A *myo*-inositol D-3 hydroxykinase activity in *Dictyostelium*

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A soluble ATP-dependent enzyme which phosphorylates *myo*-inositol has been characterized in *Dictyostelium*. The *myo*-inositol kinase activity was partially purified from amoebae by chromatography on DEAE-Sepharose and phenyl-Sepharose columns. The product of both the partially purified activity and of a crude cytosolic fraction was *myo*-inositol 3-phosphate. The partially purified preparations of *myo*-inositol kinase (a) possessed a K_m for *myo*-inositol of 120 μM (in the presence of 5 mM-ATP) and for ATP of 125 μM (in the presence of 1 μM -*myo*-inositol), (b) did not recognize allo-, epi-, muco-, neo-, *scyllo*-, 1D-*chiro* or 1L-*chiro*-inositol as substrates, (c) were competitively inhibited by three naturally occurring analogues of *myo*-inositol: 1L-*chiro*-inositol (K_i 49.5 $\pm$ 0.7 μM : the structural equivalent of *myo*-inositol, except that the D-3 hydroxy moiety is axial), D-3-deoxy-*myo*-inositol [K_i 103 $\pm$ 1 μM : (–)-viburnitol], and sequoyitol (K_i 271 $\pm$ 7 μM ; unlike 1L-*chiro*-inositol and D-3-deoxy-*myo*-inositol, this was a substrate for the kinase), and finally (d) were apparently non-competitively inhibited by *myo*-inositol 3-phosphate. The product of *myo*-inositol kinase could be detected in intact amoebae and was a substrate for the first in a series of inositol polyphosphate kinases present in *Dictyostelium* which ultimately yield *myo*-inositol hexakisphosphate. The activity of *myo*-inositol D-3-hydroxykinase in *Dictyostelium* lysates showed evidence of developmental regulation.

INTRODUCTION

myo-Inositol, the most abundant of the naturally occurring inositols (which include *myo*-inositol, 1L- and 1D-*chiro*-inositol, *scyllo*-inositol and neo-inositol; Anderson, 1972) is metabolized in several ways. The most well known is the reaction catalysed by PtdIns synthase in which *myo*-inositol is exchanged for the CMP moiety of CMP phosphatidic acid (Agranoff *et al.*, 1958). PtdIns synthase has long been considered the only enzyme in a non-plant cell that catalyses the synthesis of a *myo*-inositol-phosphate link, and hence in these situations it is presumed to be the sole source of all inositol phospholipids, PtdIns-glycans and inositol phosphates (apart from Ins3P, which can be derived from glucose 6-phosphate by Ins3P synthase). Plant cells also contain an inositol kinase activity which therefore represents an additional mechanism by which they can make inositol phosphates (Deitz & Albersheim, 1965; English *et al.*, 1966). We have recently described a series of reactions in *Dictyostelium*, catalysed by soluble ATP-dependent enzymes, that convert *myo*-inositol into InsP₆ (Stephens & Irvine, 1990). The first step in this pathway is catalysed by a *myo*-inositol kinase, and accordingly that report represented the first description of an authentic *myo*-inositol kinase in a non-plant cell. Plant-derived *myo*-inositol kinase activity is soluble and ATP-dependent, and phosphorylates the D-3 hydroxy group of *myo*-inositol (Loewus *et al.*, 1982). In amoebae, *myo*-inositol kinase represents the first, and hence a potentially regulatory, step in the synthesis of InsP₆. The situation in plant tissues is currently unclear because, although Ins3P has been shown to act as a precursor of InsP₆ in broken-cell preparations (Molanari & Hoffman-Ostenhof, 1968; Bollmann *et al.*, 1980), in one of the best-studied systems [the ripening and germinating mung-bean (*Phaseolus aureus*) seed] *myo*-inositol kinase activity is not expressed at the same time as are the enzymes responsible for catalysing at least part of the pathway synthesizing InsP₆ (Biswas *et al.*, 1978).

The experiments described in this report were designed to characterize the substrate specificity and catalytic properties of

myo-inositol D-3 hydroxykinase in *Dictyostelium* and to search for evidence of developmental and/or metabolic regulation of its activity.

MATERIALS AND METHODS

NC4, a laboratory strain of the cellular slime mould *Dictyostelium*, was co-cultured with *Klebsiella aerogenes* as described previously (Kay & Trevan, 1981). Cells were harvested from half-cleared plates into 20 mM-NaCl/20 mM-KCl/1 mM-CaCl₂/10 mM-Mes (pH 6.2, 25 °C; NS/Mes) and freed from bacteria by differential centrifugation (Kay & Trevan, 1981). Intact amoebae (2 $\times$ 10⁷/ml) were labelled with [³H]Ins (10 $\mu\text{Ci}/\text{ml}$) in a silicone-coated glass flask containing 50 ml of NS/Mes. After 4 h of continuous shaking (180 rev. min⁻¹) the amoebae were pelleted by centrifugation, the supernatant was removed and 2 ml of 3% (v/v) ice-cold HClO₄ was added to the cells. The acid-precipitated cellular debris was resuspended by vortex-mixing and then pelleted by centrifugation; the supernatant was removed, neutralized with 2 M-KOH/0.1 M-Mes/25 mM-EDTA and stored at –20 °C. The inositol monophosphates in the sample were subsequently analysed by anion-exchange h.p.l.c. using an acetate-based eluant as described previously (Stephens *et al.*, 1988), except that ammonium acetate replaced the potassium acetate used in the original work. This marginally increased the retention time of standard inositol monophosphates (by less than 10%). The use of a totally volatile eluant meant that fractions containing [³H]InsPs could be desalted by freeze-drying. Standard [¹⁴C]Ins3P, [¹⁴C]Ins2P, [³²P]Ins5P and [¹⁴C]Ins4P (prepared as described in Stephens *et al.*, 1988) were added to each sample to act as internal markers.

Enantiomeric configuration of D/L-InsP

D/L-[³H]Ins1P, extracted and h.p.l.c.-purified from either [³H]Ins-prelabelled *Dictyostelium* or *myo*-inositol kinase assays, was partially oxidized with periodate (90 min in the dark at 25 °C, with 0.5 ml of 10 mM-sodium periodate) then reduced

Abbreviation used: PMSF, phenylmethanesulphonyl fluoride.

