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PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE MAY REPRESENT THE SITE OF RELEASE OF PLASMA MEMBRANE-BOUND CALCIUM UPON STIMULATION OF HUMAN PLATELETS

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SUMMARY: Thrombin stimulation of human blood platelets caused an extensive (up to 45 %) and rapid (5-10 s) decline in endogenous phosphatidylinositol 4,5-bisphosphate (PI-P_2). Thrombin initiated an equally rapid loss of membrane-bound Ca, as indicated by the decrease in fluorescence of chlortetracycline (CTC)-loaded platelets. PI-P_2 breakdown also correlated with decreased CTC fluorescence upon use of other platelet stimuli: Arachidonate caused moderate and slow decreases in both PI-P_2 and CTC fluorescence, while ionophore only induced minimal changes. Thrombin-induced decreases in PI-P_2 content could account for release of sufficient membrane-bound Ca to raise cytoplasmic free [Ca^{2+}] to 1-2 uM, supporting the hypothesis that PI-P_2 represents the Ca-binding site involved in the stimulus-dependent increase in cytoplasmic Ca^{2+} evoked by receptor-ligand interactions.

Stimulation of human platelets with physiological (thrombin, collagen) and nonphysiological agonists (ionophores) leads to release of contents of intracellular organelles into the medium, and culminates in aggregation. These phenomena are accompanied by intracellular events: mobilization of membrane-bound Ca (1-3), phospholipid metabolism (4-11), oxygenation of arachidonate to icosanoids (12), and protein phosphorylations (11, 13).

PI, PI-P, and PI-P_2 may play an integral role in stimulus-response coupling involving Ca metabolism (14, 15). Upon cell stimulation PI-P_2 is hydrolyzed by a phospholipase C, producing diglyceride and inositol 1,4,5-trisphosphate (16, 17). The latter could act as a "second messenger" in the release of intracellular Ca (18). The well-known affinity of PI-P_2 for Ca (19, 20) suggests that PI-P_2 may be involved in intracellular mobilization of

ABBREVIATIONS: CTC, chlortetracycline; PA, phosphatidic acid; PI, phosphatidylinositol; PI-P, phosphatidylinositol 4-phosphate; PI-P_2, phosphatidylinositol 4,5-bisphosphate.

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Ca in platelets (10, 21). The present study demonstrates that in platelets PI-P_2 could represent the receptor-linked site from which bound Ca is released to raise intracellular free Ca as measured by quin-2 (22).

EXPERIMENTAL PROCEDURES

Platelet suspensions were prepared as described (6, 9), resuspended in 154 mM NaCl and adjusted to a platelet count of 10^{10}/ml.

Phospholipid studies were carried out at 37°C in a total volume of 2 ml: .5 ml platelet suspension was added to 1.4 ml buffer (75 mM Tris, 95 mM NaCl, 7 mM glucose, 3 mM KH_2PO_4, .35% fatty acid-free bovine albumin, pH 7.4); after 4 min preincubation, agonist was added in 100 ul 154 mM NaCl. Incubations were stopped and lipids extracted (6, 9, 23) by adding .5 ml EDTA/KCl (.1 M disodium EDTA/2 M KCl, pH 6.5) and 7 ml $CHCl_3$/MeOH (1:2, v/v). The lower phase was removed after adding 1.5 ml EDTA/KCl and 2 ml $CHCl_3$. The upper phase washed once with 3.5 ml $CHCl_3$, and the combined lower phases dried under N_2 (37°C) and applied in $CHCl_3$/MeOH (2:1) to TLC plates. PI-P_2 and PI-P were separated (24), and lipid phosphorus determined (6).

For CTC fluoresence measurements, platelet-rich plasma was incubated (25 min, 37°C, 50 uM CTC) (1, 25), and platelet suspensions prepared as above. Fluorescence was measured (37°C) in a Perkin-Elmer 650-10S instrument. Samples were prepared in the same proportions as for lipid analyses. Fluorescence changes were recorded without stirring in order to measure the initial effects of stimulation without superimposition of aggregation effects. Wavelengths and slitwidths were: excitation - 380 nm, 5 nm width; emission - 560 nm, 2 nm width. After preincubation to establish base line levels of CTC fluorescence, platelets were stimulated as above by rapid injection of agonist in 100 ul 154 mM NaCl.

Ionomycin was a gift from Squibb, Princeton, NJ. Chlortetracycline was purchased from Sigma, St. Louis, MO, and TLC plates from Whatman, Clifton, NJ. Other reagents were obtained as described (6, 9).

