Properties of the Inositol 3,4,5,6-Tetrakisphosphate 1-Kinase Purified from Rat Liver

REGULATION OF ENZYME ACTIVITY BY INOSITOL 1,3,4-TRISPHOSPHATE*

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Inositol 3,4,5,6-tetrakisphosphate is a novel intracellular signal that regulates calcium-dependent chloride conductance (Xie, W., Kaetzel, M. A., Bruzik, K. S., Dedman, J. R., Shears, S. B., and Nelson, D. J. (1996) J. Biol. Chem. 271, 14092–14097). The molecular mechanisms that regulate the cellular levels of this signal are not characterized. To pursue this problem we have now studied the 1-kinase that deactivates inositol 3,4,5,6-tetrakisphosphate. The enzyme was purified from rat liver 1600-fold with a 1% yield. The native molecular mass was determined to be 46 kDa by gel filtration. The \( K_m \) values for inositol 3,4,5,6-tetrakisphosphate and ATP were 0.3 and 10.6 \( \mu \)M, respectively. The kinase was unaffected by either protein kinase A or protein kinase C. Increases in Ca\(^{2+} \) concentration from 0.1 to 1–2 \( \mu \)M inhibited activity by 10–20%. Most importantly, inositol 1,3,4-trisphosphate was shown to be a potent (\( K_i = 0.2 \) \( \mu \)M), specific, and competitive inhibitor of the 1-kinase. Our new kinetic data show that typical receptor-dependent adjustments in cellular levels of inositol 1,3,4,5-tetrakisphosphate provide a mechanism by which the concentration of inositol 3,4,5,6-tetrakisphosphate is dependent on changes in phospholipase C activity. These conclusions also provide a new perspective to our understanding of the physiological importance of the pathway of inositol phosphate turnover initiated by the inositol 1,4,5-trisphosphate 3-kinase.

The agonist-mediated activation of phospholipase C and the resulting increased rate of hydrolysis of phosphatidylinositol 4,5-bisphosphate releases \( \text{Ins}(1,4,5)_P_2 \), which mobilizes cellular Ca\(^{2+} \) stores (1). The fact that the pathway by which \( \text{Ins}(1,4,5)_P_2 \) is metabolized is complex has led to the speculation that one or more of the many downstream metabolites might serve important cellular functions (2–4). Support for this opinion has come mainly from research into the actions of \( \text{Ins}(3,4,5)_P_4 \), the product of 3-kinase-directed phosphorylation of \( \text{Ins}(1,4,5)_P_2 \). As a result of many studies, there is now considerable evidence that \( \text{Ins}(3,4,5)_P_4 \) augments \( \text{Ins}(1,4,5)_P_2 \)-initiated Ca\(^{2+} \) mobilization (5). Yet the search for other signaling functions of additional inositol polyphosphates continues at several laboratories.

Attention has come to focus on a somewhat distant metabolic relative of \( \text{Ins}(1,4,5)_P_2 \), namely \( \text{Ins}(3,4,5,6)_P_4 \). The latter was first identified in mammalian cells in 1988, at which time it was noted that its levels increased substantially upon receptor-mediated activation of phospholipase C (6, 7). We subsequently proposed that \( \text{Ins}(3,4,5,6)_P_4 \) was an “orphan” second messenger (8, 9). The first indication of what the function of this messenger might be came from work by Barrett, Traynor-Kaplan, and colleagues with the T84 colon epithelial cell line (10, 11). They drew attention to a correlation during receptor activation between the bulk \( \text{Ins}P_4 \) content of the cell and the uncoupling of the customary ability of Ca\(^{2+} \) to stimulate Cl\(^{-} \) secretion. This prompted a study in which we treated T84 cells with cell-permeant analogues of inositol phosphates (12). The results indicated that \( \text{Ins}(3,4,5,6)_P_4 \) was the specific \( \text{Ins}P_4 \) isomer responsible for inhibiting Ca\(^{2+} \)-dependent Cl\(^{-} \) secretion (12). We subsequently consolidated this idea by demonstrating that the Ca\(^{2+} \)-dependent Cl\(^{-} \) conductance was directly inhibited by \( \text{Ins}(3,4,5,6)_P_4 \) when it was microinjected into the T84 cell (13). \( \text{Ins}(3,4,5,6)_P_4 \) had a similar effect when injected into CFPAC-1 cells, an adrenal carcinoma cell line deficient in cAMP-activated Cl\(^{-} \) transport (14). Moreover, the cloned calcium-activated chloride channel from bovine trachea has also been shown to be directly inhibited by \( \text{Ins}(3,4,5,6)_P_4 \) (15). In all three of these electrophysiological studies (13–15) the effects of \( \text{Ins}(3,4,5,6)_P_4 \) were very specific to that isomer, and they could not be imitated by up to 10-fold higher concentrations of other inositol polyphosphates.

