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Phosphoinositide metabolism in resting and thrombin-stimulated human platelets

Evidence against metabolic heterogeneity

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The specific radioactivity of the phosphodiester and phosphomonoester moieties of the phosphoinositides was determined in resting and thrombin-stimulated platelets that were prelabelled with [^{32}P] P_i. In the unstimulated cells, the specific radioactivity of the monoester phosphates of PIP and PIP_2 was similar. Both prolonged incubation at 37°C and upon stimulation with 0.5 U/ml of thrombin, the specific radioactivity of the monoester phosphates decreased 10–15% in both polyphosphoinositides. In the unstimulated cells, the specific radioactivity of the diester phosphates was similar in PI, PIP and PIP_2 but amounted only to 3% of the activity of the monoester groups. Prolonged incubation of the unstimulated cells as well as stimulation with thrombin induced a similar 5–6 fold increase in specific radioactivity of the diester phosphate of PI, PIP and PIP_2. The results indicate that the phosphoinositides in both resting and thrombin-stimulated platelets exist in a metabolically homogeneous pool.

Phospholipid metabolism; Inositol phospholipid; Thrombin; (Human platelet)

1. INTRODUCTION

Polyphosphoinositides constitute a minor fraction of total cell phospholipids. Nevertheless, important functions have been assigned to them such as the receptor-mediated transmembrane signalling (review [1]). In resting cells [2] as well as in stimulated cells [3–5], evidence has been presented for multiple metabolic pools of phosphoinositides. In platelets the existence of distinct metabolic pools of PA, PI and PIP_2 has been suggested [6–8]. Recently, Vickers and Mustard studied the specific [^{32}P]P_i and [^{3}H]glycerol labelling of the phosphoinositides in both resting and stimulated platelets and proposed separate pools of polyphosphoinositides [9,10].

We have recently reported that the phosphomonoester groups of both PIP and PIP_2 are in metabolic equilibrium with the ATP γ-phosphoryl in resting platelets. The specific radioactivity of the diester phosphate was similar for all three phosphoinositides but much lower than that of the monoester phosphates [11]. This could indicate metabolic equilibrium between the phosphoinositides. To evaluate further this possibility we used two approaches: (i) prolonged incubation of gel-filtered pulse-labelled platelets and (ii) thrombin stimulation of the platelets, both conditions resulting in an increase in specific radio-

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Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; GroPI, diacylated phosphatidylinositol

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activity of the diester phosphate of PI [8,12,13]. The changes in specific labelling of both diester and monoester phosphates of the phosphoinositides was studied.

2. MATERIALS AND METHODS

2.1. *Platelet isolation, labelling and incubation*

Platelet-rich plasma was prepared as described [14], and incubated for 60 min at 37°C with 0.3 mCi/ml of [^{32}P]orthophosphate ([^{32}P]P_i, carrier-free, Amersham International, code PBS.11). Subsequently, the platelets were gel-filtered into a Ca^{2+}- and phosphate-free Tyrode's solution (pH 7.25) containing 0.2% w/v serum albumin (fraction V, Miles Laboratories) and 5 mM glucose. Final platelet concentrations ranged between 3.5 and 4.2 × 10^8 cells/ml and the experiments were started within 10 min from the gel filtration.

In experiments with resting cells, gel-filtered platelets were incubated at 37°C and shaken at 100 strokes per min. To study the effect of stimulation, gel-filtered platelets were incubated at 37°C for 3 min before addition of 0.5 U/ml of thrombin (final concentration, La Roche). Duplicate samples of 4 ml of platelets were withdrawn at the times indicated for subsequent analysis.

2.2. *Phospholipid extraction and chromatography*

Each duplicate sample of platelets was extracted by 4 vols of chloroform/methanol/conc. HCl (20:40:1, by vol., 0°C) as described by Holmsen et al. [8]. Subsequently the lipids were separated by thin layer chromatography on potassium oxalate-treated silica gel plates (Merck, art. 5553) using chloroform/methanol/20% methylamine (60:36:10, by vol.) [14]. The phosphoinositide fractions were visualized by overnight radioautography (Fuji X-ray film) and scraped off the plates.

