Regulated Externalization of Phosphatidylserine at the Cell Surface

IMPLICATIONS FOR APOPTOSIS*

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The regulated loss of plasma membrane phosphatidylserine (PS) asymmetry is critical to many biological processes. In particular, the appearance of PS at the cell surface, a hallmark of apoptosis, prepares the dying cell for engulfment and elimination by phagocytes. While it is well established that PS externalization is regulated by activation of a calcium-dependent phospholipid scramblase activity in concert with inactivation of the aminophospholipid translocase, there is no evidence indicating that these processes are triggered and regulated by apoptotic regulatory mechanisms. Using a novel model system, we show that PS externalization is inducible, reversible, and independent of cytochrome c release, caspase activation, and DNA fragmentation. Additional evidence is presented indicating that the outward movement of plasma membrane PS requires sustained elevation in cytosolic Ca$^{2+}$ in concert with inactivation of the aminophospholipid translocase and is inhibited by calcium channel blockers.

Aptosis, senescence, and necrosis are distinct cell death mechanisms in normal physiology and during pathological duress. Irrespective of mechanism, and with very few exceptions (1, 2), dying cells trigger downstream events that result in the appearance of phosphatidylserine (PS)$^2$ at the cell surface. This serves as a specific recruitment signal for phagocyte docking and subsequent engulfment and degradation of the apoptotic cell (2–4). PS externalization is critical to this process, since its absence results in impaired recognition and clearance of apoptotic debris that can lead to inflammatory and autoimmune responses (5, 6).

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2 The abbreviations used are: PS, phosphatidylserine; CM, complete medium; TG, thapsigargin; JC-1, 5,5’6,6’-tetrachloro-1,1’3,3’-tetrathylbenzimidazolylcarboxylic acid iodide; Fura-2AM, Fura-2 acetoxymethyl ester; EGTA-AM, EGTA-acetoxyethyl ester; NEM, N-ethylmaleimide; PDA, pyridyldithioethylamine; C6-NBD, 6-[7-nitro-2–1,3-benzoxadiazol-4-yl]amino]hexanoyl; NBD-PS, 1-oleoyl-2-[C6-NBD]sn-glycero-3-phospho-L-serine; NBD-PC, 1-oleoyl-2-[C6-NBD]sn-glycero-3-phospho-L-sn-lysine; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone; cyt c, cytochrome c; RBC, red blood cell; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PI, propidium iodide; FACS, fluorescence-activated cell sorter; ER, endoplasmic reticulum.

Mammalian cells have an asymmetric transbilayer distribution of phospholipids such that most of the PS is localized at the inner membrane leaflet. This asymmetry is maintained by the activity of lipid-specific transporters that regulate the distribution of PS by bilayer leaflets, a process that is Ca$^{2+}$-dependent (7, 8). Interestingly, lipid transport studies in (organelle-free) erythrocytes have shown that sulfhydryl-modifying reagents do not induce the outward movement of PS (9).

These results are in sharp contrast to more recent observations demonstrating that nucleated cells externalize PS upon sulfhydryl modification (10, 11). This suggests that the appearance of PS at the cell surface is regulated by specific intracellular signaling events that are absent in erythrocytes.

It is generally accepted that PS externalization during apoptosis occurs downstream to cytochrome c (cyt c) release. There is, however, no direct evidence indicating that cyt c release, caspase activation, or DNA fragmentation is related to, or responsible for PS exposure. In this study, we show that PS externalization can be specifically induced and reversed by a mechanism that is distinct and separable from other hallmarks of apoptosis and operates through a Ca$^{2+}$-dependent mechanism that is inhibited by calcium channel blockers, suggesting involvement of L-type Ca$^{2+}$ channels.

EXPERIMENTAL PROCEDURES

Cells and Cell Lines

The human T cell leukemia cell line Jurkat and the chronic myelogenous leukemia cell line K562 were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (CM). Red blood cells (RBC) were isolated from blood collected from healthy volunteers. The blood was centrifuged and the buffy coat removed, and the RBC were washed with TBS (20 mM Tris, pH 7.4, 150 mM NaCl) containing 20 mM glucose (TBSG) and resuspended in the same buffer. RBC studies presented in this manuscript were performed in accordance with the guidelines of the Institutional Review Board.

