Evidence for the formation of inositol 4-monophosphate in stimulated human platelets

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Received 6 April 1985

Human platelets were prelabeled with [^{3}H]inositol and exposed to thrombin or vasopressin. The radioactive inositol monophosphates were separated by high-performance liquid chromatography and identified by cochromatography with unlabeled standard substances. Radioactive inositol 1-monophosphate (Ins 1-P) and inositol 4-monophosphate (Ins 4-P) were detected in unstimulated platelets and accumulated in response to thrombin or vasopressin. Ins 4-P as well as Ins 1-P increased after the formation of inositol 1,4-bisphosphate (Ins 1,4-P_2) and inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3). Lithium augmented the accumulation of Ins 1-P and Ins 1,4-P_2 in stimulated platelets, and also of Ins 4-P in platelets stimulated by vasopressin, but not by thrombin. The results indicate that Ins 1,4-P_2 formed in stimulated platelets is partly degraded to Ins 4-P. The significance of Ins 4-P as a marker molecule for the study of inositol phosphate metabolism in stimulated cells is discussed.

Platelet activation *Thrombin* *Vasopressin* *Inositol phosphate* *Lithium*

1. INTRODUCTION

Ins 1,4,5-P_3 and 1,2-diacylglycerol formed during receptor-activated breakdown of PtdIns 4,5-P_2 may serve as second messengers for release of Ca^{2+} from intracellular stores and for protein phosphorylation [1–3]. As expected for an important second messenger, the enzymic formation and degradation of Ins 1,4,5-P_3 might be tightly controlled. Recent enzymatic studies on rat liver have shown that a specific Ins 1,4,5-P_3 5-phosphatase is present in plasma membranes which degrades Ins 1,4,5-P_3 to Ins 1,4-P_2 [4,5]. Unspecific phosphatase activities present in the cytosol metabolise Ins 1,4-P_2 further to Ins 1-P and Ins 4-P, respectively [4,5]. Besides a very recent study which describes small quantities of Ins 4-P in the brains of Li^+-treated rats [6], only Ins 1-P has been found among the inositol monophosphates which increase following receptor activation of intact cells or tissues (for references see [7]). Ins 1-P may, however, also derive from PtdIns hydrolysis by phospholipase C.

We recently observed the formation of two [^{3}H]inositol monophosphates in thrombin-activated platelets which cochromatographed on descending paper chromatography and on HPLC with Ins 1-P and Ins 2-P [8]. By establishing an HPLC method which separates Ins 1-P, Ins 2-P and Ins 4-P, this study describes that besides Ins 1-P and trace amounts of Ins 2-P also Ins 4-P accumulates considerably in activated platelets.

Abbreviations: Ins 1-P, inositol 1-monophosphate; Ins 2-P, inositol 2-monophosphate; Ins 4-P, inositol 4-monophosphate; Ins 1,4-P_2, inositol 1,4-bisphosphate; Ins 1,4,5-P_3, inositol 1,4,5-trisphosphate; PtdIns, phosphatidylinositol; PtdIns 4-P, phosphatidylinositol 4-monophosphate; PtdIns 4,5-P_2, phosphatidylinositol 4,5-bisphosphate; HPLC, high-performance liquid chromatography

2. MATERIALS AND METHODS

2.1. *Materials*

LiChrosorb^RNH_2 (5 μm) columns for HPLC

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were obtained from Merck (Darmstadt). μBondapakNH$_2$ (30 × 0.39 cm) columns for HPLC were purchased from Waters Assoc. (Milford, MA). All other materials were obtained as described [8,9].

