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THE RAPID PI-TURNOVER IS NOT COUPLED WITH THE AGGREGATION IN A23187-ACTIVATED HUMAN PLATELETS

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SUMMARY: Lipid metabolisms in human platelets activated by an ionophore A23187 and thrombin were examined with reference to PI-turnover. The ionophore enhanced neither the production of diacylglycerol and phosphatidic acid nor the incorporation of [^{32}P] into phosphatidylinositol (PI) despite of induction of irreversible aggregation and arachidonate-liberation. The formation of lysoPI was observed to be significantly increased by the activation of platelets by A23187. These results, which are different from those observed in the activation by thrombin, suggest that rapid PI-turnover is not required for both aggregation and arachidonate-liberation in A23187-activated platelets and that hydrolytic activity of phospholipase A_2 on PI may be involved in the arachidonate-liberation system. It was also found that A23187 could activate platelets without the presence of extracellular Ca^{2+}.

PI-turnover [1,2] is markedly enhanced when platelets are activated by receptor-mediated agents such as thrombin [3-6] and collagen [7]. It has been thought that the PI-turnover is initiated by the rapid hydrolysis of PI to DG with a specific phospholipase C, which is followed by phosphorylation to PA with DG kinase, and then PA is finally resynthesized to PI via CDP-DG [1-7]. Although the precise role of the rapid lipid metabolism in cellular functions is not yet fully clarified, the aggregation and secretion of platelet have been known to be closely associated with the PI-turnover. Since it was proposed by Michell [1] that the turnover was related to some widespread mechanism by which the concentration of free Ca^{2+} in cytosol was increased, many experiments with platelets have been performed in order to advance the hypothesis [8-10]. In support of this contention, Serhan et al. [11] reported that PA, an intermediate in PI-turnover,

Abbreviations: PI, phosphatidylinositol; DG, diacylglycerol; PA, phosphatidic acid; CDP, cytidine diphosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PRP, platelet rich plasma; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid.

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could serve as endogenous Ca^{2+} ionophore in cells. An increment in concentration of free Ca^{2+} in cytosol may lead to phosphorylation of 20K protein [12] and 40K protein [13] and to activation of lipase which is concerned in the release of arachidonate from phospholipids [14,15].

Ionophore A23187 [16] can also induce the platelet aggregation and secretion without any interaction with receptor on plasma membrane [17]. It is a well known fact that A23187 increases the concentration of cytosolic Ca^{2+} by transporting Ca^{2+} across plasma membrane [16] and/or by releasing Ca^{2+} from intracellular storage sites [18,19]. One would thus expect that the A23187-induced elevation in Ca^{2+} concentration in the cytosol activated platelets without acceleration of PI-turnover.

In this communication, we have presented the results indicating that although A23187 did not accelerate the PI-turnover which was measured by production of DG and PA, and by incorporation of [^{32}P]phosphate into PI, the irreversible aggregation of platelets and arachidonate-liberation were induced. In addition, it was observed that phospholipase A_2 did hydrolyze not only PC and PE but also PI in A23187-activated platelets. We explored further the effect of extracellular Ca^{2+} on lipid metabolism in platelets stimulated by A23187.

MATERIALS AND METHODS

After centrifugation of the fresh blood obtained from healthy volunteers, the supernatant PRP, was incubated with 50 μCi/100 ml PRP of [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (New England Nuclear, 72Ci/mmol) or 500 μCi/100 ml PRP of [2-^{3}H] glycerol (New England Nuclear, 200 mCi/mmol) for 1 h at 37°C and then washed twice as described previously [3]. The washed non-labeled and labeled platelets were finally resuspended in a calcium-free Tris/citrate/bicarbonate buffer (pH 7.4) [20] to a final concentration of 5 x 10^9 platelets/ml and 2 x 10^9 platelets/ml, respectively. Suspensions contained <0.1 % of erythrocytes.

