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THE RAPID POLYPHOSPHOINOSITIDE METABOLISM MAY BE A TRIGGERING EVENT FOR THROMBIN-MEDIATED STIMULATION OF HUMAN PLATELETS

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SUMMARY: The metabolism of polyphosphoinositides was examined in human platelets activated by thrombin. The addition of thrombin to [^{3}H]glycerol-labeled platelets induced an initial loss and a subsequent increase of the radioactivity in phosphatidylinositol-4,5-bisphosphate (TPI) without any significant change in phosphatidylinositol-4-phosphate (DPI). A marked enhancement of [^{32}P]Pi incorporation into TPI occurred in parallel with an increase in this lipid content, which was accompanied with a conccurent decrease in phosphatidylinositol (PI). The rate of this subsequent increase in TPI was smaller than that observed in [^{3}H]arachidonic acid-labeled platelets, suggesting that formed TPI in activated platelets may contain much greater amount of arachidonate than preexisting TPI in resting platelets. These data indicate that thrombin causes a rapid change in TPI metabolism (initial degradation of preexisting TPI and subsequent production of arachidonate-rich TPI), which might be a primary candidate to modulate thrombin-induced function in human platelets.

The inositol phospholipids are quantitatively minor components in most mammalian cell membranes (1,2). Despite this fact, evidence has accumulated indicating that such lipids are of major importance for biological functions of membranes because of its rapid response to the activation of a wide variety of surface receptors (1,2). Most of receptors mobilize calcium as a second messenger and the matabolism of phosphatidylinositol (PI), known as "PI-cycle", has been reported to be a primary event of receptor-stimulated calcium mobilization (1). Further evidence in support of this contention was obtained by Serhan et al. (3) who reported that phosphatidic acid (PA), an intermediate

Abbreviations: PI, phosphatidylinositol; DPI, phosphatidylinositol-4-phosphate; TPI, phosphatidylinositol-4,5-bisphosphate; PA, phosphatidic acid; DG, diacylglycerol; PRP, platelet-rich plasma; HPTLC, hihg performance thin-layer chromatography; ATP, adenosine triphosphate.

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of PI-turnover, could serve as an endogenous calcium ionophore in various types of cells. We have recently observed that calcium influx due to thrombin-activation was associated with the production of PA and related to the mediation of intracellular responses (4).

On the other hand, although not as widely studied as PI, polyphosphoinositides (PI-4-phosphate (DPI) and PI-4,5-bisphosphate (TPI)), which are produced by enzymatic phosphorylation of PI and DPI by PI kinase and DPI kinase, respectively, have been also described to be associated with change during during various cell activations (5-12). Previous studies have shown that they are involved in the regulation of intracellular free calcium levels and cell membrane fluidity of some mammalian tissues(1,7,14,15). To our knowledge, surprisingly little is known about the effects of platelet activation on its polyphosphoinositides metabolism. Increased [^{32}P]Pi incorporation into these lipids has been shown in incubation of rabbit platelets with platelet-activating factor (16), however no information is available as yet regarding the breakdown of them. These findings prompted us to study the acute effect of thrombin on phosphoinositides metabolism in human platelets.

We report here experiments in which the metabolism of phosphoinositides was studied for [^{3}H]glycerol- or [^{3}H]arachidonic acid-prelabeled human platelets. It was demonstrated that upon thrombin-stimulation, preexisting TPI was decreased within 10 sec but then arachidonyl TPI rapidly rose with reciprocal decrease in PI. These results would offer the first evidence that the rapid modification of TPI is a primary event for initiation of platelet activation.

MATERIALS AND METHODS

Preparation of [^{3}H]glycerol- or [^{3}H]arachidonic acid-labeled human platelets: Fresh blood was obtained from healthy volunteers and then centrifuged for 10 min at 164 x g as reported previously (17,18). The resulting surpernatant, platelet-rich plasma (PRP) was incubated with [^{3}H]glycerol (30μCi/100 ml PRP) or [^{3}H]arachidonic acid (50 μCi/100 ml PRP) for 1 hr at 37°C. After labelling, the platelets were washed two times as described previously (17,18). The washed labeled platelets were finally resuspended in Tris-citrate-bicarbonate buffer (containing 1 mM $CaCl_2$, pH 7.4) (19) to a final concentration of 2 x 10^8 platelets/ml. Suspension was at least 95% pure.

Lipid analysis: Platelet suspension (1 ml) was incubated with thrombin (0.4 units) at 37°C for indicated time. The reaction was terminated by adding 4 ml of chloroform/methanol (1 : 2, v/v) and lipid extraction was carried out by a modification of the method of Bligh-Dyer (20). In experiments where TPI and DPI were to be examined, the above solvent was replaced by chloroform/methanol/conc.