Thus, \( \text{Ins}(3,4,5,6)_P_4 \) may now be considered to be an authentic intracellular signal with the potential to modulate many of the physiological processes that require chloride channel activity: salt and fluid secretion, pH balance, neurotransmission, osmoregulation, and volume-dependent metabolic effects (16–19). This has increased the importance of understanding the molecular mechanisms that regulate the synthesis and metabolic inactivation of this inositol polyphosphate. A particularly important aspect of this problem is to establish how the activation of phospholipase C is coupled to changes in cellular levels of \( \text{Ins}(3,4,5,6)_P_4 \). We have proposed that this cannot be explained as a simple mass action effect (8, 9). That is, we have argued that a receptor-mediated increase in \( \text{Ins}(3,4,5,6)_P_4 \) concentration does not reflect a nonspecific accumulation of downstream metabolites of \( \text{Ins}(1,4,5)_P_3 \) (8, 9). Instead we have proposed that agonists intervene in the activities of the \( \text{Ins}(3,4,5,6)_P_4 \) 1-kinase/\( \text{Ins}(1,3,4,5,6)_P_5 \) 1-phosphatase substrate cycle (8, 9). However, this hypothesis has developed very little in the absence of any significant progress in determining the mechanism for this proposed regulatory process. In no...
small part, this uncertainty is due to the fact that no laboratory has yet purified the 1-phosphatase, and what we know of the 1-kinase depends almost entirely on a single paper by Stephens et al. (7). In that study, the enzyme in a 100,000 × g supernatant made from rat brain was enriched approximately 35-fold by ammonium sulfate precipitation and anion-exchange chromatography (7). No kinetic information emerged from that work.

We now describe the procedures we have developed to purify the Ins(3,4,5,6)P₄ enzyme from a rat liver 100,000 × g supernatant. We have used these preparations to obtain the first kinetic description of this enzyme’s activity. We have further investigated possible mechanisms by which this enzyme might be controlled in intact cells. We conclude that Ins(1,3,4)P₃ is a physiologically important regulator of 1-kinase activity that accounts for the close relationship between phospholipase C activity and changes in intracellular levels of Ins(3,4,5,6)P₄.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Inositol was purchased from American Radiolabeled Chemicals, Inc. or Du Pont NEN. Nonradioactive 3H-Inositol, 4,5,6,7-[3H]Aminopterin was synthesized as described previously (13). The other inositol phosphates were purchased from Calbiochem or the University of Rhode Island Foundation (Kingston, RI). Bovine serum albumin, phosphocreatine, phosphocreatine kinase, heparin agarose resin (Type II), protease inhibitors, and the calmodulin kinase fragment 290–309 were all purchased from Sigma. Phenyl-Sepharose CL-4B resin and Mono Q HR10/10 column was from Pharmacia Biotech, Inc. and Matrex A dye resin was from Amicon.

