Regulation of Phosphatidylserine Transbilayer Redistribution by Store-operated Ca\textsuperscript{2+} Entry

ROLE OF ACTIN CYTOSKELETON* 

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The phosphatidylserine transmembrane redistribution at the cell surface is one of the early characteristics of cells undergoing apoptosis and also occurs in cells fulfilling a more specialized function, such as the phosphatidylserine-dependent procoagulant response of platelets after appropriate activation. Although an increase in cytoplasmic Ca\textsuperscript{2+} is essential to trigger the remodeling of the plasma membrane, little is known about intracellular signals leading to phosphatidylserine externalization. Here, the role of store-operated Ca\textsuperscript{2+} entry on phosphatidylserine exposure was investigated in human erythroleukemia HEL cells, a pluripotent lineage with megakaryoblastic properties. Ca\textsuperscript{2+} entry inhibitors (SKF-96365, LaCl\textsubscript{3}, and miconazole) inhibited store-operated Ca\textsuperscript{2+} entry in A23187- or thapsigargin-stimulated cells and reduced the degree of phosphatidylserine externalization concomitantly, providing evidence for a close link between the two processes. In cells pretreated with cytochalasin D, an agent that disrupts the microfilament network of the cytoskeleton, store-operated Ca\textsuperscript{2+} entry and phosphatidylserine externalization at the cell surface were inhibited. In a context where most of the key actors remain to be identified, these results provide evidence for the implication of both store-operated Ca\textsuperscript{2+} entry and cytoskeleton architectural organization in the regulation of phosphatidylserine transbilayer migration.

Changes in the cytoplasmic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}],\textsubscript{i}) constitute one of the main pathways by which information is transferred from extracellular signals into various intracellular compartments (1, 2). The induction of receptor-mediated cytosolic Ca\textsuperscript{2+} signals involves two interdependent and closely coupled events: (i) a rapid and transient release of Ca\textsuperscript{2+} stored in the endoplasmic reticulum, (ii) followed by slowly developing extracellular Ca\textsuperscript{2+} entry (1, 3, 4).

In many cell types, including human platelets, depletion of the intracellular Ca\textsuperscript{2+} stores induces entry of Ca\textsuperscript{2+} across the plasma membrane (5), referred to as capacitative or store-operated Ca\textsuperscript{2+} entry (SOCE) (6). Cell stimulation by Ca\textsuperscript{2+}-mobilizing agents triggers the migration of phosphatidylserine (PS) to the exoplasmic leaflet (7), an aminophospholipid sequenced in the inner leaflet of the plasma membrane of non-stimulated cells (8), followed by the shedding of small vesicles released from the plasma membrane (9, 10). These microparticles contain surface proteins and cytoplasmic components of the original cell. Thus, shedding of PS-expressing microvesicles appears to be closely associated with cell surface externalization of PS (11). The exposure of PS is a key step for the hemostatic response (11) and becomes a recognition signal for the phagocytosis of apoptotic cells (12). It has been demonstrated that exposure of PS in erythrocytes and platelets (13) as well as during apoptosis (14) is dependent on extracellular Ca\textsuperscript{2+} and that a continuously elevated [Ca\textsuperscript{2+}]\textsubscript{i}, level may be necessary to sustain the externalization process (15). Furthermore, Ca\textsuperscript{2+} influx across the plasma membrane and maximal exposure of aminophospholipids seem necessary for membrane microparticle formation in platelets (10). In a previous study (16), we have shown that an alteration of SOCE is correlated with a defective exposure of PS in cells isolated from a patient with an extremely rare inherited disorder of PS externalization, the Scott syndrome. Nevertheless, little information regarding the upstream and downstream intracellular signals leading to PS externalization or phospholipid scrambling has been provided. Although several hypotheses have been proposed regarding direct or indirect coupling mechanisms of the endoplasmic reticulum and the plasma membrane (17, 18), little is known about the intracellular signals governing SOCE or the Ca\textsuperscript{2+} channels mediating this particular form of Ca\textsuperscript{2+} entry. Recently, Rosado et al. (19) suggested that SOCE is mediated by actin cytoskeleton in human platelets.

