Biochem. J. (1989) 263, 621–624 (Printed in Great Britain)

Evidence for tight metabolic control of the receptor-activated polyphosphoinositide cycle in human platelets

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The [^{32}P]PIP_2/[^{32}P]PA and the [^{32}P]PIP/[^{32}P]PA relationships were demonstrated to be remarkably similar after stimulation of [^{32}P]P_i-prelabelled platelets for 90 s with various combinations and concentrations of agonists and inhibitors. Thus the activity of the PI and PIP kinases with the corresponding phosphomonoesterases may be tightly controlled during receptor-mediated platelet stimulation involving phospholipase C activation.

INTRODUCTION

A large number of cells and tissues, including platelets, respond to external, receptor-mediated, activation by PLC-catalysed hydrolysis of the plasma-membrane phospholipid PIP_2 (for reviews, see [1–3]). This diesteratic cleavage produces the signal molecules IP_3 and DAG, which mobilize cytoplasmic Ca^{2+} and activate protein kinase C respectively. DAG is rapidly phosphorylated to PA, which again is converted into PI through two intermediate steps. PI is successively phosphorylated to PIP and PIP_2, closing the PPI cycle.

In the present study, we have evaluated how the ^{32}P radioactivities of PIP_2, PIP and PA in [^{32}P]P_i-prelabelled platelets correlate with each other during receptor-mediated PLC activation.

MATERIALS AND METHODS

Materials

Stock solutions of bovine thrombin (Hoffmann–La Roche), PAF (Sigma), adrenaline (Sigma), hirudin (Sigma; from leeches) and acetylsalicylic acid (Sigma) were stored at −20 °C. Solutions of CP (Sigma) and CPK (Sigma; type I, rabbit muscle) were freshly prepared in Tyrode's solution. [^{32}P]P_i was obtained from Amersham (code PBS-11; carrier-free).

Platelet isolation, labelling and incubation

Platelet-rich plasma was prepared by differential centrifugation of freshly drawn human venous blood anticoagulated with ACD [4], and was incubated with [^{32}P]P_i (0.1 mCi/ml) for 1 h at 37 °C. In some experiments (Fig. 2), acetylsalicylic acid (1 mM) was added to platelet-rich plasma during the last 15 min of the preincubation. The platelets were transferred by gel-filtration through Sepharose 2B to a nominally Ca^{2+}- and P_i-free Tyrode's buffer [4]. The platelet concentration was standardized at 3.5×10^8 cells/ml. The basal extracellular concentration of ADP in GFP was determined with a luciferin–luciferase method [5] and was in the range 0.1–0.6 μM; ADP was effectively converted into ATP by CP/CPK. Suspensions of gel-filtered platelets were incubated at 37 °C without stirring for 3 min before addition of the agonist. Hirudin or adrenaline was added 30 or 10 s before thrombin respectively.

Phospholipid extraction and chromatography

Samples (0.5 ml; 1 vol.) were withdrawn from the incubation mixtures 90 s after the addition of agonist, and mixed with 4.0 vol. of chloroform/methanol/conc. HCl (20:40:1, by vol.; 0 °C). The further processing of the samples, including separation by t.l.c. in a methylamine solvent system and determination of radioactivity of the phospholipids, was performed as previously described [4]. The results are expressed as a percentage of the values for saline-only-treated controls. In a typical experiment, the 100% values (controls) of PIP_2, PIP and PA correspond to approx. 4700, 2800 and 130 c.p.m. per 0.5 ml of GFP respectively.