**Preparation of [3H]Ins(3,4,5,6)P₄—**[3H]Ins(3,4,5,6)P₄ is not available commercially. We prepared it from [3H]inositol-labeled erythrocytes isolated from 5-day-old chicks that had been incubated for 24 h with 10 mCi of [3H]inositol/ml of packed cells at 37°C (20). Incubations were quenched and neutralized (20), and the [3H]Ins(3,4,5,6)P₄ peak was isolated by HPLC (6) and desalted (12). In order to ascertain the specific radioactivity of the [3H]Ins(3,4,5,6)P₄, its mass was determined from the extent to which it inhibited partially purified Ins(1,3,4,5,6)P₅/5′ kinase (12). Most of the data described in this paper were obtained using a single batch of [3H]Ins(3,4,5,6)P₄ (specific radioactivity, 217 dpm/pmol). For this preparation, the extent of contamination of [3H]Ins(3,4,5,6)P₄ with [3H]Ins(1,4,5,6)P₃ (less than 5%) was determined using the partially purified Ins(1,4,5,6)P₃/3-kinase (12). Many preliminary experiments, including those described in Table I, were performed with a different batch of [3H]Ins(3,4,5,6)P₄ (1.5 dpm/pmol) isolated as described above but from [3H]inositol-labeled turkey erythrocytes.

**Assay of Ins(3,4,5,6)P₄ 1-Kinase**—During purification, 10–40 µl of sample were incubated at 37°C for 10–30 min in a final volume of 100 µl containing 4000 dpm of [3H]Ins(3,4,5,6)P₄ (adjusted to a concentration of 5 µM with nonradioactive substrate), 20 mM HEpes (pH 7.2), 6 mM MgSO₄, 0.4 mg/ml saponin, 100 mM KCl, 0.3 mg/ml bovine serum albumin, 2 µM InsP₃, 5 mM ATP, 10 mM phosphocreatine, 2.5 Sigma units of phosphocreatine kinase.

The final preparations of enzyme were also assayed using the above methods except that the cheleng medium did not contain InsP₃. In some cases, the free Ca²⁺ concentration was adjusted using EGTA/Ca²⁺ buffers (21).

The reaction mixture was quenched with 1 ml of ice-cold medium containing 1 mg/ml InsP₃, 0.2 µl ammonium formate, 0.1 µM formic acid. The quenched reactions were diluted to 10 ml with water and chromatographed on Bio-Rad gravity-fed columns using AG 1-X8 ion-exchange resin. In some experiments, incubations were quenched with 40 µl of 2 M perchloric acid plus 1 mg/ml InsP₃, neutralized with Freon/octylamine, and chromatographed on HPLC using a Partisil SAX column (12).

**Purification of Ins(3,4,5,6)P₄ 1-Kinase**—Preparation of 100,000 × g Supernatant—Livers obtained from 200–250 g male Sprague-Dawley rats with ice-cold saline were frozen at −80°C until use (freezing did not impair enzyme stability). All subsequent procedures (which took 5 days) were conducted at 0–4°C, and all the column chromatography procedures (except for the final Matrex Blue A stage) utilized a Pharmacia FPLC system. For each preparation, about 70 g of liver was homogenized (500–1000 rpm, five up-and-down strokes) in 140 ml of medium consisting of 20 mM Bis-Tris (pH 7.0), 1 mM EGTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 1.5 h at 100,000 × g, and the resultant supernatant was filtered (0.2 µm).

**Heparin-Agarose Chromatography**—The filtrated supernatant was loaded at about 1 ml/min to a heparin-agarose column (3.2 × 20 cm) that was equilibrated with 300 ml of Buffer A (20 mM Bis-Tris (pH 7.0), 1 mM EGTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin A) at a flow rate of 2 ml/min. After washing with 80 ml of Buffer A at 1.5 ml/min, the column was eluted at 1.5 ml/min with a linear gradient of 2.5–5.5 mM pyrophosphate in Buffer A for 300 min followed by 50 mM pyrophosphate in Buffer A for 200 min at 2 ml/min.

