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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 25 September 2013
Final form accepted: 16 April 2014
Published online: April 2014

Volume 19 (2014) pp 262-276
DOI: 10.2478/s11658-014-0195-3

Research article

MONITORING OF MEMBRANE PHOSPHOLIPID SCRAMBLING IN HUMAN ERYTHROCYTES AND K562 CELLS WITH FM1-43 – A COMPARISON WITH ANNEXIN V-FITC ¶

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Abstract: The styryl dye FM1-43 becomes highly fluorescent upon binding to cell membranes. The breakdown of membrane phospholipid asymmetry in ionophore-stimulated T-lymphocytes further increases this fluorescence [Zweifach, A. Biochem. J. 349 (2000) 255]. In this study, the capacity of FM1-43 to monitor membrane phospholipid scrambling was explored using flow cytometry in human erythrocytes and human erythrocyte progenitor K562 cells. The Ca^{2+}-dependent phosphatidylserine-specific probe annexin V-FITC was used for comparison. The presented data show that the loss of phospholipid asymmetry that could be induced in human erythrocytes by elevated intracellular Ca^{2+} or by structurally different membrane intercalated amphiphilic compounds increases the FM1-43 fluorescence two- to fivefold. The profile of FM1-43 fluorescence for various

¶ Invited paper

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Abbreviations used: A23187 – calcium ionophore; BSA – bovine serum albumin; C10E8 – octaethylene glycol mono n-decyl ether; C12E8 – octaethylene glycol mono n-dodecyl ether; C12T – dodecyltrimethylammonium bromide; C12Z – 3-(dodecyldimethylammonio)-1-propanesulphonate; CPZ – chlorpromazine hydrochloride; DMSO – dimethyl sulfoxide; FITC – fluorescein isothiocyanate; Fluo3-AM – 4-(6-acetoxymethoxy-2, 7-dichloro-3-oxo-9-xanthenyl)-4'-methyl-2,2'(ethylenedioxy)dianiline-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl) ester; FM1-43 – (n-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl) pyridinium dibromide); IONO – ionomycin; NEM – n-ethylmaleimide; PMA – phorbol 12-myristate 13-acetate; PS – phosphatidylserine; S12S – sodium dodecyl sulphate; S14S – sodium tetradecyl sulphate

treatments resembles that of phosphatidylserine exposure reported by annexin V-FITC. FM1-43 detected the onset of scrambling more efficiently than annexin V-FITC. The amphiphile-induced scrambling was shown to be a Ca^{2+}-independent process. Monitoring of scrambling in K562 cells caused by NEM-induced Ca^{2+}-release from intracellular stores and by Ca^{2+} and ionophore A23187 treatment showed that the increase in FM1-43 fluorescence correlated well with the number of annexin V-FITC-detected phosphatidylserine-positive cells. The results presented here show the usefulness of FM1-43 as a Ca^{2+}-independent marker of dissipation in asymmetric membrane phospholipid distribution induced by various stimuli in both nucleated and non-nucleated cells.

Keywords: FM1-43, Phospholipid scrambling, Annexin V-FITC, Phosphatidylserine exposure, Human erythrocytes, K562 cells, Ionophores, Amphiphiles

INTRODUCTION

Cell membrane phospholipids are normally asymmetrically distributed, with phosphatidylcholine and sphingomyelin located mainly in the outer leaflet and the aminophospholipids phosphatidylserine (PS) and the majority of the phosphatidylethanolamines in the inner leaflet. Maintenance of this transversal lipid distribution is mediated by inwardly directed aminophospholipid translocase and less specific outwardly directed floppase enzymes [1, 2].

Phospholipid scrambling (the disruption of phospholipid asymmetry in a cell membrane) is thought to occur due to inhibition of the aminophospholipid translocase and a concomitant activation of the phospholipid scramblase, which in turn catalyzes randomization of the phospholipids [3–9]. It is well known that an elevated intracellular Ca^{2+} concentration induces scrambling. This can easily be experimentally achieved by treating the cells with ionophores in the presence of Ca^{2+} [10–16]. Furthermore, in nucleated cells, treatments that induce a release of Ca^{2+} from intracellular stores also trigger lipid scrambling [17, 18]. On the other hand, it is still unclear whether scrambling induced by membrane-perturbing exogenous amphiphiles is a Ca^{2+}-dependent process.

The disruption of phospholipid asymmetry in a cell membrane results in PS exposure in the external leaflet of the membrane [3]. PS exposure promotes the assembly and activation of several enzymes of the coagulation and complement system [10, 19, 20] and serves as a recognition signal of pathological [21, 22] and old [23, 24] cells for macrophages. It is also one of the earliest detectable events in apoptosis [25, 26].

