Volume 236, number 1, 53–56 FEB 06166 August 1988

Characterization of inositol 1,3,4-trisphosphate phosphorylation in rat liver

Carl A. Hansen, Stephan vom Dahl, Benjamin Huddell and John R. Williamson

University of Pennsylvania School of Medicine, Department of Biochemistry and Biophysics Philadelphia, PA 19104, USA

Received 27 May 1988

Liver homogenates phosphorylated Ins 1,3,4-P_3 to an $InsP_4$ isomer that was distinct from Ins 1,3,4,5-P_4. This $InsP_4$ isomer accumulated in vasopressin stimulated hepatocytes prelabeled with *myo*-[^{3}H]inositol with a time course that lagged behind Ins 1,3,4-P_3 formation. The Ins 1,3,4-P_3 kinase responsible for its formation was partially purified from rat liver. The enzyme had a K_m for Ins 1,3,4-P_3 of 0.29 μM, a K_m for ATP of 141 μM and was not affected by changes in free Ca^{2+} in the physiological range. The relationship of this new $InsP_4$ isomer to the inositol phosphate signaling pathway is discussed.

Signal transduction; Inositol phosphate; Inositol-1,3,4-trisphosphate kinase; Ca^{2+}; (Liver cell)

1. INTRODUCTION

As in many other cell types, Ca^{2+} signaling in liver is initiated by an agonist-induced hydrolysis of inositol lipids in the plasma membrane to inositol phosphates and 1,2-diacylglycerol [1,2]. The inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) released in this process acts as a Ca^{2+} mobilizing second messenger by opening a Ca^{2+} channel located in a specialized intracellular compartment. Once formed, Ins 1,4,5-P_3 is either dephosphorylated to Ins 1,4-P_2 or phosphorylated to Ins 1,3,4,5-P_4 by a Ca^{2+}/calmodulin-activated 3-kinase [3–5]. Subsequent metabolism of Ins 1,3,4,5-P_4 proceeds via dephosphorylation to Ins 1,3,4-P_3, probably by the same 5-phosphomonoesterase that converts Ins 1,4,5-P_3 to Ins 1,4-P_2 [6,7]. The primary pathway of Ins 1,3,4-P_3 metabolism involves dephosphorylation to Ins 3,4-P_2 by a Li^+-sensitive inositol polyphosphate 1-phosphomonoesterase [8], an enzyme which also dephosphorylates Ins 1,4-P_2 to Ins 4-P. A minor degradative pathway is catalysed by a Li^+-insensitive 4-phosphomonoesterase, leading to Ins 1,3-P_2 [9]. Recently, Balla et al. [10,11] demonstrated that Ins 1,3,4-P_3 could be phosphorylated to Ins 1,3,4,6-P_4 in extracts of adrenal glomerulosa cells and that this new $InsP_4$ isomer accumulated in intact cells stimulated with angiotensin II. An Ins 1,3,4-P_3 6-kinase activity has also been reported to be present in crude liver homogenates [12]. In this paper, we demonstrate that this second $InsP_4$ isomer accumulates in hepatocytes after vasopressin stimulation and describe a partial purification and characterization of the Ins 1,3,4-P_3 kinase responsible for its formation.

Correspondence address: J.R. Williamson, University of Pennsylvania School of Medicine, Dept of Biochemistry and Biophysics, Philadelphia, PA 19104, USA

2. MATERIALS AND METHODS

2.1. *Measurement of inositol phosphate accumulation in hepatocytes*

The procedures used for the isolation of rat hepatocytes, labeling of the cells with *myo*-[^{3}H]inositol, and extraction of [^{3}H]inositol phosphates for HPLC analysis were as described in [13]. The HPLC method was modified as follows: a Whatman Partisil SAX column was equilibrated at a flow rate of 1.2 ml/min with 0.55 M $NH_4H_2PO_4$, pH 4.8. Following sample injection, the mobile phase was maintained isocratic for 18 min and then increased to 0.83 M $NH_4H_2PO_4$, pH 4.8, for the next 15 min. Ins 1,3,4-P_3, Ins 1,4,5-P_3, Ins 1,3,4,5-P_4 and the new $InsP_4$ isomer eluted at 11, 13, 24 and 27 min, respectively. Fractions of 0.6 ml were collected and quantified by liquid scintillation counting [13].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2.2. *Partial purification of Ins 1,3,4-P_3 kinase*

