Contributions of Ca\(^{2+}\) to Galectin-1-induced Exposure of Phosphatidylserine on Activated Neutrophils*

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Apoptotic cells redistribute phosphatidylserine (PS) to the cell surface by both Ca\(^{2+}\)-dependent and -independent mechanisms. Binding of dimeric galectin-1 (dGal-1) to glycoconjugates on N-formyl-Met-Leu-Phe (fMLP)-activated neutrophils exposes PS and facilitates neutrophil phagocytosis by macrophages, yet it does not initiate apoptosis. We asked whether dGal-1 initiated Ca\(^{2+}\) fluxes that are required to redistribute PS to the surface of activated neutrophils. Prolonged occupancy by dGal-1 was required to maximally mobilize PS to the surfaces of fMLP-activated neutrophils. Like fMLP, dGal-1 rapidly elevated cytosolic Ca\(^{2+}\) levels in Fluor-4-loaded neutrophils. An initial Ca\(^{2+}\) mobilization from intracellular stores was followed by movement of extracellular Ca\(^{2+}\) to the cytosolic compartment, with return to basal Ca\(^{2+}\) levels within 10 min. Chelation of extracellular Ca\(^{2+}\) did not prevent PS mobilization. Chelation of intracellular Ca\(^{2+}\) revealed that fMLP and dGal-1 independently release Ca\(^{2+}\) from intracellular stores that cooperate to induce optimal redistribution of PS. Ca\(^{2+}\) mobilization by ionomycin did not permit dGal-1 to mobilize PS, indicating that fMLP initiated both Ca\(^{2+}\)-dependent and -independent signals that facilitated dGal-1-induced exposure of PS. dGal-1 elevated cytosolic Ca\(^{2+}\) and mobilized PS through a pathway that required action of Src kinases and phospholipase C\(\gamma\). These results demonstrate that transient Ca\(^{2+}\) fluxes contribute to a sustained redistribution of PS on neutrophils activated with fMLP and dGal-1.

Plasma membrane phospholipids are normally asymmetrically distributed, with phosphatidylserine (PS)\(^1\) and phosphatidylethanolamine concentrated in the inner leaflet and phosphatidylcholine and sphingomyelin enriched in the outer leaflet. Tissue injury signals platelets, leukocytes, and endothelial cells to rapidly and reversibly mobilize PS, promoting coagulation, and complement activation on cell surfaces (1). Apoptotic signals cause more gradual and sustained redistribution of PS to the outer leaflet of the plasma membrane, providing a key recognition marker that enables macrophages to ingest dying cells from many tissues (2).

Resolution of acute inflammation is thought to require extravasated neutrophils to undergo apoptosis before they are phagocytosed (3–6). However, mice deficient in Fas or Fas ligand have normal numbers of circulating neutrophils (7, 8), even though binding of Fas to FasL induces apoptosis of neutrophils in vitro (9–11). Furthermore, transgenic mice expressing Bcl-2 in mature neutrophils have normal neutrophil homeostasis and unimpaired macrophage-mediated phagocytosis of neutrophils, even though the expression of Bcl-2 in neutrophils inhibits apoptosis (12). Such data argue that neutrophils that enter inflamed tissues need not undergo apoptosis to be removed by phagocytosis. Galectin-1, a member of a large family of \(\beta\)-galactoside-binding lectins (13, 14), is expressed on activated endothelial cells and secreted into perivascular matrices (15–18), where it could encounter emigrating neutrophils. We have shown that binding of dimeric galectin-1 (dGal-1) to glycoconjugates on activated neutrophils and on leukocyte cell lines mobilizes PS and promotes phagocytosis by macrophages, but does not elicit DNA fragmentation or other signs of programmed cell death (19). This suggests an alternative mechanism to expose PS, marking neutrophils for phagocytosis without inducing apoptosis.

The mechanisms for maintaining and disrupting phospholipid asymmetry at the plasma membrane are still poorly understood. An aminophospholipid translocase is believed to maintain asymmetry by transporting PS and phosphatidylethanolamine, but not phosphatidylcholine or sphingomyelin, from the outer to the inner membrane leaflet (2). Rapid disruption of asymmetry in stimulated cells requires not only inactivation of the translocase, but also activation of a phospholipid scramblase that allows net relocation of phospholipids to the outer leaflet (1). Both events require a rise in cytoplasmic Ca\(^{2+}\) levels. A family of candidate scramblase proteins has been cloned; each is a type 2 membrane protein with a Ca\(^{2+}\)-binding site in the cytoplasmic domain (1). Occupancy of this site alters the conformation of a purified scramblase, which occurs in parallel with increases in its activity (20). This suggests a structural basis for the Ca\(^{2+}\)-dependence of rapid PS relocation in activated cells.

