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Determination of Inositol 1,4,5-Trisphosphate Levels in *Dictyostelium* by Isotope Dilution Assay

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A commercial isotope dilution assay was used for the determination of Ins(1,4,5)P₃ levels in the microorganism *Dictyostelium discoideum*. Cross-reactivity in the assay was detected with extracts from cells and the medium. The compound which induced this cross-reactivity was tentatively identified as Ins(1,4,5)P₃ by (i) co-degradation with authentic [³²P]Ins(1,4,5)P₃ by three specific Ins(1,4,5)P₃ phosphatases, and (ii) co-chromatography with authentic [³²P]Ins(1,4,5)P₃ on HPLC columns. The cellular concentration was estimated as 165 ± 42 pmol/10⁸ cells, yielding a mean intracellular Ins(1,4,5)P₃ concentration of 3.3 μM. *Dictyostelium* cells secrete large amounts of Ins(1,4,5)P₃ at a rate of about 10% of the cellular content per minute, yielding about 0.13 μM extracellular Ins(1,4,5)P₃ after 15 min in a suspension of 10⁸ cells/ml. The chemoattractant cAMP induced a transient increase of the Ins(1,4,5)P₃ concentration; the data suggest an intracellular rise from 3.3 to 5.5 μM with a maximum at 6 s after stimulation. © 1989 Academic Press, Inc.

The pivotal role of Ins(1,4,5)P₃¹ as the second messenger for receptor-mediated Ca²⁺ mobilization has been firmly established in a wide variety of systems (see Refs. (1,2) for reviews). Cellular Ins(1,4,5)P₃ levels have been measured by chromatography of the water-soluble cell extract of [³H]inositol-labeled cells on HPLC columns (3–5). The procedure has several disadvantages: Many cells are not very permeable to inositol, and large amounts of [³H]inositol have to be applied to get suffi-

cient radioactivity in the substrate phosphatidyl-inositol(4,5)-bisphosphate. Second, the analysis of [³H]Ins(1,4,5)P₃ by HPLC is complex and elaborate. More important, however, the method does not provide information on the actual mass of Ins(1,4,5)P₃.

The mobilization of Ca²⁺ by Ins(1,4,5)P₃ is thought to act through a receptor which has been described in several organisms (6–8). The high affinity and specificity of these receptors for Ins(1,4,5)P₃ suggest that they may be used in an isotope dilution assay, which would detect extracted Ins(1,4,5)P₃ by competition with [³H]Ins(1,4,5)P₃ for binding to a limited number of Ins(1,4,5)P₃ binding sites. In principle, an isotope dilution assay is simple, fast, and detects molar levels of Ins(1,4,5)P₃, and cells do not have to be labeled with [³H]inositol. The main disadvantage of an isotope dilution assay is sometimes the difficulty to prove that the cross-reactivity in the assay is due to the ligand and not to other interfering compounds. In this report the recently introduced commercial Ins(1,4,5)P₃ isotope dilution assay was investigated. Methods are described for the identification of the cross-reacting compound as Ins(1,4,5)P₃, using a simple HPLC procedure and enzymes which specifically dephosphorylate Ins(1,4,5)P₃ at the 5- or 1-position. The assay was used to measure intra and extracellular Ins(1,4,5)P₃ levels in the microorganism *Dictyostelium discoideum*.

MATERIALS AND METHODS

Materials

The Ins(1,4,5)P₃ assay kit was obtained from Amersham (TRK.1000). [³²P]Ins(1,4,5)P₃ (6.03 TBq/mmol) was purchased from New England Nuclear. A preparation of Ins(1,4,5)P₃ 5-phosphatase (50-fold purified from rat brain) was kindly provided by Dr. C. Erneux, Free University of Brussels. Ins(1,4,5)P₃ 5-phosphatase and 1-phosphatase from *Dictyostelium* were isolated, separated, and partly purified by DEAE-cellulose chroma-

