

Partial purification and some properties of rat brain inositol 1,4,5-trisphosphate 3-kinase

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An enzyme which catalyses the ATP-dependent phosphorylation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] was purified approx. 180-fold from rat brain cytosol by (NH₄)₂SO₄ precipitation, chromatography through hydroxyapatite, anion-exchange fast protein liquid chromatography and gel-filtration chromatography. Gel filtration on Sepharose 4B CL gives an *M_r* of 200 × 10³ for the native enzyme. The inositol tetrakisphosphate (InsP₄) produced by the enzyme has the chromatographic, chemical and metabolic properties of Ins(1,3,4,5)P₄. Ins(1,4,5)P₃ 3-kinase displays simple Michaelis–Menten kinetics for both its substrates, having *K_m* values of 460 μM and 0.44 μM for ATP and Ins(1,4,5)P₃ respectively. When many of the inositol phosphates known to occur in cells were tested, only Ins(1,4,5)P₃ was a substrate for the enzyme; the 2,4,5-trisphosphate was not phosphorylated. Inositol 4,5-bisphosphate and glycerophosphoinositol 4,5-bisphosphate were phosphorylated much more slowly than Ins(1,4,5)P₃. CTP, GTP and adenosine 5′-[γ-thio]triphosphate were unable to substitute for ATP. When assayed under conditions of first-order kinetics, Ins(1,4,5)P₃ kinase activity decreased by about 40% as the [Ca²⁺] was increased over the physiologically relevant range. This effect was insensitive to the presence of calmodulin and appeared to be the result of an increase in the *K_m* of the enzyme for Ins(1,4,5)P₃. Preincubation with ATP and the purified catalytic subunit of cyclic AMP-dependent protein kinase did not affect the rate of phosphorylation of Ins(1,4,5)P₃ when the enzyme was assayed at saturating concentrations of Ins(1,4,5)P₃ or at concentrations close to its *K_m* for this substrate.

INTRODUCTION

Receptor-stimulated phosphodiesteratic cleavage of the plasma-membrane phospholipid phosphatidylinositol 4,5-bisphosphate produces two molecules with second-messenger functions: Ins(1,4,5)P₃, which releases Ca²⁺ ions from an intracellular store, and *sn*-1,2-diacylglycerol, which remains in the membrane and activates the Ca²⁺- and phospholipid-dependent protein kinase C (Berridge & Irvine, 1984; Nishizuka, 1984).

Ins(1,4,5)P₃ can be metabolized in two ways: 5-specific dephosphorylation to yield Ins(1,4)P₂ (Downes *et al.*, 1982; Storey *et al.*, 1984), or ATP-dependent phosphorylation, producing Ins(1,3,4,5)P₄ (Irvine *et al.*, 1986). Ins(1,3,4,5)P₄ may be subsequently dephosphorylated to Ins(1,3,4)P₃ (Batty *et al.*, 1985). The rapidity with which Ins(1,4,5)P₃ is converted into Ins(1,3,4,5)P₄ in stimulated cells and with which the concentration of Ins(1,3,4,5)P₄ declines when stimulation ceases has made it attractive to speculate that Ins(1,3,4,5)P₄ itself performs second-messenger functions (Batty *et al.*, 1986; Hawkins *et al.*, 1985). Indeed, Irvine & Moor (1986) have published some evidence to suggest that Ins(1,3,4,5)P₄ is involved in the mechanisms by which sea-urchin eggs raise an extracellular membrane after fertilization.

In their initial description of Ins(1,4,5)P₃ 3-kinase in a variety of tissues, Irvine *et al.* (1986) selected the soluble activity of rat brain for further investigation. We have produced a substantially purified preparation of this enzyme, which has allowed us to confirm and extend their initial observations and also to investigate the substrate specificity and potential mechanisms by which this important component of the pathways of inositol phosphate metabolism in cells is controlled.

MATERIALS AND METHODS

Assay of Ins(1,4,5)P₃ kinase

Samples to be assayed for Ins(1,4,5)P₃ kinase activity were incubated at 30 °C in a buffer containing 70 mM-potassium glutamate, 10 mM-Hepes, pH 7.2, 11 mM-MgSO₄, 10 mM-ATP, 30 mM-NaCl, [³H]Ins(1,4,5)P₃ (Amersham International) (10 μM; sp. radioactivity 5 d.p.m./pmol) and bovine serum albumin (10 mg/ml). Incubations were terminated by the addition of an equal volume of ice-cold HClO₄ (10%, v/v), centrifuged (2000 g), and the acid was removed from the supernatant by the method of Sharpes & McCarl (1982). EDTA was added to a final concentration of 20 mM, and the InsP₄

