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DEGRADATION OF PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE IS INSENSITIVE TO CA^{2+} MOBILIZATION IN STIMULATED PLATELETS

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SUMMARY. Thrombin stimulation of [^{32}P]-prelabeled platelets induces a rapid decrease of the radioactivity from phosphatidylinositol-4,5-bisphosphate. No significant change is observed in phosphatidylinositol-4-monophosphate. The initial, thrombin-induced decrease of phosphatidylinositol-4,5-bisphosphate is not inhibited by cytochalasin D or by compounds that interfere with the mobilization of Ca^{2+} such as 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate, the calmodulin-antagonist, trifluoperazine, prostacyclin and cyclic AMP. Our information indicates that the rapid loss of phosphatidylinositol-4,5-bisphosphate is linked to receptor activation and insensitive to Ca^{2+}-mobilization.

Activation of platelets by ADP, thrombin, collagen or adrenaline, induces typical physiological responses such as shape change, release of the contents of the alpha and dense granules and aggregation. It is generally thought that these platelet responses are effected through the mobilization of Ca^{2+} from intracellular compartments (1). The polyphosphoinositides, phosphatidylinositol-4-monophosphate and phosphatidylinositol-4,5-bisphosphate bind Ca^{2+} with high affinity (2). Since these two lipids appear to be preferentially located at the inner surface of the plasma membrane (3), it is possible that the initial release of Ca^{2+} could be from these lipids. In support of such an idea, we have recently observed that thrombin stimulation of platelets causes a rapid decrease in the phosphatidylinositol-4,5-bisphosphate content (4). We now report that the initial decrease of platelet phosphatidylinositol-4,5-bisphosphate can still be observed in the presence of different inhibitors

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(8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8)¶, trifluoperazine, cytochalasin D, prostacyclin, cyclic AMP), which block Ca^{2+}-dependent responses. We suggest that the rapid degradation of phosphatidylinositol-4,5-bisphosphate is not a consequence of the mobilization of cytosolic Ca^{2+} and could, therefore, be involved in the initial release of Ca^{2+} from platelet membranes.

MATERIALS AND METHODS

Most materials were obtained as previously described (4-7). Washed platelets were isolated as before (4-7). Platelets from 500 ml of horse blood were suspended in 20 ml of buffer containing 25 mM Hepes/1 mM EGTA (pH 7.0), 130 mM NaCl and 10 mM glucose and then incubated with carrier-free (^{32}P)orthophosphate (New England Nuclear) at 37°C for 90-120 min. Platelets were then washed with 100 ml of the buffer and gently resuspended in 20 ml of the same buffer. Triplicate samples (0.5 ml, 7 x 10^8 platelets) were preincubated for 7 min at 37°C before addition of appropriate compounds for 5 min. Thrombin (1 unit/ml) was then added for 10 sec. The reactions were stopped by adding 1.8 ml of chloroform/methanol/conc. HCl (100:200:2, v/v) and the phases separated by addition of 0.6 ml of chloroform and 0.6 ml of 2 M KCl (4). The lower chloroform phase was transferred and dried under a flow of N_2. The lipids were separated on thin layer plates (silica gel G impregnated with 1% potassium oxalate containing 2 mM EDTA) using chloroform/methanol/4 N NH_4OH (45:35:10, v/v) (4,8). The lipids were visualized by autoradiography. The R_f values for various phospholipids were as follows: 0.28, phosphatidylinositol-4,5-bisphosphate; 0.36, phosphatidylinositol-4-monophosphate; 0.44, lysophosphatidylinositol; 0.51, phosphatidic acid. The lipids were then counted by liquid scintillation in Bray's solution. The experiments presented are representative of three giving very similar results.

RESULTS AND DISCUSSION

Measurements of radioactivity and phosphorus content of platelet [^{32}P]phosphatidylinositol-4,5-bisphosphate and [^{32}P]phosphatidylinositol-4-monophosphate show that thrombin (2 units/ml) induces degradation of 15-20% of the total phosphatidylinositol-4,5-bisphosphate within 10 sec (4). The mobilization of Ca^{2+} also takes place within seconds after stimulation of platelets (9). Ionophore A23187, which is known to increase the cytosolic concentration of Ca^{2+}, induces little or no phosphatidylinositol-4,5-bisphosphate degradation (4) suggesting that the loss of phosphatidylinositol-4,5-bisphosphate is not a consequence of Ca^{2+}-mobilization. This point has been further studied by using agents which interfere with stimulus-induced Ca^{2+}-mobilization. TMB-8 (8-(Diethylamino)-octyl-3,4,5-trimethoxybenzoate), an anaesthetic-like compound,

¶Abbreviations: TMB-8, 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate; TPI, phosphatidylinositol-4,5-bisphosphate; DPI, phosphatidylinositol-4-monophosphate; LPI, lysophosphatidylinositol; PA, phosphatidic acid; LPA, lysophosphatidic acid.

