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Increased aminophospholipid translocase activity in human platelets during secretion

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Fluorescent labeled analogs of phosphatidylcholine (NBD-PC) and phosphatidylserine (NBD-PS) were used to study transport of phospholipids from the outer to the inner leaflet of the plasma membrane of human platelets. Platelets were stimulated with thrombin or Ca^{2+}-ionophore at various extracellular [Ca^{2+}]. No significant transport of NBD-PC could be observed either in resting or stimulated platelets. NBD-PS transport in platelets stimulated with thrombin (with or without extracellular Ca^{2+}), or ionophore in the presence of EGTA, was enhanced 4-fold ($t_{1/2} \approx 2$ min) compared to unstimulated controls ($t_{1/2} \approx 8$ min). Stimulation with ionophore at extracellular [Ca^{2+}] exceeding 8 μM caused a gradual decrease in inward transport of NBD-PS. At 100 μM Ca^{2+}, NBD-PS transport becomes as slow as that of NBD-PC. We conclude that platelet activation by agonists that induce secretion without appreciable shedding is accompanied by an increase in translocase activity that maintains asymmetry during fusion which occurs during exocytosis.

The plasma membrane of platelets as well as erythrocytes is characterized by an asymmetric distribution of the phospholipids [1–5]. In the presence of extracellular Ca^{2+}, platelets rapidly lose transbilayer lipid asymmetry upon stimulation by Ca^{2+}-ionophore A23187, upon treatment with sulfhydryl-oxydizing agents, by activation with a mixture of collagen and thrombin [6,9], or by addition of complement pore C_{5b}-C_9 [7]. Sims et al. [7] showed that surface exposure of phosphatidylserine in activated platelets is directly coupled to shedding of microvesicles from the plasma membrane. They suggested that fusion between apposing segments of cytoplasmic membrane surfaces, as part of the budding process, could lead to a local collapse of phospholipid asymmetry through transient formation of a phase of non-bilayer structures [8]. We have recently shown that loss of membrane phospholipid asymmetry during shedding only occurs because aminophospholipid translocase is inactive during the shedding process [9]. However, not all platelet activators induce membrane shedding, but all activators produce secretion [6,7]. This is of interest because the secretory events also involve fusion, i.e., of granule membranes with the plasma membrane, and could therefore be expected to cause randomization of phospholipids over both membrane leaflets, which does not occur to an appreciable extent. Therefore, the present study was undertaken to investigate the potential contribution of the aminophospholipid translocase in the maintenance of phospholipid asymmetry in the platelet plasma membrane during secretion.

The translocase activity of platelets was measured by the ability of these cells to translocate fluorescent analogs of phosphatidylcholine (PC) and phosphatidylserine (PS), acyl-chain labeled NBD-PC or NBD-PS (NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl), from the outer to inner leaflet of the membrane. In initial experiments, these activities are compared to those of red cells. The fluorescent analogs of PS were synthesized from NBD-PC by phospholipase D catalyzed base exchange in the presence of L-serine [10,11]. Washed human platelets and red cells were isolated from freshly drawn blood by differential centrifugation as described before [6]. Both platelets and red cells were finally resuspended at a concentration of 10^8 ml^{-1} in Hepes buffer, containing 137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes and 5 mM glucose (pH 7.5). For experiments with activated platelets, the cells (10^8 ml^{-1}) were incubated with various agonists for 5 min at 37 °C. Trans-

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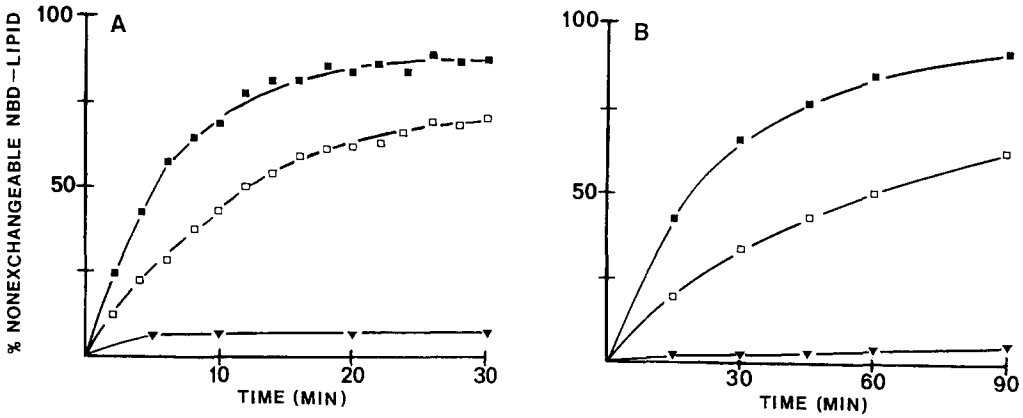