¹ Abbreviations used: Ins, *myo*-inositol; Ins1P, *D*-*myo*-inositol 1-phosphate; Ins(1,4)P₂, *D*-*myo*-inositol 1,4-bisphosphate; Ins(4,5)P₂, *D*-*myo*-inositol 4,5-bisphosphate; Ins(1,4,5)P₃, *D*-*myo*-inositol 1,4,5-trisphosphate; Ins(1,3,4)P₃, *D*-*myo*-inositol 1,3,4-trisphosphate; Ins(1,3,4,5)P₄, *D*-*myo*-inositol 1,3,4,5-tetrakisphosphate; Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; PB, 10 mM KH₂PO₄/Na₂HPO₄.

tography as described by Van Lookeren Campagne *et al.* (9).

Ins(1,4,5)P₃ Extraction

D. discoideum cells were grown, harvested, and starved for 5 h as described (10). Cells were washed three times by centrifugation for 2 min at 200g in 10 mM KH₂PO₄/Na₂HPO₄ (PB) and resuspended in this buffer at a density of 10⁸ cells/ml (equivalent to about 15 mg protein/ml). Air was bubbled through the suspension at a rate of about 15 ml air/min per milliliter cell suspension. After 10–30 min 100 μ l of the cell suspension was briefly centrifuged (5 s at 10,000g), the supernatant was added to a tube containing 100 μ l of 3.5% perchloric acid, and 100 μ l of PB and 100 μ l of 3.5% perchloric acid were added to the pellet. Tubes were incubated for about 15 min at 0°C, the lysates were neutralized with 50 μ l of KHCO₃ (50% of a saturated solution at 22°C), CO₂ was allowed to escape, and tubes were centrifuged for 2 min at 10,000g. The Ins(1,4,5)P₃ levels were determined in the supernatants with the isotope dilution assay.

Isotope Dilution Assay

The assay was performed as described by the manufacturer with some modifications. The incubation contained 20 μ l each of assay buffer, [³H]Ins(1,4,5)P₃ (2685 cpm), sample, and Ins(1,4,5)P₃ binding protein in 1.5-ml plastic tubes. After 15 min of incubation at 0°C, tubes were centrifuged for 2 min at 10,000g. The supernatant was aspirated, and the pellet was dissolved in 100 μ l of H₂O. Then 1.3 ml of scintillator (Instagel, Packard) was added and radioactivity was determined in a liquid scintillation counter.

Recovery and Identification of Ins(1,4,5)P₃

The experiments are described in detail in the figure and table legends.

RESULTS AND DISCUSSION

The recently introduced commercial assay for Ins(1,4,5)P₃ was optimized for the determination of Ins(1,4,5)P₃ levels in the microorganism *Dictyostelium*. The results of Table 1 show that under the conditions used (see Materials and Methods) the assay provides a good signal: 728 $\pm$ 58 cpm [³H]Ins(1,4,5)P₃ was bound to the binding protein in the absence of competing Ins(1,4,5)P₃ and 95 $\pm$ 3 cpm in the presence of a saturating concentration of Ins(1,4,5)P₃. Half-maximal inhibition of [³H]Ins(1,4,5)P₃ binding was observed at about 1 pmol Ins(1,4,5)P₃ per assay.

We have recently observed that [³H]inositol-labeled *Dictyostelium* cells secrete large amounts of [³H]Ins(1,4,5)P₃ (Van Haastert and Van der Kaay, in preparation). Therefore, cells were incubated for 30 min

TABLE 1
Isotope Dilution Assay for Ins(1,4,5)P₃

20 μ l sample	Radioactivity in pellet (cpm)
H ₂ O	728 $\pm$ 58
0.3 pmol Ins(1,4,5)P ₃	557 $\pm$ 21
1.25 pmol Ins(1,4,5)P ₃	353 $\pm$ 10
5.0 pmol Ins(1,4,5)P ₃	188 $\pm$ 9
75 pmol Ins(1,4,5)P ₃	95 $\pm$ 3
Extract of cells	316 $\pm$ 5
Extract of medium	195 $\pm$ 3
Extract of buffer	726 $\pm$ 45