Abbreviations used: Ins1P, *D*-myo-inositol 1-phosphate; Ins3P, *D*-myo-inositol 3-phosphate; Ins(1,4)P₂, *D*-myo-inositol 1,4-bisphosphate; Ins(4,5)P₂, *D*-myo-inositol 4,5-bisphosphate; Ins(1,3,4)P₃, *D*-myo-inositol 1,3,4-trisphosphate; Ins(1,4,5)P₃, *D*-myo-inositol 1,4,5-trisphosphate; Ins(2,4,5)P₃, *D*-myo-inositol 2,4,5-trisphosphate; Ins(1,3,4,5)P₄, *D*-myo-inositol 1,3,4,5-tetrakisphosphate; Ins(3,4,5,6)P₄, *D*-myo-inositol 3,4,5,6-tetrakisphosphate; Ins(1,3,4,5,6)P₅, *myo*-inositol 1,3,4,5,6-pentakisphosphate; GroPIns(4,5)P₂, glycerophosphoinositol 4,5-bisphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate; f.p.l.c., fast protein liquid chromatography.

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produced was separated from $\text{Ins}(1,4,5)P_3$ by anion-exchange chromatography on Bio-Rad AG1 (200–400 mesh; formate form) with ammonium formate/formic acid eluents as described in Downes *et al.* (1986).

Preparation of calmodulin and the purified catalytic subunit of cyclic AMP-dependent protein kinase

Calmodulin was purified from rat testis by the method of Dedman & Kaetzel (1983). The catalytic subunit of cyclic AMP-dependent protein kinase was purified from bovine heart by the method of Reimann & Beham (1983).

Purification of $\text{Ins}(1,4,5)P_3$ kinase

The brains of 20 male Wistar rats (180–220 g) were removed, cleared of blood clots and extraneous membranes, minced and homogenized on ice in 250 mM-sucrose/50 mM-Tris (pH 7.2)/1 mM-EGTA/1 mM- NaN_3 /12 mM- β -mercaptoethanol / leupeptin (1 $\mu\text{g}/\text{ml}$) / pepstatin (1 $\mu\text{g}/\text{ml}$) / antipain (1 $\mu\text{g}/\text{ml}$) / 50 μM -phenylmethanesulphonyl fluoride (3 ml/g wet wt.). The homogenate was filtered through a 100 μM mesh and centrifuged at 300 000 g in a Beckman L8-M preparative ultracentrifuge for 1 h. The supernatant thus obtained was subjected to $(\text{NH}_4)_2\text{SO}_4$ precipitation: proteins precipitated at concentrations between 114 and 243 g/l at 4 °C were resuspended in 5 ml of 50 mM- KH_2PO_4 /1 mM-EGTA, pH 7.2, and dialysed against a 1000-fold excess of this buffer for 2 h. The dialysed protein solution was diluted to 100 ml with dialysis buffer and applied to a column of hydroxyapatite (Bio-Gel HTP; 4.4 cm \times 8 cm), after the column was washed with 600 ml of this buffer; bound protein was then eluted with 500 mM- KH_2PO_4 /1 mM-EGTA at a flow rate of 2 ml/min. All the $\text{Ins}(1,4,5)P_3$ kinase activity was found in the high-salt eluate.

As the $\text{Ins}(1,4,5)P_3$ kinase activity was consistently eluted in the main protein peak, fractions were pooled according to their A_{280} . The peak of protein thus obtained was dialysed against 50 mM-Tris (pH 7.2)/1 mM-EGTA/1 mM- NaN_3 . The dialysed protein solution was loaded on to a Pharmacia Mono Q HR/16/10 semi-preparative anion-exchange f.p.l.c. column. The column was washed with 100 ml of loading buffer, and bound protein was eluted with a linear gradient of 0–0.7 M-NaCl in loading buffer at a flow rate of 2 ml/min. The eluate was assayed for $\text{Ins}(1,4,5)P_3$ kinase activity, and the peak fractions thus obtained were pooled and concentrated by dialysis against solid sucrose overnight.

The concentrated protein solution obtained was applied to a column of Sepharose 4B CL (3.2 cm \times 88 cm; Pharmacia), which had been equilibrated with 500 mM-NaCl/50 mM-Tris (pH 7.2)/1 mM-EGTA/1 mM- NaN_3 , and eluted with this buffer at a flow rate of 1.0 ml/min. The eluate was assayed for $\text{Ins}(1,4,5)P_3$ kinase activity, and the peak was dialysed first against Bio-Rad Biogel concentrator resin and, when concentrated, against a 1:1 (v/v) mixture of glycerol and 50 mM-Tris (pH 7.2)/1 mM-EGTA/1 mM- NaN_3 . The purified enzyme was stored at –20 °C in the presence of 5 mM-dithiothreitol, under which conditions it was found to be stable for several months.

All columns were run on the Pharmacia f.p.l.c. system.

Protein was determined by the method of Bradford (1976).