Cells undergoing apoptosis frequently require Ca\(^{2+}\) to gradually mobilize PS (21–23), although this is not a consistent requirement (24, 25). In fact, a rise in cytoplasmic Ca\(^{2+}\) inhibits DNA fragmentation and other markers of apoptosis in some cells, including neutrophils, although exposure of PS was not

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\(1\) The abbreviations used are: PS, phosphatidylserine; BAPTA/AM, O,O’-bis(2-aminoethyl)N,N,N’,N’-tetraacetic acid, tetraacetoxymethyl ester; dGal-1, dimeric galectin-1; FITC, fluorescein isothiocyanate; Fluor-4 AM, Fluor-4-acetoxymethyl ester; fMLP, N-formyl-Met-Leu-Pro; G, G-protein; HBSS, Hank’s balanced salt solution; PI, propidium iodide; PKC, protein kinase C; PLC, phospholipase C; PP2, 4-amino-5-(4-chlorophenyl)-7-((t-butyl)pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine.
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Measured (26, 27). Chelation of both intracellular and extracellular Ca\(^{2+}\) does not prevent anti-Fas antibodies from mobilizing PS on apoptotic Jurkat cells (23). Unlike normal cells, hematopoietic cells from patients with Scott syndrome do not activate scramblase in response to agonists that mobilize Ca\(^{2+}\). Yet ultraviolet irradiation, an initiator of apoptosis, activates scramblase and redistributes PS to the surface of Scott syndrome lymphoid cells (28). These data suggest that exposure of PS during apoptosis may result from scramblase activation by Ca\(^{2+}\)-dependent and/or -independent mechanisms.

Unlike the rapid, Ca\(^{2+}\)-dependent mobilization of PS on activated platelets and other blood cells, dGal-1-stimulated exposure of PS on activated neutrophils develops over hours, which is similar to the time course of PS exposure on cells undergoing apoptosis. Nevertheless, binding of dGal-1 does not induce apoptosis (19). It is therefore not clear whether dGal-1 uses mechanisms to redistribute PS like those in other systems. In this study, we examined the contribution of Ca\(^{2+}\) to the dGal-1-induced redistribution of PS on activated neutrophils.

**Materials and Methods**

**Reagents**—The chemicals and antibodies used and their sources were as follows: anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology); polyclonal anti-PLC\(γ\) 2, anti-Fgr, anti-Hck, and anti-Lyn antibodies and protein A-agarose beads (Santa Cruz Biotechnology); N\(^{N\text{-formyl-Met-Leu-Phe}}\) (fMLP), probenecid (4\(\text{-}N\text{-dipropargyl-2H-indole} \times \text{HCl} \text{(Sigma)}

Human serum albumin. Resting neutrophils were activated with 1 mM lactose, for the times indicated. In some experiments, neutrophils were treated with dGal-1 for 1, 2, 3, or 4 h. A portion of the cells was immediately analyzed by flow cytometry. Another portion was disengaged with lactose, washed, and resuspended at 107 cells/ml in HBSS with 0.5% human serum albumin. Incubation with dGal-1 progressively cleared with protein A-agarose for 30 min at 4 °C and then incubated with 10 \(\mu\)M dGal-1 in the presence or absence of the Src kinase inhibitors PP2 or PP3. Fluorescence readings were converted to molar concentrations of [Ca\(^{2+}\)]\(i\), as described (30).

In some experiments, Fluorescein- and Immunoblotting—Neutrophils were pre-treated with 2 mM di-isopropyl fluorophosphate for 30 min at 4 °C. They were then incubated with 10 \(\mu\)M dGal-1 in the presence or absence of 20 mM lactose in the presence or absence of the Src kinase inhibitors PP2 or the inactive analogue PP3 (10 \(\mu\)M) at 37 °C for the indicated interval. The cells were lysed in ice-cold lysis buffer (1% Nonidet P-40, 0.1% SDS, 50 mM Tris-\(\text{HCl} \text{pH 8.0, 150 mM NaCl, 10 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 0.5% sodium deoxycholate, 3 mM Na\(_3\text{VO}_4\), 10 \(\mu\)g/ml each of aprotinin, leupeptin, pepstatin, and soybean trypsin inhibitor, 1 mM Pefabloc SC, 1 mM di-isopropyl fluorophosphate, and 50 mM \(\text{N\text{-methyl-

**Preparation of dGal-1**—Recombinant human dGal-1 expressed in Escherichia coli was purified on lactose-Sepharose as described previously (19).

