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ACCUMULATION OF [^{3}H]-INOSITOL INTO INOSITOL POLYPHOSPHATES DURING DEVELOPMENT OF *DICTYOSTELIUM*

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SUMMARY: Inositol hexakisphosphate ($InsP_6$) is present in large amounts during the development of *Dictyostelium discoideum* although its function is unknown. We have investigated the accumulation of [^{3}H]-labelled inositol into both InsP6 and a more highly charged inositol species called $InsP_Y$ during development of amoebae on filters. We report that the pattern of [^{3}H]-labelled inositol incorporation into $InsP_6$ and $InsP_Y$ differs markedly from the pattern seen for inositol phospholipids and inositol(1,4,5)trisphosphate. Incorporation into PtdIns, PtdInsP, PtdInsP$_2$ and Ins(1,4,5)P$_3$ reached plateau values within 4 h. In contrast, incorporation into $InsP_6$ continued in an approximately linear manner for the first eight hours of development. No incorporation into $InsP_Y$ was seen during the first three hours of development at which time accumulation of [^{3}H] continued linearly in a similar manner as for $InsP_6$. Because the total cell concentration of $InsP_6$ (measured by chemical assay) changes very little during development it seems probable that incorporation of label into $InsP_Y$ after 3 h is due to a developmentally controlled metabolic switch rather than a requirement for a threshold level of its probable precursor, $InsP_6$.

Previous research in this laboratory has shown that when amoebae of Dictyostelium are labelled with [^{3}H]-Inositol while cells are developing on filters, most of the accumulated [^{3}H] is found in a large pool of $InsP_6$. Studies by Martin *et al.* (1) using NMR have provided estimates of the intracellular concentration of $InsP_6$ in strain AX2 (assuming uniform distribution throughout the cell) of about 0.7 mM. Recent evidence of Europe-Finner *et al.* (2,3) has indicated that *Dictyostelium* also forms a more highly charged inositol-containing

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ABBREVIATIONS: InsP, InsP$_2$, InsP$_3$, and $InsP_6$ represent inositol mono- , bis-, tris-, and hexakisphosphates, respectively, with assignment of phosphate position where appropriate. PtdIns4P and PtdIns(4,5)P$_2$ represent phosphatidylinositol 4-phosphate and phosphatidylinositol(4,5)bisphosphate respectively.

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compound, which is retained on a SAX HPLC column longer than $InsP_6$. This molecule we have called $InsP_Y$ and have tentatively suggested it to be a pyro-phosphate form of $InsP_6$.

In this present work we have studied the patterns of incorporation of [^{3}H]-Inositol into both polyphosphoinositides and inositol polyphosphates such as $InsP_6$ and $InsP_Y$ during development.

MATERIALS AND METHODS

Materials: D-myo-(1,2-^{3}H)Inositol (specific activity 41.1 Ci mmol^{-1}) was obtained from New England Nuclear and was passed through a Dowex 1-X8 column (formate form) prior to use so as to remove trace impurities; myo-[^{3}H]-Inositol (specific activity 99.5 Ci mmol^{-1}) stabilised with PT6-271 and D-myo-[5-^{32}P]-Inositol(1,4,5)-trisphosphate were obtained from Amersham International PLC.

Harvesting of amoebae: *Dictyostelium discoideum* strain NC4, was grown in association with *Klebsiella aerogenes* (strain OXF1) on SM nutrient agar plates (4). Amoebae were prepared by growth as lawns on bacterially inoculated agar plates under conditions permitting uniform clearing of the bacteria by the feeding amoebae or from the growing edges of clones. Amoebae were harvested from the bacterial plates at the preaggregation stage of their life-cycle, in P buffer (17 mM-Na/K phosphate buffer pH 6.1) and washed free of bacteria by repeated centrifugation at 190 x g for 2 minutes.