Note. The isotope dilution assay contained [³H]Ins(1,4,5)P₃ (2685 cpm), binding protein, and 20 μ l samples as indicated in a total volume of 80 μ l. The reaction was conducted for 15 min at 0°C and terminated by centrifugation, and the radioactivity in the pellet was determined. *Dictyostelium* cells were shaken for 30 min in phosphate buffer at a density of 2 $\times$ 10⁸ cells/ml and centrifuged, and the medium and the cell pellet were extracted with perchloric acid. The data for cells and medium were converted to Ins(1,4,5)P₃ concentrations using the standard curve, indicating 1.55 pmol/20 μ l in the cellular extract and 4.44 pmol/20 μ l in the extract of the medium. The results shown are the means and standard deviations of triplicate determinations.

in buffer, and extracts were made with perchloric acid from the cells and from the medium. The results (Table 1) demonstrate that both preparations induced cross-reactivity in the Ins(1,4,5)P₃ assay. In a control experiment, buffer without cells was extracted with perchloric acid by the same method; this preparation did not show inhibition in the Ins(1,4,5)P₃ assay.

Recovery of Ins(1,4,5)P₃

Ten picomoles of authentic Ins(1,4,5)P₃ was added to 100 μ l of cells, medium, or buffer, and the samples were extracted and processed in the Ins(1,4,5)P₃ assay. The volume of the final extracts was 250 μ l, of which 20 μ l was used in the assay, predicting a maximal recovery of 0.8 pmol of Ins(1,4,5)P₃ per assay. The cell extract produced a cross-reactivity equivalent to 1.26 pmol of Ins(1,4,5)P₃ per assay (Table 2); this figure increased to 1.97 pmol/assay when authentic Ins(1,4,5)P₃ was added before extraction, indicating a 89% recovery of the added Ins(1,4,5)P₃. In the medium 1.15 pmol/assay cross-reactivity was detected, which increased to 1.81 pmol upon addition of authentic Ins(1,4,5)P₃ (84% recovery). The extraction of plain buffer with authentic Ins(1,4,5)P₃ leads to the same high recovery (90%).

Identification of Cross-Reactivity Using Enzymes

Three Ins(1,4,5)P₃ degrading enzymes were available during this study: a 5-phosphatase, purified 50-fold from rat brain particulate fraction (kindly supplied by Dr. Erneux), and a 5-phosphatase and a 1-phosphatase, both

TABLE 2
Recovery of Ins(1,4,5)P₃

Extract	Cross-reactivity (pmol/assay)	Recovery (%)
Cells	1.26	
Cells + Ins(1,4,5)P ₃	1.97	89
Medium	1.15	
Medium + Ins(1,4,5)P ₃	1.81	84
Buffer	0.06	
Buffer + Ins(1,4,5)P ₃	0.83	90

Note. *Dictyostelium* cells (100 μ l) were incubated for 15 min at a density of 10⁸ cells/ml in phosphate buffer and briefly centrifuged. The medium and the cell pellet or buffer were extracted with 100 μ l of perchloric acid in the presence or absence of 10 pmol of authentic Ins(1,4,5)P₃. After neutralization with 50 μ l of KHCO₃ and centrifugation, the concentration of Ins(1,4,5)P₃ was determined in 20 μ l of the supernatant with the isotope dilution assay. The recovery was calculated by dividing the difference between extracts with and without added Ins(1,4,5)P₃ by the maximal recovery attainable (0.8 pmol/assay). The data shown are the means of duplicate determinations.

purified about 5-fold from *Dictyostelium* (9), and manuscript in preparation). Ins(1,3,4,5)P₄ is hydrolyzed by both 5-phosphatases at a rate similar to that of Ins(1,4,5)P₃, but is hydrolyzed at least 5-fold slower by the 1-phosphatase. None of the enzyme preparations hydrolyzed significant amounts of Ins1P, Ins(1,4)P₂, Ins(4,5)P₂, or Ins(1,3,4)P₃ (data not shown).