**Isolation, Activation, and dGal-1 Treatment of Human Neutrophils**—Human blood neutrophils, isolated as described (29), were suspended in Ca\(^{2+}\)-free-Mg\(^{2+}\) containing Hank’s balanced salt solution (HBSS) with 0.5% human serum albumin. Resting neutrophils were activated with 1 mM fMLP for 10 min at 37 °C. Resting or activated neutrophils were incubated with 10 \(\mu\)M dGal-1 at 37 °C. In the presence or absence of 20 mM lactose, for the times indicated. In some experiments, neutrophils were loaded with 10 \(\mu\)M BAPTA/AM for 30 min before treatment with fMLP or dGal-1 in the presence or absence of 2 mM EGTA. In other experiments, cells were treated with 1 \(\mu\)g of immunocyto for 10 min, washed, and then incubated with or without dGal-1 for 4 h. In other experiments, cells were treated with 2 \(\mu\)M U73122 or 10 \(\mu\)M PP2 or PP3 before or after stimulation with fMLP. Before analysis by flow cytometry, agglutination of dGal-1-treated cells was reversed by incubation with 20 mM lactose at 37 °C for 30 min. In some experiments, neutrophils were treated with dGal-1 for 1, 2, 3, or 4 h. A portion of the cells was immediately analyzed by flow cytometry. Another portion was disengaged with lactose, washed, and then further incubated for a total of 4 h before processing for flow cytometry.

**Flow Cytometry**—Neutrophils were incubated with a mixture of FITC-conjugated annexin V and PI for 15 min on ice as described (19). The cells were diluted into HBSS and analyzed immediately on a FACSscan instrument (BD Biosciences) using Cell Quest software. The fluorescence intensity for binding of both annexin V and PI was measured for the entire cell population. For most experiments, the data are represented as the percentage of cells that stained with annexin V above a threshold level, but that remained viable as assessed by staining with PI below a threshold level.

**Ca\(^{2+}\) Flux Measurements**—Resting neutrophils were loaded with 3 \(\mu\)M Fluo-4 AM at 37 °C for 30 min in the presence of 4 \(\mu\)g probenecid, an inhibitor of anion transport, to minimize dye leakage. The cells were washed with HBSS, incubated for 30 min at room temperature to allow the Fluo-4 AM dye to completely de-esterify, washed twice more, and resuspended at 106 cells/ml in HBSS with 0.5% human serum albumin. In some experiments, neutrophils were incubated with BAPTA in HBSS for 30 min along with Fluo-4 AM. Fluorescein-labeled cells (3 \(\times\) 10\(^5\)) were treated with fMLP and/or dGal-1 at 37 °C in the presence or absence of 2 mM EGTA, 2 \(\mu\)M U73122, or 10 \(\mu\)M PP2 or PP3. Fluorescence readings were obtained in a staining cell fluorometer (PerkinElmer Life Sciences LS-50) equipped with a water-jacketed cuvette holder. After obtaining the basal signal, fluorescence intensities were acquired at 0.1-s intervals in continuous 5 min with continuous stirring of the cell suspension. The cells were lysed with 0.1% Triton X-100 to determine the maximum fluorescence. The minimum fluorescence was determined by adding EGTA to the lysed cells. These fluorescence measurements were converted to molar concentrations of [Ca\(^{2+}\)]\(i\), as described (30).

In vitro experiments, Fluorescein- and Immunoblotting—Neutrophils were pre-treated with 2 mM di-isopropyl fluorophosphate for 30 min at 4 °C. They were then incubated with 10 \(\mu\)M dGal-1 in the presence or absence of 20 mM lactose in the presence or absence of the Src kinase inhibitors PP2 or the inactive analogue PP3 (10 \(\mu\)M) at 37 °C for the indicated interval. The cells were lysed in ice-cold lysis buffer (1% Nonidet P-40, 0.1% SDS, 50 mM Tris-\(\text{HCl} \text{pH 8.0, 150 mM NaCl, 10 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 0.5% sodium deoxycholate, 3 mM Na\(_3\text{VO}_4\), 10 \(\mu\)g/ml each of aprotinin, leupeptin, pepstatin, and soybean trypsin inhibitor, 1 mM Pefabloc SC, 1 mM di-isopropyl fluorophosphate, and 50 mM \(\text{N\text{-methyl-

**Results**

**Continuous Binding of dGal-1 Enhances PS Exposure on Activated Neutrophils**—Incubation with dGal-1 progressively mobilizes more PS on fMLP-activated neutrophils for at least 8 h (19). To determine whether continuous binding of dGal-1 is required to maximally expose PS, fMLP-activated neutrophils...
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were treated with dGal-1 for 1, 2, 3, or 4 h. At each interval, a portion of the cells was immediately analyzed for binding of annexin V. Another portion of the cells was incubated with lactose, washed, and then further incubated for a total of 4 h before flow analysis. The data are depicted as the percentage of cells that stained with annexin V above a threshold level, but that remained viable as assayed by staining with PI below a threshold level. The results represent the mean ± S.D. of three experiments.