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(500 μ l of 0.1 M-NaBH₄, 12 h in the dark at 25 °C) and dephosphorylated as described (Stephens *et al.*, 1989). The predominant ³H-labelled polyols recovered were identified by their relative chromatographic mobilities on a Pb²⁺ cation-exchange column as described (Stephens *et al.*, 1989). D- and L-[³H]glucitol were distinguished using a yeast-derived L-iditol dehydrogenase (Sigma). D-Glucitol (sorbitol, Sigma) is a substrate for this enzyme ($V_{\max}$ 330 nmol·min⁻¹, K_m 25 mM; see Stephens, 1990), whereas L-glucitol (prepared from L-glucose as described in Stephens *et al.*, 1988) is not a substrate. Assays were run essentially as described previously (Stephens *et al.*, 1989), with 45 μ g of D-glucitol/ml and 1000 d.p.m. of D-[¹⁴C]glucitol (Amersham) as internal standards and 2–2.5 units of polyol dehydrogenase·ml⁻¹.

Preparation of NC4 lysates

NC4 amoebae were routinely lysed by resuspending them (at 3×10^8 /ml) in 10% (v/v) glycerol/10 mM-Hepes/1 mM-EGTA/0.2 mM-phenylmethanesulphonyl fluoride (PMSF) (pH 7.5, 4 °C) and then forcing them through Whatman no. 3 filter paper [Das & Henderson (1983); less than 1% of the cells remained intact]. The resulting lysate was immediately adjusted to pH 7.0 with a solution of 1 M-Hepes (acid form) and dithiothreitol was added (1 mM final concentration). Cytosolic fractions were prepared from the lysates by centrifugation (100 000 g, 60 min, 4 °C), and the pellet was resuspended in lysis buffer (pH adjusted to 7.0 and containing 1 mM-dithiothreitol) to yield a particulate fraction.

Assay of inositol kinase

Two forms of assay were employed. When the assays contained *myo*-[³H]inositol (which was routinely passed through a 50 μ l column of Bio-Rad AG 1.8 200–400 resin, in the formate form, before its inclusion in an assay), the incubation medium (100–200 μ l total volume) had a composition as follows: 5 mM-ATP, 6 mM-MgCl₂, 1 mM-dithiothreitol, 25 mM-Hepes, 1 mM-EGTA, 80 mM-KCl and 5–50 μ Ci of [³H]Ins·ml⁻¹ (pH 7.0, 25 °C). When determining the K_m of the enzyme for CTP or ATP, the assays also contained phosphocreatine kinase (5 units·ml⁻¹), 10 mM-phosphocreatine, 12 mM-MgCl₂ and the nucleotides, added as their magnesium salts (the activity of *myo*-inositol kinase in these assays was not changed by raising the MgCl₂ concentration to 14.0 mM). Some assays were quenched by placing them in a boiling-water bath for 5 min and then processed for chromatography by sequentially adding 0.1 M-EDTA (pH 7.0, NaOH, 50 μ l), phytate hydrolysate (10 μ l containing 12 μ g of phosphorus) and 5 mM-*myo*-inositol (600 μ l). The samples were then centrifuged and their supernatants were applied to 2 cm columns of Bio-Rad AG 1.8 200–400 resin (formate form). *myo*-Inositol was eluted with 5 mM-*myo*-inositol (10 ml) and 50 mM-ammonium formate (10 ml), and [³H]InsPs were eluted with 0.2 M-ammonium formate and 0.1 M-formic acid (10 ml), and quantified by mixing the eluant with 10 ml of scintillation fluid (Packard 299) and counting the sample for radioactivity in a liquid scintillation spectrophotometer. When assays containing 1 μ Ci of [³H]Ins and no enzyme were processed in this way, 1000–2000 d.p.m. was eluted in the inositol monophosphate fraction. Alternatively, the assays were quenched by adding 3.61% (w/v) HClO₄ (600 μ l) and phytate hydrolysate (10 μ l, containing 12 μ g of phosphorus). The acid-precipitated protein was pelleted by centrifugation and neutralized with 2 M-KOH/0.1 M-Mes/25 mM-EDTA (approx. 200 μ l, resulting in the pH of the sample rising to 6). The neutralized samples were applied to 2 cm columns of anion-exchange resin and eluted as described above. This procedure, although more time-consuming, resulted in assays that contained

1 μ Ci of [³H]Ins and no enzyme, giving rise to inositol monophosphate fractions with only 200–400 d.p.m.

Inositol kinase assays with unlabelled inositols (or inositol analogues, all at 2 mM final concentration) also contained [³²P]ATP (3–6 μ Ci· μ mol⁻¹; 0.2 mM final concentration), 1 mM-MgCl₂, 25 mM-Hepes, 1 mM-EGTA, 10 mM-LiCl, BSA (1 mg·ml⁻¹) and 900 μ l of a partially purified preparation of *Dictyostelium myo*-inositol kinase (final pH = 7.0 at 25 °C) in a total volume of 1.3 ml. The assays were incubated at 25 °C for 2 h, boiled and centrifuged, and their supernatants were applied to 2 cm columns of Bio-Rad AG 1.8 200–400 resin (formate form). The columns were washed with water (10 ml), 50 mM-ammonium formate (10 ml) and 0.15 M-ammonium formate/0.1 M-formic acid (8 ml, which eluted [³²P]P_i and [³²P]InsP). The [³²P]InsP fraction was mixed sequentially with 10 M-HClO₄ (1 ml), 10% (w/v) ammonium molybdate (1 ml) and 2-methylpropan-1-ol/toluene (1:1, v/v, 20 ml). The samples were centrifuged to produce two clear phases. The upper phase was removed and replaced with fresh 2-methylpropan-1-ol/toluene (20 ml), and the samples were mixed, centrifuged and the upper phases were removed and replaced with fresh solvent three more times. The final lower phase was Čerenkov-counted in a liquid scintillation spectrophotometer. Assays containing no enzyme typically resulted in the final lower phase containing 20 000–40 000 c.p.m. Once counted, the lower phases were mixed with 7.84 mg of orthophosphoric acid, washed with 2-methylpropan-1-ol/toluene (1:1, v/v and then mixed with tri-*N*-octylamine/Freon (1:1, v/v, 1.1 vol.; Sharpes & McCarl, 1982), [¹⁴C]Ins2P, [¹⁴C]Ins4P and [¹⁴C]Ins3P and a portion of the resulting aqueous phase was analysed by anion-exchange h.p.l.c. as described above.

