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EVIDENCE FOR COUPLING OF PHOSPATIDIC ACID FORMATION AND CALCIUM INFLUX IN THROMBIN-ACTIVATED HUMAN PLATELETS

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Summary: The time-sequential relationship between Ca^{2+} flux, phospholipid metabolism and platelet activation have been examined. Thrombin-activation caused a marked enhancement in $^{45}Ca^{2+}$ influx and a decrease in extracellular Ca^{2+} concentration measured by murexide dye, which occurred in parallel with the conversion of 1,2-diacylglycerol (DG) to phosphatidic acid (PA). The incorporated $^{45}Ca^{2+}$ was located mainly in cytosolic fraction. The influx of Ca^{2+} was observed to commence prior to the onset of lysophospholipids formation and subsequent liberation of arachidonic acid. These data provide evidence which indicates a coupling between the rapid PI-turnover and the active Ca^{2+} influx, in which phosphatidic acid (PA) may serve as a Ca^{2+} ionophore.

It is generally believed that one of the earliest and most crucial step in the sequence of platelet activation is an increase in cytoplasmic Ca^{2+} concentration (1,2). An increment in concentration of free Ca^{2+} in cytosol may lead to phosphorylation of 20K (3,4) and 40K proteins (5), and also to activation of phospholipase A_2 (6,7) which is concerned in the release of arachidonic acid from phospholipids (7-10). Since the concept was proposed by Michell (11) that the PI-turnover is related to some widespread mechanism by which the concentration of free Ca^{2+} in cytosol is increased, many experiments with platelets have been performed in order to advance the hypothesis (12-14). Indeed, the PI-turnover is markedly enhanced when platelets are activated by receptor-mediated agents such as thrombin (11,15-18). It has been thought that the PI-turnover is initiated by the rapid hydrolysis of PI to 1,2-DG by a specific phospholipase C, which is followed by phosphorylation to PA by DG kinase, and then PA is finally resynthesized to PI via CDP-DG (11,15-18).

Abbreviations: PI, phosphatidylinositol; DG, diacylglycerol; PA, phosphatidic acid; CDP, cytidinediphosphate; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PRP, platelet-rich plasma; EDTA, ethylenediamine tetraacetic acid.

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Further evidence in support of the contention that PI-turnover acts to increase the free intracellular Ca^{2+} concentration was obtained by Serhan et al. (19,20) who reported that PA, an intermediate in PI-tunover, could serve as an endogenous Ca^{2+} ionophore in various cells.

Feinstein and his co-workers (14,21) examined the time-course of the intracellular release of membrane-bound Ca^{2+} employing chlortetracycline fluorescence, and found that the stimulant-induced release of intracellular membrane-bound Ca^{2+} significantly precedes the onset of secretion. However, little information has been available regarding the time sequence of Ca^{2+} flux, phospholipid turnover, and platelet activation (secretion and aggregation).

The experiments reported in this communication represent an assessment of the possible involvement of PI-turnover in Ca^{2+} flux. The time courses of the Ca^{2+} efflux monitored by murexide dye (22) and the Ca^{2+} influx measured with Ca^{2+} have been determined for thrombin-activated platelets. It was demonstrated that the conversion of 1,2-DG to PA was coincident with Ca^{2+} influx, subsequently leading to activation of phospholipase A_2. These findings would offer the first evidence which supports the contention that PI-turnover increases intracellular free Ca^{2+}.

MATERIALS AND METHODS

Lipid metabolism After differential 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, 78.2 Ci/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 two times to separate from plasma and unbound radioactive arachidonic acid and glycerol as described previously (8,18). The washed labeled platelets were finally resuspended in a calcium-free Tris/citrate/bicarbonate buffer (pH 7.4)(23) to a final concentration of 2 x 10^8 platelets/ml. Suspension contained < 0.1 % of erythrocytes.