Because fluorometry monitors Ca²⁺ fluxes only for brief periods, we used flow cytometry to determine whether dGal-1 caused a delayed rise in cytosolic Ca²⁺ in fMLP-activated neutrophils. No such rise was observed 1, 2, 3, or 4 h after addition of dGal-1 (Fig. 3). Addition of the Ca²⁺ ionophore ionomycin at all of these times increased intracellular Ca²⁺, confirming the sensitivity of the flow cytometric assay (Fig. 3). These combined results indicate that dGal-1 only transiently increases cytoplasmic Ca²⁺ in fMLP-activated neutrophils.

Extracellular Ca²⁺ Is Not Required for dGal-1-induced Mobilization of PS on Activated Neutrophils—To address whether Ca²⁺-dependent signaling contributes to the dGal-1-induced exposure of PS, we first determined whether extracellular Ca²⁺ contributed to the response. Activated neutrophils treated with dGal-1 for 4 h mobilized equivalent amounts of PS whether they were suspended in Ca²⁺- or EGTA-containing buffer (Fig. 4A). This result demonstrates that dGal-1 does not require extracellular Ca²⁺ to redistribute PS to the surface of activated neutrophils.

Both dGal-1 and fMLP Contribute to Maximal PS Exposure through Release of Ca²⁺ from Intracellular Stores—Stimulation of neutrophils with dGal-1 causes a rapid, transient rise in cytosolic Ca²⁺ that is primarily due to release of Ca²⁺ from intracellular stores. To assess the contributions of this Ca²⁺ flux to mobilization of PS, neutrophils in EGTA-containing buffer were sequentially stimulated with 1 μM fMLP and 10 μM dGal-1 in the presence or absence of 20 mM lactose. The data are representative of at least three independent experiments.

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buffer were stimulated with fMLP. A portion of the cells was then treated with dGal-1 for 4 h in EGTA. Another portion was loaded with the intracellular Ca\(^{2+}\) chelator BAPTA and then treated with dGal-1 for 4 h in EGTA. Preloading with BAPTA partially but significantly reduced the dGal-1-induced redistribution of PS to the surface of activated neutrophils (Fig. 4B). Because chelation of extracellular Ca\(^{2+}\) did not inhibit PS exposure, the effect of BAPTA must reflect its ability to chelate Ca\(^{2+}\) released from intracellular stores.

Stimulation of neutrophils with fMLP also causes a rapid, transient rise in cytoplasmic Ca\(^{2+}\) that is primarily due to release of Ca\(^{2+}\) from intracellular stores. To assess the contributions of this Ca\(^{2+}\) flux to mobilization of PS, neutrophils loaded with BAPTA were stimulated with fMLP in Ca\(^{2+}\)-containing buffer and then treated with dGal-1 for 4 h in the presence of EGTA. This treatment partially but significantly reduced the dGal-1-induced redistribution of PS compared with cells sequentially stimulated in the presence of Ca\(^{2+}\) (Fig. 4C). Because BAPTA remained in the cells during both the fMLP and dGal-1 incubations, the inhibition observed in Fig. 4C could reflect chelation of Ca\(^{2+}\) released by dGal-1 rather than by fMLP. We therefore preloaded neutrophils with BAPTA and sequentially treated the cells with fMLP and dGal-1 in the continuous presence of EGTA. This combined chelation of intracellular and extracellular Ca\(^{2+}\) markedly inhibited PS exposure (Fig. 4D). These results indicate that optimal mobilization of PS requires that both fMLP and dGal-1 release Ca\(^{2+}\), which can be derived solely from intracellular stores. The additional inhibition of PS exposure by adding EGTA to BAPTA-loaded cells during fMLP stimulation may reflect chelation of extracellular Ca\(^{2+}\) that would otherwise enter the cell after BAPTA-induced activation of Ca\(^{2+}\) channels in the plasma membrane, where it might saturate the chelating capacity of BAPTA (32, 33).