2.3. *Determination of specific radioactivity of the di- and monoester phosphates*

The phosphoinositide fractions from one of the duplicate samples were deacylated and treated with

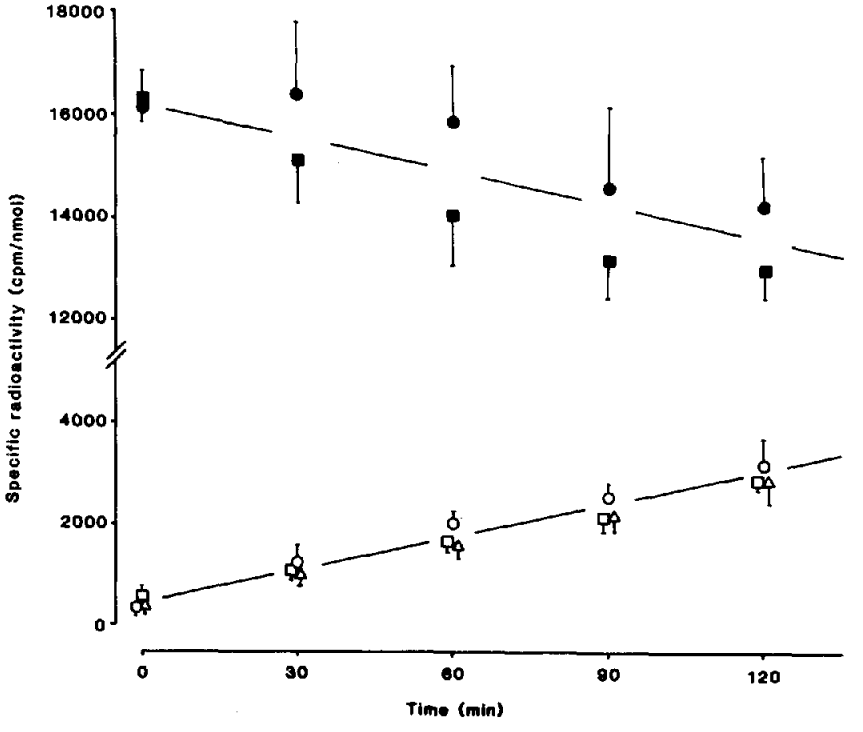

Fig.1. Effect of prolonged incubation of gel-filtered, [^{32}P]P_i labelled platelets on the specific ^{32}P radioactivity of mono- (filled symbols) and diester phosphates (open symbols) of PIP_2 (○), PIP (□) and PI (△).

Table 1

Thrombin-induced changes in mass of PIP_2, PIP and PI

	PIP_2	PIP	PI
0 s	132 ± 16	168 ± 12	1408 ± 79
15 s	148 ± 5	167 ± 25	1238 ± 20
60 s	172 ± 23	219 ± 12	1140 ± 160
180 s	173 ± 14	205 ± 13	921 ± 16

Gel-filtered, [^{32}P]P_i-prelabelled platelets were incubated at 37°C for 3 min before 180 s of stimulation with 0.5 U/ml of thrombin. The data represent means ± SD from 4 separate experiments and are expressed as nmol/10^{11} platelets

bovine alkaline phosphatase (Sigma, St. Louis, USA), as described by Koreh and Monaco [5]. GroPI from PI, PIP and PIP_2 (containing the phosphodiesters), and P_i (representing the phosphomonoesters), were separated by one-dimensional chromatography on Whatman paper no.1 for 36 h at 22°C in isobutyric acid/conc. ammonia/water/100 mM EDTA (500:21:279:8, by vol.) [11]. The spots were detected by radioautography, cut out and the radioactivity was measured by liquid scintillation counting.

The fractions from the other duplicate sample were used to determine the total mass and radioactivity of PI, PIP and PIP_2. The lipids were digested at 180°C for 30 min in 100 μl perchloric acid (70%). Inorganic phosphate was thereafter measured with a malachite-green method [15] and the radioactivity was determined from the same digests.

The specific radioactivity of the phosphodiester and phosphomonoester groups of the phosphoinositides was calculated from the values obtained for total radioactivity, total mass and relative distribution of radioactivity between the di- and monoester phosphates. The calculation for PIP_2 is based on the assumption that the radioactivity is evenly distributed over the 4- and 5-phosphomonoester groups [11].