2.2. *Measurement of [^{3}H]inositol phosphates in human platelets*

Platelet-rich plasma from 50 ml blood was centrifuged after addition of prostacyclin (300 ng/ml) and the platelets were resuspended in 1 ml of Tyrode-Hepes buffer containing 1 mM $MgCl_2$, 1 mM EGTA, apyrase (100 μg), prostacyclin (2 μg) and 2 mCi [^{3}H]inositol. The platelet suspension ($2-2.5 \times 10^9$ platelets per ml) was incubated at 37°C for 4 h in a shaking water bath. Platelets were then pelleted by centrifugation at 800 × *g* and washed once in 8 ml Tyrode-Hepes buffer containing inositol (5 mM), $CaCl_2$ (0.1 mM), $MgCl_2$ (1 mM), heparin (25 units/ml), apyrase (200 μg/ml), and prostacyclin (300 ng/ml). After centrifugation, platelets were resuspended in 1.5–2 ml Tyrode-Hepes buffer containing $CaCl_2$ (0.1 mM), $MgCl_2$ (1 mM) and apyrase (200 ng/ml). The platelet concentration was adjusted to $1-1.3 \times 10^9$ per ml. The platelet suspension was kept at 37°C for 60 min to reach equilibrium between the free [^{3}H]inositol and the [^{3}H]inositol incorporated into platelet phosphoinositides. $LiCl_2$ (10 mM final concentration) was added 60 min [10] before addition of the platelet stimulus.

Samples of platelet suspension (1.0 ml) were placed into aggregometer tubes and platelets were stirred for 3 min before exposure to thrombin (2 U/ml) or vasopressin (1 μM). Aliquots (0.2 ml) were transferred at various times into 0.75 ml of chloroform/methanol/conc. HCl (100:200:2). After addition of a mixture of unlabeled Ins 1-P, Ins 2-P, Ins 4-P, Ins 1,4-P$_2$ and Ins 1,4,5-P$_3$, the aqueous soluble inositol phosphates were extracted [9] and the samples were dried at room temperature under a stream of nitrogen.

The preparation of Ins 1-P, Ins 1,4-P$_2$ and Ins 1,4,5-P$_3$ has been described [9]. Ins 4-P was also prepared by alkaline hydrolysis of phosphoinositides [11]. Phosphoinositides (100 mg) were dissolved in 5 ml of 2 N KOH and refluxed for 30 min. Inositol phosphates were then separated by paper chromatography and eluted with water [8,9,11,12]. The inositol monophosphate band contains mainly Ins 4-P, less Ins 1-P [11] and also traces of Ins 2-P (see below).

Inositol monophosphates were separated from inositol bis- and trisphosphate by Dowex anion-exchange chromatography [8]. The dry platelet sample was dissolved in 3 ml H_2O, neutralised by addition of cyclohexylamine and applied to Dowex 1 × 8 columns (1 ml, formate form). Fraction I (30 ml H_2O) was discarded; fraction II containing inositol monophosphates (30 ml of 200 mM ammonium formate, 100 mM formic acid) was passed through Dowex HCR-W2 columns to remove NH_4^+ [8]. The eluates were then dried at 40°C by rotoevaporation. The inositol monophosphates were further separated on new HPLC columns conditioned as described [9].

I used either a Lichrosorb^{R}NH$_2$ (5 μm) or a μBondapak^{R}NH$_2$ column. Separation was carried out utilizing a 20 min isocratic elution with 50 mM ammonium acetate/acetic acid buffer, pH 4.0 (solvent A), followed by a 60 min linear gradient to 50% of solvent B (2 M ammonium acetate/acetic acid, pH 4.0) at a flow rate of 1 ml/min. Fractions were collected every 0.5 min and measured for radioactivity by liquid scintillation counting or for phosphorus [13].

In experiments in which in addition radioactive Ins 1,4-P$_2$ and Ins 1,4,5-P$_3$ were measured, the dry platelet samples were dissolved in 200 μl solvent A and directly applied to HPLC [9]. Separation was carried out utilizing a 10 min isocratic elution with solvent A, followed by a 30 min linear gradient to 25% solvent B, a 20 min linear gradient to 100% solvent B and a 20 min isocratic elution with solvent B at a flow rate of 1 ml/min. Fractions were collected every 1 min (from 1 to 25 min), every 0.5 min (from 26 to 40 min) or every 2 min (from 41 to 80 min), and measured for radioactivity or for phosphorus.