To examine the significance of SOCE on PS externalization, we have combined the detection of PS exposure and the measurement of [Ca\textsuperscript{2+}], in human erythroleukemia (HEL) cells, a megakaryoblastic cell line expressing specific receptors found in platelets (20, 21). We report here that SOCE regulates PS externalization, and cytoskeletal elements modulate both SOCE and PS transmembrane migration. The present study opens a new field of investigation of possible relationships between Ca\textsuperscript{2+} signaling and the remodeling of the plasma membrane.
Experimental Procedures

Materials—RPMI 1640 and fetal calf serum were from Life Technologies (Paisley, UK), and other cell culture reagents were from BioWhittaker (Walkersville, MD). Ca2+ ionophore A23187 and SKF-96365 (1-β-[3(4-methoxyphenyl)propoxy]-4-methoxyphenethyl-1H-imidazole hydrochloride) were obtained from Calbiochem (La Jolla, CA). Fluo-3/acetoxymethyl ester (Fluo-3) was from Molecular Probes (Eugene, OR) and d-thapsigargin (TG) from Alexis Corp. (San Diego, CA). Miconazole, cytochalasin D (CytD) and LaCl3 were products from Sigma Chemical Co. (St. Louis, MO). Human blood coagulation factors Xa and prothrombin, annexin V and annexin VFITC were, respectively, the same as those previously used in our laboratory (22, 23). Factor V was purchased from Diagnostica Stago (Asnières, France) and chromogenic substrate H-p-phenylalanine-t-piperyl-t-arginine-p-nitroanilide-dihydrochloride (Chromozyme TH) from Roche Diagnostics (Mannheim, Germany).

Cell Culture—HEL cells constitute a continuous megakaryoblastic cell line expressing markers such as thrombopoietin, thrombin receptors, and glycoprotein Ib/IIa, with some of them being found in platelets (24–26). HEL were seeded at 1 × 106 cells/ml and cultured in RPMI 1640 (free Ca2+ concentration ~ 4 mM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, at 37°C in humidified 5% CO2 atmosphere. All experiments were performed with maximal concentrations of inhibitors at which no cytotoxicity was observed.

Assessment of PS Exposure by Prothrombinase Functional Assay—Cell suspensions were centrifuged at 600 × g for 5 min, washed once in Hanks’ balanced salt solution, and resuspended at the concentration of 1 × 106 cells/ml. They were loaded with 3 μM Fluo-3/AM and 10 μM miconazole and resuspended in RPMI 1640 medium (free Ca2+ concentration ~ 1 mM) and activated with 2 μM ionophore A23187 for 10 min at 37°C, and centrifuged at 12,000 × g for 2 min to separate the microparticle-containing supernatant from the corresponding cells. Procoagulant PS exposure in stimulated cells and derived microparticles was detected, in the presence of 1 mM CaCl2, using a human prothrombinase assay. In this phospholipid is the rate-limiting parameter promoting the activation of prothrombin by factor Xa in the presence of factor Va (27). Thrombin generated by the assembled prothrombinase complex was measured using a chromogenic assay, as described elsewhere (23).

Measurements of [Ca2+]i, by Flow Cytometry—HEL cells were cultured at 106 cells/ml. They were loaded with 5 μM Fluo-3 for 30 min at room temperature and then washed twice in RPMI 1640 medium supplemented with CaCl2 (final Ca2+ concentration ~ 1 mM) and resuspended at the same concentration (1 × 106 cells/ml, 500 μl/analysis). To study Ca2+ release and Ca2+ entry separately, the experiments were performed in the presence of EGTA (1 mM), able to chelate the totality of Ca2+ in RPMI 1640 medium. Cells were then stimulated by the different agents, and Ca2+ release was measured using a FACScan Beckton-Dickinson flow cytometer and the CELLQuest software. A baseline value was obtained for each sample by fluorescence measurement for 30 s before addition of pharmacologic agents. Collection was immediately resumed, then terminated after additional 5 min (~50,000 events). Fluo-3 fluorescence was expressed in arbitrary fluorescence intensity units and plotted as FL-1 versus time. To convert these values into absolute [Ca2+]i, calibration was performed at the end of each experiment. [Ca2+]i was calculated using the equation: [Ca2+]i = Ki([F − Fmin]/[Fmax − F]), where Ki is the dissociation constant of the Ca2+-Fluo-3 complex (400 nm) (28). Fmax represents the maximum fluorescence (obtained by treating cells with 10 μM A23187), and Fmin corresponds to the minimum fluorescence (obtained for ionophore-treated cells in the presence of 1 mM EGTA). F is the actual sample fluorescence. Fluorescence intensities were expressed as the increase in fluorescence with respect to baseline fluorescence intensity before stimulation.