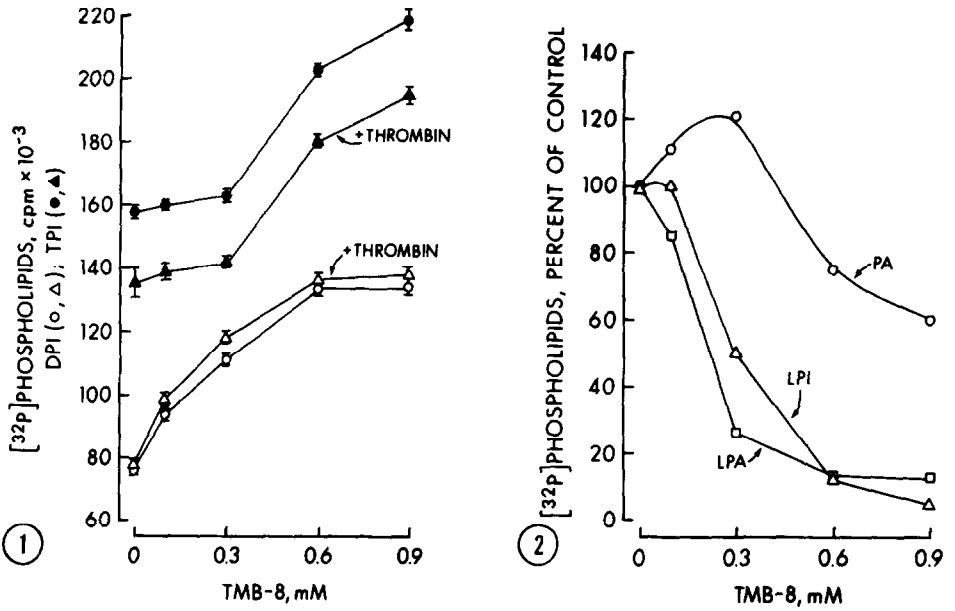

Fig. 1 - The effect of TMB-8 on thrombin-induced changes in [^{32}P]phosphatidylinositol-4,5-bisphosphate (TPI) and [^{32}P]phosphatidylinositol-4-monophosphate (DPI).

Triplicate samples (0.5 ml) of [^{32}P]platelets were preincubated with various concentrations of 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8) for 5 min before addition of thrombin (1 unit/ml) for 10 sec. The lipids were analyzed as detailed in Methods.

Fig. 2 - The effect of TMB-8 on the thrombin-induced formation of phosphatidic acid (PA), lysophosphatidylinositol (LPI) and lysophosphatidic acid (LPA).

Conditions are as in Fig. 1 except that incubations with thrombin (1 unit/ml) were for a period of 2 min. The identity of lysophosphatidylinositol produced during platelet stimulation with thrombin or ionophore A23187 has been confirmed and extensively discussed in a previous report (7).

inhibits the release reaction (10) and the liberation of arachidonic acid from membrane phospholipids (11) presumably by blocking the mobilization of Ca^{2+} from intracellular stores (12). Trifluoperazine at appropriate concentrations (10-50 μM) is a calmodulin-antagonist (13) and also blocks activation of phospholipases A_2 in platelets (14,15). Neither of these compounds is able to block the loss of phosphatidylinositol-4,5-bisphosphate induced by thrombin (1 unit/ml) (Fig. 1 and Table 1). Under the same conditions of stimulation, TMB-8 (Fig. 2) inhibits the formation of lysophospholipids produced by Ca^{2+}-dependent phospholipase A_2 activities (11). These results indicate that the loss of phosphatidylinositol-4,5-bisphosphate in stimulated platelets is not due to the internal mobilization of Ca^{2+} or to calmodulin-dependent reactions.

Table 1. The effect of Ca^{2+}, trifluoperazine and cytochalasin D on thrombin-induced changes in platelet [^{32}P]phosphatidylinositol-4,5-bisphosphate (TPI) and [^{32}P]phosphatidylinositol-4-monophosphate (DPI)

Additions	[^{32}P]TPI % of Control	[^{32}P]TPI % Net Loss	[^{32}P]DPI % of Control
None	100 ± 1.1	-	100 ± 1.5
Thrombin (1 unit/ml)	87.5 ± 1.1	12.5	100.8 ± 1.5
Ca^{2+} (1 mM)	95.9 ± 1.2	-	96.1 ± 1.5
Ca^{2+} + Thrombin	83.8 ± 1.2	12.1	96.1 ± 2.3
Trifluoperazine (50 μM)	113.4 ± 4.6	-	167.2 ± 3.5
Trifluoperazine + Thrombin	100.2 ± 2.4	13.2	162.8 ± 3.7
Cytochalasin D (10 μM)	107 ± 1.5	-	83.8 ± 2.6
Cytochalasin D + Thrombin	93 ± 2.6	14.4	86.2 ± 1.0

Triplicate samples (0.5 ml) of [^{32}P]-labeled platelet suspension were preincubated for 5 min with 1 mM Ca^{2+} or 50 μM trifluoperazine or 10 μM cytochalasin D before addition of 1 unit/ml thrombin for 10 sec. The lipids were analyzed as described in Methods. Percentages were calculated with regard to control incubations containing no additions. Net percent loss induced by thrombin was calculated in comparison to appropriate controls with trifluoperazine or cytochalasin D alone.

