Inositol polyphosphate metabolism and inositol lipids in a green alga, *Chlamydomonas eugametos*

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Swimming suspensions of *Chlamydomonas eugametos* were pelleted and homogenized, and the metabolism of inositol polyphosphates by cellular homogenates or supernatants was investigated. $\text{Ins}(1,4,5)\text{P}_3$ was dephosphorylated under physiological conditions to yield a single InsP_2 , $\text{Ins}(1,4)\text{P}_2$. In the presence of ATP it was phosphorylated to give $\text{Ins}(1,3,4,5)\text{P}_3$ as the only InsP_4 . The $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase activity was predominantly soluble, was not detectably affected by calmodulin or Ca^{2+} , and had a K_m for $\text{Ins}(1,4,5)\text{P}_3$ of $50\ \mu\text{M}$ (two orders of magnitude higher than its mammalian counterpart). $\text{Ins}(1,3,4,5)\text{P}_4$ was dephosphorylated by the cellular supernatants to $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$, and could be phosphorylated to $\text{Ins}(1,3,4,5,6)\text{P}_5$. No $\text{Ins}(1,3,4)\text{P}_3$ 6-kinase activity could be detected, and experiments with $[^3\text{H}]\text{Ins}(1,4,[^{32}\text{P}]\text{P})_3$ revealed that $\text{Ins}(1,3,4,5,6)\text{P}_5$ is formed from $\text{Ins}(1,4,5)\text{P}_3$ with little loss of the 5-phosphate, i.e. the predominant route of synthesis is probably by a direct 6-phosphorylation of $\text{Ins}(1,3,4,5)\text{P}_4$. Similar experiments with an $(\text{NH}_4)_2\text{SO}_4$ fraction of turkey erythrocyte cytosol gave essentially the same result, i.e. direct phosphorylation of $\text{Ins}(1,3,4,5)\text{P}_4$ in the 6 position is the predominant route of synthesis of InsP_5 from that InsP_4 *in vitro*. No InsP_6 formation was detected in any of these experiments, but labelling of intact *C. eugametos* with $[^3\text{H}]\text{inositol}$ revealed that the cells do synthesize InsP_6 . The lipids of *C. eugametos* cells contain PtdIns , $\text{PtdIns}(4)\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ [Irvine, Letcher, Lander, Drabak, Dawson & Musgrave (1989) *Plant Physiol.* **64**, 888–892]. Further examination of ^{32}P -labelled lipids revealed that about 20% of the PtdInsP was the $\text{PtdIns}(3)\text{P}$ isomer, and about 1% or less of the PtdInsP_2 was the $\text{PtdIns}(3,4)\text{P}_2$ isomer. The overall inositide metabolism of *C. eugametos* resembles that of a mammalian cell more closely than it does that of a plant cell or slime mould, and this suggests firstly that the known metabolism of inositol polyphosphates arose at an early time in eukaryotic evolution, and secondly that *Chlamydomonas* might prove a useful organism for genetic and comparative studies of inositide enzymology.

INTRODUCTION

As our knowledge of the complexity of inositide cellular signalling in vertebrate cells increases, and as this goes hand in hand with a proliferation of inositol phosphate isomers, many of which are of as yet unknown significance and function (for reviews see Irvine *et al.*, 1988; Shears, 1989; Berridge & Irvine, 1989), it becomes increasingly of interest and importance to examine organisms which are taxonomically (hence evolutionarily) remote from those more usually studied. This is for two reasons; firstly, elucidating indirectly the approximate time in evolution at which particular processes appeared can help to clarify interrelationships between metabolism and function. Secondly, simpler organisms can be more amenable to genetic analysis and manipulation. In the context of this paper, it is also relevant that there is increasing evidence that polyphosphoinositol lipids exist in higher plants (see, e.g., Irvine *et al.*, 1989; Coté *et al.*, 1989), and that $\text{Ins}(1,4,5)\text{P}_3$ may serve as a second messenger in them (e.g. Alexandre *et al.*, 1990; Blatt *et al.*, 1990; Gilroy *et al.*, 1990), so examination of inositide metabolism in lower plants is a useful end in itself.

