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Polyphosphoinositide synthesis in platelets stimulated with low concentrations of thrombin is enhanced before the activation of phospholipase C

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When platelets, prelabelled with [^{32}P]orthophosphate, were stimulated with thrombin (0.5 U·ml^{-1}) there was an immediate increase in the radioactivity associated with the pools of polyphosphoinositides. Only subsequent to this increase, did the radioactivity of these phospholipid pools decrease as expected from a receptor-mediated activation of phospholipase C (phosphoinositidase). Phosphorylation of diacylglycerol (one of the second messengers formed in the hydrolysis of phosphatidylinositol-bisphosphate) to phosphatidic acid took place with a lag phase of about 3–5 s. Together these experiments suggest that stimulation of kinases phosphorylating phosphatidylinositol and phosphatidylinositol-phosphate may precede or occur in parallel with activation of receptor-linked phosphoinositidase.

Phosphoinositide turnover; Phosphatidylinositol kinase; Microfilament; Profilin/actin

1. INTRODUCTION

Binding of a wide range of agonists to their cognate cell surface receptors induces increased turnover in the phosphatidylinositol-cycle (PtdIns-cycle) [1]. According to the current model, the primary response to these ligand/receptor (L/R) interactions is an activation of a phosphoinositidase hydrolysing phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) generating the two second messengers inositol(1,4,5)-trisphosphate (InsP_3) and diacylglycerol (DG) [2], the former causing release of Ca^{2+} from intracellular stores [3] and the latter activating protein kinase C [4]. The polyphosphoinositides are thought to be connected by futile cycles rapidly reestablishing any unbalance caused by signal transduction.

Many agonists activating the PtdIns-cycle also induce actin polymerization and formation of microfilament assemblies at the inner leaflet of the plasma membrane, building up new cell surface projections, membrane lamellae and spikes. In motile cells these cell surface projections are instrumental in translocating the cell. In receptor-mediated activation, resulting in mobilization of intracellular Ca^{2+}, stimulation of actin polymerization is a very early event and is followed by a generalized increase in motile activity (ref. in [5]). The unpolymerized form of actin, the profilin/actin complex, appears to be the major source of actin for this filament formation [6].

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Isolated profilin, as well as profilin in complex with actin binds strongly to PtdIns(4,5)P_2 and also has some affinity for phosphatidylinositol(4)phosphate (PtdIns (4)P). In the case of the profilin/actin complex, the interactions with the phospholipids cause release of monomeric actin which can engage in filament formation. Profilin then remains bound to the lipid. Thus the microfilament system seems to be directly linked to phosphatidylinositide turnover, with a major control at the level of the ligand/cell surface receptor interaction.

The contention that the PtdIns-cycle might be involved not only in regulating but also in driving actin polymerization was supported by results of experiments with platelets showing that agents inhibiting ligand-induced formation and hydrolysis of polyphosphoinositides greatly reduced thrombin-induced actin polymerization [5]. In addition, increasing the intracellular levels of Ca^{2+} with the use of the ionophore A_{23187}, or activating protein kinase C with phorbolesters (or both) did not cause significant actin polymerization in platelets. This suggests that the receptor-induced event, or events, triggering actin polymerization either precedes or is unrelated to the generation of the second messengers, InsP_3 and DG. The specific interaction between PtdIns(4,5)P_2 and profilin/actin seen in vitro points at this intermediate being the agent recruiting profilin/actin to the plasma membrane where the actin is made available for polymerization onto preexisting filament ends either spontaneously or by a specific actin polymerizing function.

This reasoning suggests that one of the immediate effects of the ligand/receptor interaction might be the activation of kinases generating polyphosphoinositides.

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To test this possibility we have analysed for polyphosphoinositides in samples taken during the first 10 s of stimulation of platelets with low concentrations of thrombin.

2. EXPERIMENTAL

Thrombin, prostacyclin (PGI_2), and deoxyribonuclease I (DNase I) were from Sigma. The DNase I was further purified according to Markey [9]. [^{32}P]orthophosphate was from Amersham.