H.p.l.c. analysis of inositol phosphates

Inositol phosphates were analysed by anion-exchange h.p.l.c., with a Whatman Partisil 10 SAX column eluted with a gradient of 0–1.7 M-ammonium formate, pH adjusted to 3.7 with H_3PO_4 , as described in Hawkins *et al.* (1986).

Preparation of inositol phosphates

$[^3\text{H}]\text{Ins}1P$ and $[^3\text{H}]\text{Ins}(1,4)P_2$ were purchased from New England Nuclear (Boston, MA, U.S.A.). *myo*- $[^3\text{H}]\text{-Inositol}$, $[\text{U-}^{14}\text{C}]\text{Ins}3P$ and $[^3\text{H}]\text{Ins}(1,4,5)P_3$ were from Amersham International. $[^3\text{H}]\text{Ins}(4,5)P_2$ and $[^3\text{H}]\text{Ins}(2,4,5)P_3$ were prepared by alkaline hydrolysis of $[^3\text{H}]\text{phosphatidylinositol 4,5-bisphosphate}$ (from New England Nuclear) by the method of Brockerhoff & Ballou (1961). These inositol phosphates were purified by anion-exchange h.p.l.c. and desalted by the method of Hawkins *et al.* (1986).

$[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ was prepared by phosphorylation of $[^3\text{H}]\text{Ins}(1,4,5)P_3$ with the purified rat brain $\text{Ins}(1,4,5)P_3$ kinase preparation, and was purified by anion-exchange h.p.l.c. before desalting.

$[^3\text{H}]\text{Ins}(1,3,4)P_3$ was prepared by dephosphorylation of $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ catalysed by a membrane-associated phosphomonoesterase of turkey erythrocytes (Morris *et al.*, 1987) and purified by anion-exchange chromatography on Bio-Rad AG-1 (200–400 mesh; formate form) before desalting.

$[^3\text{H}]\text{Ins}(3,4,5,6)P_4$ and $[^3\text{H}]\text{Ins}(1,3,4,5,6)P_5$ were isolated from $[^3\text{H}]\text{Ins}$ -labelled chick erythrocytes exactly as described in Stephens *et al.* (1988a).

Preparation of Ca^{2+} /EGTA buffers

The ratios of Ca^{2+} to EGTA which, when added to the basic incubation buffer, would give free $[\text{Ca}^{2+}]$ of 0–5 μM were calculated by using published association constants (Fabiato & Fabiato, 1979). The actual free $[\text{Ca}^{2+}]$ values in a series of parallel incubations were then measured by using the fluorescent indicator quin-2 (Tsien *et al.*, 1982).

RESULTS AND DISCUSSION

Purification of $\text{Ins}(1,4,5)P_3$ kinase

Table 1 shows total protein, total $\text{Ins}(1,4,5)P_3$ kinase activity and $\text{Ins}(1,4,5)P_3$ kinase specific activity throughout the series of purification steps as described in the Materials and methods section. $\text{Ins}(1,4,5)P_3$ kinase behaved as a single activity during the purification process.

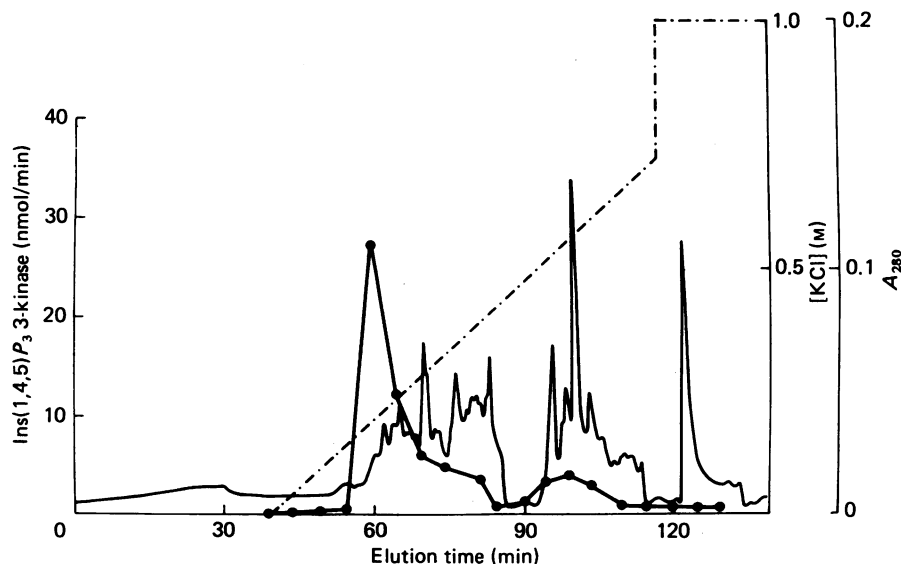
As reported by Irvine *et al.* (1986), $\text{Ins}(1,4,5)P_3$ kinase in rat brain is almost wholly cytosolic. The enzyme is precipitated by relatively low concentrations of $(\text{NH}_4)_2\text{SO}_4$ (114–243 g/l at 4 °C).