We have previously found that swimming suspensions of the biflagellate green alga *Chlamydomonas eugametos* incorporate $[^{32}\text{P}]\text{P}_i$ and *myo*- $[^3\text{H}]\text{inositol}$ readily into PtdIns , $\text{PtdIns}(4)\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ in proportions which are similar to those seen in equivalent labelling experiments using animal cells, and considerably greater than in most higher-plant tissues (Irvine *et al.*, 1989). Over the years, *Chlamydomonas* species have been well studied and many aspects of their cell biology, biochemistry and genetics are understood (Harris, 1989). Furthermore, large changes in intracellular Ca^{2+} levels have been implicated in the control of flagellar movement (Hyams & Borisy, 1978; Kamiya

& Witman, 1984) and the induction of sexual responses during mating (Claes, 1980; Bloodgood & Levin, 1983; Schuring *et al.*, 1990). It is therefore attractive to consider the possibility that an inositol phosphate/ Ca^{2+} signalling system exists in this unicellular plant. This was supported by the results from some preliminary experiments (R. F. Irvine & A. J. Letcher, unpublished work) showing that cell extracts were able to phosphorylate $\text{Ins}(1,4,5)\text{P}_3$, a reaction which may have important implications for intracellular Ca^{2+} homeostasis (Irvine *et al.*, 1988; Berridge & Irvine, 1989; Irvine, 1990). For these various reasons we considered worthwhile an investigation of inositol phosphate and inositol lipid metabolism in this organism.

MATERIALS AND METHODS

C. eugametos strain 17.17.2 (Schuring *et al.*, 1987) was grown in Petri dishes on agar-containing medium (Wiese, 1965). Cultures were maintained at 20°C in a 12 h light/12 h dark regime. An average light intensity of 2500 lx was provided by Philips TL 65W/33 fluorescent tubes. Cell suspensions were obtained by flooding 2–4-week-old cultures with distilled water overnight. The free-swimming cells were sexually competent and can be considered to be gametes.

Preparation of homogenates

Suspensions of swimming cells were combined, centrifuged briefly (1000 g, 10 min) and the concentrated cells resuspended in approx. 4 vol. of supernatant, and homogenized either by (a) Whirlimixing them with glass beads or (b) sonication. The resulting homogenate was centrifuged at 100000 g for 40 min and the supernatant divided into batches and stored frozen at -15°C . The various enzymes were stable for several months, but did not keep indefinitely.

Materials

[^3H]Ins(1,4,5) P_3 and [^3H]Ins(1,3,4,5) P_4 were from Amersham International. Ins(1,4,[^{32}P]5) P_3 was prepared as in Wreggett *et al.* (1990). Calmodulin was from Sigma. Ins(1,2,4) P_3 was prepared by acid-catalysed migration of Ins(1,3,4) P_3 (NEN) (Pizer & Ballou, 1959; Stephens *et al.*, 1991), i.e. 80 °C in 1 M-HCl for 20 min to yield an Ins P_3 which was chromatographically distinct from the starting material and yet gave altritol on periodate oxidation, reduction and dephosphorylation (Batty *et al.*, 1985). A mixture of [^3H]Ins(1,4) P_2 and [^3H]Ins(1,3) P_2 was prepared by alkaline hydrolysis of [^3H]Ins(1,3,4) P_3 (NEN) (18 h at 100 °C, conc. NH_3); only trace amounts of Ins(3,4) P_2 (Irvine *et al.*, 1987) are produced by this method (Wreggett & Irvine, 1989).

