The Rapid Formation of Inositol Phosphates in Human Platelets by Thrombin Is Inhibited by Prostacyclin*

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The biochemistry events underlying the ability of thrombin to enhance the metabolism of inositol phospholipids in human platelets have been investigated using platelets prelabeled with \[^{3}H\]inositol. Thrombin treatment caused rapid formation of radioactive inositol monophosphate (IP), inositol bisphosphate (IP2), and inositol trisphosphate (IP3) with less marked and more variable changes in the levels of radioactive inositol phospholipids. Formation of IP2 and IP3 could be detected 5 s after exposure to thrombin and before IP levels increased. Low doses of thrombin which produced only shape change in human platelets also caused significant formation of IP2 and IP3 but not IP. These results suggest that thrombin-induced platelet activation may be mediated through hydrolysis of polyphosphoinositides. The majority of IP formed presumably arises from the hydrolysis of IP2. Prostacyclin inhibited thrombin-induced formation of all three inositol phosphates.

Stimulation of platelets by thrombin is associated with rapid changes in membrane inositol phospholipids through activation of phospholipases C and A2 (Lapetina and Siess, 1983). Stimulation of phospholipase C occurs before phospholipase A2 activation (Lapetina, 1982). However, it is presently unclear which of the various inositol phospholipids are hydrolyzed by phospholipase C and to what extent. \[^{32}P\]IP2 levels decrease in platelets within the first 5 s of exposure to thrombin (Billah and Lapetina, 1983; Agranoff et al., 1983), and the immediate breakdown product of phospholipase C attack on this phospholipid, \[^{32}P\]IP3, increases within this time period (Agranoff et al., 1983). These data, therefore, demonstrate that phospholipase C directly hydrolyzes IP2 on exposure to thrombin but do not rule out the possibility that it simultaneously degrades PI and possibly IPPI. The present study has, therefore, been undertaken to further investigate the pathway of inositol phospholipid metabolism that takes place on exposure to thrombin by monitoring the rates of production of \[^{3}H\]inositol phosphates. In addition, the action of prostacyclin on thrombin-induced formation of inositol phosphates has been investigated.

EXPERIMENTAL PROCEDURES

Preparation of \[^{3}H\]inositol-labeled Human Platelets—Platelets were prepared from fresh human blood (100 ml) anticoagulated with 0.15 volume of ACD buffer (85 mM trisodium citrate, 111 mM dextrose, 71 mM citric acid). Donors had not received medication in the previous 3 weeks. Platelet-rich plasma was obtained by centrifugation at 200 X g for 20 min; it was then centrifuged at 800 X g for 15 min in the presence of prostacyclin (5 ng/ml) to yield a platelet-rich pellet. This pellet was then resuspended in 3 ml of a modified Tyrode-HEPES buffer (134 mM NaCl, 12 mM NaHCO3, 2.9 mM KCl, 0.34 mM Na-PO4, 1 mM KCl, 5 mM HEPES, 5 mM glucose, and 0.1 mM EGTA buffered to pH 7.4) containing \[^{3}H\]inositol (64 μCi/ml). Platelets were incubated at 37 °C for 3 h and then washed with 30 ml of buffer in the presence of prostacyclin (1 μg/ml). Platelets were finally resuspended in buffer without EGTA or prostacyclin, and their concentration adjusted to 8.0 X 10^6/ml using a Coulter Counter 2P (Coulter Electronics, Ltd.).

