Thrombin induces the rapid formation of inositol bisphosphate and inositol trisphosphate in human platelets

Wolfgang Siess and Helge Binder

Medizinische Klinik Innenstadt der Universitat, Ziemssenstr 1, 8000 Munchen 2, FRG

Received 9 November 1984

Human platelets prelabeled with [^{3}H]inositol were exposed to thrombin The aqueous soluble inositol phosphates were separated by anion exchange column chromatography, paper chromatography or high-performance liquid chromatography, and identified by cochromatography with authentic standard substances Thrombin immediately induces the rapid formation of inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate Accumulation of inositol-1-monophosphate and inositol-2-monophosphate occurs later after a time lag of 10 sec The results indicate that the phospholipase C induced polyphosphoinositide hydrolysis rather than the phosphatidylinositol hydrolysis is the triggering event for platelet activation, and support the concept of inositol 1,4,5-trisphosphate as putative second messenger

Platelet activation *Thrombin* *Phospholipase C* *Inositol phosphate* *Inositol phospholipid*

1. INTRODUCTION

Physiological platelet stimuli such as thrombin, collagen, ADP, platelet-activating factor, arachidonic acid and prostaglandin endoperoxides induce in platelets the degradation of inositol containing phospholipids [1–6]. This reaction, which is catalysed by phospholipase C, leads to two putative second messengers: inositol 1,4,5-trisphosphate, which can mobilise Ca^{2+} from non-mitochondrial pools, and 1,2-diacylglycerol, which stimulates protein phosphorylation by protein kinase C activation [7–9]. Both molecules have been found in stimulated platelets, and they may act synergistically to induce the physiological platelet response [2,10,11]. Recently, we have shown the close association of phospholipase C activation with each physiological platelet response, i.e., platelet shape change, release reaction and aggregation [5,6,12–15]. It is not clear which of the three inositol phospholipids – PtdIns, PtdIns4P or PtdIns4,5P_2 – is degraded initially by phospholipase C upon receptor activation [16]. Following recent studies on platelets and other cell types it seems that PtdIns4,5P_2 rather than PI is hydrolysed ([10,17–19]; for review see [7]). To address this question further, we measured the formation of the respective inositol phosphates in human platelets upon activation with thrombin.

Abbreviations: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns4,5P_2, phosphatidylinositol 4,5-bisphosphate; HPLC, high-performance liquid chromatography

2. MATERIALS AND METHODS

2.1. *Materials*

L-*myo*[2-^{3}H]inositol was purchased from NEN, and L-3-phosphatidyl[U-^{14}C]inositol and L-*myo*[U-^{14}C]inositol-1-phosphate were from Amersham. L-α-phosphatidylinositol, L-α-phosphatidylinositol 4-monophosphate, L-α-phosphatidylinositol 4,5-bisphosphate, phosphoinositides, L-α-glycerophosphate, Dowex 1 × 8 (chloride form 100–200 mesh), Dowex-HCR-W2, myoinositol 2-monophosphate and phospholipase C from *Bacillus cereus* were all obtained from Sigma. Equipment and columns for HPLC were from Waters.

Published by Elsevier Science Publishers B V (Biomedical Division)
00145793/85/$3 30

2.2. *Preparation of inositol phosphate standards*

The alkaline hydrolysis of phosphoinositides as described by Grado and Ballou [20] leads to a mixture of two isomers of inositol trisphosphate and of inositol bisphosphate which are separated by descending paper chromatography [21]. By comparison with the work of Ballou and colleagues [20,22] and Irvine et al. [23], these compounds are identified as inositol 1,4,5-trisphosphate (slow-running), inositol 2,4,5-trisphosphate (fast-running), inositol 1,4-bisphosphate (slow-running) and inositol 4,5-bisphosphate (fast-running). Inositol 1,4,5-trisphosphate and inositol 1,4-bisphosphate were also obtained by alkaline hydrolysis of authentic PtdIns4,5P_2 and PtdIns4P. Glycerophosphoinositol was prepared by mild alkaline hydrolysis from phosphatidylinositol [24]. Inositol 1,2-cyclic monophosphate was obtained by incubation of phosphatidylinositol with phospholipase C from platelet cytosol or from *B. cereus* [25,26]. Inositol 1-monophosphate and inositol-2-monophosphate were obtained by heating inositol 1,2-cyclic monophosphate at 100°C for 15 min in 0.1 N HCl [27].