Enzyme assays

Most conditions are given in the Results section, but pH values were all adjusted with Tris/maleate buffers (50 mM final concn.). Separation of inositol phosphates was by Dowex chromatography as described in Batty *et al.* (1985) or Spencer *et al.* (1990). Quenching and processing of samples, and separation of Ins P_2 and Ins P_3 isomers by h.p.l.c., were as described in Wreggett & Irvine (1989). Most of the assays (to identify products) were performed under limiting substrate conditions (i.e. apparent first order), but for kinetic studies (e.g. Fig. 4) less than 5% of the substrate was consumed in the assays.

H.p.l.c. separation of Ins P_3 isomers

This was carried out on Partisphere SAX columns, by using an internal [^{32}P]Ins(1,3,4,5,6) P_5 standard, exactly as described by Stephens & Irvine (1990) and Stephens (1990).

Preparation of turkey erythrocyte supernatant, and incubation with Ins(1,4,5) P_3

Turkey erythrocytes were prepared and lysed essentially as in Stephens *et al.* (1990), and the cytosolic supernatant was precipitated at 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$, followed by dialysis in 50 mM-Hepes/2 mM-EGTA with phenylmethanesulphonyl fluoride, antipain, leupeptin, pepstatin and mercaptoethanol. From this preparation, 316 μg of protein was incubated with a mixture of [^{32}P]Ins(1,4,5) P_3 and [^3H]Ins(1,4,5) P_3 at 37 °C in a final volume of 200 μl containing 5 mM-ATP, 10 mM-phosphocreatine, 1 unit of creatine kinase/ml, 0.1 M-KCl, 50 mM-Hepes (pH 7.0, 37 °C), 2 mM-EGTA, with Ca^{2+} and Mg^{2+} to give free concentrations of 100 nM- Ca^{2+} and 1 mM- Mg^{2+} .

RESULTS AND DISCUSSION

Polyphosphoinositol lipids in *C. eugametos*

We have previously identified PtdIns(4,5) P_2 in *C. eugametos* (a) by co-chromatography of the *myo*-[^3H]inositol-labelled lipid, deacylated, with [^{32}P]GroPIns(4,5) P_2 and (b) susceptibility of the head-group of the ^{32}P -labelled lipid (i.e. deacylated and deglycerated) to the specific Ins(1,4,5) P_3 5-phosphatase in human erythrocyte ghosts (Irvine *et al.*, 1989). We did not examine PtdIns P , however, to see whether any PtdIns(3) P (Whitman & Cantley, 1988) is present, nor did we specifically seek possible trace amounts of PtdIns(3,4) P_2 in the PtdIns(4,5) P_2 . In the present experiments we obtained poor incorporation of *myo*-[^3H]inositol into PtdIns P , and, although after removal of fatty acids and glycerol and subsequent separation of Ins P_2 isomers by isocratic h.p.l.c. (Wreggett & Irvine, 1989) we were able to detect what we believe to be Ins(1,4) P_2 and Ins(1,3) P_2 , the low incorporation made quantification and identification unreliable.

We therefore labelled *C. eugametos* for 2 or 18 h with [^{32}P] P_i and after extraction and purification of PtdIns P by t.l.c. (Irvine,

1986), it was deacylated, deglycerated and analysed by isocratic h.p.l.c. with internal ^3H -labelled Ins P_2 standards. Note that another lipid chromatographed just behind PtdIns P on t.l.c., but incorporated [^{32}P] P_i radiolabel more slowly; the product of this after deacylation and deglyceration chromatographed on h.p.l.c. just before Ins(1,3) P_2 (which is the earliest-eluted Ins P_2 in this h.p.l.c. system), and its presence illustrates the complications caused by unknown polar lipids in plant extracts (Drøbak *et al.*, 1988). A typical result from 2 h-labelled tissues is shown in Fig. 1. It is apparent that both Ins(1,3) P_2 and Ins(1,4) P_2 are probably present (confirming the [^3H]inositol data mentioned above), with approx. 20% of the PtdIns P radioactivity at both 2 h and 18 h labelling (see legend of Fig. 1) being in PtdIns(3) P . If we take this as an approximation of the mass levels, then the level of PtdIns(3) P is relatively higher than in mammalian cells (Whitman & Cantley, 1988), but lower than in yeast (Auger *et al.*, 1989), where it is approx. 50% of the total PtdIns P . We also examined the PtdIns P_2 in the ^{32}P -labelled lipids by identical techniques, and found that < 1% of the total PtdIns P_2 is the 3,4 as opposed to the 4,5 isomer (Fig. 1b), confirming our initial observation that *C. eugametos* contains mostly PtdIns(4,5) P_2 (Irvine *et al.*, 1989).