3. RESULTS

An HPLC method was developed which separates 3 inositol monophosphate isomers: Ins 1-P, Ins 2-P and Ins 4-P (fig.1). Both HPLC columns tested (the LiChrosorb^{R}NH$_2$ and the μBondapak^{R}NH$_2$ columns) could be used for the separation of the 3 inositol monophosphates. The resolution of Ins 2-P and Ins 4-P decreased,

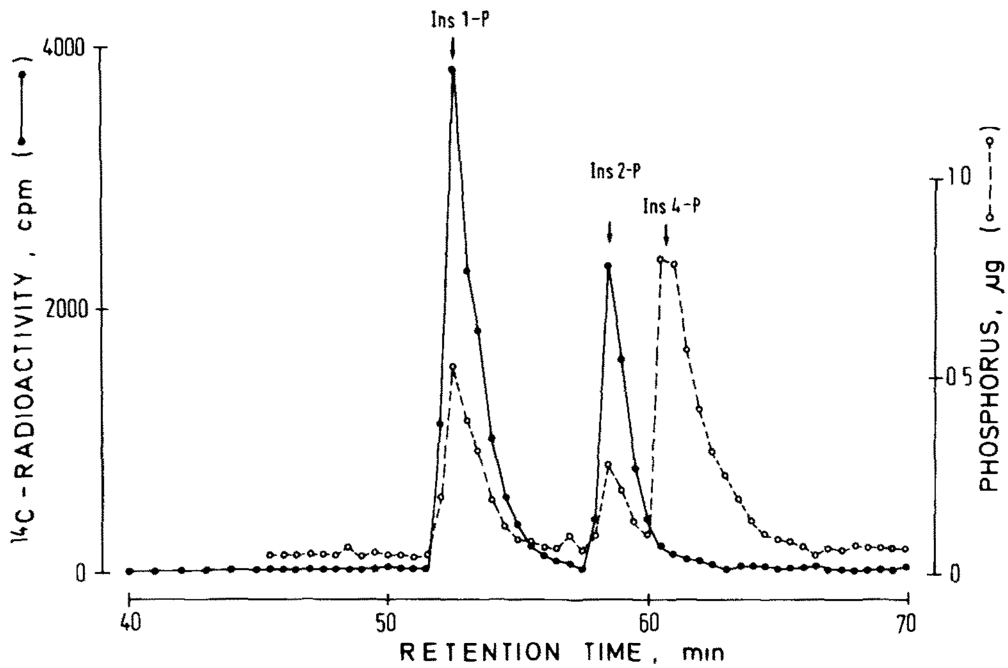


Fig.1. Separation of inositol monophosphate standards by HPLC. ^{14}C-labeled Ins 1-P and Ins 2-P (●——●) produced by acidic hydrolysis of [^{14}C]PtdIns [9,14] were mixed with unlabeled Ins 1-P, Ins 2-P and Ins 4-P (○---○) produced by alkaline hydrolysis of phosphoinositides [11] and applied to a LiChrosorb^{R}NH$_2$ column. For details see section 2.

however, with longer use of the columns. It was observed that only HPLC could reliably resolve Ins 2-P from Ins 4-P. On descending paper chromatography, using as solvent either isopropyl alcohol/ammonia/water (70:10:20) at 40°C [14] or ethanol/13.5 M NH_3 (3:2) [15], and on silica gel glass fiber sheets [16] no separation of Ins 2-P from Ins 4-P could be achieved (unpublished).

The method was applied to measure [^{3}H]inositol monophosphates in human platelets which were prelabeled with [^{3}H]inositol. Two major [^{3}H]inositol monophosphate peaks on HPLC were found which cochromatographed exactly with unlabelled Ins 1-P and Ins 4-P, respectively. Both compounds were detected in small amounts in unstimulated platelets and accumulated following platelet exposure to thrombin (figs 2,3) or vasopressin (table 1). In addition, a minor radioactive peak on HPLC was detected, which may correspond to Ins 2-P (fig.2).

Thrombin (2 U/ml) as the stronger platelet stimulus induces a larger accumulation of inositol phosphates in human platelets than vasopressin (2 μM) (table 1). Interestingly, thrombin stimulates the accumulation of Ins 1-P more than that of Ins 4-P. Vasopressin seems to increase Ins 1-P and Ins 4-P to similar levels.

