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STIMULATION OF PROTHROMBINASE ACTIVITY OF PLATELETS AND ERYTHROCYTES BY SUB-LYTIC TREATMENT WITH PHOSPHOLIPASE C FROM CLOSTRIDIUM WELCHII

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SUMMARY: Treatment of platelets or red cells with small amounts of phospholipase C from Clostridium welchii enables both cells, prior to the onset of lysis, to stimulate prothrombin conversion by coagulation factor Xa and Va in the presence of calcium. Phospholipase C treatment of both cells also exposes significant amounts of phosphatidylserine at the outer surface. The level of phosphatidic acid formed from diglycerides produced by phospholipase C action, is similar to that formed in activated platelets upon triggering the phosphatidylinositol cycle. A possible involvement of this cycle to activate platelets to become more procoagulant is discussed.

We have previously shown that stimulation of human blood platelets by the combined action of collagen plus thrombin leads to increased exposure of phosphatidylserine in the outer leaflet of the plasma membrane, presumably resulting from an induced transbilayer movement of phospholipids (1,2). This activation procedure enables the platelets to enhance the conversion of prothrombin to thrombin by a complex of coagulation factors Xa and Va in the presence of calcium. This catalytic effect is caused by increased complex formation of coagulation factors and negatively charged polar headgroups in the membrane phospholipid surface (3,4,5). Both in platelets and erythrocytes, phosphatidylserine is the major negatively charged phospholipid present but it is virtually absent from the exterior surface of the membrane (6,7,8). Its exposure at the outer surface as can occur during platelet activation, may serve to stimulate the coagulation process (9,10).

It has been shown by Mauco et al. (11) for human platelets and by Allan et al (12) for human erythrocytes that introduction of diglycerides in the membrane by the action of phospholipase C from C.welchii results in the formation of phosphatidic acid, which suggests a transbilayer movement of diglycerides

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to the inner monolayer of the membrane where diglyceride kinase and ATP are available (12,13). We have previously demonstrated (14) that incubation of human platelets with phospholipase C from C.welchii results in an increase of their ability to enhance the conversion of prothrombin to thrombin by the factor Xa-Va-complex. At that time we suspected the known lytic effect of phospholipase C from C.welchii (6,11,12) to be responsible for this phenomenon since lysis results in exposure of the inner leaflet of the plasma membrane where phosphatidylserine is located, while this lipid is not degraded by phospholipase C (6). We now show that before the onset of lysis, phospholipase C treatment of red cells and platelets enables both cells to stimulate prothrombin conversion and that this effect can be ascribed to an increased exposure of negatively charged phospholipid at the cell outer surface.

MATERIALS AND METHODS

Phospholipase C from Clostridium welchii and phospholipase A_2 from Naja naja were purified according to Zwaal et al (6). Blood coagulation factors Va, Xa and prothrombin were prepared as described in Rosing et al (3).

Blood was drawn from healthy male volunteers and collected in acid-citrate-dextrose (0.052 M citric acid/0.08 M Na_3citrate/0.183 M glucose, 1 vol of ACD to 5 vols of blood). Platelet-rich plasma and packed erythrocytes were separated by centrifugation at 200 x g for 15 min at room temperature. Erythrocytes were washed three times in 0.9% (w/v) NaCl by centrifugation at 1000 x g for 10 min. Platelets were pelleted from platelet-rich plasma at 600 x g for 15 min. The platelet pellet was carefully resuspended in a buffer containing 136 mM NaCl, 2.7 mM KCl, 2.0 mM $MgCl_2$, 25 mM glucose, 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) and 0.05% (w/v) fatty-acid free human serum albumin, pH 6.6 (Hepes-buffer). The platelets were washed twice in this buffer by centrifugation at 600 x g for 15 min. Before each centrifugation 1 vol of ACD was added to 15 vols of platelet suspension. Finally the platelets were resuspended in Hepes buffer pH 7.5. Before adding phospholipase C, platelets and erythrocytes were diluted in Hepes buffer pH 7.5 to a cell-count of $5 \times 10^7 \cdot ml^{-1}$ using a Coulter counter and Ca^{2+} was added to a final concentration of 3 mM.