RESULTS AND DISCUSSION

Thrombin addition (.3 U/10^8 platelets) initiated a rapid decrease in endogenous PI-P_2 (Fig. 1). At this level of thrombin the observed net loss of PI-P_2 averaged 30-40% of control values in 10 separate studies, and was

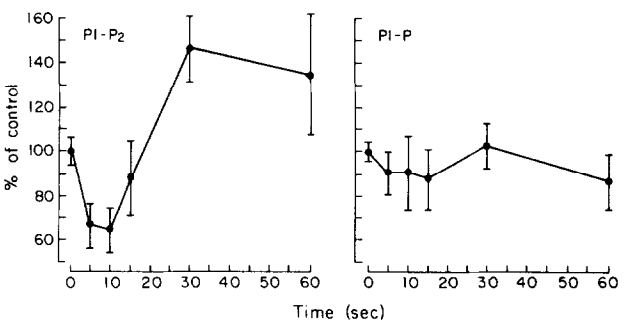

Fig. 1 Changes in endogenous PI-P_2 (left) and PI-P (right) upon stimulation with 0.3 U thrombin/10^8 platelets. A net loss of PI-P_2 of more than 35 % occurred within 5-10 s, and was followed by a rise to 150 % (zero time PI-P_2 levels were 77 pmoles/10^8 platelets). In contrast, the levels of PI-P did not change significantly at any thrombin dose used.

TABLE 1

PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE CONTENT* FOLLOWING THROMBIN STIMULATION OF WASHED PLATELETS

Thrombin, Units/10^8 Platelets	Sec Following Stimulation 5	10	15
0.02	90	90	92
0.06	79	80	95
0.20	77	74	93
0.30	61	64	82

* Percent of unstimulated control platelets.

Washed human platelets were stimulated with thrombin, lipids extracted, and PI-P_2 separated from other phospholipids and assayed for phosphorus. See Experimental Procedures for details. Results are means of 2 or more experiments each performed in triplicate.

complete within 5-10 sec. At intervals greater than 15 sec after thrombin stimulation there was rapid resynthesis of PI-P_2, resulting in a PI-P_2 level 130-150% of that of unstimulated controls. The possibility that the decrease in PI-P_2 was related to an influx of Ca^{2+} from the medium was ruled out, since both time course and extent of the PI-P_2 decrease were unaffected by addition of 5 mM EGTA. This observation extends our previous studies indicating that the initial changes in platelet PI and PA induced by thrombin were unaffected by the presence of EGTA in the medium (6, 9). Thrombin addition did not exert a major effect on PI-P content (Fig. 1).

Decreases in PI-P_2 content depended on the quantity of thrombin added, as did the time required to reach the nadir (Table 1). CTC fluorescence, an indicator of release of membrane-bound Ca, showed a similar dose-dependency of both the magnitude of the decreases in CTC fluorescence, as well as for the time required to reach the level of maximum decrease (Fig. 2).

The decrease in PI-P_2 content upon platelet stimulation was not limited to thrombin but also occurred with arachidonate (Fig. 3). However, the decrease in PI-P_2 was only moderate and occurred slowly (Fig. 3) compared to thrombin (Fig. 1). Both ionophore A23187 (2 uM), which induces breakdown of PI by a mechanism independent of phospholipase C activation (8), as well as ionomycin (2 and 5 uM, Fig. 3), had only minor effects on PI-P_2 degradation,