HCl (20 : 40 : 1, v/v) (11). Briefly, this was followed by the addition of 1 ml of chloroform and 1 ml of 2 M KCl-5 mM EDTA (or distilled water when HCl was present), and mixing. The phases were separated by centrifugation and the lower phase was collected and dried under nitrogen. The phosphoinositides were separated on high performance TLC (HPTLC), impregnated with potassium oxalate (1%), in a solvent system of chloroform/acetone/methanol/acetic acid/water (40 : 15 : 13 : 12 : 8, v/v) (10). The neutral lipids and other phospholipids were analyzed as previously described (18). Spots were identified by comigration with authentic standards. The areas corresponding to individual lipids were scraped into vials and the radioactivity was determined in a scintillation counter (Beckman LS 7500) with toluene/Triton X-100/water/2,2'-p-phenylene-bis-(5-phenyloxazole)/2,5-diphenyloxazole (800 ml : 200 ml : 50 ml : 0.24 g : 3.3 g) (4).

Measurement of [^{32}P]phosphate incorporation into inositol phospholipids: To observe the effects of thrombin on [^{32}P]Pi incorporation into platelet phosphinositides (PI, DPI and TPI), platelets suspension (2 x 10^8 platelets/ml) was incubated with [^{32}P]Pi (10 μCi) in the presence or absence of thrombin (0.4 units) for indicated time, and lipid extraction and its analysis were carried out as described above.

Materials: High performance TLC plates were purchased from E.Merck. [2-^{3}H]Glycerol (10 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (78.2 Ci/mmol) and [^{32}P]phosphate were obtained from New England Nuclear. Polyphosphoinositides (TPI and DPI), other phospholipids and diolein as the standards were from Sigma, Serdary and Tokyo Chemical Co., respectively. Thrombin (bovine) was supplied from Mochida Pharmaceutical Co.. X-O mat Medical X-ray films were obtained from Eastman Kodak Co.. All other chemicals were of reagent grade.

RESULTS

Separation of inositol phospholipids: Studies on inositol phospholipids metabolism have been somehow hampered by the cumbersomeness of separating their lipids. In this study, inositol phospholipids were separated according to the method of Jolles et al. (10) using HPTLC as described under "Materials and Methods". As shown in Fig.1, PI, DPI, and TPI were well separated each other.

Changes subsequent to thrombin-stimulation in [^{3}H]glycerol-labeled phosphoinositide: The metabolism of inositol phospholipids induced by platelet activation is shown in Fig.2 where [^{3}H]glycerol was used to label platelets. Following activation by thrombin the radioactivity in PI was decreased from 10.7 x 10^3 dpm to 5.5 x 10^3 dpm within 60 sec and remained at this level up to 5 min. A transient decrease in TPI was observed without any significant alteration in DPI. The radioactivity in TPI was decreased from 1.0 x 10^3 dpm to 0.8 x 10^3 dpm within 10 sec, and was then progressively increased with the further incubation. The other polyphosphoinositide, DPI, remained rather unchanged up to 5 min.

A rapid and transient increase in 1,2-diacylglycerol (DG) formed from PI was observed in platelets exposed to thrombin, and reached the maximum within 10

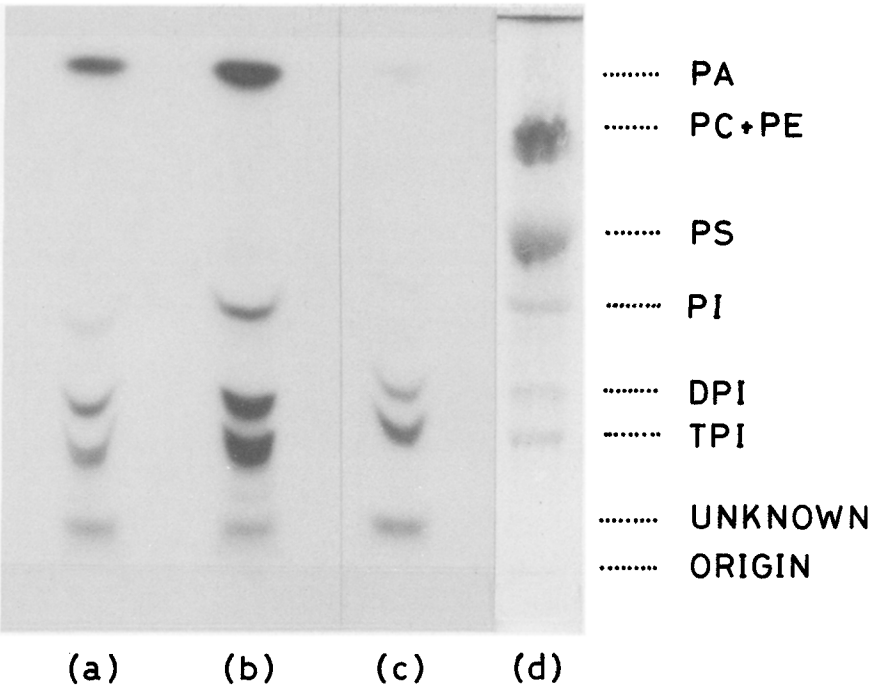
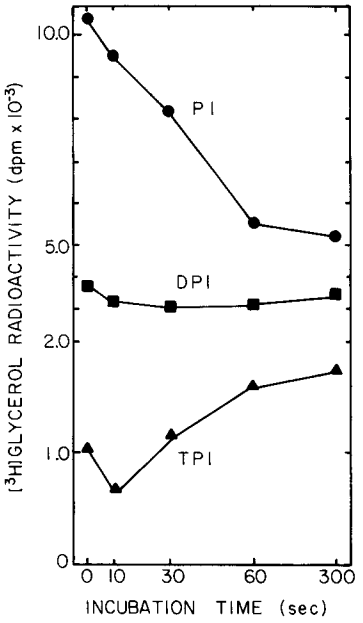