Partial purification of an inositol kinase activity from *Dictyostelium*

All of the following steps were carried out at 4 °C. A cytosolic fraction was prepared from NC4 amoebae as described above and applied to a column (6 mg of protein/ml of resin) of DEAE-Sepharose (Pharmacia) pre-equilibrated with 25 mM-Hepes, 1 mM-EGTA and 1 mM-dithiothreitol, pH 7.0 at 4 °C (buffer A). Once loaded with enzyme, the column was washed with buffer A and eluted with a rising concentration of NaCl in buffer A. The eluant was collected in fractions, and samples from each were assayed for *myo*-[³H]inositol kinase (as described above) and [³H]Ins3P phosphatase [assays contained a buffer identical with that described for *myo*-[³H]inositol kinase (see above) and 4000–10 000 d.p.m. of [³H]Ins3P (see below)]. The fractions containing *myo*-[³H]inositol kinase were pooled, and the concentration of NaCl in the sample was raised to 2 M before applying it to a column of phenyl-Sepharose (Pharmacia) pre-equilibrated with 2 M-NaCl in buffer A; approx. 1 mg of total protein was loaded per ml of column resin. The kinase activity was eluted with a falling gradient of NaCl in buffer A.

Preparation of [³H]Ins3P

myo-[³H]Inositol (100 μ Ci) was incubated with a sample of DEAE/phenyl-Sepharose-purified *myo*-inositol kinase in 200 μ l of the assay buffer defined above. The assay was stopped with HClO₄, neutralized and the [³H]Ins3P was purified on an anion-exchange h.p.l.c. column as described.

Electroporation

Amoebae were resuspended at 1×10^8 /ml in ice-cold *myo*-[³H]inositol kinase assay buffer, as described above, and permeabilized by exposing them to three discharges from a 25 μ F capacitor charged to 800 V (Gene-Pulser, Bio-Rad). This protocol resulted in optimal incorporation of [³H]Ins into lipids.

Development of *Dictyostelium*

AX2 (an axenic laboratory strain of *Dictyostelium*) was cultured (Watts & Ashworth, 1970), harvested into 16.5 mM-KH₂PO₄/3.9 mM-K₂HPO₄/2 mM-MgSO₄ and developed on agar supports [1.8 % (w/v) Oxoid C28 agar; 3.5 × 10⁸ amoebae on a 14 cm-diameter plate]. Development was assumed to start with the first wash with phosphate buffer. Cells were scraped up every 3 h (three lots of 5 × 10⁸ cells per time point) and stored at -20 °C in the form of a centrifugally packed pellet. The frozen pellets were either (a) thawed into ice-cold lysis buffer and assayed for protein, glycogen phosphorylase and cyclic AMP phosphodiesterase as described previously (Kay, 1979), or (b) thawed into ice-cold 25 mM-Hepes/1 mM-EGTA (pH 7.0, 4 °C)/1 mM-dithiothreitol/0.5 % (v/v) Triton X-100/10 % (v/v) glycerol/0.2 mM-PMSF. The lysates were vortex-mixed for 10 min (at 4 °C) and then pelleted by centrifugation in a Microfuge (3 min at maximum speed). The supernatants were assayed for protein (see below) and portions from each lysate were diluted with lysis buffer to yield 0.8 ml of solution containing soluble protein (1 mg·ml⁻¹). Each of the diluted supernatants was mixed with 3 mg of BSA. Samples of each supernatant which had been diluted to a concentration of *Dictyostelium* protein of 1 mg·ml⁻¹ were assayed for *myo*-[³H]inositol kinase, [³H]Ins3P phosphatase, [³H]Ins(1,4,5)P₃ phosphatase (see below) and [³H]Ins(1,2,4,5,6)P₅ kinase (see below). [³H]Ins(1,4,5)P₃ phosphatase was assayed in the buffer described for *myo*-inositol kinase, except that it contained 10000 d.p.m. of [³H]Ins(1,4,5)P₃ (Amersham) in each assay. A maximum of 5 % of the

[³H]Ins(1,4,5)P₃ was phosphorylated in each assay. The tritiated products of the [³H]Ins(1,4,5)P₃ assays were resolved on 2 cm columns of Bio-Rad AG 1.8 200-400 resin (chloride form; Spencer *et al.*, 1990). [³H]Ins(1,2,4,5,6)P₅ kinase was assayed in the buffer described for *myo*-inositol kinase, except that it contained 28000 d.p.m. of [³H]Ins(1,2,4,5,6)P₅ (prepared as described in Stephens, 1990). [³H]InsP₅ and [³H]InsP₆ were resolved on 12 cm columns of Bio-Rad AG 1.8 200-400 resin (chloride form) (Spencer *et al.*, 1990; L. R. Stephens, unpublished work).

Protein assay

Protein was assayed according to Bradford (1976), with BSA as a standard.

RESULTS

NC4 amoebae were permeabilized by treatment with detergents, by electroporation or by forced filtration, and then incubated under identical conditions with [³H]Ins and an ATP-containing buffer. [³H]Ins was incorporated into both inositol phosphate and inositol lipid fractions. However, the quantity of [³H]Ins incorporated into a total lipid fraction was relatively small in comparison with the amount of [³H]InsPs formed in the same assays. Furthermore, the various permeabilization strategies did not have parallel effects on [³H]InsP and [³H]Ins lipid accumulation (see Table 1). If either exogenous Ptd[¹⁴C]Ins or a crude [³H]Ins lipid extract from an assay containing Triton

Table 1. Incorporation of [³H]Ins into inositol phosphates and inositol lipids in *Dictyostelium* homogenates

NC4 amoebae were resuspended (1 × 10⁸/ml) in ice-cold 10 mM-Hepes/1 mM-EGTA/0.2 mM-PMSF (pH 7.5, 4 °C) and then either (a) lysed by forced filtration and partitioned into particulate and soluble fractions by centrifugation; 150 µl portions of these fractions were then assayed for *myo*-inositol kinase in the presence or absence of 0.25 % (w/v) Triton X-114 (as described in the text), or (b) their pH was adjusted and they were then mixed with dithiothreitol (like the subcellular fractions described above, see the Materials and methods section) and assayed for *myo*-inositol kinase directly ('cells'), after electroporation (treatment 1), or in the presence of various detergents and exogenous radiolabelled lipids. The final concentrations of detergents were 0.16 % (w/v) saponin, 0.25 % (w/v) Triton X-114 and 0.04 % (w/v) cetyltrimethylammonium bromide (CTAB), and the assays contained 2 µCi of *myo*-[³H]Ins in a total volume of 200 µl. To test whether lipids were serving as precursors of inositol phosphates, [³H]-labelled lipids, extracted (Bligh & Dyer, 1959) from an assay identical to that for treatment 5, were incubated with a suspension of amoebae (as described for treatment 5, except containing no *myo*-[³H]Ins). In a similar set of assays exogenous [¹⁴C]PtdIns was added with Triton X-114 to an assay identical with treatment 5 above (but containing no *myo*-[³H]Ins). Reactions were quenched with either 3.75 vol. of chloroform/methanol/HCl (100:200:1, by vol.) or 5 % (v/v) HClO₄. Those samples quenched with organic solvents were separated into two phases (Bligh & Dyer, 1959). The lower phase was washed twice with fresh upper phase, dried under vacuum and dissolved in scintillation fluid to quantify the total [³H]-labelled lipids [the concentrations of detergents used did not alter the recovery of [¹⁴C]PtdIns during a Bligh & Dyer (1959) extraction]. The samples that had been quenched with HClO₄ were centrifuged and the supernatants were neutralized with 2 M-KOH/0.1 M-Mes/25 mM-EDTA, and applied to small anion-exchange resin columns to separate [³H]InsPs from [³H]Ins. Data are expressed as means of duplicates and are typical of three similar experiments.