Fig. 1. Inositol phosphate head-groups derived from *C. eugametos* phospholipids

C. eugametos cells ((+) strain) were incubated for 2 h with ^{32}P as described in the Materials and methods section. (a) PtdIns P was purified by t.l.c. (Irvine, 1986) and then deacylated and the glycerol moiety removed as in Batty *et al.* (1985). The products were then analysed by h.p.l.c. (Wreggett & Irvine, 1989), with internal markers of [^3H]Ins(1,3) P_2 and [^3H]Ins(1,4) P_2 . This result was similar in three independent analyses, in two analyses of cells labelled for 18 h (though overall incorporation was higher), and in two analyses of a (–) strain at 2 h labelling. (b) Ins P_3 s derived from PtdIns P_2 of the same cells; the internal markers are [^3H]Ins(1,3,4) P_3 and [^3H]Ins(1,4,5) P_3 . Labelling the cells for 18 h produced a very similar pattern with higher incorporation of ^{32}P , and (–)-strain cells give also a similar pattern.

Fig. 2. Products of dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$

After incubation of $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ with a cellular supernatant of *C. eugametos* (see the text), the resulting inositol phosphates were analysed by h.p.l.c. (exactly as in Fig. 1). The elution positions of other InsP_2 s in parallel runs are indicated. Identical results were obtained in a parallel experiment. The internal marker (○) is $[\text{^{14}C}]\text{Ins}(1,4)\text{P}_2$.

Fig. 3. Separation of $\text{Ins}(1,2,4)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ on Partisil WAX

(a) $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ was treated with acid to cause phosphate-group migration as described in the text, and then analysed by h.p.l.c. on Partisil WAX as described in the Materials and methods section. In a similar experiment, the three ^3H -labelled peaks were collected, and submitted to periodate oxidation, reduction and dephosphorylation, and yielded respectively, in order of elution, altritol, altritol and arabitol. Thus the peaks can be identified, in order of elution, as $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,2,4)\text{P}_3$ and $\text{Ins}(2,3,4)\text{P}_3$. The internal marker (○) is $\text{Ins}(1,4,5)[\text{^{32}P}]\text{P}_3$. Also shown (b) is the elution of the InsP_3 derived from *C. eugametos* InsP_4 [in turn produced *in vitro* by phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$], by treatment of that InsP_4 with human erythrocyte ghosts in the presence of Mg^{2+} (see the Materials and methods section). Two sequential h.p.l.c. runs are depicted. For the first; ▲, InsP_3 from *C. eugametos* InsP_4 ; △, $\text{Ins}(1,4,5)[\text{^{32}P}]\text{P}_3$ standard. For the second; ●, $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ standard; ○, $\text{Ins}(1,4,5)[\text{^{32}P}]\text{P}_3$ standard.

Fig. 4. (a) pH optimum and (b) Lineweaver-Burk plot for $\text{Ins}(1,4,5)\text{P}_3$ as a substrate for *C. eugametos* $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase

Similar results were obtained for (a) in one identical experiment and for (b) five identical experiments.

Dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$

If $\text{Ins}(1,4,5)\text{P}_3$ was incubated with *C. eugametos* supernatants at 25 °C, with 2 mM- Mg^{2+} , pH 7.5, it was dephosphorylated to InsP_2 and InsP . The principal InsP_2 detected was $\text{Ins}(1,4)\text{P}_2$ (Fig. 2), based on co-chromatography with a ^{14}C -labelled $\text{Ins}(1,4)\text{P}_2$ standard (Fig. 2), and its complete destruction by periodate, under conditions where 80% recovery of inositol from $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$ internal carrier was achieved (see Irvine *et al.*, 1989). It is particularly noteworthy that only a trace of (probable) $\text{Ins}(4,5)\text{P}_2$ was detected; this is the predominant product of $\text{Ins}(1,4,5)\text{P}_3$ hydrolysis in a higher-plant tissue (*Nicotiana tabacum*; Joseph *et al.*, 1989) and also in the slime mould *Dictyostelium*, where $\text{Ins}(4,5)\text{P}_2$ is produced in equal amounts to $\text{Ins}(1,4)\text{P}_2$ (Van Lookeren Campagne *et al.*, 1988). $\text{Ins}(4,5)\text{P}_2$ has also been detected as a product of $\text{Ins}(1,4,5)\text{P}_3$ metabolism by what was probably an extracellular phosphatase found on rat enterocytes (Rubiera *et al.*, 1990). We cannot rule out the possibility that more $\text{Ins}(4,5)\text{P}_2$ was produced and further hydrolysed, but inclusion of 2 μM - $\text{Ins}(4,5)\text{P}_2$ as a 'trap' did not reveal any higher production of radiolabelled $\text{Ins}(4,5)\text{P}_2$. $\text{Ins}(1,5)\text{P}_2$ was eluted just after $\text{Ins}(1,4)\text{P}_2$ on Partisil SAX (result not shown), and would have yielded xylitol after periodate degradation (Irvine *et al.*, 1989). Thus $\text{Ins}(1,4,5)\text{P}_3$ is, at least under these conditions *in vitro*, dephosphorylated by a specific 5-phosphatase that is presumably related to the equivalent animal enzyme(s) (see Shears, 1989), though we have not explored its properties further.

Phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$

Incubation of $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ with *C. eugametos* supernatant in the presence of 5 mM-ATP and 10 mM- Mg^{2+} resulted in the formation of InsP_4 . This was found to be $\text{Ins}(1,3,4,5)\text{P}_4$ by the methods described in Batty *et al.* (1985), the key step being

Table 1. Phosphorylation of [^{32}P / ^3H]Ins(1,4,5) P_3 *in vitro* by (a) *C. eugametos* supernatants and (b) turkey erythrocyte supernatant

(a) A mixture of [^{32}P]Ins(1,4,5) P_3 (Wreggett *et al.*, 1990) and [^3H]Ins(1,4,5) P_3 (Amersham) was incubated at 25 °C for 10 or 30 min at pH 8.0, with 13 mM-Mg $^{2+}$, 6.5 mM-ATP and 20 μl of *C. eugametos* supernatant in a final volume of 150 μl . Ins P_3 , Ins P_4 and Ins P_5 fractions were separated as in Spencer *et al.* (1990). Results are means $\pm$ S.E.M. of triplicate incubations, and are typical of a series of three experiments. (b) A similar mixture of radiolabelled Ins(1,4,5) P_3 s was incubated at 37 °C and pH 7.0 with a 0–40%-(w/v)-(NH $_4$) $_2$ SO $_4$ fraction from turkey erythrocytes as described in the Materials and methods section. Results are expressed as means $\pm$ S.E.M. of triplicate incubations.

(a) *C. eugametos* supernatants

	Starting Ins(1,4,5) P_3	Ins P_4 (10 min)	Ins P_4 (30 min)	Ins P_5 (10 min)	Ins P_5 (30 min)
^3H (d.p.m.)	11049 $\pm$ 116	8844 $\pm$ 80	7656 $\pm$ 323	742 $\pm$ 85	2176 $\pm$ 238
^{32}P (d.p.m.)	9734 $\pm$ 134	8015 $\pm$ 119	6845 $\pm$ 286	645 $\pm$ 79	1954 $\pm$ 184
$^3\text{H}/^{32}\text{P}$	1.135 $\pm$ 0.004	1.102 $\pm$ 0.012	1.118 $\pm$ 0.010	1.151 $\pm$ 0.014	1.113 $\pm$ 0.018