2.3. *Preparation of platelets labeled with [^{3}H]inositol*

Platelet rich plasma was prepared from human blood (100 ml) anticoagulated with acidic citrate/dextrose [12]. Prostacyclin (300 ng/ml) was added, platelets were centrifuged and resuspended in Tyrode–Hepes buffer [12] at a platelet concentration of 2–2.5 × 10^9/ml. [^{3}H]Inositol (1 mCi) and $MnCl_2$ (3 mM) were added, and platelet suspensions were incubated in aggregometer tubes at 37°C for 90 min while stirring (250 rpm). PGE_1 (1 μg/ml) and EGTA (5 mM) were added, and after further 30 min of incubation the platelets were centrifuged and washed once in Tyrode-Hepes buffer containing 5 mM unlabeled inositol, 1 mM EGTA, 10% of plasma and prostacyclin (300 ng/ml). Platelets were finally resuspended in 10–12 ml of Tyrode-Hepes buffer containing 1 mM EGTA.

Samples of platelet suspensions (0.5 ml) were placed in aggregometer tubes, LiCl (10 mM) was added, and platelets were stirred for 2 min before exposure to thrombin (2 U/ml). Shape change and aggregation were recorded. Reactions were stopped by transferring the samples into 1.8 ml of chloroform/methanol, 1:2. Since endogenously formed inositol phosphates including inositol trisphosphate were recovered at the same rate following extraction at acidic or neutral pH, aqueous soluble inositol phosphates were extracted at neutral pH [28] in order to avoid degradation of inositol 1,2-cyclic monophosphate [27]. The samples were then frozen, lyophilised and stored at −20°C.

2.4. *Separation of inositol phosphates*

Anion exchange column chromatography on Dowex 1 × 8 columns (1 ml) was used as first step to separate the inositol phosphates. Dowex in chloride form was converted into the formate form with 8 ml of 5% formic acid and rinsed with 25 ml of H_2O. The lyophilised platelet samples were dissolved in 1 ml of H_2O and a mixture of unlabeled inositol phosphate standards (inositol 1,4,5-trisphosphate, inositol 1,4-bisphosphate, glycerophosphoinositol, inositol 1-phosphate, inositol 2-phosphate) was added. If the samples were separated subsequently on HPLC, two fractions were eluted: Inositol and some glycerophosphoinositol in 30 ml of H_2O and inositol phosphates in 14 ml of 1 M ammonium formate, 100 mM formic acid. If the samples were separated subsequently by paper chromatography, the following fractions were eluted [29]: fraction I (30 ml of H_2O); fraction II containing inositol monophosphates (20 ml of 200 mM ammonium formate, 100 mM formic acid); fraction III containing inositol bisphosphate (20 ml of 400 mM ammonium formate, 100 mM formic acid); fraction IV containing inositol trisphosphate (12 ml of 1 M ammonium formate, 100 mM formic acid). In order to remove the ammonium ions the eluates were passed through Dowex HCR-W2 columns (10 ml) followed by rinsing with 50 ml of H_2O. The eluates were then dried at 40°C by rotoevaporation ex vacuo and further separated either by descending paper chromatography [20,21] or by HPLC. Separation by HPLC was carried out on a μBondapak NH_2-column (3.9 mm × 30 cm) utilising a 20 min isocratic elution with 75 mM ammonium acetate/acetic acid followed by a 120 min linear gradient to 2 M ammonium acetate/acetic acid, pH 4.0, at a flow rate of 1 ml/min. Fractions were collected, split into two halves and either measured for ^{3}H-radioactivity by liquid scintillation counting or for

phosphorus [30]. Inositol phosphates in samples separated by paper chromatography were visualised by an ammonium molybdate spray [25]. The respective zones were cut out, eluted with 1 ml of 0.1 N NaOH and 1 ml of H_2O and measured for ^3H-radioactivity by liquid scintillation counting.

2.5. *Separation of inositol phospholipids*

Inositol phospholipids were extracted at acidic pH [19] and separated on thin layer plates impregnated with 1% potassium oxalate [31].

3. RESULTS AND DISCUSSION

The addition of thrombin to human platelets prelabeled with [^3H]inositol induces the formation of four radioactive inositol phosphates: inositol 1-monophosphate, inositol 2-monophosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate. These products were identified by cochromatography with authentic standards on HPLC or paper chromatography (fig.1,2). Both HPLC and paper chromatography separated isomers of inositol mono-, bis- and trisphosphate. Since the radioactive inositol phosphates formed in stimulated platelets cochromatographed with authentic standards in both chromatographic systems, it is evident that the formed inositol phosphates have the structures indicated above. One has to be aware, however, that the identification of any structure which is based solely on cochromatography of radioactivity with authentic compounds is not absolute [32].