Labelling of amoebae with (^{3}H)-inositol: Amoebae, suspended in P buffer at 1 - 2x10^8 cells ml^{-1}, were dispensed as 0.5ml samples onto 47mm diameter Whatman 50 filter discs supported on 47mm diameter absorbent pads (Millipore AP1004700) that were saturated with P buffer, as described by Newell & Sussman (5). After the excess liquid that had soaked through the filters had been removed, the filters and cells (without the pads) were carefully placed on a 100μl drop of D-myo-(1,2-^{3}H)inositol in the centre of a 90mm Petri dish. A thick pad of filter paper, soaked in P buffer, was attached to the underside of the Petri dish lid so as to absorb any ammonia produced by the amoebae. The Petri dishes were then placed in a humidity chamber and incubated in the dark at 22°C.

For experiments where the time course of [^{3}H]-inositol incorporation was to be determined, the cells were incubated on filters as described above for periods of 0.5 to 10 hours. The amoebae were then removed from the filters into P buffer, washed twice to remove unincorporated label and resuspended at 2.5x10^7 cells ml^{-1}. The lipids or inositol phosphates were then extracted and analysed as described previously (3).

Assay of $InsP_6$ by phosphate determination: The method used was a modification of Ames and Dubin (6). Before assay of the phosphate in $InsP_6$, samples were desalted and inorganic phosphate removed as described by Downes *et al.* (7).

RESULTS

Incorporation of [^{3}H]-inositol into phosphatidylinositols: Incorporation of [^{3}H]-labelled inositol into amoebae was determined using cells developing on filter supports (8,2). Total uptake was found to be approximately 2% of the added label after 2 h of incubation. After

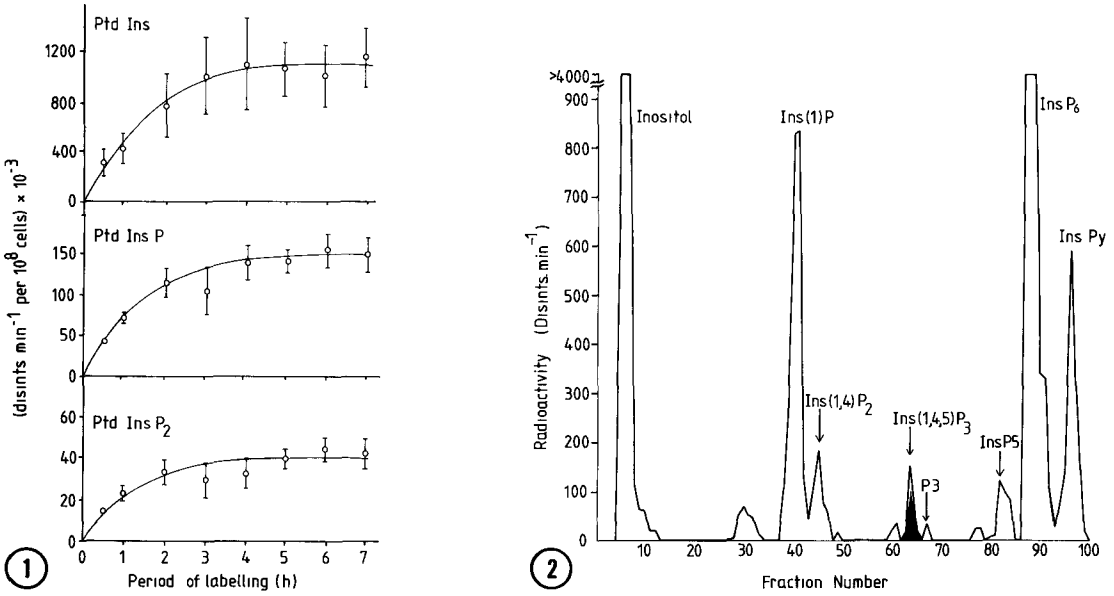

Figure 1. Time course of [^{3}H]-inositol incorporation into PtdIns, PtdInsP and PtdInsP$_2$
Amoebae were labelled on filters with [^{3}H]-inositol (specific activity 99.5 Ci mmol^{-1}) for periods of 0.5 to 7.0 hours. Samples were then harvested, washed and the lipid soluble fraction extracted. The inositol lipids were deacylated and PtdIns, PtdInsP and PtdInsP$_2$ determined as described in Methods. Results are the means of four experiment. Error bars represent S.E.M.