It should be noted that both TMB-8 (Fig. 1) and trifluoperazine (Table 1) induce increased formation of phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-4-monophosphate. These increases are due to the direct effects of these agents on the phosphorylating enzymes (unpublished observation)

Table 2. The effect of prostacyclin and dibutyryl-cAMP on thrombin-induced changes in platelet [^{32}P]phosphatidylinositol-4,5-bisphosphate (TPI) and [^{32}P]phosphatidylinositol-4-monophosphate (DPI).

Additions	Percent of Control	
	[^{32}P]TPI	[^{32}P]DPI
Thrombin (1 unit/ml)	84.7 ± 6.0 (9)	100
Prostacyclin (2 μg/ml)	94.3 ± 2.5 (9)	107
Prostacyclin + Thrombin	88.3 ± 5.1 (9)	107
Thrombin (1 unit/ml)	87.0 ± 1.4 (6)	100
Dibutyryl-cAMP (2 mM)	95.5 ± 0.7 (6)	106
Dibutyryl-cAMP + Thrombin	91.5 ± 0.5 (6)	106

Triplicate samples (0.5 ml) of [^{32}P]-labeled platelet suspension were preincubated with prostacyclin for 2 min or with dibutyryl-cyclic AMP for 5 min before addition of thrombin (1 unit/ml) for 10 sec. The lipids were analyzed as described in Methods. Results for [^{32}P]phosphatidylinositol-4,5-bisphosphate are expressed as % of control (mean ± S.D.). Number of incubations are indicated in parentheses.

Prostacyclin increases the intracellular cyclic AMP levels in platelets (16-20). Both prostacyclin and cyclic AMP inhibit secretion of platelet granules and aggregation and it is believed that cyclic AMP exerts its effects by interfering with Ca^{2+}-mobilization (15-22). Increased platelet cyclic AMP causes Ca^{2+}-sequestration and inhibits agonist-induced mobilization of Ca^{2+} as measured by chlortetracycline fluorescence technique (23). Table 2 shows that prostacyclin and dibutyryl-cyclic AMP cause only a slight inhibition of phosphatidylinositol-4,5-bisphosphate degradation. Under these conditions, prostacyclin inhibits the thrombin-induced formation of phosphatidic acid and lysophosphatidylinositol by 80% and 95%, respectively. Similar results were obtained with dibutyryl-cyclic AMP. Interestingly, each of these agents by itself causes a small, but reproducible, loss of phosphatidylinositol-4,5-bisphosphate. An inhibitory effect of cyclic AMP on phosphatidylinositol-4-monophosphate kinase, which converts phosphatidylinositol-4-monophosphate to phosphatidylinositol-4,5-bisphosphate, might explain this observation; phosphatidylinositol-4-monophosphate kinase has been shown to be modulated in brain, by a phosphorylation-dephosphorylation mechanism (24).

Another early event that occurs in thrombin-stimulated platelets is the polymerization of actin and this effect is blocked by cytochalasin D (25). Thrombin-induced phosphatidylinositol-4,5-bisphosphate degradation remains unaffected by pretreatment of platelets with cytochalasin D (Table I). Cytochalasin D alone appears to facilitate conversion of phosphatidylinositol-4-monophosphate to phosphatidylinositol-4,5-bisphosphate (Table I).

Our previous results suggest that phosphatidylinositol-4,5-bisphosphate degradation occurs very early during stimulation and may be linked to receptor activation (4). Furthermore, we now show that this transient loss of phosphatidylinositol-4,5-bisphosphate is not secondary to other early events such as Ca^{2+}-mobilization, phospholipase activation, formation of arachidonate-metabolites or actin polymerization.

Phosphatidylinositol is also rapidly degraded upon stimulation of platelets with thrombin (5,20,21,26). The degradation of phosphatidylinositol by a phosphatidylinositol-specific phospholipase C leads to the initiation of the

phosphatidylinositol-cycle. This cycle of reactions is initiated by a small increase in the cytosolic concentration of Ca^{2+} as we have recently discussed (27). Therefore, the possibility exists that the phosphatidylinositol-cycle could be a consequence of the thrombin-induced degradation of phosphatidylinositol-4,5-bisphosphate.

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