**Fig. 3. dGal-1 does not cause sustained increases of cytosolic Ca\(^{2+}\) in activated neutrophils.** Neutrophils were activated with 1 \(\mu\)M fMLP for 10 min, loaded with Fluo-4, and then incubated with 10 \(\mu\)M dGal-1 for 1, 2, 3, or 4 h. After removing bound dGal-1 with lactose, the cells were analyzed by flow cytometry. As a positive control, a portion of the cells was incubated with ionomycin and then immediately analyzed by flow cytometry. Cytosolic Ca\(^{2+}\) levels were measured by Fluo-4 fluorescence on a linear scale. The data are representative of at least three independent experiments.
activated with 1 μM fMLP for 10 min. They were then incubated with or without 10 μM dGal-1 in the presence or absence of EGTA or 20 mM lactose for 4 h. B, neutrophils in EGTA-containing buffer were activated with 1 μM fMLP for 10 min, loaded with BAPTA, and then incubated in the presence or absence of 10 μM dGal-1 in EGTA-containing buffer for 4 h. Control neutrophils were sequentially incubated with fMLP and dGal-1 in Ca^{2+}-containing buffer. Other cells were treated with dGal-1 in the presence of lactose. C, neutrophils were loaded with BAPTA, activated with 1 μM fMLP in Ca^{2+}-containing buffer for 4 h. Control neutrophils were sequentially activated with 1 μM fMLP and 10 μM dGal-1 in Ca^{2+}-containing buffer. Other cells were treated with dGal-1 in the presence of lactose. D, neutrophils were loaded with BAPTA, activated with 1 μM fMLP in EGTA-containing buffer for 10 min, and then incubated in the presence or absence of 10 μM dGal-1 in EGTA-containing buffer for 4 h. Control neutrophils were sequentially activated with 1 μM fMLP and 10 μM dGal-1 in Ca^{2+}-containing buffer. Other cells were treated with dGal-1 in the presence of lactose. E, neutrophils in Ca^{2+}-containing buffer were incubated with 1 μM ionomycin or with 1 μM fMLP for 10 min and then incubated in the presence or absence of 10 μM dGal-1 in Ca^{2+}-containing buffer for 4 h. Some cells were treated with dGal-1 in the presence of lactose. The percentage of cells expressing PS was measured by flow cytometry as in Fig. 3. The data represent the mean ± S.D. of three experiments.

**Ca^{2+} Mobilization by fMLP Is Not Sufficient to Enable dGal-1 to Mobilize PS on Activated Neutrophils**—dGal-1 causes exposure of PS on the surface of neutrophils only after they have been activated by fMLP (19). To determine whether the fMLP-mediated transient Ca^{2+} flux was sufficient to prime neutrophils for dGal-1 mobilization of PS, we treated neutrophils with ionomycin, a Ca^{2+} ionophore, for 10 min and then incubated them with dGal-1 for 4 h. Unlike fMLP treatment, ionomycin treatment did not enable neutrophils to mobilize PS in response to dGal-1 (Fig. 4E). Thus, fMLP must transmit both Ca^{2+}-dependent and Ca^{2+}-independent signals that prime neutrophils to redistribute maximal PS to the cell surface in response to dGal-1.

dGal-1 Transiently Activates Phospholipase Cγ2 in Neutrophils by an Src Kinase-dependent Mechanism—Mobilization of Ca^{2+} from intracellular stores normally occurs through the action of one or more isoforms of phospholipase C (PLC). This enzyme cleaves phosphoinositides to generate inositol triphosphates, which in turn release Ca^{2+} from the endoplasmic reticulum into the cytoplasm (34). Binding of fMLP to its G protein-coupled receptor activates a PLCβ isoform through a mechanism that does not require tyrosine phosphorylation of the enzyme (34, 35). We considered that dGal-1 might activate PLC through another mechanism. Some mediators generate signals that activate PLCγ isoforms by phosphorylation of specific tyrosine residues (34). One of these is PLCγ2, which neutrophils express (36). To determine whether binding of dGal-1 activates PLCγ2, we incubated neutrophils with dGal-1 for various periods, immunoprecipitated PLCγ2 from cell lysates, and examined the activation of the enzyme by immunoblotting with an antibody to phosphotyrosine. Binding of dGal-1 induced tyrosine phosphorylation of PLCγ2 that peaked after 1–2 min and disappeared within 5 min (Fig. 5A). Activation of PLCγ2 required that dGal-1 bind to specific glycoconjugates on the neutrophil surface, because inclusion of lactose in the media decreased the dGal-1-stimulated tyrosine phosphorylation of the enzyme (Fig. 5A).

