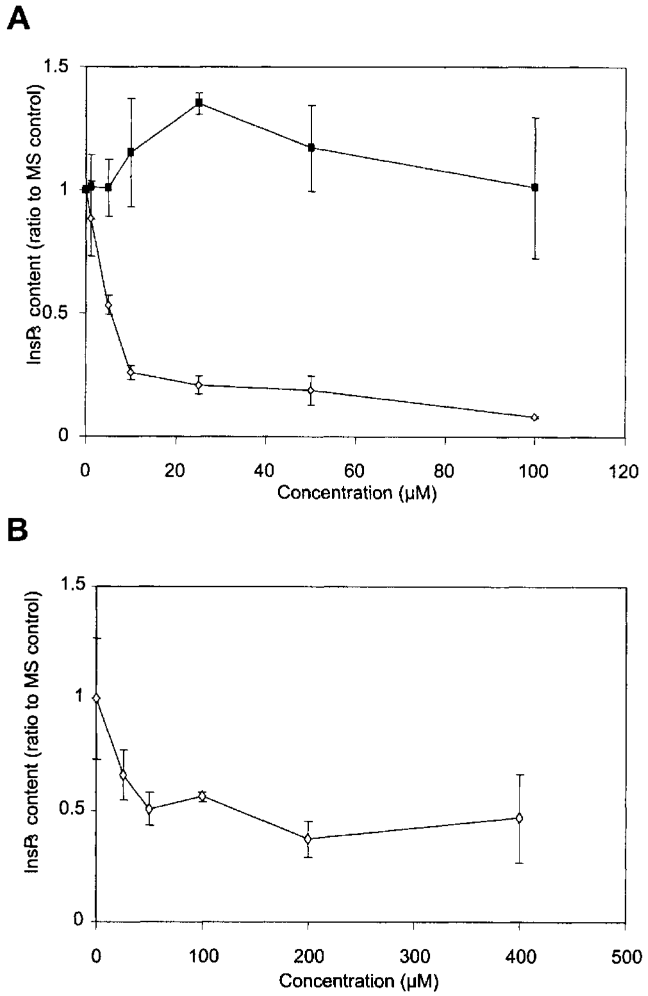

Fig. 5 Effect of inhibitors on cellular $InsP_3$ content. $InsP_3$ content in MS and inhibitor U-73122 or MS and analogue U-73433-treated cell suspensions is expressed as a ratio to the control MS-treated cells. When the cells were treated with MS salts, the $InsP_3$ content increased to 3 times the level of water control in this experiment. All the data represent means of three replications and error bars indicate standard error of the mean. (**A**) Effect of PI-PLC inhibitor U-73122 (◇) and its biologically inactive analogue U-73343 (■) at various concentrations. (**B**) Effect of Compound 48/80 at various concentrations.

the increase in IP_3 is attributable to the activation of PI-PLC, a PI-PLC-specific inhibitor U-73122 and its biologically inactive, near-identical analogue U-73343 (Powis et al., 1991[41]) were used in combination with MS salts. To monitor the viability of cells following incubation with U-73122, the Evans Blue fluorescence assay was performed (Shigaki and Bhattacharyya, 1999[44]). At concentrations below 20 µM, U-73122 did not have any effect on cell viability, even after an overnight incubation. When the cells were pre-incubated for 1 h with 10 µM U-73122, the $InsP_3$ content decreased to approximately 30% of the value for the MS treatment. Increasing the U-73122 concentration beyond 10 µM further decreased $InsP_3$ content, but the decrease was small. U-73343 did not decrease the cellular $InsP_3$ content following MS treatment (Fig. **5A**). Another common PI-PLC inhibitor, Compound 48/80 (Bronner et al., 1987[6]; Gietzen, 1983[14]), also decreased MS-induced cellular $InsP_3$ content significantly (Fig. **5B**). Although Compound 48/80 is also a calmodulin antagonist, and thus we cannot rule out secondary effects, these results indicate that it is highly likely that the MS-induced $InsP_3$ increase is caused by PI-PLC activation.

In vitro *phosphatase activities on* $InsP_3$ *are comparable in control and MS-treated cells*

Changes in $InsP_3$ content can be a result of either the change in the rate of $InsP_3$ synthesis or degradation. $InsP_3$-phosphatase activity in plants has been reported previously (Drøbak et al.,