Chromatography through hydroxyapatite was found to be essential to prevent aggregation of $\text{Ins}(1,4,5)P_3$ kinase with other proteins during anion-exchange f.p.l.c. (see Stephens *et al.*, 1988b). The large protein capacity of the hydroxyapatite column employed and the purification achieved allowed us next to use a Pharmacia Mono Q HR 16/10 semi-preparative f.p.l.c. anion-exchange column. This effective purification step is illustrated in Fig. 1. Rat brain $\text{Ins}(1,3,4,5)P_4$ phosphatase activity is almost totally particle-bound (A. J. Morris & C. P. Downes, unpublished work), and anion-exchange chromatography removes $\text{Ins}(1,4,5)P_3$ phosphatase activity.

Table 1. Purification of Ins(1,4,5) P_3 kinase

Ins(1,4,5) P_3 kinase was purified as described in the Materials and methods section. The Table shows total protein, total Ins(1,4,5) P_3 kinase activity, specific Ins(1,4,5) P_3 kinase activity and overall purification throughout the series of steps employed. The results shown are typical of three separate purifications, in which the final degree of purification of the enzyme ranged between 120- and 200-fold.

Step	Total protein (mg)	Ins(1,4,5) P_3 kinase activity (nmol/min)	Yield (%)	Specific Ins(1,4,5) P_3 kinase activity (nmol/min per mg)	Purification (fold)
300 000 g supernatant	409	303	100	0.74	1
(NH ₄) ₂ SO ₄ (113 g/l) supernatant	381	286	94	0.75	1.01
(NH ₄) ₂ SO ₄ (113–243 g/l) precipitate	132	275	90	2.08	2.81
Hydroxyapatite	81.9	246	81	3.00	4.05
Mono Q HR 16/10 (anion-exchange f.p.l.c.)	9.9	182	60	18.4	24.9
Sepharose 4B CL	1.25	112	37	89.6	121.0

**Fig. 1. Anion-exchange f.p.l.c. of Ins(1,4,5) P_3 kinase on Mono Q**

The dialysed protein solution obtained after chromatography on hydroxyapatite was applied to a Mono Q HR 16/10 anion-exchange f.p.l.c. column and eluted with a gradient of NaCl (---) as described in the Materials and methods section. The A_{280} of the eluate was monitored (—) and the eluate was assayed for Ins(1,4,5) P_3 kinase activity (●).

A minor peak of Ins(1,4,5) P_3 kinase activity was eluted at higher ionic strength than the main peak. However, this activity was not further purified or characterized in the present study.

Gel filtration and molecular-size determination of Ins(1,4,5) P_3 kinase

Fig. 2 illustrates the behaviour of Ins(1,4,5) P_3 kinase during gel filtration on a column of Sepharose 4B CL. The column was calibrated for molecular-size determinations with a variety of protein standards (Sigma). Ins(1,4,5) P_3 kinase was eluted in the position of β -amylase, indicating a native M_r of 200×10^3 . The positions of earlier- and later-eluted M_r standards are also shown.

Because of the possibility that the enzyme had suffered

proteolytic degradation, aggregation or disaggregation during the purification procedure, we also determined the M_r of Ins(1,4,5) P_3 kinase in rat brain cytosol by gel-filtration f.p.l.c. on Superose 12, which provided a value of approx. 220×10^3 , the smaller exclusion size of this gel giving a less precise estimate of M_r (results not shown).

Structure and properties of the Ins P_4 produced by the Ins(1,4,5) P_3 kinase

When analysed by anion-exchange h.p.l.c., the Ins P_4 produced by the partially purified Ins(1,4,5) P_3 kinase behaved as a single compound (Fig. 3a). When this Ins P_4 was incubated with a rat parotid-gland homogenate exactly as in Hawkins *et al.* (1986) and the products were analysed by anion-exchange h.p.l.c., a single Ins P_3 with

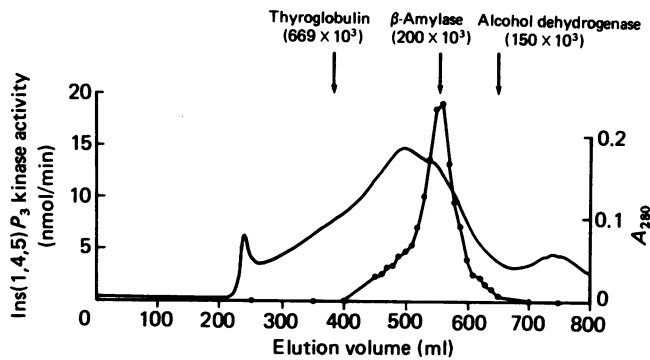


Fig. 2. Gel-filtration chromatography of $\text{Ins}(1,4,5)P_3$ kinase on Sepharose 4B CL

The peak of $\text{Ins}(1,4,5)P_3$ kinase activity obtained after anion-exchange f.p.l.c. was applied to a column of Sepharose 4B CL and eluted as described in the Materials and methods section. Protein A_{280} (—) and $\text{Ins}(1,4,5)P_3$ kinase activity (●) are shown, as are the elution positions of some M_r markers.

the chromatographic properties of $\text{Ins}(1,3,4)P_3$ was found (Fig. 3*b*).