One milliliter of platelets suspension was incubated at 37°C for indicated time with 2 units/10^9 platelets of thrombin (Sigma) or 2 μM of ionophore A23187 (Eli Lilly). To observe the [^{32}P]incorporation into PI, 1 ml of platelets suspension was activated as described above in the presence of 100 μCi of [^{32}P]phosphate (Amersham). A23187 was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mM and kept in the frozen state. The reactions were terminated by the addition of 4 ml of chloroform/methanol (1 : 2, v/v), and lipid extraction was carried out by a modification of the method of Bligh and Dyer [21] substituting 2 M KCl-5 mM EDTA mixture for water [22]. The phospholipids were separated by two dimensional chromatograpy on Silica Gel H plates containing magnesium acetate (2.5 %), using chloroform/methanol/13.5 N ammonia water (65 : 35 : 5.5, v/v) in the 1st dimension, and chloroform/acetone/methanol/acetic acid/water (3 : 4 : 1 : 1 : 0.5, v/v) in the 2nd dimension [6]. In this system lysoPI and lysoPS were in a single spot but other phospholipids were well separated. The neutral lipids were analyzed on Silica Gel G containing borate (0.4 M) in a solvent system of chloroform/acetone (96 : 4, v/v) [23]. The areas corresponding to individual lipids were scraped into vials and the

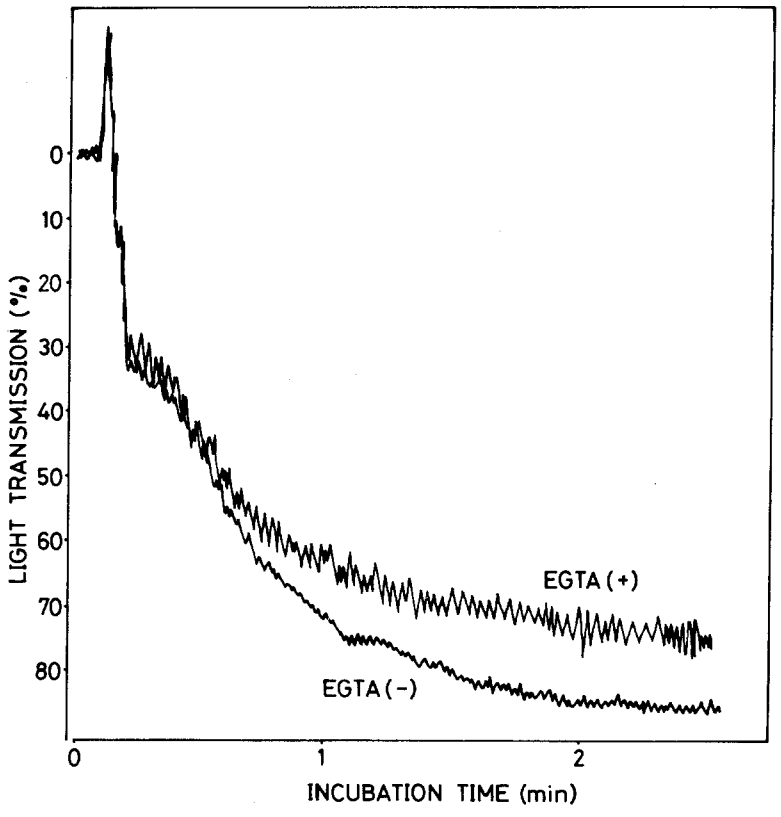

Fig. 1 A23187-induced aggregation of human platelets. PRP was activated by 2 μM of A23187 in the presence or absence of EGTA (4 mM)

radioactivity was determined in a liquid scintillation counter 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). Lipid phosphorus was assayed after charring with 50 % H_2SO_4 using the method of Rouser et al. [24]. Standard phospholipids were prepared by mixing lysoPI and lysoPS which were prepared as described in earlier report [6] with phospholipids purchased from Serdary. Triolein, diolein, monoolein and oleic acid for standard neutral lipids were obtained from Tokyo Chemical Industry Co.. All other chemicals were of reagent grade.

RESULTS AND DISCUSSION

As shown in Fig. 1, addition of ionophore A23187 at a final concentration of 2 μM to PRP produced irreversible aggregation of platelets. The presence of EGTA in PRP had no significant effect on ionophore-induced aggregation. The marked aggregation was caused even in the presence of EGTA (4 mM), suggesting that external Ca^{2+} may not participate in aggregation of platelets induced by A23187.