(b) Turkey erythrocyte supernatant

		^3H (d.p.m.)	^{32}P (d.p.m.)	$^3\text{H}/^{32}\text{P}$
	Starting Ins(1,4,5) P_3	29869 $\pm$ 146	4728 $\pm$ 24	6.317
Ins P_4 :	1 min	27342 $\pm$ 88	4449 $\pm$ 15	6.146
	5 min	27161 $\pm$ 490	4389 $\pm$ 15	6.188
	15 min	21966 $\pm$ 600	3547 $\pm$ 76	6.193
	30 min	17993 $\pm$ 227	2873 $\pm$ 29	6.263
Ins P_5 :	1 min	109 $\pm$ 3	23 $\pm$ 3	4.739
	5 min	1788 $\pm$ 14	276 $\pm$ 5	6.478
	15 min	6496 $\pm$ 22	895 $\pm$ 4	7.258
	30 min	11118 $\pm$ 185	1534 $\pm$ 28	7.25

partial dephosphorylation with alkaline phosphatase to yield an Ins P_3 which on periodate oxidation, reduction and dephosphorylation gave xylitol [and is therefore Ins(3,4,5) P_3]. Because of the partial dephosphorylation step, this method requires large amounts of Ins(1,3,4,5) P_4 (which in this context we generated easily), but we used this structural determination to test an alternative method which requires much lower levels of radioactivity.

The Ins P_4 was treated with human erythrocyte ghosts in the presence of 5 mM-Mg $^{2+}$ to yield, with > 85% recovery, an Ins P_3 . This Ins P_3 on periodate oxidation, reduction and dephosphorylation yields altritol (Batty *et al.*, 1985), which can be shown to be L- or D-altritol if required (Stephens *et al.*, 1989; Stephens, 1990), though we did not do this here, as the starting inositol phosphate was of known configuration. The Ins P_3 obtained must therefore be Ins(1,2,4) P_3 or Ins(1,3,4) P_3 , and, as the parent Ins P_4 yielded inositol after periodate degradation and was treated with the specific 5-phosphatase to give the Ins P_3 , it must be Ins(1,2,4,5) P_4 or Ins(1,3,4,5) P_4 . Fig. 3(a) shows the Ins(1,2,4) P_3 and Ins(1,3,4) P_3 do not co-chromatograph on Partisil WAX columns [note that they do on Partisil SAX with the elution protocol of Wreggett & Irvine (1989); L. R. Stephens, R. F. Irvine & A. J. Letcher, unpublished work], and so the fact that the Ins P_3 obtained by incubation with human erythrocyte ghosts from the Ins P_4 in question co-chromatographs with Ins(1,3,4) P_3 under these conditions (Fig. 3b) removes the remaining ambiguity. Thus the minimal evidence for an Ins(1,3,4,5) P_4 structure requires only that it is degraded with periodate to inositol, and that treatment with human red-cell membranes (plus Mg $^{2+}$) yields an Ins P_3 which is degraded to altritol and co-chromatographs with Ins(1,3,4) P_3 , and not Ins(1,2,4) P_3 , on Partisil WAX. Note that elimination of Ins(1,3,4,6) P_4 , on this logic, is based on it not being a substrate for the human red-cell membrane enzyme, but for certainty this can be eliminated directly by h.p.l.c. (Stephens *et al.*, 1989).

The Ins(1,4,5) P_3 3-kinase was partly characterized. We found no effect of Ca $^{2+}$ in the range 1 nM–0.1 mM, with or without added calmodulin, although we should note that neither we (Irvine *et al.*, 1986) nor Morris *et al.* (1988) found a Ca $^{2+}$ stimulation of the rat brain enzyme, even though that same enzyme clearly can be stimulated under some conditions by Ca $^{2+}$ -calmodulin (Johanson *et al.*, 1988; Takezawa *et al.*, 1988), so we cannot state unequivocally that the enzyme from *C. eugametos* is not controlled by Ca $^{2+}$. The pH optimum of the enzyme was 8 (Fig. 4a); in having activity below pH 6.5 and its sharp fall-off above pH 8 it differs from the rat brain enzyme (Irvine *et al.*, 1986; Shears, 1989).