Hemolysis of erythrocytes was measured as the release of hemoglobin, determined by the optical density at 418 nm. Lysis of platelets was measured as leakage of lactate dehydrogenase (15). Phospholipids were extracted from erythrocytes and platelets according to Bligh and Dyer (16), separated by two-dimensional thin layer chromatography and analyzed as phosphorus as described earlier (6). Methyl-phosphatidic acid, prepared from egg-phosphatidylcholine (17), was used as external standard. The rate of thrombin formation in the presence of red cells or platelets was essentially measured as described before (1). Briefly: to 300 µl of a cell suspension, 13 µl 75 mM $CaCl_2$ and 50 µl of a freshly prepared mixture containing 30 nM factor Xa and 60 nM factor Va were added. After a 2 min period to allow equilibration of the clotting factors at the cell surface, the enzymatic reaction was started by addition of 125 µl 16 µM prothrombin. After 1 min a sample was taken and immediately diluted in a buffer containing 120 mM NaCl, 50 mM Tris and 2 mM EDTA (pH 7.5) to stop the reaction. The amount of thrombin formed was determined by measuring the change in adsorbance at 405 nm per unit time produced by the action of

thrombin on the chromogenic substrate H-D-phenylalanyl-L-pipecolylarginine--p-nitronanilide dihydrochloride (S2238, AB Kabi Diagnostica, Sweden).

RESULTS AND DISCUSSION

Figure 1 shows that upon incubation with phospholipase C from C.welchii (0.01 IU/ml) both platelets and erythrocytes show an increasing ability to enhance thrombin formation before significant lysis of the cells occurs. To confirm that this activity can be ascribed to the appearance of negatively charged phospholipids at the outer surface of the cells, phospholipase C-treated cells were subsequently subjected to treatment with phospholipase A_2 from N.naja. Since the incubation with phospholipase C alone causes lysis after 30 min to both erythrocytes and platelets, cell suspensions (25 ml, $2x10^8$ cells.ml^{-1}, 10 mM Ca^{2+}) were first incubated during 10 min with phospholipase C from C.welchii (0.01 IU/ml). After this preincubation period phospholipase A_2 from N.naja (3 IU/ml) was added. Lysis remained below 10% for

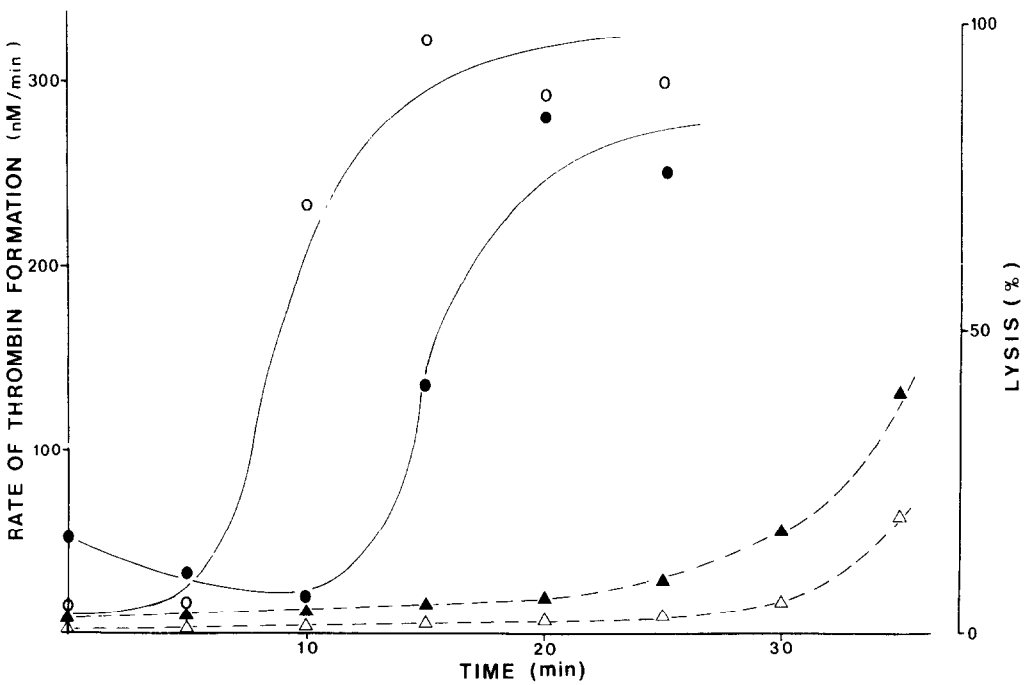

Fig. 1. Prothrombinase activity of human red cells (○) and platelets (●) induced by phospholipase C (C.welchii) treatment. Dashed lines represent lysis of erythrocytes (△) and platelets (▲). 0.01 IU/ml phospholipase C is added at t=o (1 IU is defined as the amount of enzyme able to degrade 1 μ mole of substrate per minute at 37 °C under optimal conditions (ref. 6)).

both cell preparations within 15 min following addition of phospholipase A_2, while lysis rapidly increased upon further incubation. Samples for lipid analysis were taken 15 min after addition of phospholipase A_2. Lipid degradation was stopped by adding EDTA (final concentration 15 mM) and o-phenantroline (final concentration 2 mM) and methyl-phosphatidic acid was added as an external standard prior to lipid analysis.