RESULTS

Relationship between the ^{32}P radioactivity of PIP_2, PIP and PA at increasing concentrations of thrombin

Suspensions of GFP were incubated for 90 s with increasing concentrations of thrombin. Fig. 1 shows plots of the [^{32}P]PIP_2 and [^{32}P]PIP levels versus the corresponding [^{32}P]PA level obtained with each dose of thrombin ($n = 4$). The 90 s incubation time was chosen because it produces the most extensive changes in the levels of [^{32}P]PIP_2, [^{32}P]PIP and [^{32}P]PA [4]. At low degrees of platelet activation, there was only a minimal formation of [^{32}P]PA together with a decreased level of [^{32}P]PIP_2. The corresponding level of [^{32}P]PIP, however, increased markedly above control values. As the [^{32}P]PA formation was enhanced by higher concentrations of thrombin, the [^{32}P]PIP_2 level gradually increased to an apparent plateau (approx. 130–140% of control). The ^{32}P radioactivity of PIP reached a maximal value of 170–180% of control at a corresponding [^{32}P]PA level of approx. 1000% of control; thereafter, the [^{32}P]PIP radioactivity decreased gradually towards the control

Abbreviations used: CP/CPK, phosphocreatine/creatine kinase; PPI, polyphosphoinositide; IP_3, inositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol; PA, phosphatidic acid; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PLC, phospholipase C; GFP, gel-filtered platelets.

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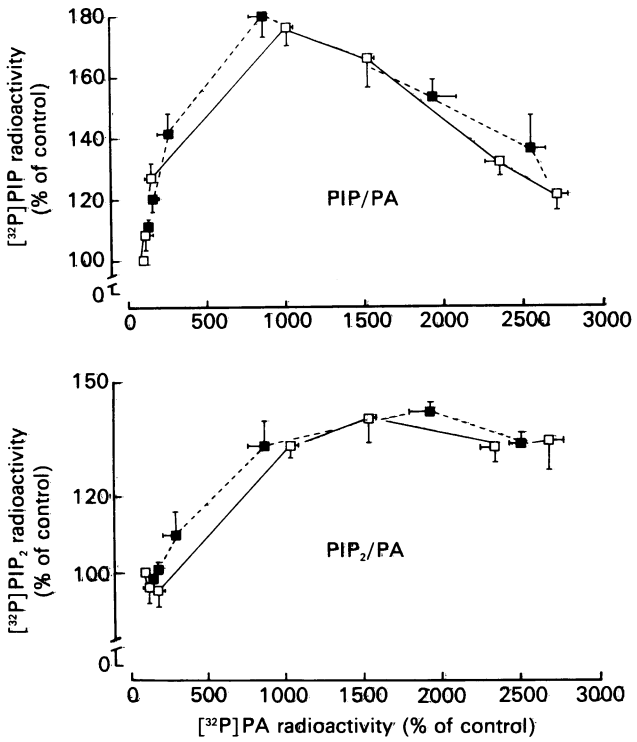

Fig. 1. Relationship between [^{32}P]PIP_2, [^{32}P]PIP and [^{32}P]PA in thrombin-stimulated platelets in the absence and presence of hirudin

[^{32}P]P_i-prelabelled platelets were stimulated with increasing concentrations of thrombin (0.01, 0.03, 0.05, 0.1, 0.3 and 0.5 unit/ml; □), or with a constant dose of thrombin (0.5 unit/ml) together with increasing concentrations of hirudin (0.25, 0.50, 1.0, 3.0, 4.0 and 5.0 units/ml; ■). The data, which represent the [^{32}P]PIP_2/[^{32}P]PA and [^{32}P]PIP/[^{32}P]PA relationships obtained with each dose of thrombin, are expressed as means ± S.D. from four independent determinations.

value as the degree of platelet stimulation was enhanced. The maximal value of the [^{32}P]PIP radioactivity almost invariably corresponded to a [^{32}P]PA formation about 1000% of control, irrespective of the changes in responsiveness to thrombin (approx. 0.05–0.1 unit/ml) between different platelet suspensions.

Effect of hirudin on thrombin-induced changes in the [^{32}P]PIP_2/[^{32}P]PA and [^{32}P]PIP/[^{32}P]PA relationships

Platelet suspensions were then stimulated with the highest dose of thrombin used (0.5 unit/ml), together with increasing concentrations of hirudin (Fig. 1), a well-known thrombin inhibitor that complexes with thrombin and thereby blocks the interaction between thrombin and its binding sites [6,7]. The addition of increasing concentrations of hirudin gradually decreased the thrombin-induced PA formation, and the corresponding changes in the ^{32}P radioactivity of PIP_2 and PIP correlated well with the data obtained with thrombin alone.