Fig. 1. The ability of platelets (A) and red cells (B) to transport NBD-lipid from the outer to inner leaflet of the plasma membrane. Platelets and red cells were incubated with either (palmitoyl-NBD)-PC or (oleoyl-NBD)-PC(▼), (palmitoyl-NBD)-PS (□) or (oleoyl-NBD)-PS (■). At each time point samples were taken to determine the fraction of NBD-lipid not removed by extraction with bovine serum albumin. Note the difference in time scale between panel A and panel B.

locase activity of the cells was measured after rapid mixing with NBD-PC or NBD-PS (1 μM final concentration) and incubation at 37°C. At different time intervals 200 μl aliquots of cell suspension were mixed with either 1 ml of Hepes buffer (pH 7.5) (to determine total fluorescence) or 1 ml of the same buffer containing 1% (w/v) bovine serum albumin (to remove NBD-lipid from the outer monolayer) and centrifuged for 3 min at $12\,000 \times g$. After solubilizing the pelleted cells in 1% Triton X-100 (2 ml) the NBD fluorescence was measured ($\lambda_{ex} = 472$ nm, $\lambda_{em} = 534$ nm). The non-exchangeable NBD-lipid remaining after extraction with bovine serum albumin was considered to be present inside the cell.

Fig. 1 shows the transport of 1-palmitoyl-2-aminocaproyl-NBD and 1-oleoyl-2-aminocaproyl-NBD-phosphatidylserine in unstimulated platelets (A) and in erythrocytes (B). It can be seen that both of the PS species were rapidly translocated, whereas the respective PC analogs were not. The halftimes required for (oleoyl-NBD)-PS and (palmitoyl-NBD)-PS to reach equilibrium transmembrane distribution in platelets are 4 min and 8 min, respectively (Fig. 1A). For red cells the halftime of translocation for (oleoyl-NBD)-PS is 15 min, which is in agreement with data presented by Schroit and coworkers [12]. (Palmitoyl-NBD)-PS is translocated with a halftime of 30 min (Fig. 1B). These results suggest that apart from a dependency on the length of the acyl chain in the 1-position of the NBD-PS, the aminophospholipid translocase is more active in platelets than in erythrocytes. To better discriminate changes in translocation rate upon stimulation of platelets, we have cho-