**Phenyl-Agarose Chromatography**—Peak fractions of enzyme activity eluted from the heparin-Sepharose column were pooled and loaded onto a Mono Q HR10/10 column previously equilibrated with 30 ml of Buffer B (50 mM Bis-Tris (pH 7.0), 1 mM EGTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin A). The column was then washed with 10 ml of Buffer B containing 1 mM MgSO₄. The flow-through was collected under gravity. Finally, 1 mg/ml bovine serum albumin and 20% glycerol were added, and the preparations were stored at −70°C until use. There was no significant loss of activity over a 6-month period.

**Gel Filtration**—The molecular weight of the partially purified Ins(3,4,5,6)P₄ 1-kinase was determined by gel filtration. Purified enzyme was concentrated using an Amicon Centricon-10 concentrator and mixed with molecular weight standards from Bio-Rad: bovine γ-globulin, chicken ovalbumin, equine myoglobin, and vitamin B₁₂. A 50–200 µl mixture was chromatographed on a Pharmacia Superdex 75 HR10/30 gel filtration column using Bis-Tris buffer (pH 7.2) containing 1 mM EGTA, 100 mM phenylmethylsulfonyl fluoride, 0.4 µM leupeptin, 0.4 µM E-64, 0.1 mM Ca²⁺, and 100 mM NaCl at a flow rate of 0.5 ml/min. 0.25-ml fractions were collected and assayed for enzyme activity.

**Assay of the Effects of Protein Kinase C and Protein Kinase A on the Ins(3,4,5,6)P₄ 1-Kinase**—A 0.4 µg aliquot of the Ins(3,4,5,6)P₄ 1-kinase preparation was incubated with 0.05 units of protein kinase C (Pierce, catalog no. 29536) or 50 units of protein kinase A (Pierce, catalog no. 29538) in a volume of 10 µl using the manufacturer’s assay kit. After 10 min of incubation at 37°C, the mixtures were assayed for 1-kinase activity.

The activities of protein kinases A and C were also determined as positive controls using colorimetric assays (Pierce, catalog no. 29517 for kinase C and catalog no. 29529 for kinase A).

**RESULTS**

**Purification of Ins(3,4,5,6)P₄ 1-Kinase**—Kinase activity during purification was assayed in the presence of 2 µM InsP₆. This had no effect on the 1-kinase (data not shown) but was used to inhibit the Ins(3,4,5,6)P₄ 3-phosphatase (22) that contaminated the kinase during the initial stages of purification. However, the InsP₆ was not included in experiments with the purified enzyme since this did not contain any Ins(3,4,5,6)P₄ phosphatase activity (data not shown).

We chose to isolate the 1-kinase from liver, a tissue in which the enzyme has relatively high specific activity (7); kinetic data we accumulated from the purified enzyme could then be compared with the inositol phosphate profiles in isolated hepatocytes (23). The rat hepatic 1-kinase is predominantly a soluble enzyme (Ref. 7 and data not shown). These crude preparations of enzyme were relatively stable when maintained at 0–4°C, but our initial attempts at further purification were thwarted by such procedures bringing about a nearly complete loss of...
Table I

| Treatment               | Activity remaining after 48 h (%) |
|-------------------------|-----------------------------------|
| Buffer only             | 2.1                               |
| 1 mM sodium pyrophosphate| 35                                |
| 2.5 mM sodium pyrophosphate| 84                                |
| 5 mM sodium pyrophosphate| 91                                |
| 10 mM sodium pyrophosphate| 100                               |
| 10 mM sodium phosphate | 41                                |
| 5 mM ammonium sulfate   | 3.5                               |
| 1 mM ATP (sodium salt)  | 28                                |
| 1 mM cAMP               | 29                                |
| 20 mM (NH₄)₂PO₄         | 22                                |
| 10 mM ammonium molybdate| 32                                |
| 1 mM sodium vanadate    | 5.1                               |
| 20 mM sodium fluoride   | 0.6                               |
| 10% glycerol            | 28                                |
| 1 mM dithiothreitol     | 8.8                               |
| 1 μM okadate acid in dimethyl sulfoxide| 24                      |
| Dimethyl sulfoxide      | 24                                |