The ability of dGal-1 to induce tyrosine phosphorylation of PLCγ2 in neutrophils suggested that dGal-1 acts proximally by stimulating either receptor or non-receptor protein tyrosine kinases. To determine the potential role of non-receptor tyrosine kinases, we treated neutrophils with the Src kinase inhibitor PP2 or its inactive analogue PP3 (37, 38). PP2, but not PP3, blocked the dGal-1-induced tyrosine phosphorylation of PLCγ2 (Fig. 5B). These data demonstrate that dGal-1 activates one or more Src kinases that in turn activate PLCγ2.

dGal-1 Activates the Src Kinases Lyn and Hck in Neutrophils—The major Src kinases in neutrophils are Lyn, Hck, and Fgr (39). To determine which of these enzymes are activated by dGal-1, resting neutrophils were treated with dGal-1 for various intervals and then lysed. Lyn, Hck, or Fgr were immunoprecipitated from the lysates with specific antibodies, and the kinase activity of each enzyme was determined by measuring the phosphorylation of a peptide acceptor using a commercial kit. Binding of dGal-1 rapidly activated both Lyn (Fig. 6A) and Hck (Fig. 6B), with peak activities observed after 1 min and a
The ability of dGal-1 to mobilize Ca\textsuperscript{2+} is mediated by PLC, and the actions of all known PLC isoforms (40, 41). U73122 blocked the Ca\textsuperscript{2+} mobilization by dGal-1. Equivalent portions of cell lysate were immunoprecipitated with an antibody to PLC\textsubscript{2}, resolved by SDS-PAGE, transferred to a membrane, and probed with an antibody to phosphotyrosine. The membrane was then stripped and reprobed with the antibody to PLC\textsubscript{2}. The data are representative of three experiments.

return to near basal levels within 60 min. The Src kinase inhibitor PP2 blocked dGal-1-induced activation of both Lyn and Hck, confirming the specificity of the kinase assay. By contrast, binding of dGal-1 did not activate Fgr, even though the anti-Fgr antibody effectively precipitated the enzyme (data not shown).

Inhibition of Src Kinases Blocks Ca\textsuperscript{2+} Mobilization by dGal-1 and Decreases dGal-1-induced Mobilization of PS on Activated Neutrophils—To determine the potential role of Src kinases in the Ca\textsuperscript{2+}-dependent mobilization of PS on activated neutrophils, we treated neutrophils with the Src kinase inhibitor PP2 or its inactive analogue PP3. PP2, but not PP3, blocked the dGal-1-mediated Ca\textsuperscript{2+} flux in resting neutrophils (Fig. 7A) and in fMLP-activated neutrophils (Fig. 7B). By contrast, PP2 had no effect on the Ca\textsuperscript{2+} flux induced by fMLP (Fig. 7B), as expected, because signaling through the fMLP receptor activates PLC\textsubscript{2}. PP2, but not PP3, also significantly inhibited the dGal-1-mediated mobilization of PS on activated neutrophils (Fig. 8). These data indicate that dGal-1 acts through Src kinases to release Ca\textsuperscript{2+} from intracellular stores, most likely by activating PLC\textsubscript{2}. This signaling pathway synergizes with fMLP-mediated Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent signals to promote maximal redistribution of PS to neutrophil surfaces.

Inhibition of PLC in Neutrophils Blocks Ca\textsuperscript{2+} Mobilization Induced by Both fMLP and dGal-1—To confirm that fMLP and dGal-1 mobilize Ca\textsuperscript{2+} through activation of PLC, we incubated neutrophils with U73122, a small molecule that inhibits the actions of all known PLC isoforms (40, 41). U73122 blocked the ability of dGal-1 to mobilize Ca\textsuperscript{2+} in resting neutrophils (Fig. 9A) and in neutrophils that had previously mobilized Ca\textsuperscript{2+} in response to fMLP (Fig. 9B). Preincubation of neutrophils with U73122 blocked Ca\textsuperscript{2+} mobilization in response to fMLP as well as dGal-1 (Fig. 9C).

Inhibition of PLC Decreases dGal-1-induced Mobilization of PS on Activated Neutrophils—To test the contribution of PLC to dGal-1-induced mobilization of PS on activated neutrophils, we first stimulated neutrophils with fMLP. A portion of the cells was then treated with dGal-1 for 4 h. Another portion was pretreated with U73122 and then treated with dGal-1 for 4 h in buffer containing EGTA to prevent U73122-mediated, secondary influx of extracellular Ca\textsuperscript{2+} (42, 43). U73122 partially but significantly reduced the dGal-1-induced redistribution of PS to the surface of activated neutrophils (Fig. 10A). Preincubation of neutrophils with U73122 before addition of both fMLP and dGal-1 caused a greater reduction in mobilization of PS (Fig. 10B). These results, which are consistent with the data from BAPTA-loaded cells (see Fig. 4, B–D), suggest that both fMLP and dGal-1 require PLC-mediated release of Ca\textsuperscript{2+} from intracellular stores for maximal mobilization of PS on neutrophil surfaces.