Figure 2. Elution profile of soluble inositol phosphates of *D.discoideum* using a Partisil SAX HPLC column
The main curve represents the [^{3}H] label in cell extracts, and the filled peak the [^{32}P] label of the InsP(1,4,5)P$_3$ added to the extracts as an internal marker. Ins(1)P and Ins(1,4)P$_2$ represent the positions of internal markers. InsP$_Y$ represents a highly charged inositol polyphosphate that elutes after InsP$_6$.

separation of lipid-soluble phosphoinositides and their deacylation, incorporation of label into phosphatidyl inositol (PtdIns), PtdInsP and PtdInsP$_2$ was determined (Fig. 1). Incorporation into these phospholipids was found to occur rapidly with plateau values being reached within 3 - 4 hours. As expected, the extent of incorporation at 4 h differed markedly between the phosphatidyl inositols, the incorporation into PtInsP being approximately 7 times less than into PtdIns and that into PtdInsP$_2$ being approximately 4-fold less than into PtdInsP$_2$.

Incorporation of [^{3}H]-inositol into lower inositol phosphates: When the soluble cell fraction of amoebae incubated with [^{3}H]-inositol was analysed by Partisil SAX HPLC for inositol phosphates (Fig. 2 shows a typical HPLC chromatograph) the pattern of incorporation of [^{3}H]-inositol into Ins(1,4,5)P$_3$ was found to be similar to that of the inositol phospholipids

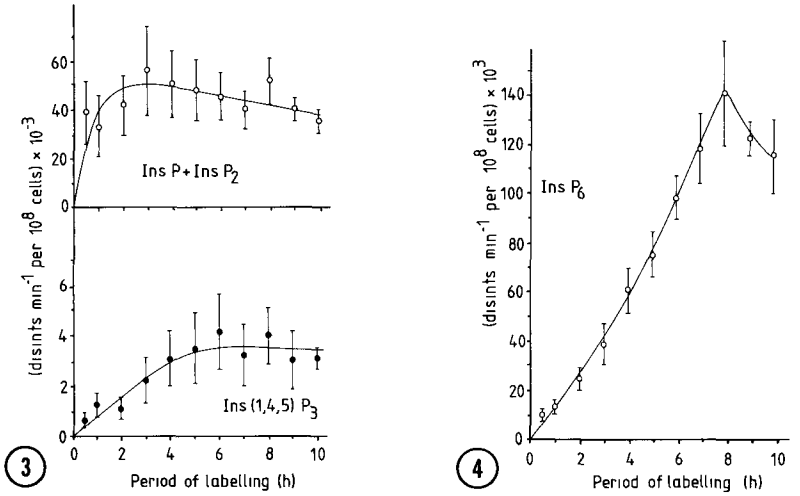

Figure 3. Time course of [^{3}H]-inositol incorporation into lower inositol phosphates. Amoebae were labelled on filters with [^{3}H]-inositol (spec. act. 99.5 ci mmol^{-1}). After periods of 0.5 to 10 h, samples were harvested, washed and their inositol phosphates extracted and separated by SAX HPLC. Results are the means of five experiments. Upper curve, inositol mono and bis phosphates; lower curve, Ins(1,4,5)P_3 . Error bars represent S.E.M.

Figure 4. Time course of [^{3}H]-inositol incorporation into $InsP_6$
Amoebae of the strain NC4 were labelled , extracted and analysed as described in the legend for Figure 3. Results are the means of five experiments. Error bars represent S.E.M.

(as expected from an intermediate that is in rapidly formed from $PtdInsP_2$) with a plateau being formed within 4 h (Fig. 3B).