Extracts from cells, the medium, or authentic Ins(1,4,5)P₃ were incubated with the enzymes for 1 h, enzyme activity was destroyed, and cross reactivity in

the Ins(1,4,5)P₃ assay was determined. The activities of these enzymes in the extracts were also determined directly by analyzing the degradation of a tracer amount of [³²P]Ins(1,4,5)P₃; this control was necessary, because the extract of the medium, and especially of the cells, contained an inhibitor of the 5-phosphatases. Nevertheless, [³²P]Ins(1,4,5)P₃ and the compound producing cross-reactivity in the Ins(1,4,5)P₃ assay were degraded with essentially the same rate. This was observed for all three enzymes, and for the extracts from cells, the medium, and authentic Ins(1,4,5)P₃. The substrate specificity of these enzymes strongly suggests that the cross-reactivity in the Ins(1,4,5)P₃ assay is derived from Ins(1,4,5)P₃ and not from other compounds.

Identification of Cross-Reactivity Using HPLC Chromatography

Extracts from cells and the medium (with an internal standard of 2.5 fmol [³²P]Ins(1,4,5)P₃) were separated by reversed phase ion-pair HPLC. The elution position of [³²P]Ins(1,4,5)P₃ was determined by Cerenkov radiation. Subsequently samples were lyophilized, and cross-reactivity in the Ins(1,4,5)P₃ assay was determined (Fig. 1). This chromatographic system was chosen because it separates many inositol polyphosphates at low ion concentrations without gradient elution (Van Haastert *et al.*, in preparation). Figure 1 demonstrates that [³²P]Ins(1,4,5)P₃ is well separated from inositol phosphates which contain less or more than three phosphates. Cross-reactivity in the Ins(1,4,5)P₃ assay was observed only in those fractions where Ins(1,4,5)P₃ was eluted; this was found for the extracts from the medium

TABLE 3
Co-degradation of Ins(1,4,5)P₃ and Cross-Reactivity by Phosphatases

Extract	Enzymes activities (% degradation)		
	Rat brain 5-phosphatase	<i>Dictyostelium</i>	
		5-Phosphatase	1-Phosphatase
A. 0.8 pmol Ins(1,4,5)P ₃	64	51	27
B. 0.8 pmol Ins(1,4,5)P ₃ + [³² P]Ins(1,4,5)P ₃	65	54	31
A. Cells	5	29	29
B. Cells + [³² P]Ins(1,4,5)P ₃	6	19	24
A. Medium	54	45	26
B. Medium + [³² P]Ins(1,4,5)P ₃	49	38	24

Note. Buffer with 0.8 pmol/20 μ l of Ins(1,4,5)P₃, cells, and medium were extracted with perchloric acid as described in the legend of Table 2. Two experiments were performed. (A) The extracts (20 μ l) were incubated with 5 μ l of 120 mM Hepes/NaOH, 6 mM EGTA, 18 mM MgCl₂, pH 7.5, and 5 μ l of the indicated enzymes from rat brain or *Dictyostelium*. After 1 h the samples were boiled for 2 min, and the Ins(1,4,5)P₃ content was determined in 20 μ l by the isotope dilution assay. (B) The incubation mixture was the same, except for the addition of 2000 cpm [³²P]Ins(1,4,5)P₃. After termination of the reaction, samples were chromatographed over Dowex columns, which were eluted with different ammonium formate concentrations to separate the products and the substrate (9). The results are presented as percentage of degradation of the compound that induces cross-reactivity in the isotope dilution assay (A), or as the percentage of degradation of authentic [³²P]Ins(1,4,5)P₃ (B).