DISCUSSION

Some activation events induce a rapid, Ca\textsuperscript{2+}-dependent redistribution of PS to the surfaces of blood cells (1). By contrast, other signals induce gradual, Ca\textsuperscript{2+}-dependent or -independent redistribution of PS to the surfaces of apoptotic cells (2). Binding of dGal-1 to fMLP-stimulated neutrophils elicits a progressive mobilization of PS that marks the cells for phagocytosis,
but this is not associated with apoptotic cell death (19). Whether this novel system for exposing PS shares mechanisms that are used by conventional activation or apoptotic pathways is not known. Here we demonstrate that fMLP and dGal-1 independently stimulate transient elevations in cytosolic Ca\(^{2+}\) that are necessary, but not sufficient, for optimal redistribution of PS on activated neutrophils.

dGal-1 mobilizes PS only on neutrophils that have been previously activated with fMLP, suggesting that both mediators transduce signals that must cooperate to alter PS distribution on the plasma membrane (19). Chemokines or other mediators that stimulate neutrophils as they emigrate from the vasculature into inflamed tissues might also cooperate with dGal-1 to mobilize PS. We observed that dGal-1, like fMLP, stimulated rapid but transient elevation in cytosolic Ca\(^{2+}\). These elevations reflect Ca\(^{2+}\) release from intracellular stores, followed by influx of extracellular Ca\(^{2+}\) across the plasma membrane. dGal-1 caused a similar transient, biphasic mobilization of Ca\(^{2+}\) in human Jurkat T cells, but the functional consequences of this Ca\(^{2+}\) mobilization were not studied (44, 45). We found that incubating neutrophils with EGTA prevented the secondary influx of extracellular Ca\(^{2+}\) elicited by both fMLP and dGal-1, but it did not prevent the mobilization of Ca\(^{2+}\) that is necessary for optimal redistribution of PS.
of PS. Thus, extracellular Ca\textsuperscript{2+} is not required for dGal-1 to redistribute PS on activated neutrophils. By contrast, chelation of intracellular Ca\textsuperscript{2+} with BAPTA partially reduced PS exposure. Treating activated cells with both BAPTA and EGTA completely prevented the PS exposure induced by dGal-1. BAPTA treatment is known to open plasma membrane channels for Ca\textsuperscript{2+} (32, 33). The addition of EGTA most likely abrogated a BAPTA-induced secondary influx of extracellular Ca\textsuperscript{2+} that would otherwise substitute for intracellular Ca\textsuperscript{2+} release. This secondary influx was not detected in our short-term fluorometry measurements of Ca\textsuperscript{2+} flux in BAPTA-treated cells. However, dyes such as Fluo-4 may not detect Ca\textsuperscript{2+} elevations in microdomains near the plasma membrane, which might affect PS distribution (25). Collectively, these data demonstrate that both fMLP and dGal-1 must increase cytosolic Ca\textsuperscript{2+}, which can be derived solely from intracellular stores, to optimally induce exposure of PS on the neutrophil surface. Mobilization of Ca\textsuperscript{2+} occurred through activation of distinct PLC isoforms, which presumably act by liberating inositol triphosphates. Signaling of fMLP through its G protein-coupled receptor is known to activate PLC\textbeta\textsubscript{3} (34, 35). Here we show that signaling of dGal-1 through an as yet unknown receptor(s) activated the Src kinases Lyn and Hck. The action of one or both of these Src kinases is required to phosphorylate tyrosines on PLC\gamma\textsubscript{2}. In turn, activated PLC\gamma\textsubscript{2} mobilizes Ca\textsuperscript{2+} that also contributes to the movement of PS to the surface of activated neutrophils. Fig. 11 presents a working model that is consistent with these data.

The Ca\textsuperscript{2+} elevations induced by fMLP and dGal-1 were transient, occurring over a few minutes, whereas the mobilization of PS progressively increased over several hours. Therefore, Ca\textsuperscript{2+} mobilized in neutrophils treated with fMLP and dGal-1 is unlikely to function simply by binding to and activating a member of the scramblase family, as may occur when some activated blood cells rapidly mobilize PS (1). Instead, it is likely that fMLP and dGal-1 initiate Ca\textsuperscript{2+}-dependent signaling pathways that contribute to redistribution of PS. Optimal mobilization of PS required that dGal-1 bind continuously to activated neutrophils for at least the 4 h measured in our experiments. Because continued binding of dGal-1 did not sustain cytosolic Ca\textsuperscript{2+} levels, signaling triggered by the transient...