When the $\text{Ins}P_3$ obtained as above was subjected to periodate oxidation, reduction and dephosphorylation as described previously (Stephens *et al.*, 1988*a*), the [^3H]polyol so produced was analysed by h.p.l.c. and was identified as altritol (for details see Stephens *et al.*, 1988*a*). The $\text{Ins}P_3$ was thus identified as D- $\text{Ins}(1,3,4)P_3$ or D- $\text{Ins}(1,2,4)P_3$ and the $\text{Ins}P_4$ from which it was derived must have been either D- $\text{Ins}(1,3,4,5)P_4$ or D- $\text{Ins}(1,2,4,5)P_4$. Treatment of the $\text{Ins}P_4$ with alkaline phosphatase, which preferentially removes lone phosphate groups rather than vicinal ones, also gave an $\text{Ins}P_3$. However, this $\text{Ins}P_3$ yielded xylitol after periodate oxidation, reduction and dephosphorylation, which is consistent with it being D- $\text{Ins}(3,4,5)P_3$, and eliminates all possible $\text{Ins}P_3$ isomers that could result from dephosphorylation of D- $\text{Ins}(1,2,4,5)P_4$. This series of experiments unambiguously identifies the product of the rat brain $\text{Ins}(1,4,5)P_3$ kinase as D- $\text{Ins}(1,3,4,5)P_4$.

Determination of the K_m values of the partially purified $\text{Ins}(1,4,5)P_3$ 3-kinase for $\text{Ins}(1,4,5)P_3$ and ATP

The partially purified enzyme was kinetically pure with respect to both substrates and product, having no $\text{Ins}(1,4,5)P_3$ or $\text{Ins}(1,3,4,5)P_4$ phosphatase activity and no ATPase activity.

The enzyme was incubated with various concentrations of $\text{Ins}(1,4,5)P_3$ and ATP, and the initial rate of production of $\text{Ins}(1,3,4,5)P_4$ was determined as explained previously. The data obtained were fitted to the Michaelis-Menten equation by non-linear regression analysis in order to calculate the K_m values for $\text{Ins}(1,4,5)P_3$ and ATP (Fig. 4). The data were fitted to the equation for a simple sequential two-substrate mechanism (Cleland, 1963). $\text{Ins}(1,4,5)P_3$ 3-kinase has a K_m for $\text{Ins}(1,4,5)P_3$ of $0.44 \mu\text{M}$. A similar value was obtained by Irvine *et al.* (1986), using rat brain cytosol as a source of the enzyme. Thus $\text{Ins}(1,4,5)P_3$ kinase has a considerably greater affinity for $\text{Ins}(1,4,5)P_3$ than do the $\text{Ins}(1,4,5)P_3$ 5-phosphatases that have been characterized (Downes *et al.*, 1982; Connolly *et al.*, 1985). The K_m obtained for ATP, $460 \mu\text{M}$, is of similar magnitude to those of other polyol and polyol