We observed that for the measurement of the various inositol phosphates one could not rely on the Dowex anion exchange column chromatography, since a small percentage of the bulk of [^3H]inositol is eluted in the inositol monophosphate fraction (see also fig.1a), and more importantly, between 10 and 25% of the inositol bisphosphate is eluted in the inositol trisphosphate fraction (unpublished). Since the amount of the radioactive inositol bisphosphate formed is high if compared to the amount of inositol trisphosphate, the use of the Dowex column chromatography would lead to false high levels for inositol trisphosphate. We therefore used the Dowex column chromatography only as first purification step of inositol phosphates before their final separation by HPLC or paper chromatography. Recovery of

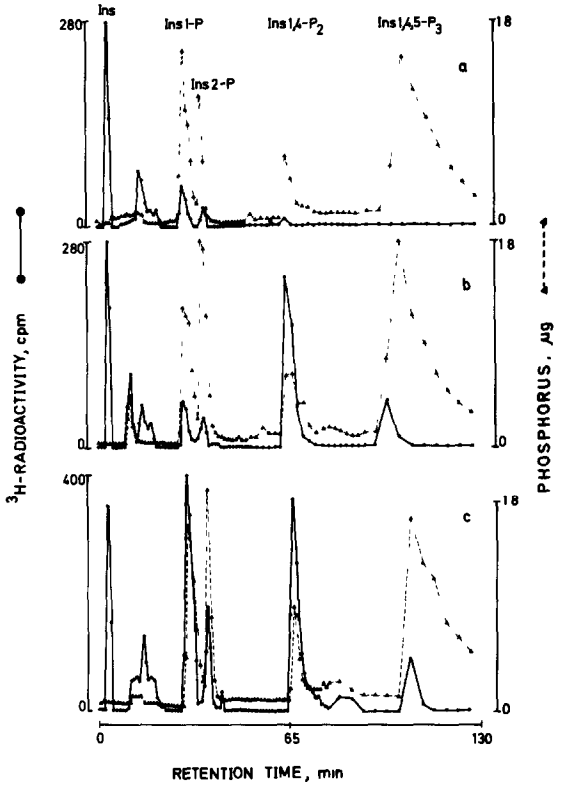

Fig 1. HPLC-separation of inositol phosphates formed in human platelets after exposure to thrombin. Human platelets were prelabeled with [^3H]inositol and either saline (a) or thrombin (2 U/ml) was added for 10 s (b) or 5 min (c). The aqueous soluble [^3H]inositol phosphates (●—●) were extracted, a mixture of unlabeled inositol phosphate standards (▲---▲) was added, and the samples were purified on Dowex column chromatography before separation on HPLC. For further details see section 2.

unlabeled inositol phosphate standards after Dowex column chromatography and HPLC was 76 ± 15% for inositol-1,4,5-trisphosphate, 66 ± 15% for inositol 1,4-bisphosphate, 69 ± 9% for inositol 1-monophosphate and 72 ± 8% for inositol 2-monophosphate (mean ± SD, n = 8).

The formation of inositol bisphosphate and inositol trisphosphate can be detected 5 s after addition of thrombin (fig.3). Inositol trisphosphate reaches its peak level at 10 s, whereas inositol bisphosphate accumulates further. In contrast, increased formation of both inositol 1-monophosphate and inositol 2-monophosphate is not detectable until 10 s after addition of thrombin. After

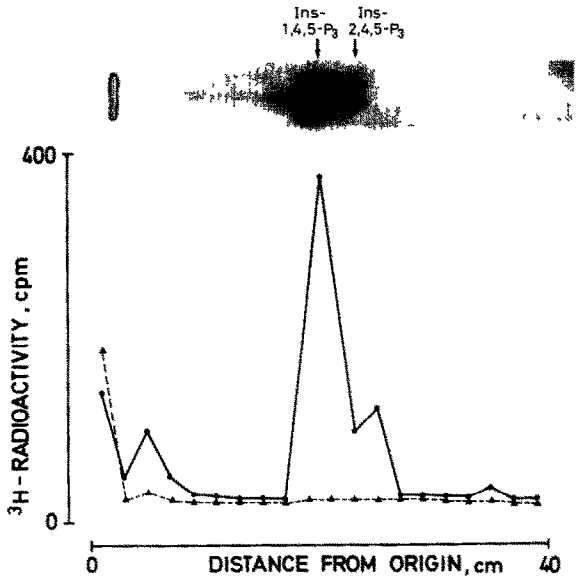

Fig 2. Formation of inositol 1,4,5-trisphosphate in human platelets after exposure to thrombin Human platelets were prelabeled with [^{3}H]inositol, and either saline (▲---▲) or thrombin (2 U/ml; ●——●) was added for 10 s. A mixture of inositol phosphates produced by alkaline hydrolysis of phosphoinositides [20] was added, pools of 5 samples were extracted and subjected to subsequent Dowex column chromatography [29] and descending paper chromatography [21]. The figure shows a radioactive profile on descending paper chromatography of the Dowex-fraction containing inositol trisphosphate (lower panel) and the cochromatography with unlabeled inositol trisphosphate standard substances (upper panel)