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**Fig. 10.** Inhibition of PLC decreases dGal-1-induced mobilization of PS on activated neutrophils. A, neutrophils in Ca\textsuperscript{2+}-containing buffer were activated with 1 \(\mu\text{M}\) fMLP for 10 min, treated with 2 \(\mu\text{M}\) U73122, and then incubated in the presence or absence of 10 \(\mu\text{M}\) dGal-1 in EGTA-containing buffer for 4 h. Control neutrophils were sequentially incubated with fMLP and dGal-1 in Ca\textsuperscript{2+}-containing buffer. Some cells were incubated in the presence of 20 mM lactose. B, neutrophils in EGTA-containing buffer were activated with 1 \(\mu\text{M}\) fMLP for 10 min, and then incubated with 10 \(\mu\text{M}\) dGal-1 for 4 h. Control neutrophils were sequentially incubated with fMLP and dGal-1 in Ca\textsuperscript{2+}-containing buffer. Some cells were incubated in the presence of 20 mM lactose. The percentage of cells expressing PS was measured by flow cytometry as in Fig. 3. The data represent the mean ± S.D. of three experiments.

**Fig. 11.** Working model of dGal-1 stimulation of PS exposure on fMLP-activated neutrophils. Activation of neutrophils through the fMLP receptor stimulates release of Ca\textsuperscript{2+} from intracellular stores through G-protein-coupled, PLC\textbeta\textsubscript{3}-induced cleavage of PIP\textsubscript{2} to inositol 1,4,5-trisphosphate and diacylglycerol. Binding of dimeric dGal-1 to a cell-surface glycoprotein receptor(s) leads to activation of a Src kinase(s), subsequent activation of PLC\gamma\textsubscript{2}, release of additional inositol 1,4,5-trisphosphate and diacylglycerol, and another transient rise in cytosolic Ca\textsuperscript{2+}. The dual transient rises in cytosolic Ca\textsuperscript{2+} are necessary but not sufficient to redistribute PS to the cell surface. The inositol 1,4,5-trisphosphate-mediated release of Ca\textsuperscript{2+} opens plasma membrane ion channels, but extracellular Ca\textsuperscript{2+} is not required to mobilize PS to the cell surface. The diacylglycerol may activate PKC\gamma, which may contribute to PS exposure by activating a phospholipid scramblase. The dashed arrows represent pathways for which signaling intermediates and/or experimental evidence remain incomplete.
rise in Ca\textsuperscript{2+} is not sufficient to optimally redistribute PS. Furthermore, Ca\textsuperscript{2+}-independent signals also contribute to PS exposure. An ionomycin-induced Ca\textsuperscript{2+} flux, unlike the fMLP stimulus, did not prime neutrophils to mobilize PS in response to dGal-1.

The kinetics of PS redistribution on activated neutrophils treated with dGal-1 resembles the gradual, progressive mobilization of PS on apoptotic cells. Some apoptotic cells use Ca\textsuperscript{2+}-dependent signals to mobilize PS, but sustained increases in cytosolic Ca\textsuperscript{2+} are usually required (25). An exception may be how another galectin, galectin-9, induces apoptosis of T cells through a Ca\textsuperscript{2+}/calpain/caspase-1 pathway (46). Transient rises in Ca\textsuperscript{2+} inhibit death of neutrophils by conventional apoptotic signals (27). By contrast, we observed that transient Ca\textsuperscript{2+} elevations promoted PS exposure on activated neutrophils exposed to dGal-1. This suggests that neutrophils can be marked for phagocytosis even when they are resistant to signals for programmed cell death.

For both apoptotic and non-apoptotic cells, the downstream events in the Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent pathways that mobilize PS require further study. At least some activated and apoptotic cells utilize PKC\(\delta\) to redistribute PS (47), and an undefined PKC helps move PS to the surface of erythrocytes (48). This is an appealing mechanism, because PKC helps move PS to the surface of erythrocytes and cooperates with other signaling pathways to cause pro-apoptotic death further demonstrates that these pro-apoptotic pathways may be separate from the mechanisms that activate death through cleavage of DNA, proteins, and other cellular substrates (50–54). The ability of dGal-1 to induce sustained redistribution of PS on the surfaces of HL-60 and MOLT-4 cells after the removal of dGal-1 (19). Cell type-specific differences in the relative levels of active scramblases and aminotranslocases may determine how readily PS exposure can be reversed.

It is increasingly clear that the mechanisms for mobilizing PS on apoptotic cells are separable from the mechanisms that actually induce death through cleavage of DNA, proteins, and other cellular substrates (50–54). The ability of dGal-1 to induce sustained redistribution of PS on activated neutrophils in the absence of apoptotic death further demonstrates that these processes are not necessarily linked (19). It will be important to determine whether dGal-1 and conventional apoptotic mediators use similar or distinct mechanisms to redistribute PS to cell surfaces. As a first step, we have shown that fMLP and dGal-1 independently induce transient mobilization of cytosolic Ca\textsuperscript{2+}, which cooperates with other signaling pathways to cause prolonged exposure of PS on the surface of activated neutrophils.
Contributions of Ca$^{2+}$ to Galectin-1-induced Exposure of Phosphatidylserine on Activated Neutrophils
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