Sulfhydryl Labeling of Cells

Jurkat and K562 cells (10$^6$/ml in CM) or RBC (2% hematocrit in TBSG) were incubated at 37 °C with the indicated concentrations of N-ethylmaleimide (NEM) or pyridyl-di-thioethylamine (PDA). After 15 min the cells were washed and resuspended in TBSG.

Calcium Loading

Jurkat and K562 cells in CM, and RBC in TBSG, were incubated at 37 °C for 1 h with A23187 at 1 and 2 μM, respectively, in
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the absence or presence of varying Ca$^{2+}$ concentrations. The ionophore was removed by centrifuging the cells through ice cold BSA (1%) followed by washing twice with TBSG.

**Apoptosis and Measurement of Apoptotic Markers**

Cells (10$^7$/ml) were triggered into apoptosis by treatment with Fas antibody (clone CH-11, 0.25 μg/ml) for 4 h at 37 °C. Cells incubated with Fas antibody or sulfhydryl reagents were tested for expression of the following apoptotic markers; for PS externalization (FITC-annexin V binding), cells (10$^8$) were incubated for 10 min at 20 °C in 0.5 ml of TBS containing 2 mM CaCl$_2$, 0.5 μM FITC-annexin V (Invitrogen) and 0.5 μl propidium iodide (PI, 1 ng/ml), followed by flow cytometry analysis. The use of annexin V to monitor PS externalization requires millimolar concentrations (PI/cell cycle analysis) - Cells (10$^6$) were fixed in 70% ethanolic containing buffer to remove excess probe. For DNA fragmentation (PI/cell cycle analysis) - cells (10$^6$) were fixed in 70% ethanolic buffer to remove excess probe.

**Lipid Transport Studies**

*Inward Lipid Transport (Flip) — RBC (2% hematocrit) and Jurkat cells (10$^6$ cells/ml) were incubated with inhibitors or sulfhydryl reagents for 15 min at 37 °C followed by incubation with 6-[β(7-nitro-2,1,3-benzoazadil-4-yl)amino]hexanoyl-1-phosphatidylerine (NBD-PS) or -phosphatidylcholine (NBD-PC) (1 μg/ml) at 37 °C. At the indicated time intervals, aliquots (150 μl) of the cell suspension were set aside for determining total NBD-lipid or centrifuged at 2,000 x g for 3 min through 1 ml of ice-cold 1% BSA to remove NBD-lipids at the outer membrane leaflet. The supernatant was discarded and the cell pellets were solubilized in 0.2% Triton X-100 in TBS for fluorescence measurements. Transport of PS to the inner leaflet was expressed as the rate of increase in NBD fluorescence in the cell pellets.

*Outward Lipid Transport (Flop) — RBC (2% hematocrit) were incubated for 1 h at 37 °C with NBD-PS (1 μg/ml) to enable transport to the inner leaflet of the cells. Residual lipid at the outer leaflet was removed by centrifuging the cells through ice-cold 1% BSA followed by washing twice with ice-cold TBSG. NBD-PS-labeled cells were then incubated with inhibitors (varying concentration), sulfhydryl reagents (0.5 mM), or A23187 (2 μM) in the presence of Ca$^{2+}$ (varying concentrations) for 1 h at 37 °C. Aliquots of the cell suspension were processed as described above for inward transport. Transport of PS to the outer leaflet was expressed as the fraction of total NBD fluorescence removed by back exchange with BSA.

**Data Analysis and Statistics**

Data were analyzed using the graphical data analysis software packages, Microsoft Excel (Seattle, WA) and SlideWrite Plus (Encinitas, CA). Data presented are mean ± S.E. of at least three independent experiments.