Treatment	¹⁴ C or ³ H content (d.p.m.)	
	Total lipid	Total inositol phosphates
Cells	5490	3202
1. Electroporated	36025	70250
2. Saponin	1200	20800
3. CTAB	849	283000
4. CTAB + saponin	—	29140
5. Triton X-114	4543	262667
6. Triton X-114; no ATP	—	9529
7. Exogenous ³ H-labelled lipid	14200	204
8. Exogenous [¹⁴ C]PtdIns	20050	476
9. No enzyme blank	—	2610
10. Cell filtrate	4250	191591
11. Cell filtrate + Triton X-114	—	193021
12. Supernatant	—	181170
13. Supernatant + Triton X-114	—	174922
14. Particulate	—	5023
15. Particulate + Triton X-114	—	7149

Fig. 1. Purification of *myo*-inositol kinase

(a) A 5 ml column of DEAE-Sepharose was loaded with 30 mg of protein derived from a *Dictyostelium* cytosolic fraction (as described in the Materials and methods section), washed with buffer A (25 mM-Hepes/1 mM-EGTA/1 mM-dithiothreitol/0.1 mM-PMSF, pH 7.0, 4 °C) and eluted with a NaCl gradient (---). Fractions (6 ml) were collected every 6 min; 150 μ l portions were assayed for inositol kinase ($\blacktriangle$) and inositol 3-phosphate phosphatase ($\circ$) and 50 μ l portions were assayed for protein ($\blacksquare$) as described in the text. The separation shown is typical of that obtained with seven independent preparations of inositol kinase. Recoveries of inositol kinase activity varied between 300 and 400 %. However, this apparent increase in activity is the result of the combination of an increase in NaCl concentration, a decrease in glycerol concentration and a loss of inositol monophosphate phosphatase activity that occurs during this purification. In the crude cytosol fraction the inositol monophosphate phosphatase activity hydrolyses approx. 10–20 % of the product of inositol kinase when assays are performed as described. Recoveries of inositol monophosphate phosphatase varied between 80 and 100 %, whereas typically, in a parallel assay with a crude cytosol fraction, a maximum of 2–3 % of the [3 H]Ins would be phosphorylated to [3 H]InsP. (b) The fractions emerging from a DEAE-Sepharose column (a) which contained the majority of the inositol kinase activity and a minimum of Ins3P phosphatase activity were pooled, their NaCl concentration was adjusted to 2 M, and then they were applied to a column of phenyl-Sepharose and eluted with a falling NaCl concentration gradient (0.6 ml $\cdot$ min $^{-1}$). Portions of 100 μ l were removed from each fraction and assayed for inositol kinase as described. Recoveries of inositol kinase activity from the phenyl-Sepharose columns ranged from 60 to 80 %; however, the enzyme activity was reversibly decreased by approx. 10 % on moving from 2 M-NaCl to 0.1 M-NaCl.

X-100, permeabilized amoebae, ATP and [3 H]Ins was added to a suspension of amoebae in assay buffer (with Triton X-100), then barely detectable amounts of 3 H or 14 C were released as inositol phosphates. The accumulation of [3 H]InsPs occurring in permeabilized amoebae incubated with [3 H]Ins was completely dependent on an exogenous supply of ATP. These data suggest the [3 H]Ins lipids formed in the assays containing permeabilized amoebae, ATP and [3 H]Ins are not the precursors of the [3 H]InsPs formed in the same assays. If force-filtered homogenates were separated into particulate and soluble fractions by centrifugation, then it was clear that the presumed inositol kinase activity was soluble. The inositol kinase was partially purified from an NC4 cytosolic fraction via DEAE and phenyl-Sepharose columns (Fig. 1). If the partially purified inositol kinase activity was assayed as described in the Materials and methods section then its product accumulated linearly both with time, for a minimum of 40–50 min, and with respect to the quantity of protein added to the assay. This was the case if the enzyme was assayed either

under zero-order conditions with respect to both of its substrates or under first-order conditions with respect to the inositol concentration, so long as less than 10 % of the total substrate had been consumed (results not shown).

The [3 H]Ins-labelled product of the partially purified *myo*-[3 H]inositol kinase activity co-chromatographed, during anion-exchange h.p.l.c., with D/L-[14 C]Ins1P but not with [14 C]Ins2P, [14 C]Ins4P or [32 P]Ins5P (results not shown). A portion of a preparation of the partially purified inositol kinases product, that was h.p.l.c.-pure and [3 H]Ins-labelled, was treated with 10 mM-sodium periodate for 90 min and then reduced and dephosphorylated (see the Materials and methods section). The overall recoveries of 3 H through these very mildly oxidizing conditions were usually in the range 40–50 %, of which the majority was in [3 H]inositol (75–90 %), with a smaller amount in [3 H]glucitol (8–20 %) and traces in [3 H]iditol and [3 H]altritol (1–2 %). Within a given set of oxidations the proportions of the [3 H]polyols that were recovered from [3 H]Ins1P (NEN) were

Fig. 2. Oxidation of D-glucitol by polyol dehydrogenase

(a) An assay containing L-iditol dehydrogenase ($2.5 \text{ units} \cdot \text{ml}^{-1}$), D- ^{14}C glucitol, $250 \mu\text{M}$ -D-glucitol and ^{3}H glucitol derived from the incomplete periodate oxidation of the D- or L- ^{3}H Ins1P formed by *myo*-inositol kinase was incubated at 25°C until its A_{340} had ceased to rise significantly faster than in a parallel assay that contained no substrate, at which point it was boiled for 3 min. The denatured protein and salt were removed from the boiled assays (as described in Stephens *et al.*, 1988) and the sample was applied to a cation-exchange resin column (Pb^{2+} mode; see the Materials and methods section; Stephens *et al.*, 1988, 1989; Stephens, 1990). The carbohydrate column was eluted with water (25°C) at $0.2 \text{ ml} \cdot \text{min}^{-1}$ and fractions were collected every 1.5 min and counted for ^{14}C ($\bullet$) and ^3H ($\circ$) radioactivity utilizing standard dual-label liquid scintillation counting techniques. The times at which the injections of pure solutions of fructose, inositol, altritol and glucitol resulted in single refractile peaks emerging in the eluant (see Stephens *et al.*, 1988) are shown, and are corrected for the difference in void volume between the points at which refractive index and radioactivity are detected. (b) Three cuvettes containing $950 \mu\text{l}$ of buffer with L-iditol dehydrogenase ($3 \text{ units} \cdot \text{ml}^{-1}$) (see Materials and methods section) were equilibrated at 25°C . After 19–25 min $50 \mu\text{l}$ of 4.5 mM -D- ($\square$) or L-glucitol ($\triangle$) or $50 \mu\text{l}$ of water ($\circ$) was added and the A_{340} of each of the assays was monitored for the following 3 h.

very similar to those obtained for the ^3H -labelled product of the inositol kinase.