Kinetic studies showed that Ins 1-P and Ins 4-P accumulate later than Ins 1,4-P_2 and Ins 1,4,5-P_3 following platelet exposure to thrombin (fig.3) and vasopressin (unpublished).

Li^+ has a profound effect on the accumulation of inositol phosphates in human platelets. In unstimulated platelets, Li^+ increased the levels of Ins 1-P and Ins 4-P but not of Ins 1,4-P_2. In platelets stimulated by thrombin, Li^+ augmented the accumulation of Ins 1-P and Ins 1,4-P_2, but not of Ins 4-P. Ins 4-P levels seemed to be even reduced in the presence of Li^+. In platelets stimulated by vasopressin, Li^+ augmented the accumulation of Ins 1-P, Ins 4-P and Ins 1,4-P_2. Li^+ had no effect on the accumulation of Ins 1,4,5-P_3 in resting or stimulated human platelets (fig.3, table 1).

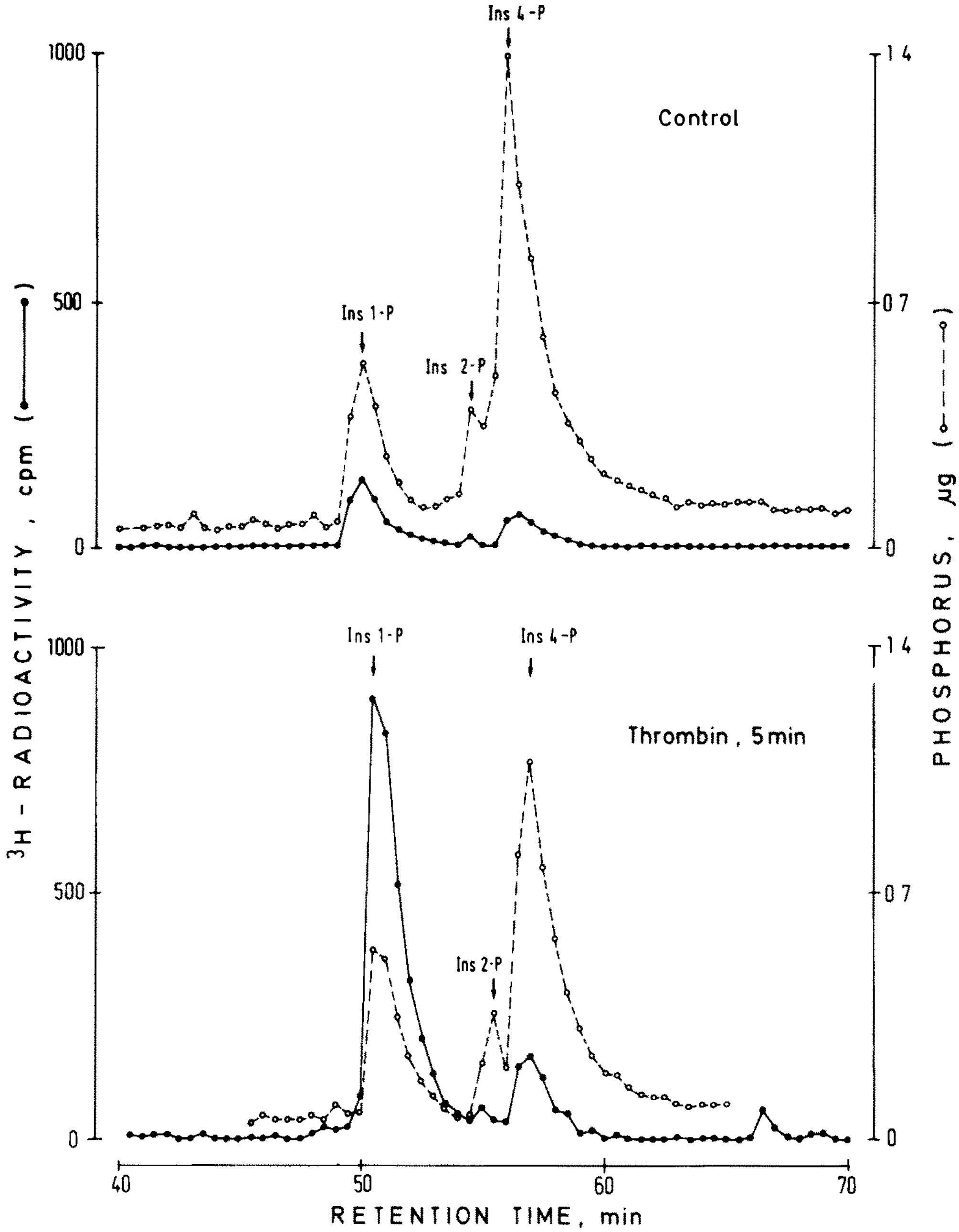
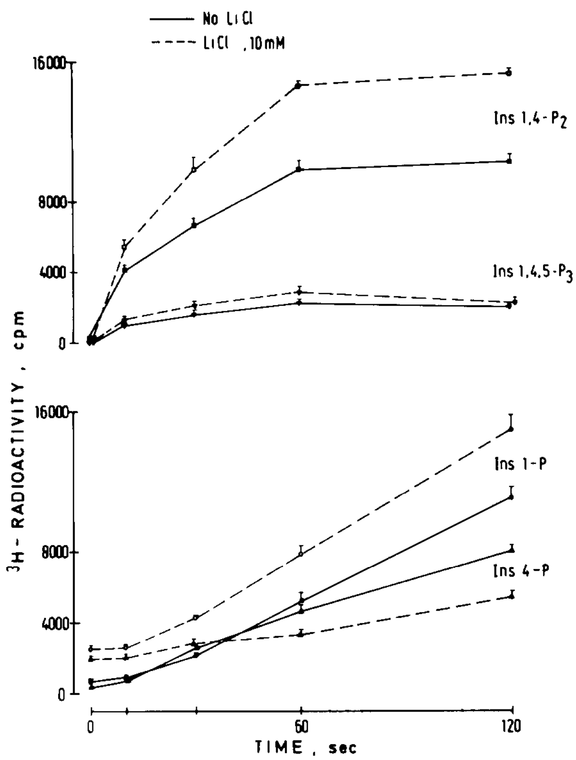