In contrast, the incorporation of label into the InsP and $InsP_2$ fractions equilibrated much more rapidly. As the individual peaks of InsP and $InsP_2$ isomers were difficult to separate from each other consistently, the combined fraction of all mono and bis inositol phosphates was used for quantitative assay. In this fraction, labelling was found to approach the maximum value within 0.5 h rather than 4 h as with Ins(1,4,5)P_3 (Fig. 3A). The majority of these rapidly formed mono and bis isomers probably represent isomers formed on the Ins(1,4,5)P_3-independent pathway to $InsP_6$ from inositol (via Ins3P and Ins(3,6)P_2) that was recently demonstrated by Stephens and Irvine (1990).

Incorporation of [^{3}H]-inositol into $InsP_6$ and $InsP_7$: The incorporation of [^{3}H]-labelled inositol into $InsP_6$ was also found to be very rapid (approximately 20 fold faster than the initial rate into Ins(1,4,5)P_3) and almost linear over 8 h of development (Fig. 4). After 8 h, total incorporation reached values that were 30 - 50 fold greater than into Ins(1,4,5)P_3.

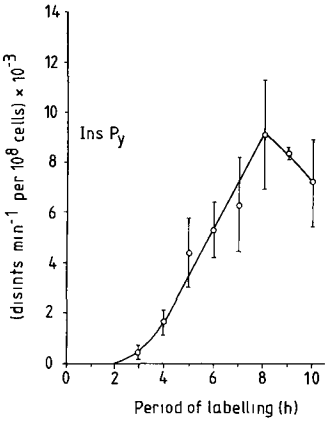

Figure 5. Time course of [^{3}H]-inositol incorporation into $InsP_Y$
Amoebae of the strain NC4 were labelled, extracted and analysed as described in the legend for Figure 3. Results are the means of five experiments. Error bars represent S.E.M.

However, incorporation of [^{3}H]-inositol into $InsP_Y$ (Fig.5) occurred only after 3 h of development and then proceeded in a similar manner to the incorporation into $InsP_6$. IP_y was also found to incorporate ^{32}P when cells were labelled with [^{32}P] phosphate (data not shown).

Changes in $InsP_6$ as measured by phosphate assay: Recent data of Stephens *et al.* (10) indicated that during development of *Dictyostelium* strain AX2 on agar supports, the cellular concentration of $InsP_6$ measured by phosphate assay increased from 7.5 to 9 nmoles mg^{-1} protein during the first 8 h of development. Using a phosphate assay of desalted $InsP_6$ separated by HPLC, we found a similarly small increase in this compound in developing amoebae of strain NC4. The cellular content of $InsP_6$ (assuming uniform distribution in the cell of volume 520 μm^3) rose by 300 μM (from 2.6 to 2.9 mM) over the period 0 to 8 h.

DISCUSSION

The aim of this study was to compare the pattern of labelling *in vivo* by [^{3}H]-inositol into $InsP_6$ and $InsP_Y$ with that into the lower charged inositol phosphates and inositol phospholipids. We report that the pattern of incorporation into $InsP_6$ and $InsP_Y$ is very different from that into $Ins(1,4,5)P_3$ and PtdIns, PtdInsP and $PtdInsP_2$. The incorporation into $InsP_6$ was linear over 8 h and was rapid. In contrast the incorporation into $Ins(1,4,5)P_3$

was slower and reached a plateau value after 4hrs. Unlike the phosphatidyl inositides and lower inositol phosphates which rapidly turn over, $InsP_6$ represents a metabolic product with a large pool that turns over relatively slowly. The function and significance for the cell of this amount of $InsP_6$ and its small but steady increase during development are not at present understood. Even less is understood about the significance of $InsP_Y$. This compound can be labelled with both ^3H and ^{32}P and resembles $InsP_6$ in its pattern of steady accumulation, except that no incorporation was detected before 3 h of development on filters. Since its likely precursor ($InsP_6$) does not change greatly in concentration over the first three hours of development, it seems unlikely that incorporation into $InsP_Y$ is dependent upon the accumulation of a threshold concentration of $InsP_6$. It seems more probable, therefore, that the change in incorporation represents a metabolic switch which is part of the developmental program.

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