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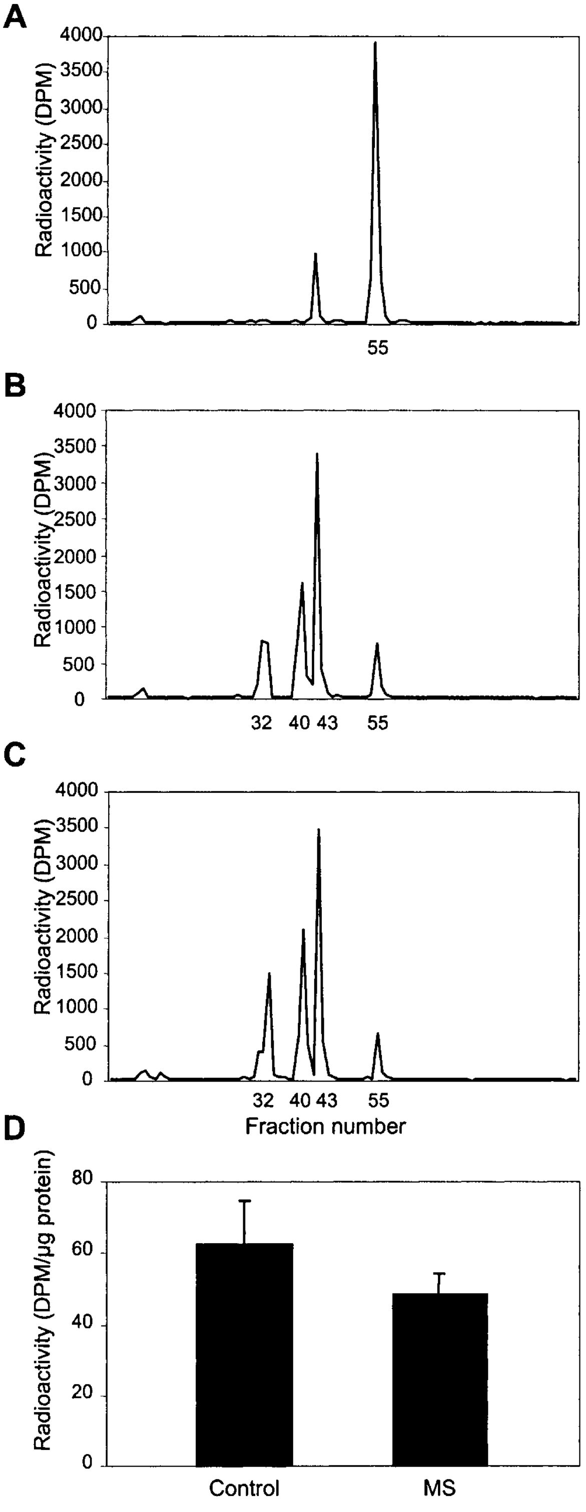

Fig. 6 HPLC profiles of inositol phosphates following incubation of [^{3}H]-InsP$_3$ in crude extracts from MS-treated or water control cells. Fractions number 55/56 and 40 correspond to InsP$_3$ and Ins(1,4)P$_2$, respectively. Fraction number 32 and 43 are most likely various isoforms of inositol monophosphates and Ins(4,5)P$_2$, respectively. (**A**) Boiled crude extract as a negative control. (**B**) Extract from water contol. (**C**) Extract from MS-treated cells. (**D**) Amount of undegraded IP$_3$. Counts for fractions 55 and 56 were combined and standardized using protein contents. The Y axis reports the remaining [^{3}H]-InsP$_3$. The data represent means of three replications and the bars are standard error of the mean.

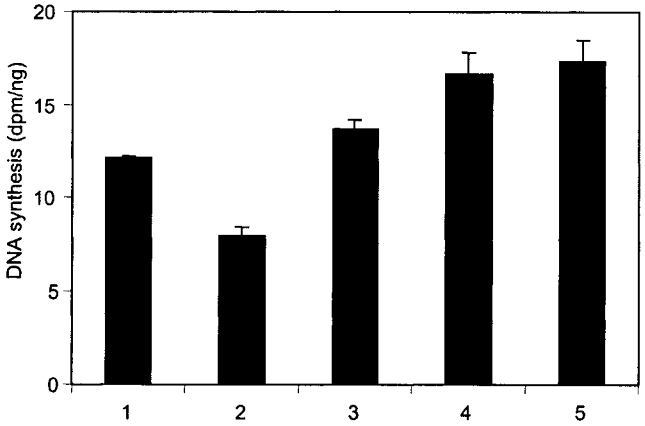

Fig. 7 PI-PLC activity and DNA synthesis. Soybean suspension cultures were treated with a PI-PLC inhibitor and/or activator, DNA was labelled *in vivo* with [^{3}H]-thymidine for 1 h after 15 h incubation with an inhibitor and/or activator. DNA synthesis was determined by calculating the ratio of tritium-labelled DNA to total DNA. Data represent means of three replications and error bars indicate standard error of the mean. 1, control; 2, 5 μM U-73122; 3, 5 μM U-73343; 4, MS salts; 5, 5 μM U-73122 and MS salts.