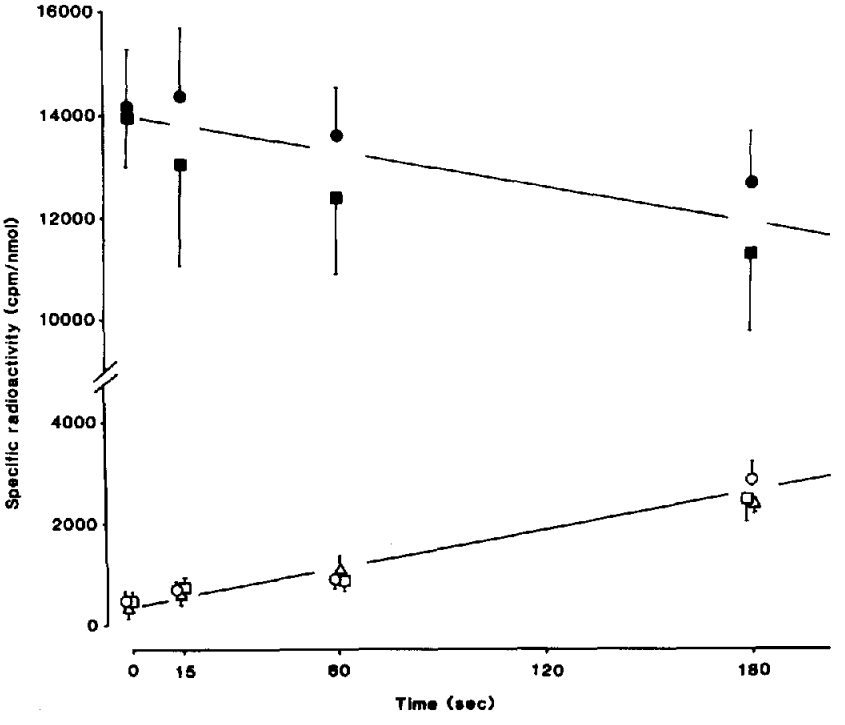

Fig.2. Effect of stimulation of gel-filtered, [^{32}P]P_i labelled platelets with 0.5 U/ml of thrombin on the specific radioactivity of mono- (filled symbols) and diester phosphates (open symbols) of PIP_2 (○), PIP (□) and PI (△).

3. RESULTS

3.1. *Unstimulated platelets*

The mass of PIP_2, PIP and PI in unstimulated platelets was 141 ± 18, 169 ± 22 and 1498 ± 129 nmol/10^{11} cells, respectively (n = 8) and remained constant during the 120 min of incubation (not shown). The specific radioactivity of both mono- and diester phosphates is shown in fig.1. For both PIP_2 and PIP, the specific radioactivity of the monoester phosphates decreased slightly during 120 min of incubation. Immediately after the gel filtration of the platelets, there was no difference in specific radioactivity of the monoesters of PIP and PIP_2. Upon prolonged incubation, the specific radioactivity of the monoesters of PIP_2 tended to be slightly higher than that of PIP. However, the difference was not significant in any point when tested with the paired Student's t-test.

In contrast to the monoester phosphates, the specific radioactivity of the diester phosphates of PIP_2, PIP and PI increased during the 120 min incubation period from about 3% to 20% of that of the monoester phosphates. There was no difference in specific radioactivity between the individual phosphoinositides (fig.1).

3.2. *Stimulated platelets*

Within 3 min of thrombin stimulation, the mass of PIP and PIP_2 increased by 30% whereas the mass of PI decreased by 40% (table 1). The specific radioactivity of the monoester phosphates of the polyphosphoinositides decreased slightly during the 3 min of stimulation (fig.2). There was no significant difference in specific radioactivity between PIP_2 and PIP. The specific radioactivity of the diester phosphate increased 6 times upon thrombin stimulation of the platelets (fig.2) and there was no significant difference between the three phosphoinositides.

4. DISCUSSION

We have previously shown that the phosphomonoester groups of PIP and PIP_2 are in metabolic equilibrium with each other and with the ATP γ-phosphoryl in unstimulated platelets [11]. The present study demonstrates that in unstimulated platelets, the diester phosphates in PI, PIP and PIP_2 are in metabolic equilibrium with each other. This indicates that there is free exchange between the polyphosphoinositides and the entire pool of PI, and suggests strongly that there is metabolic homogeneity between all phosphoinositides of unstimulated human platelets. In contrast, Vickers and Mustard [9] proposed compartmentation of PI, PIP and PIP_2 in rabbit platelets. This was based on small differences in specific [^{32}P]P_i labelling of phosphoinositides upon prolonged incubation of unstimulated platelets. However, we demonstrate in this study that under such conditions, the specific radioactivity of the diester phosphate will increase. Thus, the specific radioactivity of total PIP and PIP_2 will be affected in different ways.

The present results clearly demonstrate that the diester phosphates of all the phosphoinositides are in metabolic equilibrium with each other upon thrombin stimulation of platelets. The data further suggest that the monoesters of PIP and PIP_2 remain in equilibrium with each other during the 3 min of stimulation.