The most interesting property of the *C. eugametos* enzyme was its low affinity for Ins(1,4,5) P_3 . The K_m for this substrate is about 55 μM (Fig. 4b), which is at least two orders of magnitude higher than for the equivalent mammalian enzymes (Shears, 1989). This has intriguing implications for the evolution and function of the enzyme. The most likely function of Ins(1,3,4,5) P_4 may be to control Ca $^{2+}$ entry into cells (Irvine *et al.*, 1988; Irvine, 1990), and there is evidence (see the Introduction) that *Chlamydomonas* does regulate Ca $^{2+}$ entry. It is not possible (or advisable) to compare directly the total activity of this enzyme in *Chlamydomonas* with that in a mammalian cell, but we think that it is perhaps relevant to note that, when a supernatant was assayed at a low Ins(1,4,5) P_3 concentration (50 nM), at neutral pH and with ATP present, Ins(1,4,5) P_3 was predominantly phosphorylated rather than dephosphorylated; this is in contrast with similar experiments with rat brain (Irvine *et al.*, 1986). Also, the formation of Ins(1,3,4) P_3 indirectly from Ins(1,4,5) P_3 in these experiments was slow compared with the formation of Ins(1,3,4,5) P_4 , whereas with rat brain Ins(1,3,4) P_3 soon becomes the predominant product (Irvine *et al.*, 1986). This suggests (though no more than that) that *Chlamydomonas* may have compensated for the low affinity of its kinase for Ins(1,4,5) P_3 by having a relatively high level of activity. Also it implies that the

evolution of the high affinity of Ins(1,4,5) P_3 kinase [compared with the Ins(1,4,5) P_3 5-phosphatase] occurred subsequent to the split between animals and plants. Examination of Ins(1,4,5) P_3 kinase in other organisms might throw further light on this question.

Metabolism of Ins(1,3,4,5) P_4

When Ins(1,3,4,5) P_4 was presented to supernatants or homogenates of *C. eugametos* with no ATP present, h.p.l.c. analysis revealed the formation of both Ins(1,3,4) P_3 and Ins(1,4,5) P_3 . At pH 7.5 and 2 mM-Mg²⁺, the latter predominated. Thus in addition to the expected 5-phosphatase activity [predicted from the presence of Ins(1,4,5) P_3 5-phosphatase; Batty *et al.*, 1985; Connolly *et al.*, 1987], there is an active 3-phosphatase; a similar activity has been described in several mammalian systems (for references see Shears, 1989), but it is not known whether Ins(1,3,4,5) P_4 is the major substrate. Ins(1,3,4,5,6) P_5 can act as a substrate for a 3-phosphatase in animal cells (Stephens & Downes, 1990), and there is a very potent inhibitor of Ins(1,3,4,5) P_4 3-phosphatase in the cytoplasm (Hodgson & Shears, 1990), which could be Ins P_5 .

When ATP was included in the incubation, we also detected formation of Ins(1,3,4,5,6) P_5 [identified as that isomer by chromatography in an h.p.l.c. system which separates Ins P_5 isomers (Stephens, 1990), and with an internal [³²P]Ins(1,3,4,5,6) P_5 standard (results not shown)]. By analogy with animal cells (Irvine *et al.*, 1988; Shears, 1989), one would expect the route of synthesis to involve a 6-phosphorylation of Ins(1,3,4) P_3 , but we were not able to detect any Ins(1,3,4) P_3 6-kinase activity in the supernatants under these conditions. Furthermore, as the data in Table 1(a) show, when Ins(1,4,5) P_3 was used labelled in the 5-phosphate and the inositol ring [as a mixture of Ins(1,4,[³²P]5) P_3 and [³H]Ins(1,4,5) P_3], the ³²P/³H ratios in Ins P_5 indicate that essentially no 5-dephosphorylation occurred during the conversion of Ins(1,4,5) P_3 into Ins(1,3,4,5,6) P_5 [presumably via Ins(1,3,4,5) P_4].