Flow cytometry analysis—PS probing was achieved using annexin VFITC (140 nm final concentration). Incubation at room temperature was allowed to proceed for 10 min before data acquisition. In some cases, cells activated in the presence of 1 mM CaCl2 with 2 μM Ca2+ ionophore A23187 for 10 min at 37°C before addition of annexin VFITC. In another set of experiments, cell-derived microparticles were separated from activated cells by centrifugation at 400 × g for 5 min at room temperature. Under this condition, 100% of cells were eliminated as well as the larger cell fragments. Annexin VFITC was added to the microparticle-containing supernatant. Samples were analyzed using the FACScan flow cytometer. The forward light scatter and fluorescence channels were set at logarithmic gains. The forward light scatter setting was E-01. The identification of cell and microparticle populations was performed as previously already described (29).

Results

Changes of [Ca2+]i—To investigate the changes of [Ca2+]i, HEL cells were loaded with the Ca2+-binding fluorophore Fluo-3, and emission fluorescence shifts were monitored by flow cytometry. As shown in Fig. 1, stimulation by the Ca2+ ionophore A23187 evoked Ca2+ signal with two characteristic phases: a rapid rise in [Ca2+]i, within a few seconds, followed by a sustained phase of elevated [Ca2+]i, lasting several minutes, which resembles Ca2+ entry. To determine whether this sustained Ca2+ entry was reflected Ca2+ entry, cells were treated with SKF-96365, an agent known to block store-operated Ca2+ channels (30) and other Ca2+ channels. SKF-96365 by itself significantly increased (p < 0.05) basal fluorescence intensity, [Ca2+]i, basal values being 43 ± 5 nM, 87 ± 10, and 103 ± 27 nM in the absence and in the presence of 3 and 10 μM SKF-96365, respectively. At 1 μM, SKF-96365 did not significantly modify Ca2+ response induced by calcium ionophore A23187 (Table I). As illustrated in Fig. 1, SKF-96365 treatment reduced the amplitude of the [Ca2+]i sustained phase in HEL cells stimulated with A23187 in the presence of external Ca2+ in the medium (~1 mM). SKF-96365 was responsible for a dose-dependent inhibition of Ca2+ entry between 3 and 10 μM, with
reductions of ~13 and 20%, respectively (Table I). Above 10 μM SKF-96365, cytotoxicity precluded further observations (not shown).

In platelets, A23187 has been reported to induce Ca²⁺ release from intracellular stores, and after sustained depletion, voltage-independent Ca²⁺ influx across the plasma membrane becomes activated (7). To confirm which is the pathway affected by SKF-96365 in HEL cells, the same experiments were repeated in the presence of LaCl₃, an inhibitor of divalent cationic channels (31), and miconazole, an imidazole compound able to inhibit SOCE (32). No significant difference was detected in the basal fluorescence intensity after LaCl₃ treatment ([Ca²⁺]), basal values were 43 ± 6 and 48 ± 12 nM, in the absence and in the presence of LaCl₃, respectively). However, miconazole increased significantly (p < 0.05) the basal fluorescence intensity ([Ca²⁺]), basal value was 76 ± 10 nM). As illustrated in Table I, LaCl₃ (30 μM) or miconazole (10 μM) treatment reduced Ca²⁺ entry by ~20% and 15%, respectively. Collectively, these data suggest that the sustained phase of elevated [Ca²⁺], induced by A23187 in HEL cells is due to SOCE activation.

**Effect of Ca²⁺ Entry Inhibitors on PS Externalization**—In the absence of stimulation (Fig. 2A), HEL cells and the corresponding supernatants showed a basal PS-dependent prothrombinase activity of 0.01 ± 0.005 and 0.02 ± 0.008 NIH units of thrombin generated per min/ml/10⁶ cells, respectively. Following treatment with 3 and 10 μM SKF-96365, the activities increased ~2-fold, testifying to a weak ability of this agent to perturb PS asymmetric distribution by itself. After Ca²⁺ ionophore treatment, prothrombinase activity was ~12-fold and ~3.5-fold enhanced in cells and corresponding supernatants, respectively. SKF-96365 significantly (p < 0.05) affected the development of prothrombinase activity induced by Ca²⁺ ionophore with an inhibition of ~40 and 48% in the presence of 3 and 10 μM SKF-96365, respectively. The same behavior was observed in the corresponding supernatants.