Five perfused rat livers were homogenized (1:4, w/v) in 20 mM Tris-maleate, pH 7.0, 5 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of aprotinin and pepstatin A with ten 10 s bursts of a polytron at half maximum speed. The homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant recentrifuged at 85000 × *g* for 90 min. The 85000 × *g* supernatant was subjected to $(NH_4)_2SO_4$ fractionation and the pellet from the 35–55% cut was resuspended in 25 ml of 20 mM Hepes, pH 7.4, 150 mM KCl, 1 mM $MgCl_2$ and 1 mM dithiothreitol (column buffer). The resuspended pellet was applied to a Sephacryl S-300 column (5 cm × 50 cm) equilibrated with the same buffer and eluted at a flow rate of 40 ml/h. The fractions containing Ins 1,3,4-P_3 kinase activity were pooled and applied to a DEAE-Sepharose column (1.6 cm × 15 cm) equilibrated with column buffer. The column was eluted with a 300 ml linear gradient of 150–600 mM KCl in column buffer, with Ins 1,3,4-P_3 kinase activity eluting at approx. 450 mM KCl.

2.3. *Ins 1,3,4-P_3 kinase assay*

The standard assay buffer for Ins 1,3,4-P_3 kinase activity consisted of 20 mM Hepes, 110 mM KCl, 10 mM NaCl, 6 mM $MgCl_2$, 5 mM ATP, 1.0 mM EGTA, 10 mM LiCl, 0.05% bovine serum albumin and 7.5 μM [^{3}H]Ins 1,3,4-P_3 (100 dpm/pmol) at pH 7.2. Assays were conducted at 37°C, initiated by the addition of enzyme and quenched by the addition of formic acid (0.1 M final concentration). Product was separated from substrate on minicolumns of Dowex AG1-X8 (0.5 cm × 0.5 cm) and quantified by liquid scintillation counting as previously described [13]. To determine the pH dependence, 20 mM Mes, 20 mM Mops and 20 mM Hepes were substituted for the 20 mM Hepes and the pH adjusted between 6 and 9. The effect of free Ca^{2+} on enzyme activity was performed by adding various amounts of $CaCl_2$ to the buffer and the resulting free Ca^{2+} concentration determined using a Ca^{2+}-sensitive electrode. Protein concentration was determined by the method of Bradford [14].

2.4. *Materials*

Ins 1,4,5-P_3 was obtained from Behring Diagnostics. [^{3}H]Ins 1,4,5-P_3 was from New England Nuclear. *myo*-[^{3}H]Inositol was obtained from American Radiochemicals. Unlabeled and tritiated Ins 1,3,4-P_3 were synthesized from Ins 1,4,5-P_3 using the purified 3-kinase [15] and Ins 1,3,4,5-P_4 5-phosphomonoesterase [7]. Dowex AG1-X8 was from Bio-Rad. DEAE-Sepharose and Sephacryl S-300 were from Pharmacia. All other reagents were from Sigma Chemicals or Fisher Scientific.