In these incomplete oxidations of ^{3}H inositol monophosphates, the amount of ^{3}H inositol that is finally recovered is an indication of the amount of unoxidized ^{3}H InsP remaining when the reaction is quenched with NaBH_4 . The preponderance of ^{3}H glucitol in the straight-chain ^3H -labelled products recovered

Fig. 3. *Dictyostelium myo*-inositol kinase for ATP and CTP

DEAE-phenyl-Sepharose-purified *Dictyostelium*-derived *myo*-inositol kinase was incubated with various concentrations of CTP ($\bullet$) and ATP ($\circ$) in the presence of $1 \mu\text{M}$ - ^{3}H *myo*-inositol. Under the conditions of these assays, the resulting changes in free Mg^{2+} concentration were insignificant; see the Materials and methods section. No more than 4% of the total substrate was converted to product. The data are derived from two independent experiments (one for ATP and one for CTP), and each point is the mean of two determinations. One further experiment yielded similar results for both CTP and ATP. The apparent K_m values for ATP and CTP were approx. $125 \mu\text{M}$ and $833 \mu\text{M}$ respectively.

Fig. 4. Inhibition of *Dictyostelium myo*-inositol kinase by Ins3P

The apparent K_m of a partially purified preparation of *Dictyostelium myo*-inositol kinase for *myo*-inositol was determined in the presence of various concentrations of Ins3P ($\bullet$, $500 \mu\text{M}$; $\blacktriangle$, $150 \mu\text{M}$; $\blacklozenge$, $50 \mu\text{M}$; $*$, $0 \mu\text{M}$). Assay conditions were as described in the text. A maximum of 3% of the initial substrate was converted to product. The data are means ($n = 2$) and were replicated in one further experiment (using $50 \mu\text{M}$ - and $200 \mu\text{M}$ -Ins3P). The results were fitted by eye and are consistent with Ins3P being a non-competitive inhibitor of ^{3}H Ins kinase.

is an indication that the D-3–4 and D-5–6 carbon–carbon bonds of ^{3}H Ins1P are the favoured initial sites of oxidation by periodate. Presumably, the presence of the D-1 or L-1 phosphate hinders oxidation of the *cis*-related hydroxy groups which are usually the preferred site of attack for periodate (and would yield D- or L- ^{3}H iditol).

Fig. 5. Interaction of the inositols and some of their analogues with *Dictyostelium myo-inositol kinase*

DEAE-Sephacel-purified *Dictyostelium myo-inositol kinase* was incubated with 1.5 μCi of *myo*- ^3H inositol (under first-order conditions with respect to the *myo*-inositol concentration) and various concentrations of other inositols or their analogues. The quantity of ^3H InsP formed in 30 min was quantified as described in the text. Each point is a mean of two determinations. Note that neo-inositol saturates aqueous solutions at room temperature at approx. 0.72 g/litre (4 mM). Compounds tested, in order of decreasing inhibitory potency, were: $\circ$, L-*chiro*-inositol; $\diamond$, (-)-viburnitol; $\blacktriangle$, *myo*-inositol; $\blacklozenge$, sequoyitol; ∇ , D/L-4-amino-*myo*-inositol; ∇ , 2-fluoro-*myo*-inositol; $\blacktriangledown$, 1-fluoro-*scyllo*-inositol; $\odot$, 3-deoxy-3-fluoro-*myo*-inositol; $\triangle$, epi-inositol; $+$, *scyllo*-inositol; $\bullet$, 1D-*chiro*-inositol; $\star$, (-)-bornesitol; $\times$, muco-inositol; $*$, quebrachitol; $\triangle$, 4-deoxy-*myo*-inositol; $\square$, 2-methoxy-*myo*-inositol; $\blacksquare$, allo-inositol; $\star$, (+)/(-)-bornesitol. The incorporation in the absence of any analogues ($\odot$) and in the absence of added enzyme ($\bullet$) are also shown.

D-Glucitol, but not L-glucitol, is a substrate for L-idoitol dehydrogenase (polyol dehydrogenase, EC 1.1.1.14; see the Materials and methods section and Fig. 2), and upon oxidation it yields D-fructose (Fig. 2). If ^3H glucitol derived from the partial oxidation of ^3H Ins1P and D- or L- ^3H Ins1P (product of the partially purified inositol kinase) were mixed with D- ^{14}C glucitol and incubated with L-idoitol dehydrogenase (under first-order conditions with respect to the polyol substrate mixture), then the D- ^{14}C glucitol and the ^3H glucitol derived from ^3H Ins1P were oxidized to ^{14}C fructose and ^3H fructose respectively. The ^3H glucitol derived from the product of *myo*-inositol and ^{14}C glucitol derived from authentic ^{14}C Ins3P were not oxidized (results not shown). This suggests that the product of *myo*-inositol kinase yields L- ^3H glucitol and is therefore ^3H Ins3P. This conclusion, that the original ^3H InsP from

which the L- ^3H glucitol was derived was ^3H Ins3P, is supported by the following. (a) The expectation that a D-3 phosphate would afford more protection to the D-3-4 carbon-carbon bond than the D-5-6 carbon-carbon bond. It would appear from the total oxidation of the ^3H glucitol from ^3H Ins1P and the total resistance to oxidation of the ^3H glucitol from the *myo*-inositol kinase-derived ^3H Ins1P that this protection is effectively complete. (b) The observation that authentic ^{14}C Ins3P yielded L- ^{14}C glucitol.