One milliliter of platelets suspension was incubated at 24°C for indicated time with 2 units thrombin (Mochida Pharmaceutical Co.) per 10^9 platelets. 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 - Dyer (24). The phospholipids were separated by two dimensional chromatography on silica gel H plates containing magnesium acetate (2.5 %), using chloroform/methanol/13.5 N ammonia water (65 : 35 : 6, v/v) in the 1st dimension, and chlorofom/acetone/methanol/acetic acid/water (3 : 4 : 1 : 1: 0.5, v/v) in the 2nd dimension (8). In this system, lysoPI and lysoPS were localized in a single spot but other phospholipids were well separated. The neutral lipids were analyzed on silica gel G plates containing borate (0.4 M) in a solvent system of chloroform/acetone (96 : 4, v/v) (25). 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) (scintillation cocktail A).

Release reaction PRP was incubated with 0.2 μCi/100 ml PRP of [^{14}C]-hydroxytriptamine (serotonin) (New England Nuclear, 58.5 mCi/mmol) or 2μCi/100 ml PRP of $^{45}Ca^{2+}$ (Amersham) at 37°C for 20 min. Labeled platelets were washed two times as described above. After 200 μl of platelet suspension (2 x 10^8 cells) was activated by 2 units thrombin/10^9 platelets at 24°C for appropriate incubation time, reactions were terminated by the addition of 800 μl of ice-cold 10 mM EDTA-buffer and then spun at 1,645 x g for 2 min at 4°C. The radioactivity in the resulting supernatant was counted with toluene/Triton X-100/2,2'-p-phenylene-bis-(5-phenyloxazole)/2,5-diphenyloxazole (1 l : 0.5 l : 0.2 g : 4 g) (scintillation cocktail B). The extracellular Ca^{2+} concentration was determined by the murexide dye method (23). Washed platelets (2 x 10^8 cells) were incubated with thrombin and murexide dye (0.8 mM) and absorbance changes were traced.

Measurement of $^{45}Ca^{2+}$ uptake To observe the $^{45}Ca^{2+}$ incorporation into platelets by $^{45}Ca^{2+}$-continuous labeling, platelet suspension (2 x 10^8 cells) was activated by thrombin (2 units/10^9 platelets) in the presence of 8 nCi of $^{45}Ca^{2+}$ and reactions were stopped as described above. The activity of $^{45}Ca^{2+}$ uptake was also measured by $^{45}Ca^{2+}$ -pulse labeling. Platelet suspension (2 x 10^8 cells) was incubated with thrombin for indicated time. At 5 sec before the termination of reaction, $^{45}Ca^{2+}$ (8 nCi) was added to the reaction mixture, and reactions at different intervals were terminated by adding cold 10 mM EDTA as described above. After cells were separated by centrifuging at 4°C, the resulting pellets were washed two times with cold 10 mM EDTA. The radioactivity of cells was determined in a scintillation counter using cocktail B.

Platelet fractionation The platelet suspension was quickly frozen using dry ice-acetone. After thawing, the disrupted platelets were spun at 40,000 x g for 60 min, and the resultant supernatant was subsequently subjected to centrifugation at 100,000 x g for 60 min.

RESULTS AND DISCUSSION

As shown in Fig. 1 B, stimulation of platelets by thrombin caused influx of extracellular $^{45}Ca^{2+}$. When thrombin was added to washed platelets in the presence of $^{45}Ca^{2+}$, an accumulation of cellular $^{45}Ca^{2+}$ took place and reached plateau with further incubation. The total cellular $^{45}Ca^{2+}$ is balanced by both the influx of extracellular $^{45}Ca^{2+}$ and the subsequent efflux of infused $^{45}Ca^{2+}$. The progressively enhancing cellular $^{45}Ca^{2+}$ suggests that the influx overcomes the efflux. In fact, as illustrated in Fig. 1 C, the activity of Ca^{2+} uptake by activated platelets, which was measured by $^{45}Ca^{2+}$ -pulse labeling, was observed to markedly increase and reach a maximum at 20 sec after exposure to thrombin. Furthermore, the incorporated $^{45}Ca^{2+}$ was found to be located mainly in cytosolic fraction (Table I). These findings demonstrated that the enhanced influx of