1991[10]; Joseph et al., 1989[22]; Martinoia et al., 1993[31]; Memon et al., 1989[32]). We examined whether there is any difference in phosphatase activities on InsP$_3$ between the water control and MS-treated cells by using the method of Joseph et al. (1989[22]). When D-*myo*-[^{3}H]-inositol 1,4,5-trisphosphate was added to crude extracts to assay phosphatase activities, the HPLC profiles of inositol phosphates were similar (Figs. **6B,C**). There was also no significant difference in the amount of InsP$_3$ content between the two treatments (Fig. **6D**). However, the amount of undegraded InsP$_3$ in the water control cells was greater than that in the MS-treated cells (Fig. **6D**), indicating that the rate of InsP$_3$ degradation is slightly higher in the MS-treated cells. Therefore, the increased InsP$_3$ content is unlikely to be the result of decreased degradation by phosphatase in MS-treated cells.

Effect of PI-PLC activity on DNA synthesis

Activation of PI-PLC following nutrient treatments suggests the possible involvement of this enzyme in physiological responses related to cell growth. Therefore, we tested whether PI-PLC modulates DNA synthesis, a cell growth-related response. We used the PI-PLC-specific inhibitor U-73122 at 5 μM to examine the effect of the inhibition of the enzyme on DNA synthesis. We applied this inhibitor at this low concentration because above 10 μM concentration the inhibitor causes cell death. The effect of the MS treatment on DNA synthesis was detected only after 8 h incubation (data not shown). Thus, we measured DNA synthesis 16 h after the various treatments. The incorporation of [^{3}H]-thymidine into DNA was significantly reduced, to 65.5% of the control, following treatment with U-73122, whereas DNA synthesis was not decreased in the cells treated with the analogue U-73343 (Fig. **7**). When the cells were treated with MS salts, DNA synthesis increased approximately 37.1% over control. The inhibition of DNA synthesis by U-73122 was completely abolished by co-treatment with MS salts (Fig. **7**). The MS-induced DNA replication was not reduced by the inhibitor use, because the amount of cellu-

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lar $InsP_3$ contents in this co-treatment of MS salts and U-73122 was actually 50% higher than the cellular $InsP_3$ concentration in the water control. This result suggests that a certain level of PI-PLC activity is sufficient for DNA synthesis.

Discussion

We have demonstrated through two independent approaches, i) HPLC analysis and ii) radio-receptor assay, an increase in cellular $InsP_3$ content in response to replenishment of 5-day-old soybean cell cultures with nutrients. The induction of $InsP_3$ content following MS treatment can be reduced or partly abolished by the use of U-73122, a PI-PLC-specific inhibitor extensively used in recent studies on plant PI-PLCs (Knight et al., 1997[24]; Koch et al., 1998[25]; Pingret et al., 1998[40]; Staxén et al., 1999[52]). Therefore, the nutrient-induced $InsP_3$ increase is most likely the result of PI-PLC activation. Inhibition of the $InsP_3$-specific phosphatase activity in nutrient-treated cells could also result in increased accumulation of $InsP_3$. For example, dephosphorylation of exogenously added D-*myo*-[^{3}H]-inositol 1,4,5-trisphosphate has been documented in different plant species (Drøbak et al., 1991[10]; Joseph et al., 1989[22]; Martinoia et al., 1993[31]; Memon et al., 1989[32]). Our study indicates that there may be an increase rather than decrease in phosphatase activity in MS-treated cells, and thus it is very unlikely that $InsP_3$-phosphatases play any role in increasing $InsP_3$ contents in MS-treated cells. The increases in IP_2 content in phosphatase assays may be due to the accumulation of dephosphorylated $InsP_3$ (Table **1**). Alternatively, increase in $InsP_2$ content could result from the use of phosphatidylinositol 4-phosphate as a substrate by the activated PI-PLC (Ettlinger and Lehle, 1988[11]; Kamada and Muto, 1994[23]; Morse et al., 1987[34]).

Analysis of a PI-PLC mutant of the slime mold, *Dictyostelium discoideum,* indicated that $InsP_3$ could be produced by a PI-PLC-independent pathway (Drayer et al., 1994[9]). In a subsequent report it was shown that both *Dictyostelium discoideum* and rat liver tissues carry a phosphatase capable of producing $InsP_3$ (Van Dijken et al., 1995[53]). In plants, however, such an alternative pathway for $InsP_3$ production has not been documented. In fact, based on a short-term non-equilibrium labelling experiment using permeabilized protoplasts of *Commelina communis,* Brearley et al. (1997[5]) concluded that $InsP_3$ is derived from the metabolism of PIP_2 by PI-PLC. Thus, we conclude from the data of inhibitor studies and phosphatase analyses that, most likely, the increases in $InsP_3$ content in nutrient-treated cells is caused by the activation of PI-PLC acitivity.