Measurement of \[^{3}H\]inositol Phosphates and \[^{3}H\]inositol Lipids in Association with Platelet Activation—Platelets were prewarmed to 37 °C and samples (0.5 ml) placed in aggregometer tubes and stirred for 1 min at 37 °C in a Chronolog aggregometer. In some experiments, prostacyclin and/or isobutylmethylxanthine were present during this period. Thrombin in varying concentrations was then added and the reaction stopped at various times by transferring the platelet suspension into tubes containing 1.88 ml of chloroform:methanol:HCl (100:200:2) by means of a Pasteur pipette. Light transmission was recorded throughout the experiment and displayed on a chart recorder. Decreased light transmission represents the shape change of platelets from a disc to a sphere, while increased light transmission reflects platelet aggregation. For experiments in which only \[^{3}H\]inositol phosphates were measured, chloroform (0.62 ml) and water (0.62 ml) were then added and the tubes vortexed and centrifuged at 800 X g for 10 min. The upper phase contained the water-soluble inositol phosphates, and portions (1.5 ml) were transferred to test tubes and water (2.5 ml) added. The inositol phosphates were then separated on Dowex 1 anion exchange columns as previously described by Berridge (1983). The following fractions were collected: \[^{3}H\]inositol and \[^{3}H\]hypocobalaminositol (16 ml of 60 mM ammonium formate, 5 mM dисodium tetraborate); IP (16 ml of 200 mM ammonium formate, 100 mM formic acid); IP2 (20 ml of 400 mM ammonium formate, 100 mM formic acid); IP3 (12 ml of 1 M ammonium formate, 100 mM formic acid). Control studies using labeled standards had shown that this elution pattern yields 100% recovery of all these inositol phosphates. Radioactivity in portions (8 ml except for \[^{3}H\]IP1 (4 ml plus 4 ml of H2O)) of these fractions was determined by scintillation counting in the gel phase using 60% (v/v) Aquasol and corrected for all loss factors. For experiments in which inositol phospholipids and inositol phosphates were both measured, the samples were divided and one-half was treated as above, and the other half treated with chloroform (0.31 ml) and 2 M KCl (0.31 ml), vortexed, and centrifuged at 800 X g for 10 min. The use of 2 M KCl enables better extraction of the polyphosphoinositides. The inositol lipids in these samples are located in the lower phase, and portions (0.8 ml) of this were dried under nitrogen and resuspended in chloroform (0.05 ml). The inositol phospholipids were then separated on oxalate-impregnated Silica Gel G-25 thin-layer chromatography plates developed in chloroform, methanol, 4 N ammonium hydroxide (45:45:10, v/v) as previously described (Billah and Lapetina, 1982). The lipids were visualized by autoradiography and characterized by co-elution with nonlabeled standards detected with iodine. The lipids were then scraped and counted by liquid scintillation and corrected.

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1 The abbreviations used are: IP2, phosphatidylinositol 4,5-bisphosphate; IP, inositol monophosphate; IP2, inositol bisphosphate; IP3, inositol trisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; HPLC, high-performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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for all loss factors. In most cases, results are shown from one experiment which is typical of 3-6 experiments performed under similar conditions.

**Characterization of the Inositol Phosphates**—The various fractions eluted from the Dowex columns were characterized by comparison with standards on Whatman No. 1 paper developed in n-propyl alcohol-ammonium water (5:4:1) for 24 h (Gräe and Ballou, 1961), TLC-SA glass fiber sheets developed in ethanol-ammonia (3:2) (Hokin-Neaverson and Sadeghian, 1976) and by HPLC. The HPLC apparatus consisted of a Waters Associates model 6000A solvent delivery system and a UGK injector. Separations were performed on a Whatman analytical SAX column utilizing a 30-min gradient from 0.02 to 0.80 M ammonium phosphate, pH 3.35, at a flow rate of 1 ml/min unless stated otherwise. Fractions (1 ml) were collected and counted by liquid scintillation using 2 ml of Aquasol.

**Materials**—32P-labeled IP2 and IP3 were produced by incubating ghosts of prelabeled red blood cells with Ca2+ (Downes et al., 1982). [3H]inositol 1-phosphate were obtained by incubating phosphatidyl[3H]inositol (270 mCi/mmol, Amersham International) with phospholipase C (Sigma) in Tyrode-HEPES buffer for 30 min at 37 °C; the water-soluble inositol phosphates were separated on Dowex 1 anion exchange columns and elution with water. Amersham International and pretreated before use by passage through a Dowex 1 anion exchange column and elution with water. Thrombin, PIP, PIP2, and lysophosphatidylinositol were from Sigma. Prostacyclin was from The Wellcome Research Laboratories, Beckenham, and the Dowex 1 anion exchange resin (100-200 mesh) was obtained from Bio-Rad. TLC-SA glass fiber sheets were from Gelman, Ann Arbor, MI, and Silica Gel G-25 thin-layer chromatography plates were from Brinkmann, New York.

**RESULTS**

**Thrombin Induces Rapid Formation of Inositol Phosphates**—Thrombin (1 unit/ml) induced rapid formation of radioactive IP, IP2, and IP3 in human platelets labeled with [3H]inositol (Figs. 1 and 7). Formation of [3H]IP2 and [3H]IP3 could be detected 5 s after stimulation (Figs. 1 and 7) and before [3H]IP levels increased (Figs. 1 and 7). Similar results were obtained in the presence of Li+ (10 mM) (Fig. 1A) which blocks breakdown of IP to free inositol in many tissues (Berridge et al., 1982) including the platelet (compare Fig. 1, A and B); Li+ (10 mM) had no effect on the shape change or aggregation response produced by thrombin. The increase in IP3 peaked after 10–20 s and was much smaller than the increment in IP2 which continued to rise throughout the first 2 min of stimulation (Figs. 1, 2, and 7). 