3. RESULTS AND DISCUSSION

In the presence of ATP, addition of 7.5 μM [^{3}H]Ins 1,3,4-P_3 to a crude liver homogenate resulted in the formation of an $InsP_4$, as first reported by Shears et al. [12]. Initial examination of this $InsP_4$ isomer by HPLC, using a previously published elution protocol [13], indicated that it comigrated with Ins 1,3,4,5-P_4. However, as the pH of the mobile phase was increased from 3.7 to 4.8, its retention time increased relative to that for Ins 1,3,4,5-P_4. Using the modified HPLC elution method, two $InsP_4$ isomers were detected in vasopressin-stimulated hepatocytes. These corresponded to Ins 1,3,4,5-P_4 and the $InsP_4$ produced in cell homogenates from Ins 1,3,4-P_3. As shown in fig.1, Ins 1,4,5-P_3 accumulated very rapidly following the addition of vasopressin, peaking within 30 s. Increases of Ins 1,3,4,5-P_4 and Ins 1,3,4-P_3 were slightly slower, reaching their maximum values by 1 min. Increased formation of the new $InsP_4$ isomer was detectable within 30 s, but it accumulated much more slowly than the other inositol phosphates and continuously increased over the 15 min experimental period. This time course was consistent with the $InsP_4$ isomer originating from Ins 1,3,4-P_3. Total accumulation of the new $InsP_4$, assessed as cpm above control, was small, reaching at best 50% of that of Ins 1,3,4,5-P_4.

Lithium greatly enhances the agonist-induced accumulation of certain inositol phosphates, particularly Ins 1,3,4-P_3, by inhibiting at least 2 enzymes, inositol monophosphate 1-phosphomonoesterase [17] and inositol polyphosphate 1-phosphomonoesterase [16]. Fig.2 shows that there was

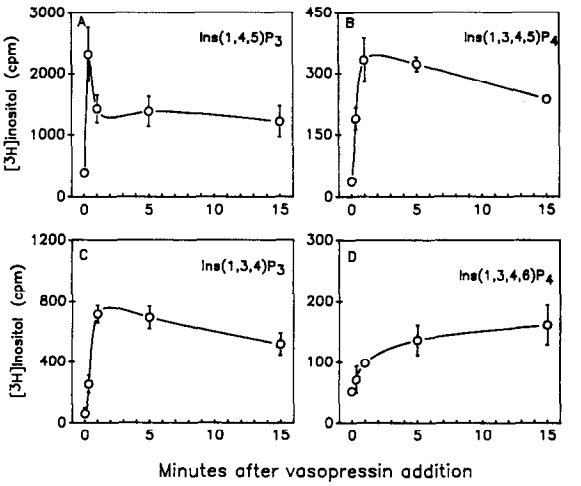

Fig.1. Time course of inositol phosphate accumulation in *myo*-[^{3}H]inositol prelabeled hepatocytes following addition of 40 nM vasopressin. 10 mg dry wt of cells were quenched by addition of perchloric acid (4% final) [13] at the above times after vasopressin addition. Each point represents the mean ± SE of three separate experiments. The assignment of $InsP_4$ as Ins 1,3,4,6-P_4 is based on the studies of others [11,12].

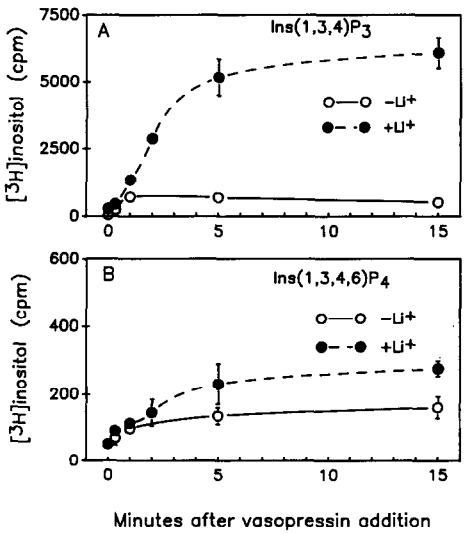

Fig.2. Effect of LiCl on the time course of Ins 1,3,4-P_3 and Ins 1,3,4,6-P_4 accumulation in *myo*-[^{3}H]inositol prelabeled hepatocytes following addition of 40 nM vasopressin. Cells were preincubated for 10 min with 10 mM LiCl. 10 mg dry wt of cells were quenched by addition of perchloric acid (4% final) [13] at the above times after vasopressin addition. Each point represents the mean ± SE of three separate experiments. The assignment of $InsP_4$ as Ins 1,3,4,6-P_4 is based on the studies of others [11,12].

little effect of LiCl on the accumulation of the new $InsP_4$ isomer, despite a ten-fold increase in its precursor, Ins 1,3,4-P_3. Since the partially purified Ins 1,3,4-P_3 kinase was not affected by LiCl (see below), these data suggest that the concentration of Ins 1,3,4-P_3 produced in vasopressin-stimulated hepatocytes, approx. 2 μM [1], was near saturating for the Ins 1,3,4-P_3 kinase.