Prothrombinase activity measurements were also performed in the presence of LaCl₃ and miconazole. LaCl₃ interfered in this assay, probably because it was able to form a coordinate complex with PS (33), making the latter inaccessible for the assembly of the coagulation factors at the cell surface. As shown in Fig. 2B, miconazole also decreased the development of prothrombinase activity induced by A23187 in HEL cells; however, the inhibition (34%, p < 0.05) was lower than that observed with 10 μM SKF-96365. Prothrombinase activity was reduced accordingly in the corresponding supernatants.

To establish whether microparticles from HEL cells released after A23187 treatment bear exposed PS at their surface, cells and/or corresponding supernatants were incubated with annexin VFITC, a widely used probe for PS (Fig. 3). Unstimulated HEL cells presented a basal level of fluorescence concerning highest fluorescence intensities. This was indeed also the case for microparticle shedding (Fig. 3A). In agreement with prothrombinase assay (Figs. 2A, 2B, and 4C), A23187 induced extensive PS exposure in cells (Fig. 3C), as shown by the marked shift of ~99% of the cells to the upper right region, i.e. that of the highest fluorescence intensities. This was indeed also the case for microparticle shedding (Fig. 3F), as evidenced by the large proportion of annexin VFITC-positive events in the lower right region (91 ± 2%). Hence, microparticles virtually stemming from activated cells bear accessible PS and are therefore procoagulant.
TG-evoked Ca\(^{2+}\) Influx—To establish whether the Ca\(^{2+}\) entry inhibitors affect Ca\(^{2+}\) release from intracellular stores or SOCE, HEL cells were initially stimulated with TG (1 \(\mu\)M), an inhibitor of reticulum endoplasmic Ca\(^{2+}\)-ATPases, in the presence of EGTA (1 mM) in the extracellular medium (Fig. 4A). Under these conditions, the addition of 1 \(\mu\)M TG to Fluo-3-loaded cells evoked a transient and slow elevation in [Ca\(^{2+}\)], indicative of SOCE. At 1 \(\mu\)M, SKF-96365 had no effect on Ca\(^{2+}\)-evoked response in TG-exposed cells (not shown). Relative to controls, the magnitude of SOCE upon Ca\(^{2+}\) addition was significantly reduced (~20\%) in 3 or 10 \(\mu\)M SKF-96365-treated cells after stimulation with 1 \(\mu\)M TG (Fig. 4, A and B).

As shown above with SKF-96365, both LaCl3 and miconazole did not affect Ca\(^{2+}\) release from intracellular stores (Table II). After Ca\(^{2+}\) reinduction, these inhibitors were able to significantly reduce SOCE (~19 and 12\%, respectively).

**DISCUSSION**

The present study provides evidence that PS externalization is related to Ca\(^{2+}\) entry in HEL cells, and more specifically to a particular way of Ca\(^{2+}\) entry, referred to as SOCE. In addition, we show that both events, SOCE and PS exposure, are regulated, at least in part, by actin cytoskeleton.

Like platelets and many other nonexcitable cells (5, 36), HEL cells undergo SOCE, as observed here after CaCl\(_2\)-induced response in TG-treated cells. Three common inhibitors of SOCE, SKF-96365, LaCl\(_3\), and miconazole (30–32, 37), partially inhibited capacitative Ca\(^{2+}\) entry in HEL cells without affecting Ca\(^{2+}\) release from intracellular stores. At the actual concentrations used, miconazole inhibited less efficiently A23187- and TG-induced Ca\(^{2+}\) entry compared with SKF-96365 and LaCl\(_3\). However, in several other studies (37, 38), it has been observed that the inhibitory effect of these compounds differs in magnitude depending on the cell type and concentration.