Activity. For example, if the enzyme was concentrated by ammonium sulfate precipitation, 98% of activity was lost within 48 h (Table I). Fortunately, we discovered that certain phosphate-containing compounds and some phosphatase inhibitors were able to preserve enzyme activity to varying extents (Table I). Among them, inorganic pyrophosphate was the most effective. The disadvantage of including pyrophosphate was that it prevented the enzyme from binding to many of the column resins that otherwise might have facilitated the purification of the enzyme. Eventually, we found that an adequate compromise was to load the crude supernatant onto a heparin-agarose column in the absence of pyrophosphate and then use this compound to elute the enzyme from the column as a single peak (Fig. 1A, Table II). These procedures brought about an approximately 20-fold purification with an apparent yield of 67%.

Pyrophosphate was also present during the next stage of purification of the enzyme by phenyl-Sepharose chromatography (Fig. 1B, Table II). However, during further purification steps (Fig. 1C, Table II) the enzyme again became unstable at 0–4°C, apparently for a different reason since pyrophosphate now offered no protective effect (a fact that also prevented us from using the purified enzyme to explore the mechanism of action of pyrophosphate). We did discover that enzyme activity could be preserved by the addition of excess protein (e.g. 1 mg/ml bovine serum albumin). It is possible that the enzyme was now being adversely affected by its inevitable dilution during purification. Neither glycerol (up to 50% (v/v)) nor polyvinylpyrrolidone (up to 0.5% (w/v)) were effective substitutes for the albumin. We attempted to compensate for this particular difficulty by scaling up our preparations to increase the concentration of the purified 1-kinase. We were then able to further purify the enzyme by anion-exchange chromatography (Fig. 1C, Table II) and Matrex Blue A dye-ligand chromatography (Table II). The final preparations, which were approximately 1600-fold pure with a 1% yield, were stored at -70°C with the addition of 1 mg/ml bovine serum albumin plus 20% (v/v) glycerol; there was no significant loss of activity for at least 6 months.

Properties of the 1-Kinase—We found that the enzyme had high affinity for both Ins(3,4,5,6)P₄ and ATP; the Kₘ value of the 1-kinase for Ins(3,4,5,6)P₄ was estimated to be 0.36 ± 0.01 μM (mean ± S.E. from three determinations, Fig. 2A). The kinetic properties of the 1-kinase activity in any tissue have not previously been described. Our determinations were made possible by the development of procedures for the chemical synthesis of enantiomerically pure Ins(3,4,5,6)P₄ (13) plus our isolation of relatively high specific activity [³H]Ins(3,4,5,6)P₄ from [³H]inositol-labeled chick erythrocytes (see “Experimental Procedures”). The Kₘ for ATP was estimated to be 10.6 ± 0.08 μM (mean ± S.E. from three determinations, Fig. 2B). The native molecular mass of the enzyme was estimated by gel filtration to be 46 ± 1 kDa (mean ± S.E. from three determinations, Fig. 3).

Regulation of 1-Kinase Activity—The most important goal of this study was to gain insight into how the 1-kinase might be regulated in vivo, particularly when phospholipase C is activated. We found that the enzyme was unaffected by its co-incubation with either protein kinase A or protein kinase C (data not shown; for details, see “Experimental Procedures”). We also investigated the effect on the 1-kinase of the changes in [Ca²⁺] that would be expected to occur in cells upon activation of phospholipase C. The enzyme activity was slightly reduced (–10 to 20%) at 1–2 μM Ca²⁺ to relative to the activity at 0.1 μM (data not shown). This small effect, which would only be expected to occur during the brief period at which a cellular Ca²⁺ transient attained its peak value, was not influenced (data not shown) by the further addition of either 5 μM calmodulin or 10 μM calmodulin antagonist (kinase fragment 290–309 (24)).