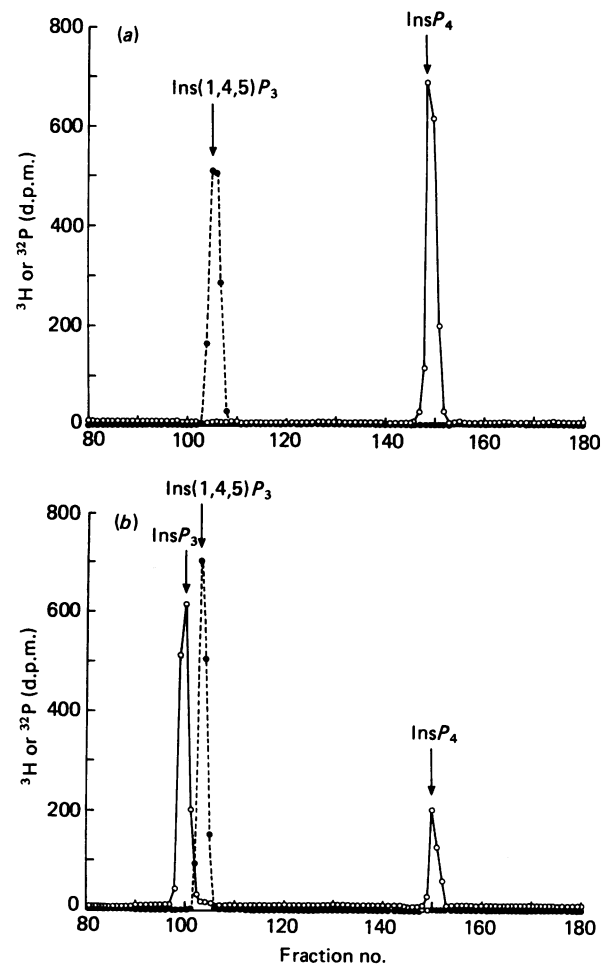


Fig. 3. Metabolism of $\text{Ins}P_4$ by a rat parotid-gland homogenate

$[^3\text{H}]\text{Ins}P_4$ prepared by phosphorylation of $\text{Ins}(1,4,5)P_3$ catalysed by the partially purified $\text{Ins}(1,4,5)P_3$ kinase was incubated with a rat parotid-gland homogenate for 0 min (a) or 10 min (b) as described in the Materials and methods section. The incubations were terminated, a standard of $\text{Ins}(1, [^{32}\text{P}]4, [^{32}\text{P}]5)P_3$ was added and inositol phosphates were separated by anion-exchange h.p.l.c. on Partisil 10 SAX. ^3H (○) and ^{32}P (●) radioactivities were detected by liquid-scintillation counting.

phosphate kinases for their substrates (see Barman, 1969). When double-reciprocal plots of the initial velocities obtained by varying each substrate concentration at fixed concentrations of the other were constructed, and then their slopes and intercepts replotted against the reciprocal of the concentration of the latter substrate, a series of converging lines was obtained (results not shown). This indicates that, in common with other kinases, binding of both substrates to the enzyme, forming a ternary complex, must occur in order to catalyse phosphate transfer (see Cleland, 1963).

Substrate specificity of $\text{Ins}(1,4,5)P_3$ 3-kinase

Partially purified $\text{Ins}(1,4,5)P_3$ 3-kinase was incubated with a variety of inositol phosphates and *myo*-inositol under the conditions described in the Materials and methods section. All incubations contained inositol phosphates at concentrations of 0.01 – $0.10 \mu\text{M}$, well below the K_m of the enzyme for $\text{Ins}(1,4,5)P_3$. The incubations

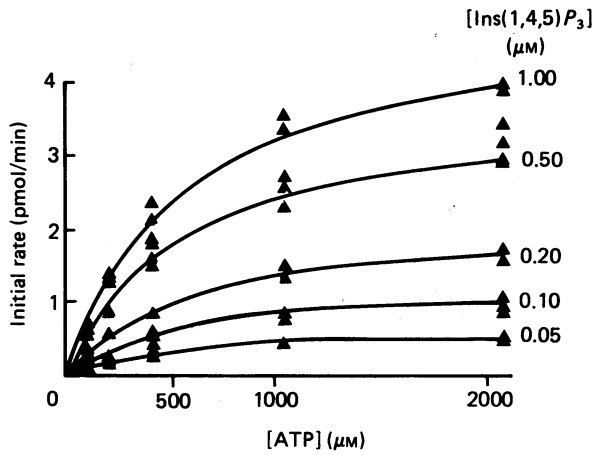


Fig. 4. Determination of the K_m values of the partially purified $\text{Ins}(1,4,5)P_3$ kinase for $\text{Ins}(1,4,5)P_3$ and ATP

Partially purified $\text{Ins}(1,4,5)P_3$ kinase was incubated with various concentrations of $\text{Ins}(1,4,5)P_3$ and ATP, and the initial rate of formation of $\text{Ins}(1,3,4,5)P_4$ was determined as described. The variation of initial rate with the concentration of ATP at increasing concentrations of $\text{Ins}(1,4,5)P_3$ is shown (\blacktriangle).

were terminated, neutralized as described in the Materials and methods section, and the samples were analysed for conversion of the starting inositol phosphates into inositol phosphates of increased polarity by anion-exchange chromatography on Bio-Rad AGI (200–400 mesh; formate form) as described by Downes *et al.* (1986), except that 2.0 M-ammonium formate/0.1 M-formic acid was used for elution of $\text{Ins}P_5$ (Stephens *et al.*, 1988b). $\text{Ins}(1,3,4,5,6)P_5$ was assayed for phosphorylation to $\text{Ins}P_6$ by anion-exchange h.p.l.c. (Stephens *et al.*, 1988a), as these inositol phosphates are inseparable by anion-exchange chromatography on small Bio-Rad AGI

columns with ammonium formate/formic acid eluents. The results obtained are shown in Table 2. The limits with which we could detect phosphorylation of inositol phosphates varied with the amounts of radioactivity in each incubation, but in all cases phosphorylation could have been detected had it occurred at a rate one-twentieth that of $\text{Ins}(1,4,5)P_3$.