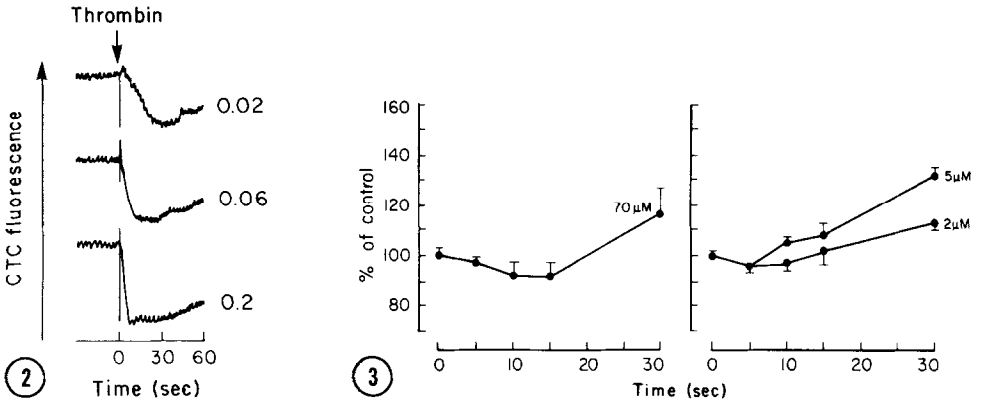

Fig. 2 CTC-fluorescence decreases initiated by thrombin stimulation. Both the extent of the fluorescence decrease as well as the time required to reach the nadir were dose dependent. The maximum decrease in fluorescence was 4.9, 5.9, and 6.2 % of control (100 %) fluorescence for each of the thrombin concentrations (indicated in U/10^8 platelets) utilized.

Fig. 3 Effects of arachidonate (left) and ionomycin (right) addition on endogenous PI-P_2 levels. While arachidonate induced a slow and small decrease in PI-P_2, ionomycin addition did not result in significant decreases. At later times an increase in PI-P_2 content similar to that seen with thrombin (compare Fig. 1) was observed. The arachidonate and ionomycin concentrations take binding to albumin into account.

although the concentrations employed induce rapid aggregation and arachidonate oxygenation (data not shown).

The capacity of ionomycin, arachidonate, and thrombin to reduce platelet PI-P_2 content was compared to their effect on CTC fluorescence (Fig. 4). Again, a very similar pattern emerged: While thrombin's action was rapid, and caused large decreases in both PI-P_2 content and CTC fluorescence, arachidonate was only moderately active, and ionomycin displayed little activity.

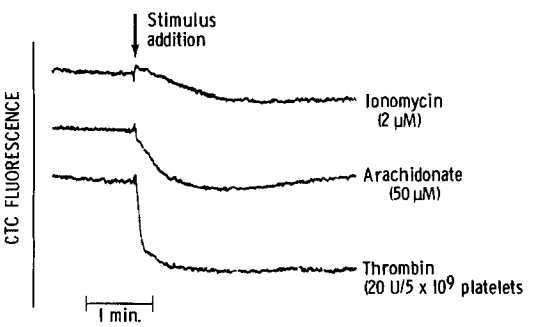

Fig. 4 Effects of ionomycin, arachidonate, or thrombin addition on CTC-fluorescence. Changes initiated by ionomycin were minimal. Decreases caused by arachidonate addition were only moderate and slow. In contrast, decreases in CTC fluorescence induced by thrombin were rapid and extensive.

CONCLUSIONS

The similarities between the decreases in endogenous platelet PI-P_2 content and the decreases in membrane-bound Ca monitored by CTC fluorescence suggest that PI-P_2 represents the site of membrane-bound Ca. Recently, membrane-associated charged phosphate groups have been implicated in CTC fluorescence (26). The present data suggest that, in the case of platelets, PI-P_2 could provide these membrane-associated phosphate groups in a stimulus-sensitive configuration, rendering membrane-bound Ca available for release upon cell stimulation. An alternative, or perhaps supplementary, explanation for the close correlation between the decrease in CTC fluorescence and the decrease in platelet PI-P_2 content might be a "second messenger" function for inositol 1,4,5-trisphosphate. This was suggested by Streb et al, since addition of inositol 1,4,5-trisphosphate, which is produced from PI-P_2 by phospholipase C, to cells with permeabilized plasma membranes resulted in release of Ca from intracellular, non-mitochondrial stores (18). However, the CTC response of human neutrophils to degranulating concentrations of fMLP is retained in cytoplasts, neutrophils devoid of intracellular organelles (27). This observation strongly suggests that the CTC response originates at the plasma membrane, and not the endoplasmic reticulum.

The hypothesis that PI-P_2 represents the receptor-linked site from which Ca is released requires that sufficient PI-P_2 is degraded upon stimulation to raise cytosolic free Ca to 1-2 uM (22). A 45 % decline in PI-P_2 content is equivalent to 35×10^{-5} fmoles Ca/platelet, yielding a total concentration of cytosolic Ca of .35 mM (at a cytosolic volume of 1 fL/ platelet). The actual concentration of free Ca will be much lower, due to the high content of Ca-binding proteins in the platelet cytoplasm. Thus the hypothesis that PI-P_2 represents the membrane-associated Ca-binding site is in agreement with reported measurements of platelet intracellular free Ca using quin-2 (22).

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