[^{32}P]PIP_2/[^{32}P]PA and [^{32}P]PIP/[^{32}P]PA relationships in platelets stimulated with combinations of different agonists

Suspensions of aspirin-treated GFP were stimulated

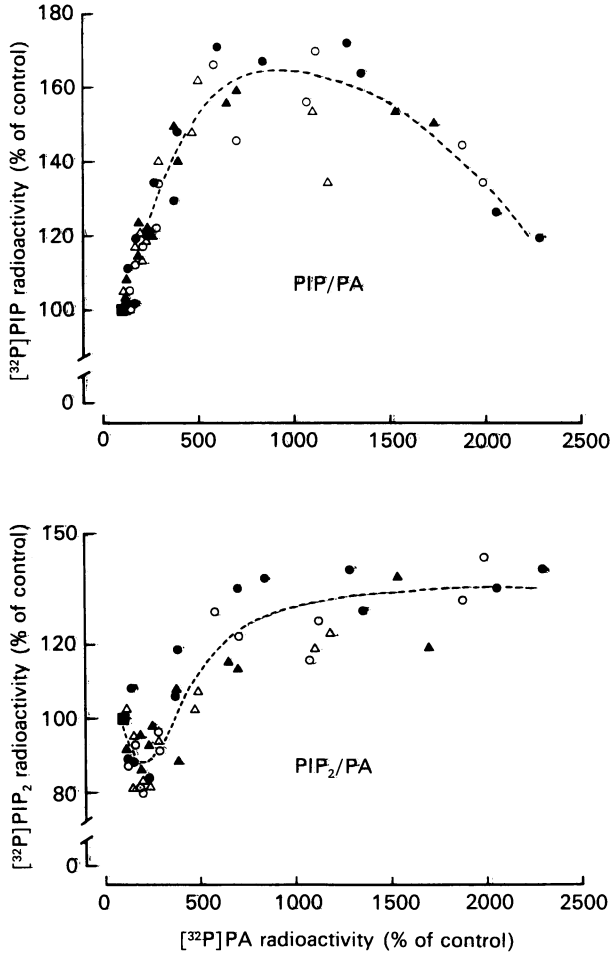

Fig. 2. Relationship between [^{32}P]PIP_2, [^{32}P]PIP and [^{32}P]PA in multi-agonist-treated platelets

Suspensions of aspirin-treated platelets were stimulated by increasing concentrations of thrombin (0.01, 0.03, 0.05, 0.08, 0.15 and 0.5 unit/ml) with or without adrenaline (●, ▲, or ○, △, respectively; 4 μM) in the absence (○, ●) or presence (△, ▲) of CP (5 mM)/CPK (10 units/ml). The Figure shows all determinations from a single experiment which is representative of three identical independent experiments.

by thrombin in the absence or presence of adrenaline and CP/CPK (Fig. 2). In the absence of the ADP-removing enzyme system CP/CPK, the platelet suspension contained potentiating concentrations of extracellular ADP (see the Materials and methods section). The presence of ADP or adrenaline may enhance thrombin-induced PLC activation [4,8,9]. The multi-agonist-induced changes in the relative levels of [^{32}P]PIP_2, [^{32}P]PIP and [^{32}P]PA were identical with those obtained with thrombin alone. In addition, a similar pattern was found with the PLC-stimulating agonist platelet-activating factor alone [10] (50–1000 nM) or plus thrombin (results not shown). Moreover, the thrombin-induced [^{32}P]PIP_2/[^{32}P]PA and [^{32}P]PIP/[^{32}P]PA relationships were the same with or without the cyclo-oxygenase inhibitor acetylsalicylic acid (Fig. 2 versus Fig. 1 respectively), i.e. in the absence or presence of PLC-activating prostaglandins and thromboxanes produced by the platelets themselves [11,12].