Fig.2. HPLC separation of [^{3}H]inositol monophosphates in control and thrombin-stimulated platelets. Human platelets prelabeled with [^{3}H]inositol were exposed to saline or thrombin (2 U/ml) while stirring (1100 rpm) for 5 min at 37°C. The aqueous soluble [^{3}H]inositol phosphates were extracted [9], unlabeled inositol monophosphate standards were added, and the inositol monophosphate fraction obtained by Dowex anion-exchange chromatography was further separated on HPLC. The fractions were measured for radioactivity of the platelet [^{3}H]inositol phosphates (●——●) and for phosphorus (○---○) of the added inositol monophosphate standards.



← Fig.3. Time course of formation of [^{3}H]inositol phosphates in thrombin-stimulated platelets in the presence (– – –) or absence (——) of Li^+. Human platelets prelabeled with [^{3}H]inositol were kept at 37°C for 60 min in buffer with (open symbols, dashed line) or without (filled symbols, solid line) LiCl (10 mM). Platelets were then exposed to thrombin (2 U/ml) for various times. The aqueous soluble inositol phosphates were extracted, unlabeled inositol phosphate standards were added, and the inositol phosphates were separated by HPLC (see [9] and section 2).

4. DISCUSSION

This study describes the presence of a [^{3}H]inositol monophosphate in human platelets which can be separated from Ins 1-P and Ins 2-P and cochromatographs with Ins 4-P on HPLC. The cochromatography with an authentic standard gives evidence for the formation of [^{3}H]Ins 4-P, but does not prove its structure. [^{3}H]Ins 4-P is present in resting platelets and accumulates in platelets activated by thrombin or vasopressin. Ins 4-P, similar to Ins 1-P increases after a time lag of 10–15 s and after the formation of Ins 1,4-P_2 and Ins 1,4,5-P_3 in platelets stimulated by thrombin (fig.3) or vasopressin (unpublished).