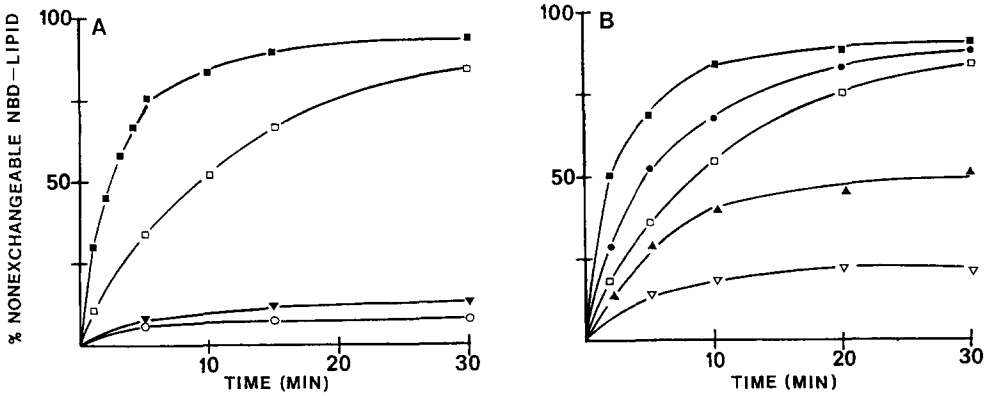

Fig. 2. The ability of platelets to translocate (palmitoyl-NBD)-lipid from the outer to inner leaflet of the plasma membrane after treatment with thrombin or A23187. (A) Activation of platelets with thrombin (4 nM) for 5 min at 37°C was followed by an incubation with NBD-PC (▼) or NBD-PS (■). Non-stimulated platelets were also incubated with NBD-PC (○) or NBD-PS (□). (B) Platelets, 60 min preincubated with the calpain inhibitor MDL 28170 (100 μM), were not stimulated (□) or treated with A23187 (1 μM) at 37°C in the presence of 1 mM EGTA (■), 6 μM Ca^{2+} (●), 8 μM Ca^{2+} (▲) or 100 μM Ca^{2+} (▽). A free Ca^{2+} concentration of 6 or 8 μM was obtained by adding various amounts of $CaCl_2$ to Hepes buffer containing 30 mM EGTA (pH 7.5), and the Ca^{2+} concentration was adjusted using a Ca^{2+}-selective electrode. After 5 min NBD-PS was added to the incubations. At different time intervals, aliquots were taken to determine the fraction of non-exchangeable NBD-lipid.

sen the palmitoyl-derivative of NBD-PS. Upon stimulation with thrombin a 4-fold increase in inward transport of NBD-PS is seen as compared to control platelets ($t_{1/2} = 2$ and 8 min, respectively) (Fig. 2A). The effect induced by thrombin is independent of extracellular Ca^{2+} since the same results were found in the presence of 1 mM Ca^{2+} or 1 mM EGTA. No appreciable transport of NBD-PC was found, indicating that the specificity of the translocase for aminophospholipid is maintained.

The effect of intracellular [Ca^{2+}] on translocase activity of platelets was investigated by varying the extracellular Ca^{2+} concentration in the presence of Ca^{2+}-ionophore. Calpain mediated inactivation of aminophospholipid translocase [9] was prevented by preincubating the platelets for one hour with 100 μM MDL 28170, a specific inhibitor for calpain (kind gift from Merrell Dow Research Institute). Fig. 2B shows that treatment of platelets with A23187 in the presence of EGTA (1 mM) causes an increase in NBD-PS transport which is almost identical to that observed for platelets stimulated with thrombin. With increasing extracellular [Ca^{2+}] a decline in rate as well as the extent of NBD-PS transport is observed at about 8 μM Ca^{2+}. At 100 μM Ca^{2+}, the rate of NBD-PS transport becomes indistinguishable from that of NBD-PC.

The actual mechanism by which Ca^{2+} inhibits aminophospholipid translocase is still unknown. Bitbol and co-workers [13] have studied the role of bivalent cations on aminophospholipid translocase activity in red blood cells and reach the conclusion that inhibition by Ca^{2+} is partly due to ATP depletion caused by the increased consumption by the calcium pump. On the other hand, they also demonstrate a direct inhibitory effect of cytosolic Ca^{2+}, presumably affecting the ATPase site of the transporter. The explanation for increased translocase activity observed at low intracellular [Ca^{2+}] remains obscure. It might be speculated to be due to an increased number of transporter molecules derived from intracellular membranes which become incorporated into the plasma membrane as a result of the secretory event.

In general, cytosolic [Ca^{2+}] as measured by fluorescent Ca^{2+}-chelators, increases upon platelet activation from about 100 nM to 1–2 μM [14]. This elevation in cytosolic Ca^{2+} which occurs upon stimulation with thrombin as well as with ionophore at low extracellular [Ca^{2+}] is sufficient to evoke secretion in platelets. However, no significant shedding or loss of membrane phospholipid asymmetry is seen under these conditions [7,15]. In view of the present findings, we propose that the increased aminophospholipid translocase activity strongly contributes to the maintenance of the asymmetric distribution of membrane phospholipids during the fusion events which accompany secretion. A further rise in cytosolic Ca^{2+} mediated by Ca^{2+}-ionophore and elevated extracellular Ca^{2+} suppresses aminophospholipid transport. Under these circumstances, fusion-induced scrambling of membrane phospholipids can no longer be prevented by the translocase because it is inactive [9]. It has been shown that in addition to secretion, ionophore in the presence of 1 mM Ca^{2+} also results in shedding of microvesicles from the plasma membrane [7,9]. All these fusion events could contribute to loss of lipid asymmetry under conditions where translocase activity is suppressed [9].

We propose that a major function of the aminophospholipid translocase is to maintain membrane phospholipid asymmetry in cells during processes which involve membrane fusion. This transport activity might, therefore, be of significant importance in cells which undergo endo- or exocytosis, cell division, and fusion events which occur during membrane biogenesis.

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