From the point of view of our efforts to understand how the Ins(3,4,5,6)P₄ 1-kinase is regulated, a particularly important result (see “Discussion”) was the potency with which this enzyme was inhibited by Ins(1,4,5)P₃ (Fig. 4); the inhibition was determined by a Dixon plot to be competitive (Fig. 4), and the estimated Kᵣ value was 0.2 ± 0.01 μM (mean ± S.E., n = 3; Fig. 4, Table III). The inhibition of 1-kinase by Ins(1,3,4)P₃ was relatively specific; Ins(1,4,5)P₃, Ins(1,3,4,5)P₄, Ins(1,3,4,6)P₄, and Ins(1,3,4,5,6)P₅ were also competitive inhibitors, but they all acted with considerably lower potency (Table III).

Discussion

In order to fully discern the actions of second messengers we need to determine how both their accumulation and metabolic inactivation are regulated. This generally requires the purification and characterization of the enzymes responsible for these metabolic control processes. This is the approach we have taken to increase our understanding of the receptor-regulated mechanisms that control metabolic turnover of Ins(3,4,5,6)P₃, a novel cellular signal (see the Introduction). Thus, we have purified the Ins(3,4,5,6)P₄ 1-kinase activity 1600-fold from a hepatic supernatant with a 1% yield. (The only previously described purification of this enzyme yielded a 35-fold enriched sample from a rat brain supernatant (7).)

The novel kinetic data that we have obtained with this purified enzyme have been particularly revealing concerning the mechanisms by which receptor-mediated activation of phospholipase C causes cellular Ins(3,4,5,6)P₃ levels to increase. For example, we have discovered that the 1-kinase has relatively high affinity for Ins(3,4,5,6)P₄ (Kₘ = 0.36 μM). In addition, we have determined that Ins(1,3,4,5)P₅ is a potent inhibitor of the 1-kinase, with a Kᵣ of 0.2 μM. Since intracellular levels of Ins(1,3,4,5)P₅ are about 1 μM under basal conditions (28), inhibition of the 1-kinase by this polyphosphate is physiologically relevant. Inhibition by Ins(1,3,4)P₄ was also relatively specific.
other inositol polyphosphates were inhibitors but at unphysiologically high concentrations (Table III), with perhaps one exception. In intact cells some slight inhibition of 1-kinase activity by \( \text{Ins}(1,3,4,5,6)P_5 \) is also possible since its steady-state levels (22, 23) are close to its \( K_i \) value of 15 \( \mu M \). However, levels of this particular isomer do not change substantially during activation of phospholipase C (4, 9), so this polyphosphate will not contribute to receptor-mediated regulation of the 1-kinase.

It is of further significance that \( \text{Ins}(1,3,4)P_3 \) is a downstream metabolite of \( \text{Ins}(1,4,5)P_3 \) (4). Thus, the intracellular levels of \( \text{Ins}(1,3,4)P_3 \) accumulate severalfold whenever phospholipase C is activated. This raises the possibility that it is these receptor-mediated changes in \( \text{Ins}(1,3,4)P_3 \) levels that, by inhibiting 1-kinase, link phospholipase C activity to elevations in \( \text{Ins}(3,4,5,6)P_4 \) levels. To further explore this idea, we fitted our kinetic data to Equation 1 (see Ref. 22).

\[
v = \frac{V_{max} \times [S]}{[S] + K_m \times (1 + ([I_1]/K_{I_1}) + ([I_2]/K_{I_2}))} \tag{Eq. 1}
\]