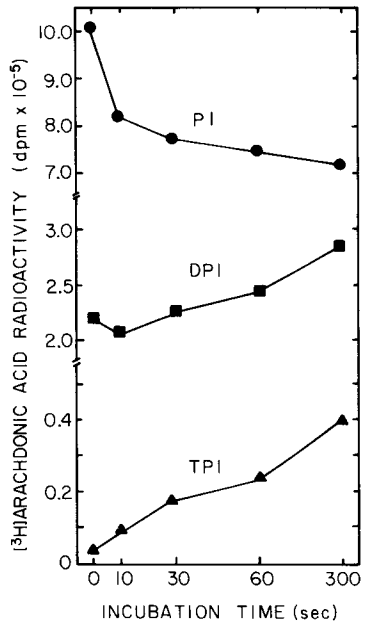
Fig.1 Autoradiographs (a, b and C) after extraction and thin-layer chromatography of [^{32}P]Pi-incorporated platelet lipid, and separation of authentic phospholipids (d). Human platelets suspension (2 x 10^8 platelets/ml) was incubated with [^{32}P]Pi (10 μCi) in the presence of thrombin (0.4 units) for 30 sec (a) and 5 min (b), or in the absence of thrombin for 5 min (c) at 37 °C. PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; DPI, PI-4-phosphate; TPI, PI-4,5-bisphosphate.

sec (17,18). The initial loss of TPI might result from its rapid conversion to 1,2-DG via TPI phosphodiesterase, as reported by Michell (21).

Changes subsequent to thrombin-stimulation in [^{3}H]arachidonic acid-labeled phosphoinositides: When [^{3}H]arachidonic acid-labeled platelets were incubated with thrombin, a rapid breakdown of PI was observed which was similar to that found in the [^{3}H]glycerol-labeled platelets. A marked enhancement of radioactivity in polyphosphoinositides (DPI and TPI) followed a concurrent reduction of radioactivity in PI. The increase rate of [^{3}H]arachidonate in these lipids was however much greater than that in [^{3}H]glycerol-labeled platelets. This difference may be due to the preexisting polyphosphoinositides before activation which contain only small amount of arachidonate. These findings suggest that thrombin-activation induced the rapid conversion of



(2)



(3)

Fig.2 Time-course of the effects of thrombin on [^{3}H]glycerol-labeled phosphoinositides. [^{3}H]Glycerol-labeled human platelets (2 x 10^8 platelets) was incubated with thrombin (0.4 units) at 37°C for indicated time. Each value is the mean of two experiments performed in duplicate. ●, phosphatidylinositol (PI); ■, PI-4-phosphate; ▲, PI-4,5-bisphosphate.

Fig.3 Time-course of the effects of thrombin on [^{3}H]arachidonic acid-labeled phosphoinositides. Platelets were prelabeled with [^{3}H]arachidonic acid instead of [^{3}H]glycerol. All other conditions and symbols are the same as those described in the legend to Fig.2. Each value is the mean of two experiments performed in duplicate.

arachidonate-rich PI (22) to arachidonyl polyphosphoinositides by phosphorylation with ATP.