DISCUSSION

Tight metabolic control of the PPI cycle

In human platelets, the formation of [^{32}P]PA from DAG indirectly monitors PLC activation. Therefore, the present results clearly demonstrate that at a certain degree of PLC activation, measured as [^{32}P]PA formation, there is a tightly corresponding level of [^{32}P]PIP_2 and [^{32}P]PIP, irrespective of the agonist–receptor system which induces activation of PLC, and irrespective of intrinsic responsiveness of the platelets (i.e. of platelets from different donors). The ^{32}P radioactivity of these two PPI metabolites is regulated through the activity of several enzymes, including PLC and the PI and PIP kinases with corresponding phosphomonoesterases. In unstimulated platelets, PI, PIP and PIP_2 are subject to rapid continuous phosphorylation and dephosphorylation [13], and changes in the [^{32}P]PIP_2 and [^{32}P]PIP radioactivity upon thrombin stimulation reflect alterations in the relative mass balance between these metabolites [13,14]. Thus our present data indicate that the activities of the PPI kinase and phosphomonoesterase pathways are closely correlated with the receptor-controlled PLC activation.

Since this close correlation of labelling ratios was apparently independent of the stimulatory conditions used, we suggest that the regulation of the PPI kinases and corresponding phosphomonoesterases is a direct or indirect consequence of changes in the concentrations of the PLC reaction products. The receptor-controlled activation of PLC produces the signal molecules IP_3 and DAG, which again may lead to specific changes in intracellular physical parameters such as cytosolic Ca^{2+} and H^+ concentrations (for reviews, see [1,2,15,16]). These alterations may regulate or affect the activity of the PPI kinases and corresponding phosphomonoesterases. The rate and extent of these physical changes vary with the potency of the inducing stimulus, and could thereby constitute a PLC-mediated gain control of the PPI enzyme activities. Indeed, results from a recent kinetic analysis of PI turnover in NRK fibroblasts argue that regulation of the PPI kinases may be important for determining both cellular IP_3 and DAG levels [17]. Moreover, the different PPI metabolites may exert negative or positive feedback control, as has been indicated by the finding that PIP_2 inhibits the PIP kinase reaction [18,19]. Such a feedback control may participate in the regulation of the enzymes [17], and could be important if the different PPI metabolites and enzymes were physically closely related to the receptor-controlled PLC, i.e. organized as a complex of enzymes, as has been shown for the fatty acid synthetase and pyruvate dehydrogenase multienzyme complex (for review, see [20]). It is probably unlikely that the PPI enzymes are directly controlled by receptor occupancy (see above).

Adrenaline potentiates agonist-induced PLC activation in human platelets, although it apparently does not activate PLC by itself [4,9,21]. It is therefore important that the presence of adrenaline during thrombin-induced stimulation only changes the curves as if a higher dose of thrombin were used. This finding indicates that adrenaline directly or indirectly only affects PLC activation without having specific effects on other steps in the PPI metabolism (see below).

Use of the [^{32}P]PIP_2/[^{32}P]PA and [^{32}P]PIP/[^{32}P]PA relationships during evaluation of platelet PPI metabolism

Platelets are a model system frequently used to study the PPI cycle and PLC activation. Our present results may be of importance during interpretation of [^{32}P]PPI data from these cells. As an example, some recent articles probably demonstrate misinterpretation of [^{32}P]PPI results obtained with platelets (e.g. [21,22]: in [22], the authors have not taken into consideration that at high degrees of cellular stimulation the [^{32}P]PIP level gradually decreases; the conclusions from data obtained with the aminoglycoside neomycin have therefore proved to be invalid [23].

The most important use of our present data, however, may be the evaluation of the mode of action of different platelet inhibitors and stimulators. In short, drugs which interfere with specific steps in the PPI metabolism (e.g. inhibition of PIP kinase) should alter the shape of the agonist-induced [^{32}P]PIP_2/[^{32}P]PA and [^{32}P]PIP/[^{32}P]PA relationships, whereas compounds which only directly or indirectly affect the PLC activation (e.g. hirudin; see above) should not change these relationships.

The present investigation was supported in part by the Norwegian Research Council for Science and the Humanities (NAVF) and the Family Blix Foundation.

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Received 20 June 1989/9 August 1989; accepted 17 August 1989