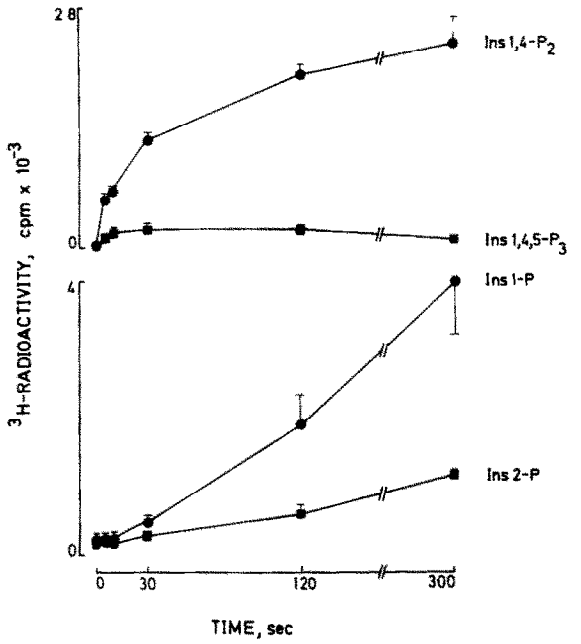

Fig 3. Time-course of inositol phosphate formation in human platelets after addition of thrombin Human platelets were prelabeled with [^{3}H]inositol Incorporation of ^{3}H into phosphoinositides of 0.5 ml platelet suspension was 92691 cpm for PtdIns, 18835 cpm for PtdIns4P and 4490 cpm for PtdIns4,5P_2. Platelet suspensions were stimulated with thrombin (2 U/ml) for various times, a mixture of unlabeled inositol phosphate standards was added and ^{3}H-labeled inositol phosphates were separated by subsequent Dowex column chromatography and HPLC as shown in fig 1.

that time lag inositol 1-monophosphate and inositol 2-monophosphate show a continuous, large accumulation (fig.3, table 1). The formation of inositol 2-monophosphate in addition to inositol 1-monophosphate could reflect the transient formation of inositol 1,2-cyclic-monophosphate [27]. Similar time courses of inositol phosphate formation upon specific cell activation have been

Table 1
Thrombin-induced formation of inositol phosphates in human platelets

Additions	^{3}H-Radioactivity (cpm)		
	Ins P_1	Ins 1,4-P_2	Ins 1,4,5-P_3
None	305 ± 78	24 ± 13	1 ± 2
Thrombin, 10 s	301 ± 63	461 ± 206	55 ± 27
Thrombin, 5 min	6117 ± 1953	1653 ± 299	44 ± 22

Platelets prelabeled with [^{3}H]inositol were exposed to thrombin (2 U/ml). Inositol phosphates were separated by subsequent anion exchange column chromatography and paper chromatography (see section 2) Values are mean ± SD of 5 experiments

reported for other cell types prelabeled with [^{3}H]inositol ([33,34]; for ref. see [7]).

The results indicate polyphosphoinositide hydrolysis, but also provide evidence for PtdIns hydrolysis in stimulated platelets. If the rate of conversion of labeled inositol phospholipids to the corresponding inositol phosphates 5 min after addition of thrombin (2 U/ml) is calculated, there is a conversion rate of 1.5 ± 0.6% for PtdIns4,5P_2, of 20 ± 8% for PtdIns4P and of 10 ± 3% for PtdIns (mean ± SD from 3 experiments). This may indicate that there is only a small pool of radioactive PtdIns4,5P_2 – if compared to PtdIns4P and PtdIns – which is degraded by phospholipase C. These results, however, do not exclude the pathway proposed recently by which PtdIns is refueling PtdIns4P and PtdIns4,5P_2, which is then hydrolysed to inositol trisphosphate and degraded to inositol bisphosphate and monophosphate [7,18,29,33].