Osmosis-induced PI-PLC activation is well documented in plants (Heilmann et al., 1999[16]; Kamada and Muto, 1994[23]; Knight et al., 1997[24]). Srivastava et al. (1989[51]) reported increased $InsP_3$ content in storage tissue slices of beet *(Beta vulgaris),* and roots of sorghum *(Sorghum bicolor)* and mung bean *(Vigna radiata)* transferred to 0.2 M mannitol. Activation of the phosphoinositide signalling pathway in response to hyperosmosis was also shown in *Arabidopsis* seedlings by Knight et al. (1997[24]). In their study, 0.666 M mannitol caused a sharp increase in calcium concentration, presumably due to increases in the phosphoinositide levels. The increase in Ca^{2+} concentration was reduced by the pretreatment of seedlings with 50 μM U-73122. In our study, a lower concentration of mannitol (87.6 mM) resulted in an increase in IP_3 contents. 2-deoxyglucose, an analogue of glucose, produced a similar result. However, glucose showed a consistently higher increase in $InsP_3$ contents than that produced by 2-deoxyglucose or mannitol. The additional increases in $InsP_3$ content over the basic increase due to osmotic changes caused by either mannitol or 2-deoxyglucose are likely to be a nutritional effect of glucose. The increase in IP_3 content caused by MS salts, on the other hand, appears to be mainly due to nutritional effects, or related to the regulation of the enzyme by calcium or phosphate, and is not likely to be due to the osmotic effect of the chemicals because the two most abundant salts in MS medium, KNO_3 (18.8 mM) and NH_4NO_3 (20.6 mM), exhibited smaller contributions to the increases in $InsP_3$ content than some of the less abundant salts (especially $CaCl_2$ and KH_2PO_4; 3.4 mM and 1.2 mM, respectively) in the MS medium.

We have demonstrated in this study that the increase in $InsP_3$ contents is associated with new DNA synthesis. Use of the PI-PLC-specific inhibitor U-73122 at a very low concentration (5 μM) significantly reduced the basal level of DNA synthesis. Evans Blue fluorescence cell death assay showed that, at this low concentration, U-73122 did not cause any cell death. Furthermore, the inhibitory effect of U-73122 on DNA synthesis was completely abolished when cells were co-supplemented with MS salts. MS salts promote DNA synthesis. MS-induced $InsP_3$ content most likely compensates for the necessary cellular $InsP_3$ concentration that is reduced by U-73122. The analogue U-73343 did not inhibit DNA synthesis. These results suggest that the rate of DNA synthesis is controlled, at least in part, by the phosphoinositide signalling pathway.

The regulation of cell growth by PI-PLC is well established in mammals (Berridge, 1993[3]). For examples, microinjection of PI-PLCβ or γ promoted DNA synthesis in fibroblast cells (Smith et al., 1989[49]), whereas microinjection of PI-PLCγ-specific antibody inhibited PI-PLC-induced DNA synthesis (Smith et al., 1990 a[48]). Suppression of PI-PLCβ, γ and δ with antisense mRNA resulted in reduced cell growth in rats (Nebigil, 1997[37]). There are also many reports showing a positive role of PI-PLC in cancer progression (Beekman et al., 1998[2]; Smith et al., 1998[47]; Yang et al., 1998[56]). It has been documented that mutation in the *PLC1* gene in haploid *Saccharomyces cerevisiae* is either lethal or leads to a growth defect, depending on the genetic background of the yeast strain (Flick and Thorner, 1993[12]; Yoko-o et al., 1993[55]). In plants, growth retardation of wheat roots due to aluminium toxicity was attributed to the inhibition of PI-PLC (Jones and Kochian, 1995[21]). Recently, Perera et al. (1999[38]) suggested that sustained $InsP_3$ increase is a signal for pulvinus cell elongation in maize in response to gravistimulation. In our investigation increases in $InsP_3$ content were also observed for a sustainable period. It could be possible that continuous signalling is essential for plant growth to take place. Alternatively, stimulation of the phosphoinositide signal pathway may have an important metabolic role, vital for plant growth.

Considering these previous reports and the results from our present study, the involvement of PI-PLC in cell growth appears to be universal across kingdoms. Contrary to the possible role of $InsP_3$ in cell growth, bacterial infection has recently been shown to cause depletion in $InsP_3$ contents in soybean cells (Shigaki and Bhattacharyya, 2000[45]). In infected tissues, presumably, the constitutive pathway involved in cell growth

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is inhibited to channelize cell metabolites to meet the new demands for the synthesis of defence compounds. We speculate that the regulation of PI-PLC may be one of the important steps in the use of cell metabolites either for cell growth or in the synthesis of defence compounds.

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