Of the inositol phosphates tested that are known to occur in cells, only $\text{Ins}(1,4,5)P_3$ was phosphorylated. $\text{Ins}(3,4,5,6)P_4$, which Stephens *et al.* (1988b) have shown to be phosphorylated to produce $\text{Ins}(1,3,4,5,6)P_5$ by a kinase activity present in rat brain cytosol, was not a substrate. $\text{Ins}(4,5)P_2$ and $\text{GroPIns}(4,5)P_2$ were phosphorylated much more slowly than $\text{Ins}(1,4,5)P_3$ (approx. one-eighteenth and one-tenth of the first-order rate), presumably producing $\text{Ins}(3,4,5)P_3$ and $\text{GroPIns}(3,4,5)P_3$ respectively. Irvine & Moor (1986) have employed $\text{Ins}(2,4,5)P_3$ to investigate the role of phosphorylation of $\text{Ins}(1,4,5)P_3$ in the raising of a fertilization membrane by sea-urchin eggs. These workers surmised that, although $\text{Ins}(2,4,5)P_3$ is only about one-fifth as potent as $\text{Ins}(1,4,5)P_3$ in Ca^{2+} -mobilization assays, the proximity of the phosphorylated 2-hydroxyl group to the 3-hydroxyl might make $\text{Ins}(2,4,5)P_3$ a very poor substrate for $\text{Ins}(1,4,5)P_3$ 3-kinase, and claimed that $\text{Ins}(2,4,5)P_3$ was not phosphorylated when incubated with a rat brain cytosolic fraction. The results in Table 2 confirm this conclusion.

When ATP[S], GTP or CTP, at concentrations of up to 50 mM, replaced ATP, no phosphorylation of $\text{Ins}(1,4,5)P_3$ could be detected (results not shown).

Effects of Ca^{2+} , calmodulin and cyclic AMP-dependent phosphorylation on the activity of $\text{Ins}(1,4,5)P_3$ 3-kinase

When the partially purified $\text{Ins}(1,4,5)P_3$ 3-kinase was incubated with a low concentration of $\text{Ins}(1,4,5)P_3$ (10 nM) and the first-order rate of phosphorylation of $\text{Ins}(1,4,5)P_3$ determined, kinase activity decreased by about 40% as the measured $[\text{Ca}^{2+}]$ was increased over

Table 2. Substrate specificity of $\text{Ins}(1,4,5)P_3$ kinase

Partially purified $\text{Ins}(1,4,5)P_3$ kinase was incubated with a variety of inositol phosphates and *myo*-inositol (see the Materials and methods section). After the incubations had been stopped, inositol phosphates were separated by anion-exchange chromatography on Bio-Rad AGI (200–400 mesh) with ammonium formate/formic acid eluents and detected by liquid-scintillation counting. The data shown are means ($n = 2$) of ^3H (d.p.m.) in each inositol phosphate fraction.

Inositol phosphates tested	^3H or ^{14}C radioactivity in each inositol phosphate fraction (d.p.m.)											
	Ins		InsP		InsP ₂		InsP ₃		InsP ₄		InsP ₅	
	Zero time	15 min incubation	Zero time	15 min incubation	Zero time	15 min incubation	Zero time	15 min incubation	Zero time	15 min incubation	Zero time	15 min incubation
Ins	5043	5011	112	131	5	10	0	1	24	34	19	22
Ins1P	117	103	1772	1736	0	0	6	0	23	31	15	20
Ins3P	39	42	1047	1185	29	27	25	28	18	17	21	21
Ins(1,4)P ₂	19	16	46	38	1831	1893	0	12	25	23	18	18
Ins(4,5)P ₂	13	20	6	16	2062	1818	1	189	19	22	20	20
GroPIns(4,5)P ₂	5	10	88	75	119	151	1841	1406	49	397	17	19
Ins(1,3,4)P ₃	16	28	23	23	11	9	1097	1029	60	65	24	28
Ins(2,4,5)P ₃	16	14	23	23	11	9	1490	1450	38	39	10	12
Ins(1,4,5)P ₃	43	50	26	34	166	170	6852	1411	43	5314	23	50
Ins(1,3,4,5)P ₄	10	8	14	13	2	12	1	2	1647	1732	22	14
Ins(3,4,5,6)P ₄	18	9	15	15	0	2	0	0	1627	1668	29	25