The rapid formation of inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate in stimulated platelets indicates that PtdIns4P and PtdIns4,5P_2 are hydrolysed before PtdIns by phospholipase C. From the present results it seems that both PtdIns4P and PtdIns4,5P_2 are degraded initially following platelet exposure to thrombin. The rapid, but restricted formation of the potent Ca^{2+}-mobiliser inositol 1,4,5-trisphosphate may indicate that the enzymatic hydrolysis of PtdIns4,5P_2 is tightly controlled. Another possible regulation step could be the activation of a specific phosphomonoesterase which degrades inositol 1,4,5-trisphosphate to inositol 1,4-bisphosphate [35]. The study of the enzymatic regulation of the formation and inactivation of inositol 1,4,5-trisphosphate may give further insights into the mechanism of platelet activation.

ACKNOWLEDGEMENTS

We are grateful to Ingeborg Drossel for the careful preparation of the manuscript and Professor Peter C. Weber for his kind support of the study.

REFERENCES

[1] Lapetina, E.G. and Cuatrecasas, P. (1979) Biochim Biophys. Acta 573, 394–402.
[2] Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580–587.
[3] Broekman, M.J., Ward, J.W. and Marcus, A J. (1980) J. Clin. Invest. 66, 275–283.
[4] Lloyd, J V , Nishizawa, E.E. and Mustard, J.F. (1973) Br J. Haematol. 25, 77–99.
[5] Siess, W , Siegel, F.L. and Lapetina, E.G (1983) J. Biol. Chem. 258, 11236–11242.
[6] Siess, W., Bohlig, B., Weber, P.C. and Lapetina, E.G. (1984) Blood, in press
[7] Berridge, M.J (1984) Biochem. J. 220, 345–360.
[8] Streb, H., Irvine, R.F., Berridge, M J. and Schulz, I (1983) Nature 306, 67–69.
[9] Nishizuka, Y. (1983) Trends Biochem. Sci. 8, 13–16.
[10] Agranoff, B.W., Murthy, P. and Seguin, E.B. (1983) J. Biol. Chem. 258, 2076–2078.
[11] Kaibuchi, K., Takai, Y , Sawamura, M., Hoshijima, M., Fujikura, T. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 6701–6704.
[12] Siess, W., Cuatrecasas, P. and Lapetina, E.G. (1983) J Biol. Chem 258, 4683–4686.
[13] Siess, W., Weber, P.C. and Lapetina, E.G. (1984) J. Biol. Chem. 259, 8286–8292.
[14] Siess, W., Siegel, F.L. and Lapetina, E.G. (1984) Biochim. Biophys Acta, in press.
[15] Lapetina, E.G and Siess, W. (1983) Life Sci. 33, 1011–1018.
[16] Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81–147.
[17] Abdel-Latif, A.A., Akhtar, R.A. and Hawthorne, J.N. (1977) Biochem J. 162, 61–73.
[18] Michell, R.H., Kirk, C.J., Jones, L.M., Downes, C. and Creba, J.A. (1981) Philos. Trans. R. Soc. London, Ser. B 296, 123–137.
[19] Billah, M.M and Lapetina, E.G. (1982) J. Biol. Chem. 257, 12705–12708.
[20] Grado, C. and Ballou, C.E. (1961) J. Biol. Chem. 236, 54–60.
[21] Desjobert, A. and Petek, F. (1956) Bull. Soc Chim Biol. 38, 871–883.
[22] Tomlinson, R.V. and Ballou, C.E. (1961) J. Biol. Chem. 236, 1902–1911
[23] Irvine, R F., Brown, K D. and Berridge, M.J. (1984) Biochem. J. 221, 269–272.
[24] Billah, M.M. and Lapetina, E.G. (1982) J Biol. Chem 257, 5196–5200.
[25] Siess, W. and Lapetina, E.G. (1983) Biochim Biophys. Acta 752, 329–338.
[26] Michell, R.H. and Allan, D. (1975) FEBS Lett 53, 302–304

[27] Pizer, F L and Ballou, C.E. (1958) J. Am Chem. Soc 85, 915–921.
[28] Bligh, E.G and Dyer, W.J. (1959) Can J Biochem. Physiol. 37, 911–917
[29] Berridge, M.J , Dawson, R M C., Downes, D.P., Heslop, J.P. and Irvine, R.F (1983) Biochem J 212, 473–482
[30] Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
[31] Jolles, J., Schrama, L H and Gispen, W H. (1981) Biochim. Biophys. Acta 666, 90–98
[32] Irvine, R.F , Letcher, A.J , Lander, D.J and Downes, C.P (1984) Biochem J. 223, 237–243.
[33] Berridge, M.J. (1983) Biochem J 212, 849–858
[34] Thomas, A P , Alexander, J and Williamson, J R (1984) J. Biol. Chem. 259, 5574–5584
[35] Downes, C.P , Mussat, M.C. and Michell, R H. (1982) Biochem. J 203, 169–177