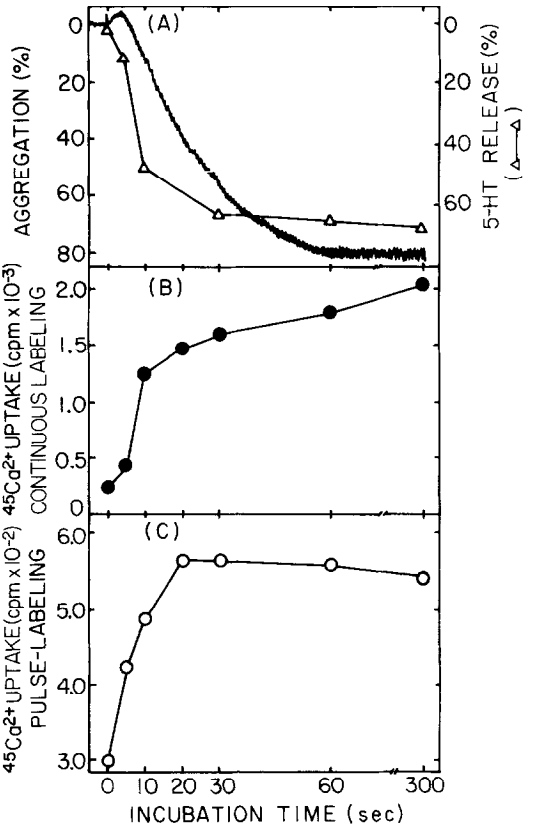

Fig. 1 Time course of thrombin-stimulated aggregation (A), serotonin (5-HT) secretion (A), $^{45}Ca^{2+}$ uptake measured by continuous labeling (B), and $^{45}Ca^{2+}$ uptake measured by pulse-labeling (C). Human platelets (2 x 10^8 cells) were incubated with throm (0.4 units) for indicated time at 24°C. Other conditions were described under "MATERIALS AND METHODS".

Table I

Distribution of Incorporated $^{45}Ca^{2+}$ in Human Platelet Fractions

Homogenate	40,000 x g Pellet	100,000 x g Pellet	100,000 x g Supernatant
95165	8169 (8.9)	2312 (2.5)	80947 (88.2)

cpm (%)

Thrombin $^{45}Ca^{2+}$ Reaction termination

0 5 10 sec

Washed human platelets (10^{10} cells/0.2 ml) were suspended in calcium-free Tris/citrate/bicarbonate buffer (pH 7.4). After 5-sec incubation of platelets (0.2 ml) with thrombin (5 units), 30 nCi of $^{45}Ca^{2+}$ was added and the reaction was terminated by adding of cold 10 mM EDTA-buffer after more 5-sec incubation. The $^{45}Ca^{2+}$ -labeled platelets were lysed by freezing-thawing and centrifuged as described under "MATERIALS AND METHODS". The radioactivity in each fraction was measured in a scintillation counter. Each value is the mean of two separate measurements.

extracellular Ca^{2+} may lead to an increase in the cytosolic free Ca^{2+} rather than in the membrane-bound Ca^{2+}.

The change in the extracellular Ca^{2+} level was determined with the murexide dye method (23). Thrombin caused the release of Ca^{2+} from dense granules into medium, which occurred coincidently with the secretion of serotonin, a constituent of dense granules (27). At 20 sec after stimulation, when the activity of $^{45}Ca^{2+}$ uptake reached a maximum, a decline in the secreted extracellular Ca^{2+} level monitored with murexide was observed (data not shown). This appears to reflect a reentry of secreted Ca^{2+} into platelets.

To ascertain whether the observed Ca^{2+} influx is associated with phospholipid turnover, platelets were labeled with [^{3}H]arachidonic acid or [^{3}H]glycerol. When [^{3}H]arachidonate-labeled platelets were incubated with thrombin at 24°C, the radioactivity of 1,2-DG was increased from 7 x 10^{2} dpm to 1.2 x 10^{4} dpm within 20 sec, and was then progressively decreased with further incubation (Fig. 2 A). A marked enhancement of radioactivity in PA, converted from 1,2-DG by DG kinase, followed a concurrent decrease in radioactivity in 1,2-DG (Fig. 2 A). Time-sequential production of 1,2-DG and PA, key intermediates in the PI-turnover, suggest an acceleration of PI-turnover. We have previously reported that thrombin enhanced the uptake of [^{32}P] into PI (15). Formation of lysophospholipids, induced by phospholipase A_2 activation, is shown in Fig. 2 B where [^{3}H]glycerol was used. Following platelet activation by thrombin, the radioactivities in lysoPC and lysoPE remained rather unchanged up to 30 sec but then rose gradually. The formation of PA and the enhancement of Ca^{2+} uptake were closely associated, which is in good agreement with the hypothesis that PA may act as an endogenous ionophore in various cells (19,20). An increment of cytosolic free Ca^{2+} caused by the enhanced Ca^{2+} influx appeared to stimulate the formation of lysophospholipids, indicating activation of phospholipase A_2 which requires Ca^{2+} (6), accompanied by arachidonic acid liberation (Fig. 2 A) and aggregation (Fig. 1 A). We have shown that ionophore A23187 capable of activating platelets without interaction with the surface receptor fails to accelerate PI-turnover (15). Taken together, these findings