**RESULTS**

*Effect of Sulfhydryl-modifying Agents on PS Asymmetry — Human RBC, the RBC progenitor cell line, K562, and Jurkat cells were tested for their ability to externalize PS when incubated with sulfhydryl reactive reagents. Fig. 1A shows that about 60% of the Jurkat cells treated with NEM or PDA (12) expressed PS as determined by their ability to bind annexin V. K562 cells responded similarly; however, the same reagents were without effect on mature red cells (Fig. 1B). The inability of RBC to respond to PDA or NEM was not due to a specific loss in outward PS transport activity since elevation of intracellular Ca$^{2+}$ with calcium ionophore (A23187) resulted in PS externalization (Fig. 1B).
Effect of Sulfhydryl-modifying Agents on the Appearance of Apoptotic Markers—Execution of programmed cell death results in the sequential display of several biochemical markers that include PS externalization, cyt c release, and activation of caspases and DNase. To determine whether treatment with sulfhydryl reagents triggers any of these apoptosis related processes, the effect of NEM and/or PDA on these events were investigated. Fig. 2 shows that incubation of Jurkat cells (and K562 cells; data not shown) with PDA or NEM did not result in the release of cyt c from the mitochondria into the cytosol (Fig. 2A) or trigger DNA fragmentation (Fig. 2B). Interestingly, while Fas antibody induced PS externalization was blocked by the pan caspase inhibitor z-VAD-fmk, sulfhydryl reagent triggered PS exposure was unaffected by this inhibitor (Fig. 2C). These data raise the possibility that pathways that lead to PS externalization are distinct from those that activate other hallmarks of apoptosis. Taken together, these results suggest that sulfhydryl modification can be a useful tool for analyzing mechanisms of PS exposure without triggering the apoptotic cascade.

PS Externalization Is Regulated by Cytosolic Ca\textsuperscript{2+} Levels—Studies with RBC have shown that elevated cytosolic Ca\textsuperscript{2+} leads to the appearance of PS at the cells outer leaflet (13, 14). To determine whether the observed effects of sulfhydryl modification is a result of altered cytosolic Ca\textsuperscript{2+}, Jurkat cells were incubated with the cytosolic Ca\textsuperscript{2+} probe, Fura-2AM, and alterations in Ca\textsuperscript{2+} levels were assessed by real-time fluorescence
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FIGURE 3. Thiol reagents and Fas antibody induce elevated intracellular Ca\(^{2+}\) and PS externalization. A, Jurkat cells were incubated with NEM (A, control) or NEM (B, 0.5 mM) and changes to cytosolic Ca\(^{2+}\) monitored in real time. Broken traces represent deviation from mean (n = 5). B, unlabeled (△) and Fura-2-labeled (●) Jurkat cells were incubated with the indicated concentrations of NEM for 15 min at 37 °C. The unlabeled cells were then tested for FITC-annexin V binding by FACS, and the Fura-2-labeled cells were assessed for cytosolic Ca\(^{2+}\). Inset, annexin V binding and cytosolic Ca\(^{2+}\) levels in Jurkat cells incubated with EGTA-AM (100 μM) prior to the addition of NEM. C, Jurkats cells were incubated with Fas antibody in the absence (closed symbols) or presence (open symbols) of zVAD-fmk (20 μM). At different time intervals cells were incubated with FITC-annexin V (△, △) or Fura-2AM (●, ○) to evaluate PS externalization by FACS or cytosolic Ca\(^{2+}\) by fluorescence spectroscopy respectively. D, phycoerythrin-labeled annexin V binding (red) and Calcium Green-1 fluorescence (green) in control and apoptotic Jurkat cells following incubation with Fas antibody for 5 h. Data shown are representative of three independent experiments.