Little is known about the intracellular signals leading to PS externalization. Our results from the present and previous (16) studies strongly suggest that an alteration of SOCE is correlated with a reduced PS exposure. Indeed, Ca\(^{2+}\) entry inhibitors, SKF-96365 and miconazole, which inhibit store-operated Ca\(^{2+}\) channels, are also able to significantly reduce PS exposure. Ca\(^{2+}\) entry inhibitors also reduced prothrombinase activity in the supernatants, suggesting a Ca\(^{2+}\) dependence for PS externalization. Our results from the present and previous (16) studies strongly suggest that an alteration of SOCE is correlated with a reduced PS exposure. Indeed, Ca\(^{2+}\) entry inhibitors, SKF-96365 and miconazole, which inhibit store-operated Ca\(^{2+}\) channels, are also able to significantly reduce PS exposure. Ca\(^{2+}\) entry inhibitors also reduced prothrombinase activity in the supernatants, suggesting a Ca\(^{2+}\) dependence for PS externalization.
microvesicle release. These inhibitors were deliberately used at low concentration to prevent artificial perturbation of the lipid organization in the plasma membrane, because this may happen in the presence of membrane-binding molecules (8).

After stimulation, HEL cells and microparticles were both strongly labeled by annexin V-FITC, confirming that a major fraction of microparticles bore exposed PS (91 ± 2%). Similar results were obtained in platelets (10, 39, 40), red blood cells (41), monocytes (29), and endothelial cells (42). This suggests that most if not all shed vesicles express PS at their surface and may therefore disseminate membrane-associated procoagulant activity.

The actin microfilaments of the cytoskeleton form a complex network, providing the structural basis for simultaneous interactions between multiple cellular structures. For example, the actin-based cytoskeleton is involved in the control of ion channel activity across the plasma membrane of different cell types (43, 44). In the present study, CytD treatment inhibited A23187-induced Ca\(^{2+}\) responses and both Ca\(^{2+}\) release and entry evoked by TG. Similar observations have been reported in astrocytes after agonist stimulation, suggesting that disruption of microfilaments inhibits receptor/Gi-protein interaction (45). However, here we can exclude this hypothesis, because HEL cells were stimulated by two agents, A23187 and TG, involving distinct signaling pathways. It is likely that the cytoskeleton regulates Ca\(^{2+}\) signaling at different stages of Ca\(^{2+}\) release and SOCE. In addition, the results concerning the effect of CytD on Ca\(^{2+}\) release and/or Ca\(^{2+}\) entry are conflicting. Ribeiro et al. (46) and Patterson et al. (47) have observed that CytD did not block TG-induced Ca\(^{2+}\) entry in NIH 3T3 or smooth muscle cells, respectively. Other authors have observed that CytD did not alter the agonist-induced Ca\(^{2+}\) release in vascular endothelial cells, but Ca\(^{2+}\) entry was reduced (48).

Moreover, in human platelets, Ca\(^{2+}\) stores released by TG were not affected by CytD, but SOCE was greatly reduced by cytoskeletal depolymerization (19). These discrepancies are probably related to a wide variability in the cytoskeletal intrinsic properties as a function of the cell type. Thus, the fact that cytoskeleton disruption attenuates A23187- and TG-induced Ca\(^{2+}\) release and entry in HEL cells indicates that cytoskeletal integrity or closely associated processes are essential for a normal coupling between Ca\(^{2+}\) stores and SOCE.

The role of cytoskeletal reorganization in PS exposure and microparticle formation has been emphasized by several groups who studied the relationships between both events in platelets. Inhibition of actin polymerization by CytD affected the shedding of membrane vesicles but had no effect on the redistribution of PS between the two leaflets of the platelet plasma membrane (49). Other authors (50–53) suggest that...
platelet cytoskeletal proteins are involved in the regulation of membrane lipid asymmetry. However, in the present study, we have observed that the diminution of Ca\(^{2+}\) influx across plasma membrane induced by CytD results in a significant reduction of PS exposure with almost no effect on microparticle shedding. Here, the partial inhibition suggests that the cytoskeleton plays a modulatory role rather than a mandatory one in this particular Ca\(^{2+}\) signaling pathway regulating PS exposure at the HEL cell surface. Recently, it has been observed that the actin-cytoskeleton is also able to modulate the uptake of membrane-derived microparticles by cells (54). These findings, when added to the results of the present study, suggest that cytoskeletal elements actively participate in the intercellular traffic of membrane proteins and, more generally, in the transcellular exchange of biological information.

HEL cells express platelet-specific membrane glycoproteins (26) and possess a strong ability of PS externalization following stimulation. Hence, such cells represent a useful model to investigate platelet properties. Among the latter, PS-depend-ent procoagulant response has an essential physiologic signif-

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