Upon stimulation of WRK-1 cells, there is evidence for both a hormone-insensitive and -sensitive pool of PIP_2 [5]. In addition, evidence from studies on brain membranes and pancreatic islets, has suggested the existence of stimulus-insensitive pools of PI [3,4]. In contrast, there is evidence for metabolic homogeneity of the polyphosphoinositides of rat hepatocytes [16]. Our present data show that stimulation of platelets with thrombin induces a considerable increase in the specific radioactivity of the diester phosphate of all the phosphoinositides. While this increase may appear insignificant compared to the absolute radioactivity in the monoester phosphates, it may explain the 6% increase in specific activity of PIP_2 reported by Vickers et al. [10]. The presented data are supported by Dangelmaier et al. [17] who found that IP_3 produced upon thrombin stimulation had similar specific ^{32}P-radioactivity as compared to that of PIP_2. Holmsen et al. [8] showed that only 20% of total PIP_2 was susceptible to thrombin-induced hydrolysis in ATP-depleted platelets. However, we have recently shown that a 10-fold decrease in available ATP only reduces phosphate turnover in the polyphosphoinositides by a factor of 3 [18]. It is therefore possible that the ATP level during the metabolic inhibition was still sufficient to maintain the actual level of PIP_2.

Billah and Lapetina [7] found evidence for different functional pools of PI, which was based on a comparison of the degradation of PI in thrombin- and ionophore-stimulated platelets. However, functional pools of PI may as well be the result of a different distribution of the enzymes involved rather than physically isolated pools of PI.

Both upon stimulation and upon prolonged incubation of unstimulated platelets, there is a small decrease in specific radioactivity of the monoester groups. These latter have previously been shown to be in equilibrium with the ATP γ-phosphoryl [11], and changes in the specific labelling of ATP will thus immediately be reflected in the monoester phosphates. Incubation of unstimulated platelets has previously been shown to induce a slight decrease in specific radioactivity of ATP [12], and upon stimulation, isotopic dilution of ATP may occur by agonist-induced liberation of unlabelled inorganic phosphate [19]. This may explain the observed decrease in specific radioactivity of the phosphomonoester groups.

In conclusion we here give evidence against metabolic compartmentation of the phosphoinositides in both resting and thrombin-stimulated platelets.

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REFERENCES

[1] Berridge, M.J. (1985) Sci. Am. 253, 124–134.
[2] Muller, E., Hegewald, H., Jaroszewick, K., Cumme, G.A., Hoppe, H. and Frunder, H. (1986) Biochem. J. 235, 775–783.
[3] Labarca, R., Janowsky, A. and Paul, S.M. (1985) Biochem. Biophys. Res. Commun. 132, 540–547.
[4] Rana, R.S., Kowluru, A. and MacDonald, M.J. (1986) Arch. Biochem. Biophys. 245, 411–416.
[5] Koreh, K. and Monaco, M.E. (1986) J. Biol. Chem. 261, 88–91.
[6] Holmsen, H., Dangelmaier, C.A. and Holmsen, H.-K. (1981) J. Biol. Chem. 256, 9393–9396.
[7] Billah, M.M. and Lapetina, E.G. (1982) J. Biol. Chem. 257, 11856–11859.
[8] Holmsen, H., Dangelmaier, C.A. and Rongved, S. (1984) Biochem. J. 222, 157–167.
[9] Vickers, J.D. and Mustard, J.F. (1986) Biochem. J. 238, 411–417.
[10] Vickers, J.D., Kinlough-Rathbone, R.L. and Mustard, J.F. (1986) Biochem. J. 237, 327–332.
[11] Verhoeven, A.J.M., Tysnes, O.-B., Aarbakke, G.M., Cook, C.A. and Holmsen, H. (1987) Eur. J. Biochem., in press.
[12] Tysnes, O.-B., Verhoeven, A.J.M. and Holmsen, H. (1986) Biochim. Biophys. Acta 889, 183–191.
[13] Vickers, J.D., Kinlough-Rathbone, R.L. and Mustard, J.F. (1984) Biochem. J. 219, 25–31.
[14] Tysnes, O.-B., Aarbakke, G.M., Verhoeven, A.J.M. and Holmsen, H. (1985) Thromb. Res. 40, 329–338.
[15] Vickers, J.D., Kinlough-Rathbone, R.L. and Mustard, J.F. (1982) Blood, 1247–1250.
[16] Palmer, S., Hawkins, P.T., Michel, R.H. and Kirk, C.J. (1986) Biochem. J. 238, 491–499.
[17] Dangelmaier, C.A., Daniel, J.L. and Smith, B. (1986) Anal. Biochem. 154, 414–419.
[18] Verhoeven, A.J.M., Tysnes, O.-B., Horvli, O., Cook, C.A. and Holmsen, H. (1987) J. Biol. Chem., in press.
[19] Fukami, M.H., Dangelmaier, C.A., Bauer, J.S. and Holmsen, H. (1980) Biochem. J. 192, 99–105.