FIGURE 4. Reversal of thiol-induced PS externalization. A, time-lapse FACS analysis of FITC-annexin V binding to Jurkat cells incubated with 0.5 mM PDA (upper panel) or 0.5 mM NEM (lower panel). Reversal of PS externalization was initiated by the addition of dithiothreitol (4 mM) at ~30 min. Data presented are representative of three independent experiments. B and C, kinetics of PS externalization (FITC-annexin V binding) (B) and cytosolic Ca\(^{2+}\) (Fura-2 fluorescence) in NEM (△) and PDA (●)-treated cells (C) following the addition of dithiothreitol. Data presented in B and C are representative of three independent experiments.

spectroscopy following addition of the thiol reagents. Fig. 3 shows that treatment of the cells with NEM resulted in a rapid elevation in cytosolic Ca\(^{2+}\) (Fig. 3A) that correlated with an increase in the fraction of annexin V-positive cells (Fig. 3B). This Ca\(^{2+}\) is from intracellular stores and not from the medium, since similar changes in Ca\(^{2+}\) levels were obtained with cells incubated in EGTA containing buffer (data not shown). The importance of intracellular Ca\(^{2+}\) to PS externalization is further emphasized by the observation that incubation of cells with the cell-permeable calcium chelator, EGTA-AM, reduced both cytosolic Ca\(^{2+}\) levels and the fraction of annexin V-positive cells by ~50% (Fig. 3B, inset).

To determine whether PS externalization during apoptosis is Ca\(^{2+}\)-dependent, Jurkat cells were incubated with Fas antibody in the presence and absence of z-VAD-fmk. The cells were analyzed for both PS externalization and cytosolic Ca\(^{2+}\) at the indicated time intervals. Fig. 3, C and D, show that the increase in annexin V-positive cells correlated with the increase in cytosolic free Ca\(^{2+}\) and that both events were blocked when the incubation was carried out in the presence of z-VAD-fmk. It should be noted that while the maximal increase in cytosolic Ca\(^{2+}\) during apoptosis is ~50% lower than with NEM, the fraction of annexin V-positive cells remain the same. This is likely attributed to differences in the kinetics and mechanism of Ca\(^{2+}\) release with NEM and Fas antibody and due to prolonged incubation times (1–6 h for Fas antibody versus 15 min for NEM) that might render the plasma membrane permeable to Ca\(^{2+}\) as apoptosis ensues.

Since PDA is a reducible thiol-disulfide exchange reagent, PDA-induced disulfides should regenerate to free sulfhydryls by treatment with reducing agents. Fig. 4, A–C, show that PDA-induced, but not NEM-induced, PS exposure and elevation in cytosolic Ca\(^{2+}\) were both reversed with dithiothreitol.
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Effect of thiolation on directional PS transport

- The data presented above suggest that elevated intracellular Ca$^{2+}$ plays a critical role in the vectorial transport of PS to the cell surface. To investigate the role of intracellular Ca$^{2+}$ on inward lipid movements, RBC were loaded with Ca$^{2+}$ by incubation at 37 °C for 1 h with A23187 (2 μM) and Ca$^{2+}$ (200 μM). These cells were then incubated with NBD-PC or NBD-PS, and internalization of the lipids was monitored at 37 °C by back exchange to BSA at the indicated time intervals. Fig. 6 shows that, similar to NEMmediated inhibition of NBD-PS transport (Fig. 6A), elevated intracellular Ca$^{2+}$ levels also inhibited inward PS movement (Fig. 6B) albeit with concomitant scrambling of both lipid species. This is clearly evident by the fact that transbilayer distributions of both PS and PC leveled off at ~50%. This effect was clearly Ca$^{2+}$-dependent since cells incubated with A23187 in the presence of increasing Ca$^{2+}$ (0.1–5 μM) demonstrated a concomitant decrease in the fraction of PS transported to the inner leaflet (Fig. 6C). Beyond 10 μM Ca$^{2+}$, however, inhibition of inward movement was superseded by activation of scrambling that resulted in complete randomization of NBD-PS across both leaflets. It should be noted that unlike the inward movement, outward NBD-PS transport increases as a function of Ca$^{2+}$ concentration until a 50% distribution of the lipid across the bilayer is achieved (21).

To determine the relationship between thiolation, Ca$^{2+}$, and PS externalization, control and NEM-treated RBC were incubated with A23187 and increasing amounts of Ca$^{2+}$. Similar to data reported earlier (22), pretreatment of RBC with NEM reduced the concentrations of Ca$^{2+}$ required for half-maximal PS externalization by ~2-fold (Fig. 6D).