The Ins 1,3,4-P_3 kinase was partially purified using the steps summarized in table 1. This procedure resulted in a 50-fold increase in its specific activity and the elimination of the Li^+-insensitive Ins 1,3,4-P_3 4-phosphomonoesterase activity. Remaining Ins 1,3,4-P_3 1-phosphomonoesterase activity was prevented by the addition of 10 mM LiCl, which had no effect on the Ins 1,3,4-P_3 kinase activity.

The relationship of Ins 1,3,4-P_3 concentration to the reaction velocity for the Ins 1,3,4-P_3 kinase is shown in fig.3. From 3 separate preparations of enzyme, the K_m for Ins 1,3,4-P_3 was 0.29 ± 0.06 μM (mean ± SE) and the K_m for ATP, in the presence of 7.5 μM Ins 1,3,4-P_3 and 5 mM $MgCl_2$, was 141 ± 11 μM. Chelation of Mg^{2+} by excess EDTA completely inhibited $InsP_4$ formation, indicating that MgATP was the actual substrate. The enzyme exhibited a broad pH profile, with the highest activity expressed between pH 6.6 and 7.5 (not shown).

Unlike the Ca^{2+}/calmodulin activated Ins 1,4,5-P_3 3-kinase, Ins 1,3,4-P_3 kinase activity was not affected by increasing free Ca^{2+} concentration over the physiological range (10^{-8} to 10^{-5} M). As

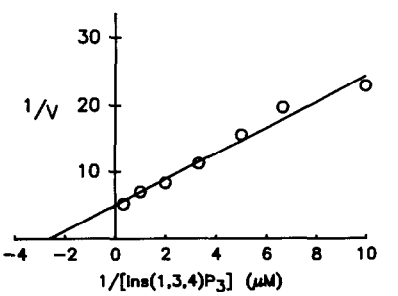

Fig.3. Representative double reciprocal plot of the substrate velocity relationship of the partially purified Ins 1,3,4-P_3 kinase. Assays were carried out as described in section 2, except the concentration of substrate, Ins 1,3,4-P_3, was varied. Units of velocity were fmol/min per mg protein.

Table 1

Partial purification of Ins 1,3,4-P_3 kinase from rat liver

Purification step	Total activity (pmol/min)	Total protein (mg)	Specific activity (pmol/min per mg)	Recovery (%)	Purification (-fold)
85000 × *g* supernatant	9300	7280	1.3	100	1
35–55% $(NH_4)_2SO_4$ fraction	7250	2630	2.8	78	2.2
Sephacryl S-300	940	56	16.8	10	12.9
DEAE-Sepharose	322	5	64.4	4	49.5

the free Ca^{2+} concentration was further increased, the activity of the enzyme decreased, with a 50% inhibition occurring at approx. 500 μM free Ca^{2+}. This pattern of inhibition by Ca^{2+} is similar to that observed with the purified platelet [18] and brain [7] soluble 5-phosphomonoesterases. Furthermore, as shown in separate experiments, the Ins 1,3,4-P_3 kinase did not bind to a calmodulin affinity column in the presence of Ca^{2+}, while most of the Ins 1,4,5-P_3 kinase activity, present in the same sample, was retained by the matrix and eluted only upon removal of Ca^{2+} by excess EGTA.