Here, \( v \) is the reaction rate for the 1-kinase under conditions in which \( [S] \) represents the concentration of \( \text{Ins}(3,4,5,6)P_4 \) (1 \( \mu M \) in resting cells (12)), \( [I_1] \) is the concentration of \( \text{Ins}(1,3,4)P_3 \), and \( [I_2] \) is the concentration of \( \text{Ins}(1,3,4,5,6)P_5 \) (i.e. 15 \( \mu M \)).
the values of the $K_m$ for Ins(3,4,5,6)P$_4$ are from Fig. 2, and the $K_i$ values of the two inhibitors are given in Table III. If we take the concentration of Ins(1,3,4)P$_3$ ([I$_1$]) to be 1 $\mu$M under basal conditions (25), Equation 1 predicts that the 1-kinase would be operating at 28% of its maximal capacity in unstimulated cells.

We next evaluated what would be the effect of a typical, receptor-mediated 5–10-fold rise in the concentration of Ins(1,3,4)P$_3$ (12,23,25). Using Equation 1 we estimated that if levels of this trisphosphate accumulated to either 5 or 10 $\mu$M, this would decrease the velocity of the kinase to 9 and 5% respectively, of its maximal capacity. Thus, these two physiologically relevant agonist-dependent changes in the concentration of Ins(1,3,4)P$_3$ would be predicted to inhibit the 1-kinase by 68 and 82%, respectively. This would perturb the poise of the Ins(3,4,5,6)P$_4$ 1-kinase/Ins(1,3,4,5,6)P$_5$ 1-phosphatase substrate cycle (8, 9) so that levels of Ins(3,4,5,6)P$_4$ would accumulate due to the ongoing activity of the Ins(1,3,4,5,6)P$_5$ 1-phosphatase. Our kinetic data also predict that during the poststimulatory phase competition between Ins(3,4,5,6)P$_4$ and Ins(1,3,4)P$_3$ would be a major factor in determining the rate at which Ins(3,4,5,6)P$_4$ returns to basal levels.

Therefore, we conclude that a major function of Ins(1,3,4)P$_3$ is to provide a mechanism by which activation of phospholipase C can induce changes in cellular levels of Ins(3,4,5,6)P$_4$. In this way phospholipase C may be considered to have both positive and negative input into Cl$^-$ ion channel activity: initial stimulation by Ca$^{2+}$ followed by inhibition by Ins(3,4,5,6)P$_4$. The

**TABLE III**

| Inhibitor          | $K_i$ (mM) |
|--------------------|------------|
| Ins(1,3,4)P$_3$    | 0.2 ± 0.01 (3) |
| Ins(1,3,4,5)P$_4$  | 9 ± 1 (3)    |
| Ins(1,3,4,6)P$_4$  | 14.5 ± 0.2 (4) |
| Ins(1,3,4,5,6)P$_5$| 15 ± 0.3 (3) |
| Ins(1,4,5)P$_3$    | 48 ± 1 (4)   |

**FIG. 2.** Substrate affinity of the Ins(3,4,5,6)P$_4$ 1-kinase. 0.09 $\mu$g of purified 1-kinase was incubated for 30 min as described under “Experimental Procedures” except that either the Ins(3,4,5,6)P$_4$ was adjusted as indicated (panel A) or the MgATP concentration was adjusted as indicated (panel B). Reactions were quenched, neutralized, and chromatographed on either gravity-fed ion-exchange columns or by HPLC as described under “Experimental Procedures.” Data are from a single experiment that was typical of three experiments. The $K_m$ and $V_{max}$ values were calculated by nonlinear regression using Sigmaplot, version 3. For Ins(3,4,5,6)P$_4$ ($n$ = 3), $K_m$ = 0.36 ± 0.01 $\mu$M, $V_{max}$ = 5.9 ± 0.1 pmol/min/µg of protein. For ATP ($n$ = 3), $K_m$ = 10.6 ± 0.08 $\mu$M, $V_{max}$ = 5.8 ± 0.2 pmol/min/µg of protein.