Thiol Reagent-induced PS Externalization Is Inhibited by L-type Ca$^{2+}$ Channel Blockers—Since plasma membrane L-type channels are involved in Ca$^{2+}$ transport into the cytosol, and elevated cytosolic Ca$^{2+}$ is central to increasing the cell sur-

### TABLE 1

|                | Inward$^a$ | Outward$^b$ |
|----------------|------------|-------------|
| RBC            | 39.4 ± 7.5 | 6.3 ± 1.5   |
| Jurkat         | 46.5 ± 1.0 | 18.2 ± 8.5  |
| Control        | 2.6 ± 0.1  | ND$^a$      |
| NEM            | 1.9 ± 0.3  | ND          |

$^a$ t = 15 min.

$^b$ p < 0.01.

$^c$ p ≥ 0.01.

$^d$ ND, not determined.

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**PS Externalization Requires Sustained Elevation of Cytosolic Ca$^{2+}$**—The data presented above suggest that a direct association exists between elevated cytosolic Ca$^{2+}$ levels and PS externalization. Interestingly, thapsigargin (TG) mediated release of Ca$^{2+}$ from the ER into the cytosol (15) failed to yield annexin V positive cells. Comparison of changes to cytosolic Ca$^{2+}$ levels with Fura-2 revealed that while NEM treatment elicited a sustained increase in cytosolic Ca$^{2+}$, treatment with TG produced a transient increase that returned to basal levels within ~8 min (Fig. 5A). The sustained presence of Ca$^{2+}$ in the cytosol following NEM treatment could be due to compromised mitochondrial buffering capacity as a result of loss in mitochondrial membrane potential ($\Psi_{\text{mt}}$) (16–19). Indeed, cells labeled with JC-1 exhibited a significant decrease in $\Psi_{\text{mt}}$ when incubated with NEM but not with TG (Fig. 5B).

**Thiol Reagents Inhibit Inward PS Transport**—To investigate the possible differential effects of thiol reagents on directional (inward and outward) PS transport, the movement of exogenously supplied NBD-PS from the outer-to-inner leaflet was monitored in control and NEM-treated RBC and Jurkat cells. Table 1 shows that the inward movement of NBD-PS is blocked in both cell types. As shown previously (12), NEM had no effect on the outward movement of NBD-PS in RBC. Outward movements of NBD-PS could not be monitored in Jurkat cells, since after internalization virtually all the lipid was metabolized to other lipid species (mostly NBD-phosphatidic acid) (20).

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**Inhibition of Inward PS Transport by Ca$^{2+}$**—The data presented above suggest that elevated intracellular Ca$^{2+}$ plays a critical role in the vectorial transport of PS to the cell surface. To investigate the role of intracellular Ca$^{2+}$ on inward lipid movements, RBC were loaded with Ca$^{2+}$ by incubation at 37 °C for 1 h with A23187 (2 μM) and Ca$^{2+}$ (200 μM). These cells were then incubated with NBD-PC or NBD-PS, and internalization of the lipids was monitored at 37 °C by back exchange to BSA at the indicated time intervals. Fig. 6 shows that, similar to NEMmediated inhibition of NBD-PS transport (Fig. 6A), elevated intracellular Ca$^{2+}$ levels also inhibited inward PS movement (Fig. 6B) albeit with concomitant scrambling of both lipid species. This is clearly evident by the fact that transbilayer distributions of both PS and PC leveled off at ~50%. This effect was clearly Ca$^{2+}$-dependent since cells incubated with A23187 in the presence of increasing Ca$^{2+}$ (0.1–5 μM) demonstrated a concomitant decrease in the fraction of PS transported to the inner leaflet (Fig. 6C). Beyond 10 μM Ca$^{2+}$, however, inhibition of inward movement was superseded by activation of scrambling that resulted in complete randomization of NBD-PS across both leaflets. It should be noted that unlike the inward movement, outward NBD-PS transport increases as a function of Ca$^{2+}$ concentration until a 50% distribution of the lipid across the bilayer is achieved (21).