Fresh platelet concentrates from human volunteers was obtained from the Blood Centre, Södersjukhuset, Stockholm. Platelets were prepared by gel filtration as described previously [5]. After gel filtration the platelets were radiolabelled with [^{32}P]orthophosphate (10 μCi/ml) for 90 min at room temperature on a rocking table. Aliquots of the platelet suspension (0.5 ml containing 10^8–10^9 corpuscles) were activated by addition of thrombin (0.5 units · ml^{-1}) for times indicated in figure legends. The stimulation was stopped by addition of 1 ml ice-cold metanol/HCl_{conc} (10:1). Lipids were extracted, and analyzed by thin-layer chromatography followed by autoradiography and scintillation counting as described previously [5]. Actin pools were determined by the DNase I inhibition assay as described by Blikstad et al. [10].

Platelets that are in a resting state as judged by their high levels of unpolymerized actin (>60% of total actin) incorporate only small amounts of [^{32}P]orthophosphate into phosphatidic acid (Ptd A) and PtdIns during a 90 min labelling period (no agonist added), whereas the PtdInsP and $PtdInsP_2$ pools become labelled. Thus in resting platelets the turnover in the PtdIns-cycle is very slow. Slightest activation of the platelets, however, results in a decreased G/F-actin ratio, and in the appearance of radiolabel in PtdA and PtdIns as well as polyphosphoinositides. In the present experiments only batches of platelets that incorporated low levels of radioactivity into Ptd A were used.

Some incorporation of radioactivity in the poly-phosphoinositides occurred in the absence of agonist also after the 90 min radiolabelling period, albeit at a slower rate. Therefore unstimulated platelets were analyzed before and after each experiment to allow the correction of the data with respect to this background incorporation.

3. RESULTS

To follow the thrombin-induced changes in the polyphosphoinositide and PtdA pools, platelets were incubated with thrombin (0.5 units · ml^{-1}) for short periods of time. As shown in fig.1A, the amount of [^{32}P]orthophosphate incorporated in PtdInsP and $PtdInsP_2$ increased initially reaching a maximal value at about 5 s. At about 10 s after thrombin addition, the radioactivity associated with the $PtdInsP_2$ pool began to decrease rapidly.

The radioactivity in the PtdInsP pool remained elevated for a longer time period than the $PtdInsP_2$ pool, although also in this case the associated radioactivity decreased in amount when the platelets had been stimulated for more than 10 s. This might be due either to a decreased synthesis rate, or increased use of PtdInsP to form $PtdInsP_2$, which then is hydrolyzed or by direct breakdown of PtdInsP.

Phosphorylation of diacylglycerol to phosphatidic acid took place with a lag phase of about 5 s, suggesting that the formation of PtdInsP and $PtdInsP_2$ does not occur secondarily to the $PtdInsP_2$ hydrolysis. A series

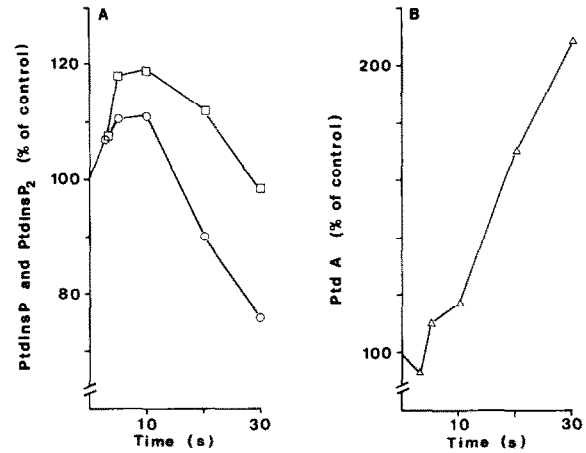

Fig.1. Effect of thrombin stimulation (0.5 units · ml^{-1}) on the ^{32}P-radiolabelling in $PtdInsP_2$, PtdInsP and Ptd A pools. Prelabelled platelets were stimulated for the times indicated. Panel A shows the net changes in PtdInsP (□) and $PtdInsP_2$ (○); panel B shows the changes in Ptd A (△).

of experiments in which the changes in the polyphosphoinositide pools caused by thrombin stimulation for between 1 and 60 s with special emphasis on the first 10 s was performed using several batches of platelets. The results are summarized in table 1. In all cases an initial increase in the radioactivity associated with the polyphosphoinositide pools was observed.