The rapid formation of inositol phosphates by thrombin confirms the observation previously made by Agranoff et al. (1983) using [32P]orthophosphate-labeled platelets. However, in contrast to the present results, Agranoff et al. (1983) did not detect an early or marked formation of [32P]IP2. The explanation for this difference between the two studies is not known but does not appear to be related to the different radiolabels used, as we have observed rapid (within 5 s)
formation of $[^{32}P]IP_2$ in platelets exposed to thrombin (not shown).

Confirmation that the increases in radioactivity observed were due to stimulated production of the various inositol phosphates was investigated using HPLC. The various fractions eluted from the Dowex columns were each found to consist of one main peak of radioactivity which co-chromatographed with its corresponding standard (Figs. 2 and 3). This strongly suggests that thrombin stimulates the formation of inositol 1-phosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-trisphosphate in human platelets. These results were confirmed by paper (Grado and Ballou, 1961) and thin-layer chromatography as described by Downes et al. (1982). [$^3H$]inositol trisphosphate was prepared from human platelets challenged with thrombin (1 unit/ml) for 2 min and separated from other labeled compounds using Dowex anion exchange chromatography (see "Experimental Procedures"). Samples of both inositol trisphosphates were co-applied to a high-performance liquid chromatography apparatus, and separations were performed on a Whatman analytical SAX column using a 20-min gradient from 0.4 to 0.8 M ammonium phosphate, pH 3.5, at a rate of 1 ml/min. Fractions (0.5 ml) were collected and counted by liquid scintillometry; window settings were set such that there was no spillover in radioactivity between the $^3H$ and $^{32}P$ channels for the amount of radioactivity applied.

The action of thrombin (1 unit/ml) on inositol phospholipid levels was more variable. Other workers had previously demonstrated that thrombin induced an initial decrease in [$^{32}P$]PIP2 followed by an increase (Billah and Lapetina, 1982; Agranoff et al., 1983). However, for platelets prelabeled with [$^3H$]inositol and stimulated with thrombin (1 unit/ml), there was no consistent pattern, and both initial increases and decreases in [$^3H$]PIP2 were observed (not shown). This is presumably a reflection of marked variability between experiments in the amount of [$^3H$]inositol incorporated into the lipids, similar to that previously observed by Berridge (1983).

Formation of Inositol Phosphates Correlates with Shape Change and Aggregation—The formation of all three [$^3H$]inositol phosphates by thrombin was dose related and correlated with the concentration curve for aggregation (Fig. 4).

Doses of thrombin (0.01–0.02 unit/ml) sufficient to produce only shape change produced significant formation of [$^3H$]IP2 and [$^3H$]IP3 (Fig. 5).

Prostacyclin Blocks Thrombin-induced Formation of Inositol Phosphates—The effect of increasing doses of prostacyclin on the action of thrombin (0.1 unit/ml) on platelets is shown in Fig. 6. Prostacyclin caused a dose-related decrease in thrombin (0.1 unit/ml)-induced formation of all three [$^3H$]inositol phosphates, and this was accompanied by a complete loss of aggregatory response and increased formation of [$^3H$]IP1 (Fig. 6). In 4 out of 5 experiments, a maximally effective dose of prostacyclin did not fully block thrombin (0.1 unit/ml)-induced formation of [$^3H$]inositol phosphates as exemplified in Fig. 6, but in one other experiment complete inhibition was observed. Against higher doses of thrombin (1 unit/ml) a maximally effective dose of prostacyclin only decreased the rate of aggregation and had much less of an inhibitory effect.

FIG. 3. Co-chromatography of [$^{32}P$]inositol 1,4,5-trisphosphate from human platelets challenged with thrombin. [$^{32}P$]inositol 1,4,5-trisphosphate was prepared from human erythrocytes as described by Downes et al. (1982). [$^3H$]inositol trisphosphate was prepared from human platelets challenged with thrombin (1 unit/ml) for 2 min and separated from other labeled compounds using Dowex anion exchange chromatography (see "Experimental Procedures"). Samples of both inositol trisphosphates were co-applied to a high-performance liquid chromatography apparatus, and separations were performed on a Whatman analytical SAX column using a 20-min gradient from 0.4 to 0.8 M ammonium phosphate, pH 3.5, at a rate of 1 ml/min. Fractions (0.5 ml) were collected and counted by liquid scintillometry; window settings were set such that there was no spillover in radioactivity between the $^3H$ and $^{32}P$ channels for the amount of radioactivity applied.

FIG. 4. Dose-response curve for thrombin-induced formation of [$^3H$]inositol phosphates. Platelets were prelabeled with [$^3H$]inositol and stimulated with various doses of thrombin for 30 s. Each point is the mean of duplicate determinations, and this experiment is representative of two others. Example traces of thrombin-induced shape change and aggregation are shown in the uppermost part of the figure. ⊢. IP; ○. IP2; △. IP3.