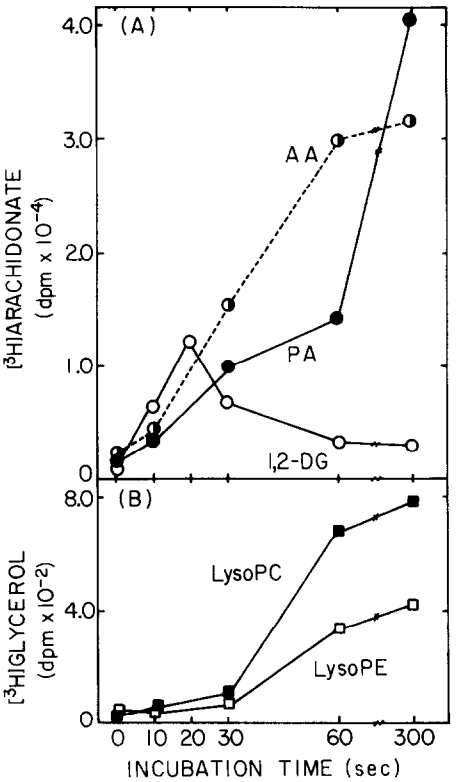

Fig. 2 Time course of thrombin-stimulated formation of 1,2-DG and PA (A), accumulation of arachidonate and metabolites (AA) (A), and production of lysoPC and lysoPE (B). [^{3}H]arachidonic acid- or [^{3}H]glycerol-labeled human platelets (2 x 10^8 cells) were incubated with thrombin (0.4 units) for indicated time at 24°C. Other conditions were described under "MATERIALS AND METHODS".

suggest that the receptor-mediated activation initiates PI-turnover allowing Ca^{2+} influx which in turn activates a phospholipase A_2 resulting in the arachidonic acid liberation as well as aggregation.

On the other hand, Feinstein et al. (21,22) have reported, through the use of recording of chlortetracycline fluorescence, that an intracellular release of membrane-bound Ca^{2+} occurs in 0.75 to 1.8 sec whereas secretion commenced 4-8 sec later. The recent work using Arsenazo III by Rebecchi et al. (27) demonstrated that thyrotropin-releasing hormone induced the prolactin secretion from rat pituitary cells by mobilizing Ca^{2+} from a sequestered cellular pool(s), which occurred without any measurable Ca^{2+} uptake. Studies with several types of cells such as leukocyte, lymphocyte,hepatocyte and chromaffine cell have shown the activation-coupled intracellular Ca^{2+} mobilization (28-31), and the concept was thus proposed that secretion does depend on mobilization

from intracellular pool-site(s) rather than influx of extracellular Ca^{2+}. Under the experimental conditions employed in this study, we were unable to measure the intracellular Ca^{2+} mobilization from pool(s) and the Ca^{2+} influx at msec order.

While a theory has been proposed by Michell (11) that PI-turnovery plays a universal role in the mobilization of Ca^{2+} in cytosol, evidence has been presented that the receptor-activation causes a transient increase in phospholipid methylation which is followed by an influx of $^{45}Ca^{2+}$ and the release of histamine in mast cells (32,33).

Thus it may be concluded that one of the most important initial step for stimulus-secretion coupling is an increase in cytoplasmic Ca^{2+} concentration by either PI-turnover or PE-methylation, dependent on the type of cells. These findings obtained in this communication support the contention that PI-turnover may be an eligible candidate for a role of increasing free cytosolic Ca^{2+}.

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