As shown in Table I, preincubation with phospholipase C allows extensive degradation of phosphatidylserine by subsequently added phospholipase A_2. Some 35 to 40% of the phosphatidylserine in both cells can be hydrolyzed while cell lysis remains below 10%. It should be emphasized that phosphatidylserine is no substrate for phospholipase C from C.welchii and also that this phospholipid is not degraded upon incubation of platelets (1) or red cells (6) with phospholipase A_2 alone. Since in intact erythrocytes and platelets phosphatidylserine is almost exclusively located in the inner leaflet of the plasma membrane (6,7,8), the data suggest that substantial amounts of phosphatidylserine become exposed at the exterior surface of platelets and erythrocytes during phospholipase C treatment. However, the amount of phosphatidylserine degraded by phospholipase A_2 cannot be directly interpreted as the actual amount of this lipid exposed at the outer surface. Cell lysis, which inevitably occurs, may precede a complete degradation of the outer monolayer.

TABLE I

Degradation of phospholipids in human platelets and erythrocytes by the combined action of phospholipase C (C.welchii) and phospholipase A_2 (N.naja).

Phospholipid	Erythrocytes	Platelets
Phosphatidylserine	33-39	35-43
Sphingomyelin	29-40	27-33
Phosphatidylcholine	15-27	25-40
Phosphatidylethanolamine	18-21	16-19

Range of degradation obtained from 4 individual experiments is expressed as percentage of the amount of each lipid class present in non-treated cells. Data are corrected for cell lysis assuming complete phospholipid degradation in the population of lysed cells. Lysis was always below 10%.
Phosphatidylserine is only degraded by phospholipase A_2, since it is no substrate for C.welchii phospholipase C. Sphingomyelin is no substrate for phospholipase A_2, its degradation being solely produced by phospholipase C. Phosphatidylcholine and phosphatidylethanolamine are attacked by both enzymes.

Moreover, appearance of phosphatidylserine in the outer leaflet presumably results from a transbilayer movement and it is likely that this process proceeds during phospholipase A_2 treatment. Also, it cannot be excluded that transbilayer movement is influenced by the phospholipase A_2 treatment per sé.

The increased exposure of negatively charged phosphatidylserine at the outer surface of both cells following phospholipase C treatment can explain their ability to enhance prothrombin conversion. It may also be possible that the formation of phosphatidic acid, resulting from diglyceride kinase action on diglycerides formed by phospholipase C, contributes to this effect (18). Under our experimental conditions, treatment of both red cells and platelets with 0.01 IU of phospholipase C for 10 min results in the formation of 2-4% of phosphatidic acid (expressed as percentage of total lipid phosphorus). This amount is insufficient to fully explain the enhancement of prothrombinase activity, even assuming that all phosphatidic acid formed in the inner leaflet would be subject to transbilayer movement.

It has been shown that diglyceride formation in bacterial membranes induces phospholipid flip-flop (19,20). Our results suggest that transbilayer movement of phospholipids (particularly phosphatidylserine) occurs after formation of diglycerides and phosphatidic acid in blood cell membranes. A possible involvement of ceramides formed from sphingomyelin by phospholipase C action can be ruled out, since treatment of the cells with sphingomyelinase does not lead to an increased exposure of phosphatidylserine towards exogenously added phospholipase A_2 (6,8). Also, sphingomyelinase treatment of red cells and platelets does not significantly increase their ability to enhance prothrombin conversion (data not shown).

Diglycerides and phosphatidic acid can produce local disturbances in the bilayer structure by the formation of intra-bilayer-inverted-micelles, which can induce transbilayer movement of phospholipid (21). Also, changes in physical properties of artificial membranes have been described upon replacing phosphatidylinositol by diglycerides (22). In situ, diglycerides and phosphatidic acid can be formed in many cells from phosphatidylinositol by the action

of endogenous phosphatidylinositol-specific phospholipase C and diglyceride kinase (23). In activated platelets, this can lead to a phosphatidic acid level close to 2% of total lipid phosphorus (24,25,26), which is very similar to the amount of phosphatidic acid formed during a 10 min incubation period of the cells with 0.01 IU/ml of C.welchii phospholipase C. Therefore, diglycerides and phosphatidic acid formed in the PI-cycle of activated platelets may produce pertubations to the bilayer structure that evoke transbilayer movement of phospholipids, leading to an increased exposure of phosphatidylserine in the outer leaflet. This process is of physiological importance since it would switch on the platelets to become more procoagulant.

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