FIG. 5. Levels of [$^3H$]inositol phosphates in platelets following shape change induced by thrombin. Platelets were prelabeled with [$^3H$]inositol and stimulated with thrombin in concentrations (0.01–0.02 unit/ml) which produced only shape change in human platelets (see example traces). [$^3H$]inositol phosphates were measured after 30 s. Indications of statistical significance were made using a Wilcoxon rank test.
Fig. 6. Effect of increasing concentrations of prostacyclin on the stimulation of human platelets by thrombin. Platelets were prelabeled with [3H]inositol and incubated with thrombin (0.1 unit/ml) for 30 s in the presence of various concentrations of prostacyclin. Each point represents the mean ± S.E. of duplicate determinations. This experiment is representative of four others that yielded similar results. Example traces of prostacyclin blockade of thrombin-induced aggregation are shown in the upper-right part of the figure. A, [3H]inositol lipids. ■, PI; ●, PIP; △,PIP2; V, lysophosphatidylinositol (LPI). B, [3H]inositol phosphates. ■, inositol monophosphate; ●, inositol bisphosphate; △, inositol trisphosphate.

Fig. 7. Time course of formation of inositol phosphates by thrombin in the absence and presence of prostacyclin. Platelets were prelabeled with [3H]inositol and incubated with thrombin (1 unit/ml) for various times in the presence of prostacyclin (PGI₂, 100 ng/ml). Each point is a single determination, and this experiment is representative of two others that yielded similar results. Example traces of prostacyclin decreasing the rate of thrombin-induced aggregation are shown in the lower right of the figure. □, ■, IP; ○, ●, IP2; △, ▲, IP3. —, no prostacyclin; ---, plus prostacyclin (100 ng/ml).

Fig. 8. Synergism by prostacyclin and isobutylmethylxanthine (IBMX) in the inhibition of platelet activation by thrombin. Platelets were labeled with [3H]inositol and stimulated with thrombin (0.1 unit/ml) in the presence of various potential inhibitors. Each point represents the mean ± S.E. of duplicate determinations. Example traces are also shown at the top of the figure and correspond to the drug combinations shown at the base of the columns. This experiment is representative of two others that yielded similar results.

DISCUSSION

The present study has demonstrated that thrombin is able to rapidly stimulate formation of [3H]IP2 and [3H]IP3 on the formation of [3H]inositol phosphates (Fig. 7). The action of prostacyclin on thrombin-induced platelet aggregation and inositol phosphate formation was markedly potentiated by isobutylmethylxanthine, a cyclic AMP phosphodiesterase inhibitor, strongly suggesting that prostacyclin was acting through cyclic AMP formation (Fig. 8).
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In early times, that thrombin does not induce phospholipase C-mediated hydrolysis of [3H]PI and that, therefore, changes in the level of [3H]PI presumably reflect its conversion to PIP and PIP2. Others have previously observed similar pathways of inositol phospholipid metabolism following receptor activation in other tissues (Berridge, 1983; Downes and Wusteman, 1983; Martin, 1983; Aub and Putney, 1984).

Prostacyclin and cyclic AMP have been shown to inhibit the activation of platelets by a wide range of stimuli including thrombin and platelet-activating factor (Billah and Lapetina, 1983; Lapetina and Siegel, 1983). The action of prostacyclin appears to be mediated through cyclic AMP production (Lapetina et al., 1977), and this is supported in the present study by the observed synergism with isobutylmethylxanthine, a cyclic AMP phosphodiesterase inhibitor. The present study has shown that prostacyclin also decreases the formation of all three inositol phosphates by thrombin. This inhibitory action implies that prostacyclin is inhibiting the action of phospholipase C either directly or indirectly. One possible site of an indirect action is to deplete the levels of PIP2 in the membrane by either inhibiting the kinase responsible for its synthesis or activating the membrane-bound phosphomonoesterase that hydrolyzes PIP2 to PIP (Irvine, 1982). A similar mechanism could be presented for the synthesis of PIP from PI. Possible support for such a mechanism is that prostacyclin causes an increase in [3H]PI levels in the membrane with no apparent increase in [3H]PIP or [3H]PIP2 levels following exposure to thrombin (see Fig. 6). Alternatively, cyclic AMP may exert a direct inhibitory action on phospholipase C.

In conclusion, it would appear that, as for other tissues, the pathway of receptor-mediated metabolism of inositol phospholipids in the platelet is through phospholipase C-induced hydrolysis of polyphosphoinositides rather than PI. The inhibition of thrombin-stimulated production of inositol phosphates by prostacyclin suggests an inhibitory site of action either directly or indirectly at the level of phospholipase C.

REFERENCES