Ins 4-P may serve as unique marker molecule for the study of inositol phosphate metabolism in stimulated cells. Among the inositol phosphates detected in stimulated human platelets [8–10,17], Ins 4-P is the only compound which cannot be formed by phospholipase C-induced hydrolysis of phosphoinositides. Ins 4-P most probably derives from degradation of Ins 1,4-P_2 by the action of non-specific soluble phosphatases [4]. Ins 1,4-P_2 is

Table 1

Effect of Li^+ on the accumulation of inositol phosphates in human platelets induced by thrombin or vasopressin

Addition	LiCl (10 mM)	^{3}H radioactivity (cpm)			
		Ins 1-P	Ins 4-P	Ins 1,4-P_2	Ins 1,4,5-P_3
None	–	473	564	264	22
	+	3390	2427	282	10
Thrombin (4 U/ml)	–	11560	6824	3564	521
	+	24080	5799	14480	584
Vasopressin (2 μM)	–	1272	1384	596	160
	+	11886	7095	2510	204

Platelets prelabeled with [^{3}H]inositol were incubated for 60 min at 37°C with or without 10 mM LiCl. Stimuli were added for 4 min. Inositol phosphates were separated by HPLC (see section 2) formed either by phospholipase C-induced breakdown of PtdIns 4-P or from Ins 1,4,5-P_3 by the action of a specific Ins 1,4,5-P_3 5-phosphatase [4,5,8,18]. According to the latter pathway Ins 4-P might ultimately derive from Ins 1,4,5-P_3. The presence of Ins 4-P in stimulated platelets indicates that the inositol monophosphates found in stimulated cells may not only derive from PtdIns hydrolysis but also from the stepwise degradation of inositol polyphosphates [4].

The observed effects of Li^+ on the accumulation of inositol phosphates are in agreement with enzymatic studies in rat liver [4,5,18] and with studies of thrombin-stimulated human platelets [17] and muscarinic receptor activation in rat brain and parotid gland [19,20]. They differ, however, sharply from results obtained in rabbit platelets [10]. Here, Li^+ augmented markedly the accumulation of Ins 1-P and Ins 1,4-P_2 in stimulated platelets which is explained by the sensitivity of the responsible phosphatase activities to Li^+ [4]. Accumulation of Ins 4-P is augmented in vasopressin- but not in thrombin-stimulated platelets. Since vasopressin is a weaker platelet agonist and induces less accumulation of Ins 4-P than thrombin, it seems that the degradation of Ins 4-P is only inhibited by Li^+, if the Ins 4-P levels are low. The accumulation of Ins 1,4,5-P_3 in stimulated platelets was barely affected by Li^+ which corresponds to the insensitivity of the Ins 1,4,5-P_3 5-phosphatase to Li^+ [4,5,18]. The observation that Li^+ induces an accumulation of Ins 1-P and Ins 4-P in non-stimulated platelets may indicate that both inositol monophosphates are produced and degraded in resting platelets. Interestingly, Li^+ does not induce an accumulation of Ins 1,4-P_2 in resting platelets.

The results reveal interesting differences in the action of thrombin and vasopressin on the accumulation of inositol phosphates in human platelets. Thrombin induces a higher increase of Ins 1-P than of Ins 4-P whereas vasopressin stimulates Ins 1-P and Ins 4-P levels to a similar degree. Since according to enzymatic studies the phosphatase activities remove the 1- and 4-phosphate groups of Ins 1,4-P_2 at similar rates, the similar levels of Ins 1-P and Ins 4-P in vasopressin-stimulated platelets could indicate that both compounds are formed entirely from Ins 1,4-P_2. In contrast, the relatively higher accumulation of Ins 1-P compared to Ins 4-P in thrombin-activated platelets may lead to the conclusion that PtdIns is also hydrolysed. The results show that the detection and the determination of Ins 4-P gives further insights into the metabolism of inositol phosphates and inositol phospholipids in stimulated cells.

ACKNOWLEDGEMENT

I thank Ingeborg Drössel for the careful preparation of the manuscript.

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