D- or L- ^3H Ins1P was the predominant (greater than 95 % of the total) ^3H InsP in both intact ^3H Ins-prelabelled NC4 amoebae and a crude homogenate of NC4 amoebae incubated with an ATP-regenerating system and ^3H Ins (see the Materials and methods section; the unknown ^3H InsPs were resolved on an anion-exchange h.p.l.c. column with ^{14}C Ins3P, ^{14}C Ins2P, ^{14}C Ins4P and ^{32}P Ins5P internal standards; results not shown). H.p.l.c.-purified preparations of D- or L- ^3H Ins1P from both intact ^3H Ins-prelabelled amoebae or the purified *myo*-inositol yielded L-glucitol upon partial periodate oxidation, reduction and dephosphorylation (results not shown), suggesting that the inositol kinase activity detected in homogenates is forming the same product as the activity that had been partially purified, and, furthermore, that intact amoebae contain Ins3P.

The K_m of the partially purified *myo*-inositol D-3 hydroxykinase activity for *myo*-inositol was $118 \pm 10 \mu\text{M}$ (mean $\pm$ S.E.M. for four independent preparations in the presence of 5 mM-ATP) and the K_m for ATP was $125 \mu\text{M}$ (in the presence of 1 μM -*myo*-inositol; Fig. 3). Of the other nucleotides tested (ITP, GTP, UTP and TTP), only CTP appeared to substitute for ATP (K_m 833 μM ; Fig. 3). The rate-enhancing effect of CTP on *myo*-inositol kinase was lost in the presence of saturating concentrations of ATP, suggesting that CTP binds to the same site as does ATP.

Ins3P decreased the incorporation of ^3H Ins into ^3H Ins3P, and this inhibition was of a non-competitive nature (Fig. 4).

A range of other inositols and inositol analogues were added to assays measuring the rate of conversion of *myo*- ^3H inositol into ^3H InsP (Fig. 5). Any decrease in the rate of incorporation is an indication that the test substance is influencing, or interacting with, the *myo*-inositol-binding site, and could result from it being an inhibitor (competitive or otherwise) or a substrate. The most potent compound tested was 1L-*chiro*-inositol (a naturally occurring inositol which possesses essentially the same structure as *myo*-inositol, except that the D-3 hydroxy group is axial rather than equatorial), followed by (-)-viburnitol (a naturally occurring cyclohexane pentol; 3-deoxy-*myo*-inositol) and sequoyitol (a naturally occurring compound; 5-O-methyl-*myo*-inositol).

1L-*chiro*-inositol and *myo*-inositol were 100 times more potent than any of the other inositols tested, and as it is likely that at least some of these inositols were contaminated with 1 % (w/w) *myo*-inositol, this is probably a minimum estimate of the selectivity of the enzyme. *cis*-inositol was not tested, but the lack of effect of inositols which contained only some of the differences between *cis*- and *myo*-inositol suggests that *cis*-inositol would not have been recognized by the inositol kinase. A more detailed examination of the three most potent inhibitors of ^3H Ins incorporation into ^3H Ins3P revealed that they operated in a competitive manner (Fig. 6), with the K_i values for 1L-*chiro*-inositol, (-)-viburnitol and sequoyitol of $49.5 \pm 0.7 \mu\text{M}$, $103 \pm 1 \mu\text{M}$ and $271 \pm 7 \mu\text{M}$ respectively. Although these data are all manifest as decreases in the incorporation of ^3H Ins into ^3H Ins3P, this could result from the competitor being either an inhibitor or a substrate. To distinguish between these two possibilities, assays were designed to measure the rate of incorporation of radioactivity from ^{32}P ATP into putative InsP-like products in the presence of partially purified *myo*-inositol

Fig. 6. Interactions of *myo*-inositol kinase with various substrates

(a) The apparent K_m of a DEAE-purified preparation of *Dictyostelium myo*-inositol kinase for *myo*-inositol was determined in the presence of various concentrations of 1L-*chiro*-inositol (■, 300 μ M; ▲, 150 μ M; △, 100 μ M; ◆, 50 μ M; ▼, 20 μ M; *, 0 μ M). The assays each contained between 1.5 and 10 μ Ci of [3 H]Ins (enzyme blanks were included in each experiment which contained precisely equivalent quantities of [3 H]Ins) and buffer as described in the text. The assays were stopped after 30 min, at which time a maximum of 5%

Table 2. Activity of *Dictyostelium myo*-inositol kinase with various substrates

DEAE-Phenyl-Sepharose purified *Dictyostelium myo*-inositol kinase was incubated with ATP (final specific radioactivity of 5.8 Ci/mol) and various potential substrates. After 2 h the assays were quenched and the 32 P radioactivity in an inositol monophosphate fraction was determined (see the Materials and methods section). The data presented are means $\pm$ S.E.M. ($n = 3$) and were replicated in one further experiment.

Substrate	Product	
	32 P (c.p.m.)	(nmol)
Blank	20 624 $\pm$ 820	0
2 mM-(–)-Viburnitol	22 240 $\pm$ 618	0.25
2 mM-L-2- <i>chiro</i> -Inositol	20 643 $\pm$ 805	0.003
2 mM- <i>myo</i> -Inositol	85 311 $\pm$ 347	10.2
2 mM-Sequoyitol	122 087 $\pm$ 958	16.0

kinase and 2 mM-*myo*-inositol, 1L-*chiro*-inositol, (–)-viburnitol or -sequoyitol. The results are summarized in Table 2. In the presence of 2 mM-1L-*chiro*-inositol or (–)-viburnitol, only background counts were obtained, whereas in the presence of the same concentration sequoyitol or *myo*-inositol, 32 P-labelled metabolites accumulated. When further analysed by anion-exchange h.p.l.c. the assays containing *myo*-inositol were found to contain an additional 32 P-labelled peak [above the control, 1L-*chiro*-inositol and (–)-viburnitol samples] which co-migrated with [14 C]InsP₃; assays containing sequoyitol contained an extra 32 P-labelled peak which was eluted just before [14 C]InsP₃ (Fig. 7). These results suggest that *myo*-inositol, 1L-*chiro*-inositol, (–)-viburnitol and sequoyitol can all bind reversibly to the same recognition site on *myo*-inositol D-3 hydroxykinase, but that only *myo*-inositol and sequoyitol can act as substrates.