It is known that arachidonate is released from membrane phospholipids and immediately converted to prostaglandins and thromboxanes when platelets undergo

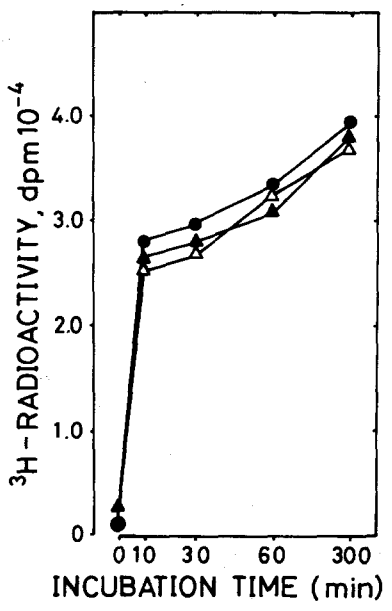

Fig. 2 Effects of A23187 and thrombin on the accumulation of arachidonate and its metabolites in human platelets. Washed, [^{3}H]arachidonate-labeled platelets (2 x 10^9/ml) were suspended in Tris/citrate/bicabonate buffer (pH 7.4) containing 1 mM EGTA. One milliliter of platelet suspension was exposed to 2 μM A23187 in the presence (▲) or absence (△) of $CaCl_2$ (3 mM), or to 4 units thrombin (●), for indicated time at 37°C. Reaction termination and lipid analysis were carried out as described under "Materials and Methods". Each value is the mean of duplicate determinations.

irreversible aggregation [26,27]. As illustrated in Fig. 2, A23187 increased the radioactivity of arachidonate metabolites in [^{3}H]arachidonate-labeled platelets both in the presence and absence of extracellular Ca^{2+} as well as thrombin did. These results (Fig. 1 and Fig. 2) provide evidence that extracellular Ca^{2+} is not required for aggregation and arachidonate-liberation in A23187-treated platelets.

Fig. 3 represents the time course of formation of 1,2-DG and PA which are key intermediates in the PI-turnover, in A23187-activated platelets. When [^{3}H]arachidonate-labeled platelets were incubated with A23187 (2 μM) in the presence or absence of extracellular Ca^{2+}, the radioactivity of 1,2-DG was increased from 5 x 10^2 dpm to 2 x 10^3 dpm within 30 sec, and was then progressively decreased with the further incubation. The rate of increase of 1,2-DG was, however, by one-fifth when compared with that of 1,2-DG formation induced by thrombin (Fig.3 a). A marked enhancement of the radioactivity of PA was observed in thrombin-activated platelets, whereas no significant change did occur in A23187-activated platelets

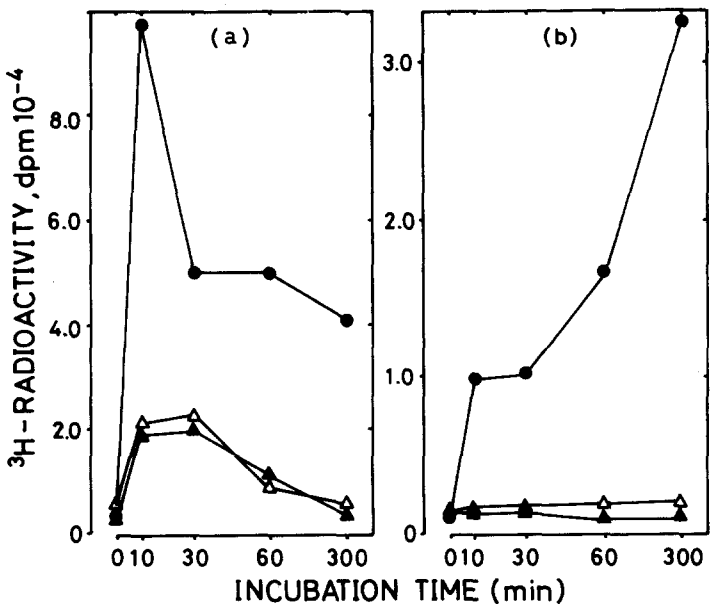

Fig. 3 Effects of A23187 and thrombin on the formation of 1,2-DG (a) and PA (b) in human platelets. Washed, [^{3}H]arachidonate-labeled platelets (as in Fig. 2) were incubated with 2 μM A23187 in the presence (▲) or absence (△) of $CaCl_2$ (3 mM), or with 4 units thrombin (●), for indicated time at 37°C. All other conditions were described in legend for Fig. 2. Each value is the mean of duplicate determinations.

(Fig. 3 b). This finding was also confirmed by phosphorus assay (data not shown). Ineffective production of 1,2-DG and PA in A23187-activated platelets agree with the results reported by Rittenhouse-Simmons [27] and Lapetina et al. [28,29], respectively.