To determine the relationship between thiolation, Ca$^{2+}$, and PS externalization, control and NEM-treated RBC were incubated with A23187 and increasing amounts of Ca$^{2+}$. Similar to data reported earlier (22), pretreatment of RBC with NEM reduced the concentrations of Ca$^{2+}$ required for half-maximal PS externalization by ~2-fold (Fig. 6D).

**Thiol Reagent-induced PS Externalization Is Inhibited by L-type Ca$^{2+}$ Channel Blockers**—Since plasma membrane L-type channels are involved in Ca$^{2+}$ transport into the cytosol, and elevated cytosolic Ca$^{2+}$ is central to increasing the cell sur-
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FIGURE 6. Effect of thiolation and Ca2+ on inward PS transport in RBC. A, control and NEM-treated RBC were incubated at 37 °C with NBD-PS or NBD-PC. At the indicated times, aliquots were centrifuged through BSA (1%) to extract the NBD-lipids remaining at the outer leaflet. The cell pellets and un-extracted aliquots of the cell suspension (total NBD-lipid) were solubilized in 0.2% Triton X-100. Inward lipid transport is expressed as the fraction of NBD-lipid not extracted by BSA. Data are representative of three independent experiments. A, B, RBC were incubated for 1 h at 37 °C with 2 μM A23187 and 200 μM Ca2+. At the indicated time intervals, the ionophore was removed by extraction with BSA. The effect of intracellular Ca2+ on inward lipid transport was determined as described above. A, control PS transport; B, control PC transport; C, A23187-Ca2+-treated PS transport; D, A23187-Ca2+-treated PC transport. Data are representative of three independent experiments. C, RBC were incubated for 60 min at 37 °C with 2 μM A23187 and the indicated Ca2+ concentrations. Inward lipid transport was determined following incubation for 60 min at 37 °C with NBD-PS (A) or NBD-PC (B) as described above. Data are mean ± S.E. (n = 3). D, control (■) and NEM-treated (●) RBC were incubated for 60 min at 37 °C with 2 μM A23187 and increasing concentrations of Ca2+. PS externalization was determined by FACS analysis of FITC-annexin V binding. Data shown are mean ± S.E. (n = 5).

面 pools of PS, we determined whether L-type channel blockers affected sulphydryl reagent-induced PS externalization. Jurkat cells were incubated with several structurally distinct inhibitors of L-type Ca2+ channels prior to the addition of NEM (1 mM). After 30 min, PS externalization was determined by FITC-annexin V binding. Data are mean ± S.E. (n = 4). A, verapamil; B, nifedipine; C, diltiazem. D. Fura-2-loaded cells were incubated with verapamil or nifedipine (100 μM) for 20 min and changes in cytosolic Ca2+ levels monitored in real-time following addition of NEM. E, control cells (no NEM); F, verapamil alone; G, nifedipine alone; H, NEM; I, verapamil + NEM; J, nifedipine + NEM. C, RBC containing NBD-PS in the inner leaflet were incubated for 20 min at 37 °C with nifedipine (●) or diltiazem (○) at the indicated concentrations prior to addition of NEM (1 mM), A23187 (2 μM), and Ca2+ (25 μM). After 60 min at 37 °C, the cells were extracted with 1% BSA to remove externalized NBD-PS. PS externalization is expressed as the fraction of total NBD-PS that was extracted by BSA. Data shown are mean ± S.E. (n = 5).

since the inhibitors did not affect the inward movement of NBD-PS (data not shown) their effects were not a result of direct inhibitor-lipid interactions.