**FIG. 3.** Gel filtration of the Ins(3,4,5,6)P$_4$ 1-kinase. An aliquot of 1-kinase was analyzed by gel filtration as described under “Experimental Procedures” using the following standards: bovine γ-globulin, 158,000; chicken ovalbumin, 44,000; equine myoglobin, 17,000; vitamin B$_12$, 135,000. The recovery of 1-kinase was 80%. The data shown are from one experiment that was typical of three experiments; the average molecular mass was 46 ± 1 kDa.

**FIG. 4.** Dixon plot of inhibition of the Ins(3,4,5,6)P$_4$ 1-kinase by Ins(1,3,4)P$_3$. 0.087 $\mu$g of enzyme was incubated for 30 min with various concentrations of Ins(3,4,5,6)P$_4$ and Ins(1,3,4)P$_3$ as indicated. Reactions were quenched, neutralized, and chromatographed on gravity-fed ion-exchange columns as described under “Experimental Procedures.” The data shown are from one experiment that was typical of three experiments.

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idea that \( \text{Ins}(1,3,4)P_3 \) serves an important physiological function and amends a long-standing belief that it is merely an inactive degradation product of \( \text{Ins}(1,4,5)P_3 \). However, this role for \( \text{Ins}(1,3,4)P_3 \) is ultimately served by the activity of the \( \text{Ins}(1,4,5)P_3 \)-3-kinase. Thus, both the function of the 3-kinase and the selective pressures that caused it to evolve (see Ref. 5) should no longer be interpreted purely in terms of regulating the levels of \( \text{Ins}(1,4,5)P_3 \) and \( \text{Ins}(1,3,4,5)P_4 \) to control \( \text{Ca}^{2+} \) mobilization.

It is entirely possible that this action of \( \text{Ins}(1,3,4)P_3 \) by itself is all that is necessary to elevate levels of \( \text{Ins}(3,4,5,6)P_4 \) in vivo. In any case, the identification of this process adds a remarkable new feature to our understanding of the control of signaling functions of inositol polyphosphates. These results also consolidate our original hypothesis (8, 9) that receptor-dependent changes in \( \text{Ins}(3,4,5,6)P_4 \) levels are not just a consequence of mass action effects dependent on alterations in levels of its metabolic precursor, i.e., \( \text{Ins}(1,3,4,5,6)P_5 \). Moreover, our data exclude a substantial contribution of some other potential regulatory processes to the control of 1-kinase. For example, the enzyme activity was not affected by either protein kinase A or protein kinase C. We also investigated whether the changes in cellular \( \text{Ca}^{2+} \) that occur upon receptor activation would affect 1-kinase activity. When free \( \text{Ca}^{2+} \) in our assays was raised from 0.1 to 1–2 \( \mu \text{M} \), there was a slight inhibition of enzyme activity (10–20%), but this seems insufficient to substantially contribute to the control of 1-kinase activity in vivo. Indeed, we have previously shown that \( \text{Ins}(3,4,5,6)P_4 \) levels in primary cultures of rat hepatocytes did not change in response to thapsigargin (which elevates cytosol \( \text{Ca}^{2+} \)). The high affinity of the kinase for ATP (\( K_m = 10.6 \mu \text{M} \)) indicates that the activity of the hepatic enzyme in vivo is unlikely to be affected by physiologically relevant changes in ATP levels, which are maintained in the millimolar range (26).

Drugs that modify the actions and metabolism of \( \text{Ins}(3,4,5,6)P_4 \) might improve treatment for certain medical conditions that result from perturbations to transmembrane \( \text{Cl}^- \) transport, such as cystic fibrosis, and heart arrhythmia. The new information we have provided concerning the properties of the 1-kinase may improve the scope for pharmacological intervention, since they identify the enzymes of \( \text{Ins}(1,3,4)P_3 \) metabolism as additional, potential therapeutic targets. Finally, our goal of sequencing and cloning the 1-kinase will help us understand the properties of this enzyme at a molecular level.

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