FIG. 1. Co-elution of Ins(1,4,5) P_3 and cross-reactivity on HPLC column. Extracts (300 μ l) from *Dictyostelium* cell and the medium were prepared as described in the legend of Table 1 and mixed with 350 cpm [32 P]Ins(1,4,5) P_3 (2.5 fmol) and 300 μ l 0.1 M tributylammonium phosphate, pH 6.5. This mixture was chromatographed on a reversed phase LiChrosorp 10RP18 column which was eluted isocratically at a flow of 1 ml/min with 1 mM tributylammonium phosphate, 25 mM KH_2PO_4/KOH , 15% methanol, pH 6.5. Fractions of 0.85 ml were collected in which the ^{32}P -radioactivity was determined by Cerenkov radiation. The samples were then lyophilized, dissolved in 200 μ l 0.1 M Tris, and 20 μ l was used to determine the cross-reactivity in the Ins(1,4,5) P_3 isotope dilution assay. Data were calculated as picomoles Ins(1,4,5) P_3 per 20- μ l assay. The arrows indicate the elution of IP_1 (Ins1P), P_1 (orthophosphate), IP_2 (Ins(1,4) P_2), and IP_4 (Ins(1,3,4,5) P_4).

(Fig. 1A) and from cells (Fig. 1B). It should be noted that the amount of [32 P]Ins(1,4,5) P_3 added is at least 1000-fold less than the amount of Ins(1,4,5) P_3 detected in the assay. The recovery of the cross-reactivity was calculated, taking into account the amount of picomoles applied to the column and the portion of the column eluent that was used in the assay (10%). About 36 pmol cross-reactivity from the cellular extract was applied to the column, and about 30 pmol was recovered; these figures are for the medium respectively 117 and 136 pmol, indi-

cating essentially complete recovery of the applied material.

Thus the compounds in the extract from cells and the medium that induce cross-reactivity in the highly specific Ins(1,4,5) P_3 assay were degraded at the same rate as Ins(1,4,5) P_3 by specific enzymes and co-eluted with Ins(1,4,5) P_3 on a HPLC column. This is regarded as sufficient evidence for their identity being Ins(1,4,5) P_3 .

cAMP-induced Ins(1,4,5) P_3 Formation in *Dictyostelium*

Having established that the cross reactivity is due to Ins(1,4,5) P_3 , it becomes possible to estimate the concentration of Ins(1,4,5) P_3 in the cells and in the medium. Four experiments were performed with 5-h-starved cells that were extensively washed and then incubated for 15 min at a density of 10^8 cells/ml. The cellular Ins(1,4,5) P_3 content was 165 ± 42 pmol/ 10^8 cells; this yields an intracellular concentration of 3.3 μ M, assuming an intracellular volume of 50 μ l/ 10^8 cells and a homogeneous distribution of Ins(1,4,5) P_3 inside the cell. The Ins(1,4,5) P_3 level in the medium was 130 ± 10 pmol/ml, yielding an extracellular Ins(1,4,5) P_3 concentration of 0.13 μ M.

cAMP is a chemoattractant for *Dictyostelium* cells (11). Addition of cAMP to sensitive cells induced a small

FIG. 2. cAMP-stimulated Ins(1,4,5) P_3 formation in *Dictyostelium*. Cells were incubated for 15 min at a density of 10^8 cells/ml and stimulated with 10^{-6} M cAMP (final concentration). At the times indicated 100 μ l of the cell suspension was added to 100 μ l of perchloric acid, and Ins(1,4,5) P_3 levels were measured in the neutralized extracts by isotope dilution assay. Samples of the cell suspension were briefly centrifuged before and 1 min after stimulation with cAMP, and Ins(1,4,5) P_3 levels were determined in the medium; about 50% of the total Ins(1,4,5) P_3 was extracellular and did not change after stimulation with cAMP. The results shown are the means and standard deviations of three experiments with duplicate determinations after and quadruplicate determinations before stimulation with cAMP. The 100% values were 286, 348, and 341 pmol/ 10^8 cells.