Enhanced incorporation of [^{32}P]phosphate into phosphoinositides during stimulation by thrombin: The effects of thrombin on the incorporation of [^{32}P]Pi into inositol phospholipids are shown in Fig.1 and Fig.4. Incubation of platelets with [^{32}P]Pi in the presence of thrombin prompted 2-fold greater incorporation for TPI and DPI, and 5-fold for PI than their respective controls. Interestingly there was little incorporation of [^{32}P]Pi into PI and polyphosphoinositides up to 30 sec and 10 sec, respectively, but shortly thereafter substantial amounts of radioactivity were noted in them (Fig.4). Also in resting platelets, [^{32}P]Pi was incorporated into DPI and TPI. This

suggests that the polyphosphoinositides undergo a very high and rapid metabolic turnover, especially with respect to the monoester-phosphates attached to the inositol ring.

DISCUSSION

Incubation of human platelets with thrombin for 10 sec induced a rapid decrease in TPI (Fig.2). This initial loss of TPI was transient and preceded the enhanced incorporation of [^{32}P]Pi into TPI. After 10 sec, when [^{32}P]Pi incorporation into this lipid appeared to increase as has also been observed with the action of certain stimuli on other cell systems (7,10-13), the level of TPI rose gradually. The re-synthesized TPI might be rich in arachidonic acid because of the markedly greater rate of rise in [^{3}H]arachidonic acid-labeled TPI than that in [^{3}H]glycerol-labeled TPI (Fig.2 and 3). On the other hand, the initial loss of TPI seems to be concequence of the activation of TPI phosphodiesterase to convert to 1,2-DG.

While the receptor-activation stimulates PI-specific phospholipase C to accelerate PI-turnover (hydrolysis of PI, production of 1,2-DG and PA, and resynthesis of PI) in thrombin-treated platelets (17,24-27), PI is also degraded

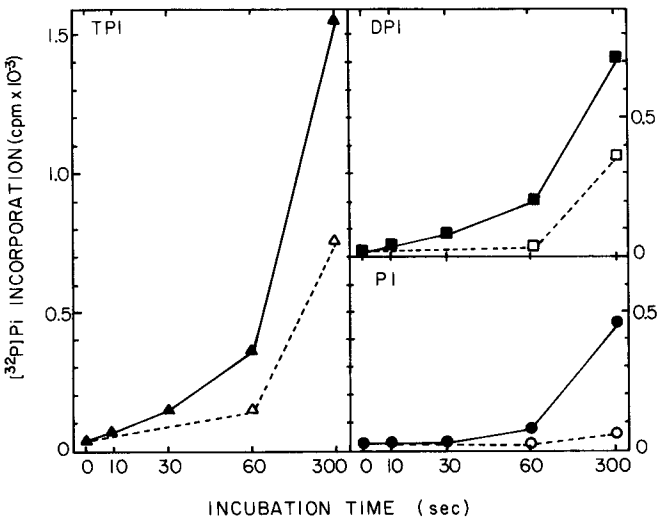


Fig.4 Time-course of [^{32}P]Pi incorporation into phosphoinositides during stimulation by thrombin. Platelets suspension (2 x 10^8 platelets/ml) were incubated with [^{32}P]Pi (10 μCi) in the presence (closed symbol) or absence (open symbol) of thrombin (0.4 units) at 37°C for indicated time. Symbols are the same as in Fig.2. Each value is the mean of two experiments performed in duplicate.

by phospholipase A_2 (23). The true significancs of this 'PI-turnover' remains unclear, but 1,2-DG activates a calcium-dependent protein kinase (28), and PA acts as a potential ionophore for calcium in thrombin-activated human platelets (4). In addition, a substantial rise in lysoPI was detected in thrombin-activated horse platelets (23) and A23187-activated human platelets (17), indicating the presence of PI hydrolyzing phospholipase A_2. Calculations based on both the loss of radioactivity of PI (Fig.2 and 3) and the increase in that of 1,2-DG and PA (intermediates of PI-turnover) (17,18) indicate that the majority of degradation of PI is compensated by the so-called 'PI -cycle'. However, such formation of TPI by time-sequential action of PI and DPI kinases is, though less markedly but significantly, observed also in resting platelets,probably offering evidence that TPI metabolism may be moderately operating in the native platelets unexposed to stimuli.

Although the biological significance of TPI remains unknown, it has been known that TPI binds calium more avidly than mono- and di-phosphoinositides (1). Farese et al. (7) suggested that ACTH-induced increases in adrenal polyphosphoinositides might directly control various metabolic processes by perturbating the plasma membrane or other intracellular membranes. Likewise, it was most recently reported that the conversion of TPI to DG could open the calcium gate (21).

Thus it would be concluded from the results obtained in the present study that the TPI metabolism may trigger for initiation of platelet activation, presumably by affecting mobilization of free calcium or membrane perturbation.

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