From the known inositol phosphate metabolism in animals (e.g. Shears, 1989), this is a rather unexpected observation, but an identical experiment using an (NH₄)₂SO₄ fraction derived from a turkey erythrocyte cytosol preparation gives a very similar result (Table 1b). Thus *in vitro* both *C. eugametos* and turkey erythrocytes convert Ins(1,3,4,5) P_4 into Ins(1,3,4,5,6) P_5 predominantly by a direct 6-phosphorylation. However, it should be noted that (a) isotope-incorporation data from intact avian erythrocytes do not indicate that this is the predominant route of Ins(1,3,4,5,6) P_5 synthesis in control or stimulated cells (Stephens & Downes, 1990; Stephens *et al.*, 1990) and (b) in avian erythrocytes Ins(1,3,4) P_3 6-kinase is at best 100-fold less active than Ins(1,4,5) P_3 3-kinase (Stephens *et al.*, 1988), so, if similar relative activities of these two enzymes were present in *C. eugametos*, the former activity would not have been detected in our assays. Thus at present we cannot be sure if and how intact algae cells synthesize Ins(1,3,4,5,6) P_5 from Ins(1,4,5) P_3 , but the overall similarity of *C. eugametos* and avian erythrocytes with respect to their inositol phosphate metabolism, including the data in Table 1, suggests that they may use routes of similar complexity *in vivo* (see, e.g., Stephens & Downes, 1990).

Ins P_6

In vitro, we could not detect any significant synthesis of Ins P_6 from [³H]inositol, Ins(1,4,5) P_3 or Ins(1,3,4,5) P_4 . We therefore instead labelled whole cells with *myo*-[³H]inositol, and analysed the acid-soluble compounds by h.p.l.c. on WAX columns. Incorporation of [³H]inositol was poor, even over 24 h, and there was no detectable radioactivity in Ins P_2 or Ins P_3 , a small incorporation into an Ins P_4 which did not co-chromatograph

with an internal [³²P]Ins(1,3,4,5) P_4 standard, no detectable incorporation into any Ins P_5 [i.e. in the region of an internal [³²P]Ins(1,3,4,5,6) P_5 standard], and a tritiated compound was detected with the chromatographic properties of Ins P_6 . Overall, the pattern resembled superficially (but with a much lower incorporation of label) that obtained from slime moulds labelled with *myo*-[³H]inositol (Stephens & Irvine, 1990), in that only the Ins P and (putative) Ins P_6 fractions incorporated significant levels of radioactivity; this suggests that, if Ins(1,4,5) P_3 or Ins(1,3,4,5) P_4 are used as second messengers by *C. eugametos*, they are present probably at much lower mass levels than Ins P_6 .

Conclusions

It is clear from the above discussions that, superficially at least, the inositide metabolism in swimming *C. eugametos* gametes resembles that in animal (including avian) systems more closely than it does that in higher plants, *Dictyostelium* or yeast. This may make this organism, with its well-defined genetics and physiological background (Harris, 1989), very useful for investigating the function and evolution of various aspects of inositide metabolism. The data also imply that the complex metabolism of inositol phosphates that we know in animal systems (Irvine *et al.*, 1988; Shears, 1989) may have evolved for the most part shortly after the appearance of eukaryotes, and one could therefore argue that, if a particular enzyme or branch of metabolism of inositol phosphates is missing in any particular animal or higher plant, it has been deleted (as non-functional) rather than never having evolved in the first place. Such considerations may help in the future to throw light on some aspects of the functions and metabolism of inositol phosphates.

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