DISCUSSION

It is generally accepted that the appearance of PS on cell surfaces is an early indication of apoptosis. There are, however, several reports of transient PS expression in viable non-apoptotic cells (1, 2). This purports the existence of PS externalization mechanisms that are not under direct apoptotic control. In an effort to define the mechanism responsible for PS externalization, we developed an inducible experimental system that triggers transbilayer PS movements. We show that PS externalization can occur through a mechanism that is independent of cyt c release, caspase activation, and DNA fragmentation, events unique to apoptosis. In contrast, PS externalization during apoptosis requires caspase activity since Jurkat cells treated with z-VAD-fmk fail to yield annexin V positive cells following incubation with Fas antibody. Although these data might indicate that apoptosis and thiolation-dependent PS externalization occur through independent mechanisms, it is possible that caspase activation results in ER and/or mitochondrial stress leading to the release of Ca2+ to the cytosol during apoptosis (24, 25). Thiolation may directly activate a stress response that leads to the surge of Ca2+ into the cytosol, thereby circumventing any requirement of caspases (Fig. 8). Such a mechanism could explain the observed independence of PS externalization from cyt c release and DNA fragmentation since both of these steps are caspase-dependent (25–27). While caspases might not directly contribute to PS externalization our data does not rule out the possibility or other proteases playing a role in this process.
Resting cells have an intracellular Ca\(^{2+}\) distribution where most of the cation is sequestered in the ER with only nanomolar amounts (\(\sim 20\) nm for Jurkat cells) present in the cytosol. One of the early events following the induction of apoptosis is the release of ER Ca\(^{2+}\) into the cytoplasm. Although this signaling is crucial to progression of the cell death cascade, it is not clear whether the ER and/or other intracellular organelles have any direct role in PS externalization. This is underscored by observations that RBC, cells that lack intracellular organelles, do not undergo classical apoptosis; yet externalize PS as a phagocyte recognition signal (4). Using RBC and Jurkat cells as a model, we showed that the inability of RBC to externalize PS in response for Ca\(^{2+}\) required to activate outward PS transport. This observation is significant because the concentrations of intracellular Ca\(^{2+}\) achieved under physiologic conditions are unlikely to reach the submillimolar concentrations needed to achieve complete scrambling of lipids across the bilayer. Indeed, the requirement for Ca\(^{2+}\) is significantly reduced by thiol inactivation. Thus, the data presented here support a model in which PS externalization is under the control of two distinct processes: (i) Ca\(^{2+}\)- and/or thiol-dependent inactivation of the PS translocase that moves aminophospholipids from the outer to the inner membrane leaflet and (ii) Ca\(^{2+}\)-dependent activation of outward transport through an L-type Ca\(^{2+}\)-dependent channel. The inactivation of Ca\(^{2+}\)-ATPase inhibitor, TG, also led to an increase in cytosolic Ca\(^{2+}\) but failed to result in PS externalization. Analysis of \(\Psi_{\text{mt}}\) and cytosolic Ca\(^{2+}\) in NEM- and TG-treated cells showed that, while NEM resulted in a significant drop in \(\Psi_{\text{mt}}\) and sustained levels of cytosolic Ca\(^{2+}\), TG had no effect on \(\Psi_{\text{mt}}\) and resulted in a rapid increase in cytosolic Ca\(^{2+}\) that was restored to basal levels within \(\sim 8\) min. This suggests that the rapid restoration of cytosolic Ca\(^{2+}\) to basal levels was due to its sequestration to the mitochondria (16–19), thereby precluding any effect on PS externalization.

The ability of L-type Ca\(^{2+}\) channel blockers to inhibit thiol-induced PS externalization suggests that these channels play a role in transbilayer PS movements. It is unlikely that the inhibition was due to reduced activity of the ryanodine receptors that promote Ca\(^{2+}\) efflux from the ER (29, 30) since changes to bulk cytosolic Ca\(^{2+}\) levels following addition of NEM were similar in control and in verapamil-treated cells. Moreover, inhibition could not be attributed to direct association between positively charged amphipathic compounds and membrane phospholipids since control experiments with chlorpromazine failed to result in inhibition of NEM-induced PS externalization. Furthermore, our conclusions are based on three structurally distinct (nifedipine, dihydropyridines; verapamil, phenylalkyl amines; diltiazem, benzothiazepines) L-type channel blockers, all of which inhibited NEM-induced PS externalization to different degrees. The efficacy of inhibition might be related to differential partitioning of the various compounds into the plasma membrane bilayer (31–33).