The levels of *myo*-inositol D-3 hydroxykinase, total Ins(1,4,5)P₃ phosphatases, Ins3P phosphatase and InsP₃ kinase activities were measured during the development of *Dictyostelium* from amoebae through to fruiting bodies (Fig. 8). In marked contrast with the levels of InsP₃ kinase and total Ins(1,4,5)P₃ phosphatase, *myo*-inositol D-3 hydroxykinase activity dropped transiently during the post-aggregatory phases of mound and tipped mound formation.

of the substrate had been converted to [3 H]InsP. Each point is the mean of two independent determinations; when fitted by eye they suggest that 1L-*chiro*-inositol is a competitive inhibitor with an apparent K_i of $49.5 \pm 0.7 \mu$ M. (b) In essence the assays were as described. The apparent K_m of *myo*-inositol kinase for inositol was determined in the presence of different concentrations of (–)-viburnitol (■, 300 μ M; ▲, 150 μ M; △, 100 μ M; ◆, 50 μ M; ▼, 20 μ M; *, 0 μ M) and [3 H]Ins (2–10 μ Ci/assay). The assays were quenched after 30 min, at which time a maximum of 3% of the starting material was phosphorylated. The data are means of duplicate determinations and when fitted by eye suggest that (–)-viburnitol is a competitive inhibitor of *myo*-inositol kinase, with an apparent K_i of $103 \pm 1 \mu$ M. (c) The apparent K_m of the preparation of DEAE-purified *Dictyostelium*-derived *myo*-inositol kinase for *myo*-inositol was determined in the presence of several different concentrations of sequoyitol (○, 600 μ M; ■, 300 μ M; □, 200 μ M; △, 100 μ M; ◇, 40 μ M; *, 0 μ M) as described for (a) and (b). A maximum of 3% of the substrate was converted to product. The data points are means ($n = 2$) and when fitted by eye suggest that sequoyitol is a competitive inhibitor with an apparent K_i of $271 \pm 7 \mu$ M.

Fig. 7. Chromatographic properties of sequoyitol monophosphate

The ^{32}P -labelled products formed in assays containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, a sample of partially purified *Dictyostelium myo*-inositol kinase and 2 mM-sequoyitol (c), 2 mM-*myo*-inositol (b) or water (a) (assays containing 2 mM-1L-*chiro*-inositol or 2 mM-(–)-viburnitol were indistinguishable from the water blanks) were desalted and mixed with $^{14}\text{C}[\text{Ins}3\text{P}]$, $^{14}\text{C}[\text{Ins}2\text{P}]$ and $^{14}\text{C}[\text{Ins}4\text{P}]$ and resolved on an anion-exchange h.p.l.c. column as described in the Materials and methods section. A ^{32}P -labelled peak (○) that was co-eluted with $^{14}\text{C}[\text{Ins}3\text{P}]$ (●) was present in the assay containing *myo*-inositol (b). An additional ^{32}P -labelled metabolite, which was eluted before $^{14}\text{C}[\text{Ins}3\text{P}]$, was present in all of the samples including the enzyme blanks. In the sample containing 2 mM-sequoyitol (c) the major ^{32}P -labelled metabolite was eluted significantly before $^{14}\text{C}[\text{Ins}3\text{P}]$ and is presumably ^{32}P sequoyitol monophosphate.

DISCUSSION

In this report we have described some of the properties of an inositol kinase activity found in *Dictyostelium*. The inositol kinase will phosphorylate the D-3 substitution site of *myo*-inositol, yielding $\text{Ins}3\text{P}$. In *Dictyostelium* $\text{Ins}3\text{P}$ is the substrate for an enzyme in a pathway culminating in the synthesis of $\text{Ins}P_6$ (Stephens & Irvine, 1990). Although $\text{Ins}P_6$ has been found in every animal and plant cell that has been investigated to date, its synthesis from *myo*-inositol has only been defined in *Dictyostelium*. Just how widespread is this pathway, and therefore *myo*-inositol D-3 hydroxykinase, remains to be established. An inositol kinase activity has already been described in plant cells (English *et al.*, 1966), the product of which is also $\text{Ins}3\text{P}$ (Loewus *et al.*, 1982). As $\text{Ins}3\text{P}$ has been shown to act as a precursor of $\text{Ins}P_6$ in plant tissue in at least one situation (Molanari & Hoffman-Ostenhof, 1968), and although none of the intermediates were defined, this may be a pathway analogous to that described in *Dictyostelium*. However, in at least one well-studied system (the developing and germinating mung-bean seedling), inositol kinase is not expressed at the same time as is

Fig. 8. Changes in *myo*-inositol kinase activity during the development of *Dictyostelium*

An exponentially proliferating axenic strain of *Dictyostelium* was starved of nutrients, plated out on a solid support and allowed to complete its full developmental cycle (see the Materials and methods section). Samples of cells were harvested, lysed at intervals and assayed for glycogen phosphorylase (Δ), cyclic AMP phosphodiesterase (■), protein, *myo*-inositol kinase (▲; 100 μl of lysate was mixed with 100 μl of assay buffer containing 1.5 μCi of $^3\text{H}[\text{Ins}]$ and incubated for 20 min), $\text{Ins}3\text{P}$ phosphatase (○; 100 μl of lysate was mixed with 100 μl of assay buffer containing 18 000 d.p.m. of $^3\text{H}[\text{Ins}3\text{P}]$ and incubated for 20 min), total $\text{Ins}(1,4,5)\text{P}_3$ phosphatase (□; 50 μl of lysate was mixed with 150 μl of assay buffer containing 8600 d.p.m. of $^3\text{H}[\text{Ins}(1,4,5)\text{P}_3]$ and incubated for 30 min) and $\text{Ins}P_6$ kinase (●; 100 μl of a 1:400 dilution was mixed with 100 μl of assay buffer containing 14 200 d.p.m. of $^3\text{H}[\text{Ins}(1,2,4,5,6)\text{P}_6]$, i.e. 28 400 d.p.m. of D/L- $^3\text{H}[\text{Ins}(1,2,4,5,6)\text{P}_6]$, and incubated for 30 min). All assay buffers were prepared as described in the Materials and methods section, and the Ins kinase and $\text{Ins}3\text{P}$ phosphatase assays both contained 10 mM-LiCl. The assays were run at 25 °C and were under first-order conditions with respect to the ^3H -labelled substrates; they were then quenched with HClO_4 and processed for chromatography as described in the Materials and methods section. Data are presented as mean activities assayed at a particular time ($\pm$ S.E.M., $n = 6$) as a percentage of the activity detected in cells after development for 45 min (these are based on results from three independent batches of lysates) or as mean activities ($n = 2$, top panel) detected in a parallel set of lysates. The activities at 45 min were calculated from assays in which the following amounts of product had accumulated: Ins kinase, $46\,505 \pm 3059$ d.p.m.; $\text{Ins}3\text{P}$ phosphatase, 240 ± 10 d.p.m.; $\text{Ins}P_6$ kinase, 2020 ± 37 d.p.m.; $\text{Ins}(1,4,5)\text{P}_3$ total phosphatase, 369 ± 21 d.p.m.

the enzymic machinery which produces $\text{Ins}P_6$ (Biswas *et al.*, 1978).