The new $InsP_4$ isomer formed in this study is most likely Ins 1,3,4,6-P_4, since it has been shown to be the product of Ins 1,3,4-P_3 phosphorylation in liver [12] and adrenal glomerulosa cell homogenates [11]. The demonstration that its accumulation is agonist-sensitive not only in adrenal glomerulosa cells [10,19], but also in liver, suggests that enhanced formation of Ins 1,3,4,6-P_4 is a general response to stimulation by Ca^{2+}-mobilizing agonists. Cells possess at least two routes for dephosphorylation of Ins 1,3,4-P_3, both with substantially greater capacity than this phosphorylation pathway, making it an unlikely disposal route for Ins 1,3,4-P_3. Balla et al. [11] state that Ins 1,3,4,6-P_4 can be converted to $InsP_5$ by adrenal glomerulosal cell extracts. Phosphorylation of Ins 1,3,4-P_3 to Ins 1,3,4,6-P_4 may thus represent an important route for synthesis of higher phosphorylated inositol phosphates. Similarly, it may be involved in coordinating the functions of the inositol phosphates of the Ca^{2+}-mobilizing signal transduction pathway with the functions of the more slowly metabolized higher phosphorylated inositol phosphates, which may also have physiological signaling roles [20].

Acknowledgements: This work was supported by NIH grant DK-15120 (J.R.W.) and DK-19525 (C.A.H.). C.A.H. is a recipient of NRSA HL-07146.

REFERENCES

[1] Williamson, J.R. and Hansen, C.A. (1986) in: Biochemical Actions of Hormones (Litwack, G. ed.) vol.14, pp.29–50, Academic Press, New York.
[2] Putney, J.W., jr (1987) Am. J. Physiol. 252, G149–G157.
[3] Biden, T.J., Comte, M., Cox, J.A. and Wollheim, C.B. (1987) J. Biol. Chem. 262, 9437–9440.
[4] Yamaguchi, K., Hirata, M. and Kuriyama, H. (1987) Biochem. J. 244, 787–791.
[5] Rhu, S.H., Lee, S.Y., Lee, K.-Y. and Rhee, S.G. (1987) FASEB J. 1, 388–393.
[6] Connolly, T.M., Bansal, V.S., Bross, T.E., Irvine, R.F. and Majerus, P.W. (1987) J. Biol. Chem. 262, 2146–2149.
[7] Hansen, C.A., Johanson, R.A., Williamson, M.T. and Williamson, J.R. (1987) J. Biol. Chem. 262, 17319–17326.
[8] Inhorn, R.C., Bansal, V.S. and Majerus, P.W. (1987) Proc. Natl. Acad. Sci. USA 84, 2170–2174.
[9] Bansal, V.S., Inhorn, R.C. and Majerus, P.W. (1987) J. Biol. Chem. 262, 9444–9447.
[10] Balla, T., Guillemette, G., Baukal, A.J. and Catt, K.J. (1987) J. Biol. Chem. 262, 9952–9955.
[11] Balla, T., Guillemette, G., Baukal, A.J. and Catt, K.J. (1987) Biochem. Biophys. Res. Commun. 148, 199–205.
[12] Shears, S.B., Parry, J.B., Tang, E.K.Y., Irvine, R.F., Michell, R.H. and Kirk, C.J. (1987) Biochem. J. 246, 139–147.
[13] Hansen, C.A., Mah, S. and Williamson, J.R. (1986) J. Biol. Chem. 261, 8100–8103.
[14] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[15] Johanson, R.A., Hansen, C.A. and Williamson, J.R. (1988) J. Biol. Chem. 263, in press.
[16] Inhorn, R.C. and Majerus, P.W. (1987) J. Biol. Chem. 262, 15946–15952.
[17] Hallcher, L.M. and Sherman, W.R. (1980) J. Biol. Chem. 255, 10896–10901.
[18] Connolly, T.M., Bross, T.E. and Majerus, P.W. (1985) J. Biol. Chem. 260, 951–958.
[19] Balla, T., Guillemette, G., Baukal, A.J. and Catt, K.J. (1988) J. Biol. Chem. 263, 4083–4091.
[20] Vallejo, M., Jackson, T., Lightman, S. and Hanley, M.R. (1987) Nature 330, 656–658.