It has previously been shown that the ATP binding cassette (ABC) transporter, ABC1, promotes Ca\(^{2+}\)-induced PS exposure at the cell surface (34). It is, however, unlikely that ABC1 is responsible for our observations since its activity is insensitive to Ca\(^{2+}\) channel blockers (35). The activity of other ABC transporter superfamily members, including P-glycoprotein and MRP (36), on the other hand, are inhibited by verapamil. These transporters, however, operate through Ca\(^{2+}\)-independent mechanisms (37). Taken together, these observations argue in favor of a direct role for the L-type channel in PS externalization.

Finally, we demonstrated that thiolation and cytosolic Ca\(^{2+}\) inhibit transport of PS from the outer to the inner leaflet and that thiolation significantly lowers the threshold for Ca\(^{2+}\) to activate outward PS transport. This observation is significant because the concentrations of intracellular Ca\(^{2+}\) achieved under physiologic conditions are unlikely to reach the submillimolar concentrations needed to achieve complete scrambling of lipids across the bilayer. Indeed, the requirement for Ca\(^{2+}\) is significantly reduced by thiol inactivation. Thus, the data presented here support a model in which PS externalization is under the control of two distinct processes: (i) Ca\(^{2+}\)- and/or thiol-dependent inactivation of the PS translocase that moves aminophospholipids from the outer to the inner membrane leaflet and (ii) Ca\(^{2+}\)-dependent activation of outward transport through an L-type Ca\(^{2+}\)-dependent channel. The inactivation of Ca\(^{2+}\)-ATPase inhibitor, TG, also led to an increase in cytosolic Ca\(^{2+}\) but failed to result in PS externalization. Analysis of \(\Psi_{\text{mt}}\) and cytosolic Ca\(^{2+}\) in NEM- and TG-treated cells showed that, while NEM resulted in a significant drop in \(\Psi_{\text{mt}}\) and sustained levels of cytosolic Ca\(^{2+}\), TG had no effect on \(\Psi_{\text{mt}}\) and resulted in a rapid increase in cytosolic Ca\(^{2+}\) that was restored to basal levels within \(\sim 8\) min. This suggests that the rapid restoration of cytosolic Ca\(^{2+}\) to basal levels was due to its sequestration to the mitochondria (16–19), thereby precluding any effect on PS externalization.

The ability of L-type Ca\(^{2+}\) channel blockers to inhibit thiol-induced PS externalization suggests that these channels play a role in transbilayer PS movements. It is unlikely that the inhibition was due to reduced activity of the ryanodine receptors that promote Ca\(^{2+}\) efflux from the ER (29, 30) since changes to bulk cytosolic Ca\(^{2+}\) levels following addition of NEM were similar in control and in verapamil-treated cells. Moreover, inhibition could not be attributed to direct association between positively charged amphipathic compounds and membrane phospholipids since control experiments with chlorpromazine failed to result in inhibition of NEM-induced PS externalization. Furthermore, our conclusions are based on three structurally distinct (nifedipine, dihydropyridines; verapamil, phenylalkyl amines; diltiazem, benzothiazepines) L-type channel blockers, all of which inhibited NEM-induced PS externalization to different degrees. The efficacy of inhibition might be related to differential partitioning of the various compounds into the plasma membrane bilayer (31–33).

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4 These data, however, do not rule out possible differences in localized Ca\(^{2+}\) gradients at the interface of the cytoplasm and plasma membrane inner leaflet (where PS is localized).
the PS translocase by sulfhydryl reagents could be due to “masking” of protein sulfhydryls critical to PS transport. In principle, such a mechanism could be operative physiologically by regulated oxidation of specific protein sulfhydryls or by their conjugation with glutathione (38). The importance of Ca\(^{2+}\) in redox activation (39–41) combined with our observations that Ca\(^{2+}\) activates and inactivates outward and inward PS movement, respectively, makes oxidative control a potential mechanism for the exposure of PS during apoptosis.

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