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/10⁶ cells, yielding a mean intracellular Ins(1,4,5)P₃ concentration of 3.3 pM.

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 nM extracellular Ins(1,4,5)P₃ after 15 min in a suspension of 10⁶ 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 pM with a maximum at 6 s after stimulation.

The pivotal role of Ins(1,4,5)P₃ 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 sufficient radioactivity in the substrate phosphatidylinositol(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-
were centrifuged for 2 min at 10,000 g. The supernatant was aspirated, and the pellet was dissolved in 100 μl of plastic tubes. After 15 min of incubation at 0°C, tubes contained 20 μl 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 10,000 g. 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 μl of each sample, [3H]Ins(1,4,5)P₃ (2885 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 10,000 g. The supernatant was aspirated, and the pellet was dissolved in 100 μl 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 ± 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 ± 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 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.

**Identification of Cross-Reactivity Using Enzymes**

Ten picomoles of authentic Ins(1,4,5)P₃ was added to 100 μl 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 μl, of which 20 μl 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%).

**TABLE 1**

<table>
<thead>
<tr>
<th>20 μl sample</th>
<th>Radioactivity in pellet (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>728 ± 58</td>
</tr>
<tr>
<td>0.3 pmol Ins(1,4,5)P₃</td>
<td>557 ± 21</td>
</tr>
<tr>
<td>1.25 pmol Ins(1,4,5)P₃</td>
<td>353 ± 10</td>
</tr>
<tr>
<td>5.0 pmol Ins(1,4,5)P₃</td>
<td>188 ± 9</td>
</tr>
<tr>
<td>75 pmol Ins(1,4,5)P₃</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>Extract of cells</td>
<td>316 ± 5</td>
</tr>
<tr>
<td>Extract of medium</td>
<td>195 ± 3</td>
</tr>
<tr>
<td>Extract of buffer</td>
<td>726 ± 45</td>
</tr>
</tbody>
</table>

Note. The isotope dilution assay contained [3H]Ins(1,4,5)P₃ (2885 cpm), binding protein, and 20 μl samples as indicated in a total volume of 80 μl. 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 × 10⁶ 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 1.97 pmol/20 μl in the cellular extract and 4.44 pmol/20 μl in the extract of the medium. The results shown are the means and standard deviations of triplicate determinations.
TABLE 2
Recovery of Ins(1,4,5)P₃

<table>
<thead>
<tr>
<th>Extract</th>
<th>Cross-reactivity (pmol/assay)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>1.26</td>
<td>89</td>
</tr>
<tr>
<td>Cells + Ins(1,4,5)P₃</td>
<td>1.97</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>1.15</td>
<td>84</td>
</tr>
<tr>
<td>Medium + Ins(1,4,5)P₃</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>0.06</td>
<td>90</td>
</tr>
<tr>
<td>Buffer + Ins(1,4,5)P₃</td>
<td>0.83</td>
<td></td>
</tr>
</tbody>
</table>

Note. Dictyostelium cells (100 μl) were incubated for 15 min at a density of 10⁶ cells/ml in phosphate buffer and briefly centrifuged. The medium and the cell pellet or buffer were extracted with 100 μl of perchloric acid in the presence or absence of 10 pmol of authentic Ins(1,4,5)P₃. After neutralization with 50 μl of KHCO₃ and centrifugation, the concentration of Ins(1,4,5)P₃ was determined in 20 μl 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.

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.
cAMP-induced Ins(1,4,5)P₃ Formation in Dictyostelium

Having established that the cross reactivity is due to Ins(1,4,5)P₃, it becomes possible to estimate the concentration of Ins(1,4,5)P₃ 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 10⁶ cells/ml. The cellular Ins(1,4,5)P₃ content was 165 ± 42 pmol/10⁶ cells; this yields an intracellular concentration of 3.3 μM, assuming an intracellular volume of 50 μl/10⁶ cells and a homogeneous distribution of Ins(1,4,5)P₃ inside the cell. The Ins(1,4,5)P₃ level in the medium was 130 ± 10 pmol/ml, yielding an extracellular Ins(1,4,5)P₃ concentration of 0.13 μM.

CAMP is a chemoattractant for Dictyostelium cells (11). Addition of CAMP to sensitive cells induced a small (Fig. 2) and from cells (Fig. 1B). It should be noted that the amount of [³²P]Ins(1,4,5)P₃ added is at least 1000-fold less than the amount of Ins(1,4,5)P₃ 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, indicating 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)P₃ assay were degraded at the same rate as Ins(1,4,5)P₃ by specific enzymes and co-eluted with Ins(1,4,5)P₃ on a HPLC column. This is regarded as sufficient evidence for their identity being Ins(1,4,5)P₃.
transient rise of the Ins(1,4,5)P$_3$ levels with a maximum at about 6 s after stimulation. Basal levels were about 325 pmol/10$^9$ cells which increased to 440 pmol/10$^9$ cells. Since Ins(1,4,5)P$_3$ is not degraded in the extracellular medium (9), the transient rise in Ins(1,4,5)P$_3$ is probably due to intracellular accumulation; this hypothesis was supported by analyzing the extracellular Ins(1,4,5)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 Ins(1,4,5)P$_3$ concentration from 160 to 275 pmol/10$^9$ cells, or from 3.3 to 5.5 $\mu$M.

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

Ins(1,4,5)P$_3$ induces Ca$^{2+}$ mobilization and the accumulation of cGMP levels in Dictyostelium (12,13). Until recently, experiments using [3H]inositol-labeled cells have not firmly established a receptor-mediated accumulation of Ins(1,4,5)P$_3$ levels, because cells are not very permeable to [3H]inositol, and intracellular [3H]inositol is metabolized to water soluble compounds which are not InsP$_x$ (in which $x =$ 1 to 6) but co-migrate with Ins(1,4,5)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 [3H]inositol as Ins(1,4,5)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 Ins(1,4,5)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 Ins(1,4,5)P$_3$, and no information becomes available about the complex metabolism of Ins(1,4,5)P$_3$, and inositol.

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

ACKNOWLEDGMENTS

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REFERENCES