transient rise of the $\text{Ins}(1,4,5)\text{P}_3$ levels with a maximum at about 6 s after stimulation. Basal levels were about $325 \text{ pmol}/10^8$ cells which increased to $440 \text{ pmol}/10^8$ cells. Since $\text{Ins}(1,4,5)\text{P}_3$ is not degraded in the extracellular medium (9), the transient rise in $\text{Ins}(1,4,5)\text{P}_3$ is probably due to intracellular accumulation; this hypothesis was supported by analyzing the extracellular $\text{Ins}(1,4,5)\text{P}_3$ levels before and 1 min after cAMP stimulation, which were essentially identical (data not shown). These data suggest that cAMP induced a transient increase of the mean intracellular $\text{Ins}(1,4,5)\text{P}_3$ concentration from 160 to $275 \text{ pmol}/10^8$ cells, or from 3.3 to $5.5 \mu\text{M}$.

The target of intracellular $\text{Ins}(1,4,5)\text{P}_3$ is probably an $\text{Ins}(1,4,5)\text{P}_3$ receptor involved in Ca^{2+} mobilization from nonmitochondrial stores. This process takes place at a half-maximal $\text{Ins}(1,4,5)\text{P}_3$ concentration of 0.1 – $1 \mu\text{M}$ (see (1)). The high intracellular $\text{Ins}(1,4,5)\text{P}_3$ concentration in *Dictyostelium* may indicate compartments of intracellular $\text{Ins}(1,4,5)\text{P}_3$, which is also suggested by the extensive secretion of $\text{Ins}(1,4,5)\text{P}_3$ in this organism. The rise of $\text{Ins}(1,4,5)\text{P}_3$ levels after cAMP stimulation, although statistically significant, is relatively small with only a 35% increase over total $\text{Ins}(1,4,5)\text{P}_3$ levels and a 70% increase over intracellular levels. However, the increase of $\text{Ins}(1,4,5)\text{P}_3$ amounts to $2.2 \mu\text{M}$ over basal levels. This increase could be very significant if it would occur in a compartment that has submicromolar $\text{Ins}(1,4,5)\text{P}_3$ concentrations.

$\text{Ins}(1,4,5)\text{P}_3$ induces Ca^{2+} mobilization and the accumulation of cGMP levels in *Dictyostelium* (12,13). Until recently, experiments using $[^3\text{H}]$ inositol-labeled cells have not firmly established a receptor-mediated accumulation of $\text{Ins}(1,4,5)\text{P}_3$ levels, because cells are not very permeable to $[^3\text{H}]$ inositol, and intracellular $[^3\text{H}]$ inositol is metabolized to water soluble compounds which are not InsP_x (in which $x = 1$ to 6) but co-migrate with $\text{Ins}(1,4,5)\text{P}_3$ on anion exchange columns (14). Only using a reversed phase ion-pair HPLC system have we been able to identify a metabolite of $[^3\text{H}]$ inositol as $\text{Ins}(1,4,5)\text{P}_3$. The radioactivity in this peak increases after cAMP stimulation with the kinetics and magnitude similar to those reported in the present study. It is evident that the detection of $\text{Ins}(1,4,5)\text{P}_3$ levels by isotope dilution assay is superior above detection by HPLC in respect to time, convenience, and radioactive hazard. It should be noted, however, that this method detects only $\text{Ins}(1,4,5)\text{P}_3$, and no information becomes available

about the complex metabolism of $\text{Ins}(1,4,5)\text{P}_3$ and inositol.

Dictyostelium cells secrete large amounts of $\text{Ins}(1,4,5)\text{P}_3$; we have estimated that cells secrete about 10% of their cellular $\text{Ins}(1,4,5)\text{P}_3$ levels per minute. We face the exciting possibility that, next to cAMP, also $\text{Ins}(1,4,5)\text{P}_3$ may have an extracellular function in this organism. The introduction of the commercial $\text{Ins}(1,4,5)\text{P}_3$ assay kit and some of the control experiments presented in this report should speed up the elucidation of the intricate role of $\text{Ins}(1,4,5)\text{P}_3$ in sensory transduction.

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