the range 10 nM–1 μ M. This inhibition was half-maximal at approx. 0.2 μ M- Ca^{2+} , was unaffected by the addition of calmodulin to a final concentration of 10 μ g/ml, and appeared to be readily reversible on addition of EGTA to a final concentration of 10 mM, suggesting that it is not the result of some Ca^{2+} -dependent covalent modification of the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase, such as proteolysis or phosphorylation (results not shown). In order to investigate the nature of the inhibition by Ca^{2+} , the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase was incubated at $[\text{Ca}^{2+}]$ of 10 nM and 2 μ M, with a concentration of $\text{Ins}(1,4,5)\text{P}_3$ close to its K_m for this substrate or with a saturating concentration of $\text{Ins}(1,4,5)\text{P}_3$ (0.4 and 10 μ M respectively). The initial rate of phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ was determined, and was found to be lowered by increasing free $[\text{Ca}^{2+}]$ only when the K_m concentration of $\text{Ins}(1,4,5)\text{P}_3$ was employed, suggesting that the effect of Ca^{2+} ions is to increase the K_m of the enzyme for $\text{Ins}(1,4,5)\text{P}_3$ (Table 3). The explanation for and role of this inhibitory effect of Ca^{2+} are unclear. Certainly, the observed effects suggest that the enzyme might be subject to only very modest degrees of inhibition during cellular stimulation, resulting in increases in cytosolic free $[\text{Ca}^{2+}]$ from around 0.15 μ M to a maximum of about 1 μ M.

Inhibition by Ca^{2+} in the sub-micromolar concentration range does not seem to be a universal characteristic of the InsP_3 kinase activities that have been documented in other tissues. For example, phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ by permeabilized insulinoma cells (Biden & Wollheim, 1986) or adrenal glomerulosa cells (Rossier *et al.*, 1986) was accelerated by Ca^{2+} . Biden *et al.* (1987) have isolated a Ca^{2+} - and calmodulin-activated $\text{Ins}(1,4,5)\text{P}_3$ kinase from the soluble fraction of insulinoma cells, and Yamaguchi *et al.* (1987) described a similar enzyme in smooth muscle. In addition we (Morris *et al.*, 1987) have described a Ca^{2+} - and calmodulin-activated $\text{Ins}(1,4,5)\text{P}_3$ kinase that appears to be an intrinsic membrane protein present in turkey erythrocytes. It is possible that the rat brain $\text{Ins}(1,4,5)\text{P}_3$ kinase described in the present paper has lost its

sensitivity to Ca^{2+} /calmodulin during the isolation procedure, perhaps as a result of limited proteolysis. Alternatively, it seems likely that there will be many different forms of $\text{Ins}(1,4,5)\text{P}_3$ kinase, each having distinct patterns of regulation, tissue and subcellular distribution, just as there are multiple forms of the phosphodiesterases that metabolize cyclic AMP.

Preincubation of the partially purified $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase with 10 mM-MgATP and the purified catalytic subunit of cyclic AMP-dependent protein kinase did not alter its activity when assayed at a saturating or close-to- K_m concentration of $\text{Ins}(1,4,5)\text{P}_3$ (Table 3). These results do not, of course, discount the possibility that the enzyme is phosphorylated with a more complex effect on its catalytic activity, since the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase preparation is not sufficiently pure to investigate any phosphoproteins produced by using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

Concluding remarks

Although not homogeneous, the partially purified $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase has proved useful in studying the properties of this enzyme. The absolute specificity of the partially purified enzyme preparation for $\text{Ins}(1,4,5)\text{P}_3$ and, in particular, its selectivity for this inositol trisphosphate over the $\text{Ins}(1,3,4)\text{P}_3$ isomer make it particularly suitable for use as a selective and sensitive assay for $\text{Ins}(1,4,5)\text{P}_3$. Preliminary experiments in our laboratory using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ have succeeded in measuring $\text{Ins}(1,4,5)\text{P}_3$ at concentrations as low as 0.5 pmol (A. J. Morris & C. P. Downes, unpublished work).

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Table 3. Effect of Ca^{2+} , calmodulin and preincubation with the catalytic subunit of cyclic AMP-dependent protein kinase on the activity of partially purified $\text{Ins}(1,4,5)\text{P}_3$ kinase

Partially purified $\text{Ins}(1,4,5)\text{P}_3$ kinase was incubated with $\text{Ins}(1,4,5)\text{P}_3$ at concentrations of 0.4 and 10 μ M at $[\text{Ca}^{2+}]$ of 10 nM and 1.8 μ M and after preincubation with the purified catalytic subunit of cyclic AMP-dependent protein kinase. The rate of production of $\text{Ins}(1,3,4,5)\text{P}_4$ was determined as described in the Materials and methods section. The results shown are means \pm S.E.M. ($n = 3$).

	$[\text{Ins}(1,4,5)\text{P}_3]$ (μ M)	Rate (pmol/min)
No addition	0.4	0.57 \pm 0.01
	1.0	1.01 \pm 0.06
+Cyclic AMP-dependent protein kinase	0.4	0.60 \pm 0.06
	1.0	0.97 \pm 0.10
$[\text{Ca}^{2+}]$ (< 10 nM)	0.4	0.55 \pm 0.10
	+ calmodulin (10 μ g/ml)	1.0
$[\text{Ca}^{2+}] = 1.8 \mu$ M,	0.4	0.21 \pm 0.02
	+ calmodulin (10 μ g/ml)	1.0

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