Addition of thrombin to platelets, prelabelled with [^{32}P]orthophosphate for 10 min at 37°C, has been shown to enhance the uptake of [^{32}P]orthophosphate into the platelets within 30 s. This increases the specific activity of ATP and polyphosphoinositides [12], something which must be taken into account when evaluating the results discussed above. Comparison of the time course of the increase in specific activity in ATP [12] with the time courses of the increases in the

Table 1

Changes in ^{32}P-radiolabelled PtdInsP and $PtdInsP_2$ pools caused by stimulation of prelabelled platelets with thrombin (0.5 units · ml^{-1})

Time (s)	PtdInsP (% of control)	$PtdInsP_2$ (% of control)
1	108 (1)	106 (1)
2	111 ± 2 (2)	108 ± 2 (2)
3	108 ± 2 (5)	108 ± 4 (5)
4	102 (1)	108 (1)
5	110 ± 8 (2)	105 ± 5 (2)
6	101 (1)	100 (1)
7	104 ± 9 (3)	99 ± 5 (3)
10	114 ± 6 (2)	104 ± 6 (2)
15	103 ± 3 (2)	99 ± 3 (2)
20	112 (1)	90 (1)
30	101 ± 6 (4)	86 ± 8 (4)
40	113 (1)	92 (1)
60	111 (1)	96 (1)

The result is expressed as percent of the unstimulated control, the number of experiments is indicated within brackets

radioactivity associated with the poly-phosphoinositide pools described here suggest that the latter is a more rapid response. This conclusion is corroborated by the presence of a distinct lag phase in the labelling of Ptd A, since it is known that the specific activity in Ptd A is in equilibrium with the γ-phosphate of ATP [13].

The apparent discrepancy between the present results and those reported earlier, where thrombin was found to cause an immediate and rapid decrease in the polyphosphoinositide pools [11], is explained by the difference in thrombin concentration used in stimulating the platelets. Increasing the concentrations of thrombin made it increasingly difficult to catch the initial rise in the radioactivity in the polyphosphoinositide pools, and with 5 units $\cdot$ ml^{-1} of thrombin which is the concentration usually used, only the phase of rapid decrease was seen (data not shown).

Finally, thrombin activation also led to an initial increase in the radiolabelling of the PtdInsP and PtdInsP$_2$ pools in an experiment in which platelets were labelled with [^{32}P]orthophosphate prior to gel filtration (data not shown).

4. DISCUSSION

The results presented here indicate that binding of thrombin to its cognate receptors on the platelet surface causes a rapid activation of kinases increasing the rate of synthesis of polyphosphoinositides, and that this effect precedes the degradation of these inositides. This is consonant with the idea that events generating polyphosphoinositides might be involved in the recruitment of profilin/actin for microfilament formation which was the starting point for these experiments.

Indications of receptor-mediated activation of kinases forming polyphosphoinositides were also seen in experiments with thrombin-treated fibroblasts [14] and platelets [5] where hydrolysis of PtdInsP$_2$ was inhibited with neomycin.

In the case of the β-receptor for platelet-derived growth factor which also mediates a signal activating actin polymerization [15], a direct association with a PDGF-activated PtdIns kinase has recently been demonstrated [16]. This kinase phosphorylates the 3′-hydroxyl of the inositol ring of PtdIns giving rise to PtdIns(3)P, PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$, which in the cell appear in parallel to the classical PtdIns(4)P and PtdIns(4,5)P$_2$. Whether these newly discovered polyphosphoinositides also constitute a significant fraction of the polyphosphoinositides formed in thrombin-stimulated platelets, and if they like the 4′-phosphorylated inositides bind to profilin and dissociate the profilin/actin complex is not yet known.

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