A23187 was far less effective than thrombin in producing 1,2-DG, PA and intermediates in the PI-turnover, though comparable amounts of arachidonate metabolites were accumulated (see Fig. 2). These observations prompted us to propose that the PI-turnover is blocked in A23187-activated platelets. The turnover is usually measured by means of incorporation of [^{32}P] into PI. In fact, as shown in Table I, A23187 did not stimulate [^{32}P]incorporation into PI, whereas thrombin enhanced the uptake of [^{32}P] into PI by 5.6-fold. Small accumulation of 1,2-DG produced in response to A23187 could be explained by the finding of Takenawa and Nagai [30], who have found that the activity of PI-specific phospholipase C was augmented by unsaturated fatty acids, especially arachidonate. Upon activation of platelets by A23187, arachidonate was released from phospholipids (Fig. 2) and subsequently the

Table I

Effects of A23187 and Thrombin on the [^{32}P] Incorporation into PI of Human Platelets

Stimulants	Incorporation into PI (cpm/2 x 10^9 platelets)	Increase (%)
None	957.8	100
A23187 Ca^{2+}(+)	1102.8	115
Thrombin	5327.6	556

Washed platelets (2 x 10^9/ml) were suspended in Tris/citrate/bicarbonate buffer (pH 7.4) containing 1 mM EGTA. One milliliter of sample was incubated with 2 µM A23187 + $CaCl_2$ (3 mM), or with 4 units thrombin, for 10 min at 37°C in the presence of 100 µCi of [^{32}P]phosphate. Reaction termination and PI separation were carried out as described under "Materials and Methods". Results are presented as the average of two experiments performed in duplicate.

phospholipase C might be to some extent stimulated by arachidonate, leading to formation of 1,2-DG.

The increase of lysophospholipids, induced by phospholipase A_2 activation, is shown in Fig. 4 where [^{3}H]glycerol was used to label platelets. Following activation by A23187 the radioactivity of lysoPC, lysoPE and lysoPI *plus* lysoPS rose

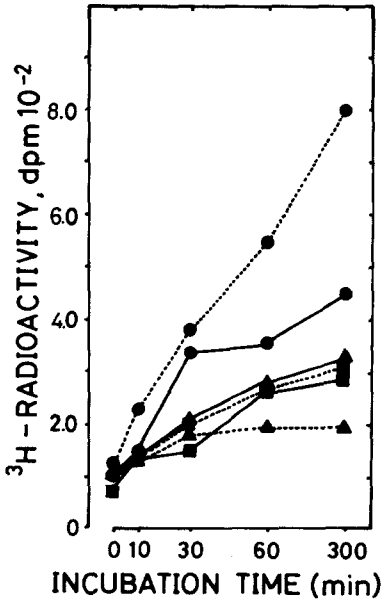

Fig. 4 Time course of lysophospholipids production after exposure of human platelets to A23187 or thrombin. Washed, [^{3}H]glycerol-labeled platelets (2 x 10^9/ml) were incubated with 2 µM of A23187 (—) without extracellular Ca^{2+} or 4 units of thrombin (·····) at 37°C for indicated time. Reaction termination was carried out as described under "Materials and Methods". ● , lysoPC; ■ , lysoPE; ▲ , lysoPS and lysoPI. Each value is the mean of two experiments performed in duplicate.

gradually, whereas the increase of lysoPI *plus* lysoPS was much smaller in thrombin-activated platelets. Although lysoPI and lysoPS were not separable with the solvent system employed in this study, the increase of lysoPI *plus* lysoPS may reflect that of lysoPI since A23187 was found to promote the hydrolysis of substantial amounts of PI but not PS (data not shown). The increment of lysoPC content in A23187-activated platelets was smaller than that in thrombin-activated platelets, but there was some significant increase of lysoPI in the former platelet. This implies that phospholipase A_2 exhibits its activity on not only PC and PE but also PI in A23187-activated platelets.

The observations obtained from the present study indicate that the rapid PI-turnover is coupled with the platelet aggregation induced by such stimulants as thrombin and collagen acting on the plasma membrane receptor, and that, in contrast, A23187 capable of activating platelets without an interaction with the receptor, does not cause any enhancement of PI-turnover. It would thus be concluded that in the A23187-stimulated platelets phospholipase A_2 substitutes for a specific phospholipase C in hydrolysis of PI in A23187-activated platelets.

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