A single claim to have detected inositol kinase activity in an animal cell has been published (but without any supporting data; Deitz & Albersheim, 1965). This preliminary description of a mammalian inositol kinase activity was based on the ability of a crude protein fraction from bovine liver to convert *myo*-inositol into a compound with a chromatographic mobility like that of $\text{Ins}P$. There are a number of practical difficulties involved in characterizing such a step in a typical mammalian cell. If the

levels of the putative Ins kinase are low, then other highly active enzymes will tend to mask its impact. For example, in homogenates [^3H]Ins will be rapidly incorporated into [^3H]PtdIns, [^3H]PtdInsP and [^3H]PtdInsP₂, and then phospholipase C, A and D activities will release some of the ^3H from these lipids as water-soluble [^3H]Ins-labelled metabolites with similar (in some cases identical) chromatographic properties to those of the putative product of an inositol kinase. Furthermore, inositol monophosphate phosphatase is a soluble and highly active enzyme in all mammalian tissues and will hydrolyse the product of a putative inositol kinase. In assays with *Dictyostelium* lysates this problem can be decreased by including LiCl in the incubation buffer (the inositol kinase activity in *Dictyostelium* is LiCl-insensitive; results not shown); however, this strategy is not water-tight, as the uncompetitive nature of the inhibition inflicted on inositol monophosphatase by LiCl means that the extent of inhibition obtained is relatively poor at the low InsP concentrations typically found in these assays. Until a mammalian inositol kinase has been characterized, the routine inclusion of LiCl in assay buffers designed to screen for an inositol kinase activity would require that the assumption that the mammalian enzyme is also LiCl-insensitive is correct.

The ability of the inositol-binding site of *myo*-inositol D-3 hydroxykinase to discriminate between *myo*-inositol and the remaining inositols that were tested, with the exception of 1L-*chiro*-inositol, suggests that all of the hydroxy groups in *myo*-inositol, apart from the D-3 moiety, are vital for the binding interaction. This can be understood by considering the difference in structure between those inositols which do not interact with the inositol-binding site of *myo*-inositol D-3 hydroxykinase, and *myo*-inositol and 1L-*chiro*-inositol. For example, neo-inositol is structurally equivalent to *myo*-inositol, except that its 5-hydroxy group is of the opposite orientation; hence, as neo-inositol does not interact with the inositol-binding site, the 5-hydroxy group of *myo*-inositol must make a vital contribution to the substrate recognition process. Using similar logic, the difference between *scyllo*-inositol (not bound) and *myo*-inositol is that the 2-hydroxy group of the former is equatorial; hence this group must be important. The failure of *myo*-inositol kinase to recognize (a) allo-inositol suggests that the 4- and 5-substitution sites of *myo*-inositol are involved (these are indistinguishable in this context from the 5- and 6-hydroxy groups); (b) muco-inositol implicates either the 3- and 4- hydroxy groups (or groups 1 and 6; see above) or the 1- and 3-hydroxy groups; (c) epi-inositol implicates the 4-hydroxy or the 6-hydroxy group; and (d) 1D-*chiro*-inositol suggests the involvement of the 1-hydroxy group. The fact that 1L-*chiro*-inositol does interact with *myo*-inositol D-3 hydroxykinase implies that the D-3 hydroxy group of *myo*-inositol is not directly involved in binding. This last observation is supported by the fact that (–)-viburnitol (3-deoxy-*myo*-inositol) is also an effective competitive inhibitor (although, as would be anticipated, it is not a substrate of *myo*-inositol D-3 hydroxykinase), whereas 4-deoxy-*myo*-inositol is not. Interestingly though, replacement of the D-3 hydroxy with fluorine (fluorine is slightly smaller than a hydroxy group and is commonly used as a space-filling substitute), which yields 3-deoxy-3-fluoro-*myo*-inositol, results in a large decrease in binding affinity. Both 1L-*chiro*-inositol and (–)-viburnitol are naturally occurring compounds, and although the former is most commonly found in higher plants in the form of its 1L-2-*O*-methyl and 1L-3-*O*-methyl derivatives [L-(–)-quebrachitol and L-(–)-pinitol respectively]. Members of the family Compositae characteristically contain substantial amounts in its unsubstituted form; (–)-viburnitol has been found in five families of dicotyledons (Anderson, 1972). These distributions are reminiscent of a number of secondary metabolites that accumulate in small

related clusters of plants that are considered to be insecticidal. If (–)-viburnitol and 1L-*chiro*-inositol are also cytotoxic, their ability to inhibit *myo*-inositol D-3 hydroxykinase may have a part to play in this.

Dictyostelium amoebae do not contain a detectable Ins(1,4,5)P₃ 3-hydroxykinase activity (van Haastert *et al.*, 1989; and see above) and therefore lack a major mechanism by which mammalian cells produce Ins3P. However, they do contain both an inositol 3-phosphate synthase activity and PtdIns3P (L. R. Stephens, unpublished work). The contribution that Ins3P synthase makes to the turnover of cellular Ins3P has never been established because of the difficulty in constructing assays capable of quantifying the flux of material through glucose 6-phosphate into Ins3P in homogenates or crude cytosolic fractions. Similarly, the importance of PtdIns3P turnover, or that of any of its metabolites, to the synthesis of Ins3P has not been established. Hence *myo*-inositol D-3 hydroxykinase currently represents the only well-defined input to the pool of Ins3P in *Dictyostelium* and therefore to the synthesis of InsP₆.

The fall in inositol kinase activity that occurs during development in *Dictyostelium* is over a time scale such that it could reflect changes in either its specific activity or its concentration. The fact that the levels of its activity fall only transiently could be interpreted to mean that the enzyme has been covalently modified and that this results in a change in activity which persists in the lysate. Varying the free Ca²⁺ concentration (0.1–10 μM) in crude cytosol fractions did not alter the inositol kinase activity detected in a standard assay, suggesting that a Ca²⁺-regulated phosphorylation or dephosphorylation is not responsible for the loss of activity. The decrease in levels of inositol kinase correspond in time to the post-aggregatory processes of mound and tipped mound formation, stages in the development of *Dictyostelium* in which the intracellular and intercellular signals controlling cellular metabolism are still relatively poorly understood.

The inositol isomers and analogues used in this work were generously provided by Professor S. J. Angyal, Professor L. Anderson and Professor A. P. Kozikowski, and Dr. N. Baggett, Dr. J. Piggott and Dr. M. Voyle. L. R. S. is a Babraham Research Fellow.

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Received 30 April 1990/18 July 1990; accepted 9 August 1990