The externalization of PS can be detected using fluorescently labeled annexin V, a reagent that preferentially binds to the PS head group [27–30]. A major disadvantage of this reagent is that millimolar concentrations of extracellular Ca^{2+} are required for the PS binding to occur. Increased intracellular Ca^{2+} levels due to uncontrolled Ca^{2+} entry can initiate diverse activities in a cell. High

cytoplasmic Ca^{2+} concentrations activate lipid scramblase [31] and block the cooperative action of phospholipid translocase and floppase. An elegant way to circumvent this problem is to use a label that reports scrambling without the need for high levels of extracellular Ca^{2+}. In addition, the rate of annexin V binding to PS is slow, which automatically excludes its usage in kinetics studies.
Since the breakdown in phospholipid asymmetry and apoptosis are important biological events, less error-prone phospholipid scrambling monitoring tools are needed [32, 33]. The primary objective of this study was to examine the cationic styryl dye FM1-43 as an indicator of membrane phospholipid scrambling in human erythrocytes and erythrocyte progenitor K562 cells. This dye, which is widely used as a probe for vesicle trafficking [34, 35], has a high affinity for lipids and is unable to cross the membrane, i.e. it partitions only into the outer leaflet of membranes [35, 36]. It was earlier shown that fluorescence of FM1-43 significantly increases in lymphocytes stimulated with ionomycin [16, 33].
Although it is not clear how FM1-43 responds to scrambling, it has been reported that FM1-43 identifies cells that expose PS, and that this might be due to the lipid-packing density changes that accompany phospholipid scrambling [33]. In order to further explore the ability of FM1-43 to monitor scrambling, we used human erythrocytes, in which scrambling can be induced by treatment with Ca^{2+}/ionophore [12, 14, 15, 31], and amphiphilic compounds [37]. To compare results between non-nucleated and nucleated cells, we performed similar experiments on K562 cells. We discuss the usefulness of monitoring membrane phospholipid scrambling with FM1-43.

MATERIALS AND METHODS

Sodium tetradecyl sulphate (S14S), sodium dodecyl sulphate (S12S), and 3-(dodecyldimethylammonio)-1-propanesulphonate (C12Z) were from Calbiochem. Octaethylene glycol mono n-decyl ether (C10E8) and octaethylene glycol mono n-dodecyl ether (C12E8) were from Fluka. N-ethylmaleimide (NEM), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), ionomycin (IONO), ionophore A23187, and dodecyltrimethylammonium bromide (C12T) were from Sigma. Phorbol 12-myristate 13-acetate (PMA) was from Sigma. Chlorpromazine hydrochloride (CPZ) was from Merck. Annexin V-FITC was from Bender MedSystems GmbH, and FM1-143 was from Molecular Probes. Fluo3-AM was from Invitrogen.
Blood was drawn from the authors by venipuncture into heparinized tubes and then centrifuged (10 min, 3000 × g). The buffy coat was removed. The erythrocytes were washed 3 times in buffer A (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2x6H_2O$, 10 mM glucose, at pH 7.4) supplemented with 1.8 mM $CaCl_2x2H_2O$. Erythrocytes were stored (1.65 x 10^9 cells/ml) at 4ºC and used within 36 h.
For incubation without Ca^{2+}, erythrocytes were washed twice in buffer B (buffer A supplemented with 1.8 mM $MgCl_2x6H_2O$) with 0.1 mM EGTA and twice in

buffer B. Annexin V-FITC binding was performed in buffer C (buffer A supplemented with 3.8 mM $CaCl_2x2H_2O$). The erythroleukemia cell line K562 was cultured at 37ºC in RPMI 1640 (GIBCO, Invitrogen Corp.) with 5% Fetal Calf Serum (FCS, GIBCO) supplemented with 2 mM L-glutamine, 10 U/ml penicillin G sodium salt and 10 μg/ml streptomycin sulphate (GIBCO) [38].

Following pretreatment with NEM (10 mM, RT, 30 min, two washes), the erythrocytes (1.65 x 10^8 cells/ml) were incubated in buffer A or B at 37ºC for 60 min with the ionophores ionomycin (0.3 μM) or A23187 (1 μM), or with amphiphilic compounds at sublytic concentrations where they exhibit maximum protection against hypotonic hemolysis [39]: S14S (23 μM), S12S (50 μM), C12E8 (44 μM), C12T (300 μM), and C12Z (263 μM). C10E8, CPZ, and PMA were used at the sublytic concentrations 215 μM, 100 μM, and 15 μM, respectively. K562 cells (1 x 10^6 cells/ml) were incubated in buffer A or B at 37ºC for 15 min with NEM (0.5 mM) or ionophore A23187 (2 μM) and subsequently washed 3 times in the same buffer (450 x g, 3 min).

Notably, in this study two different properties of NEM were utilized. It is an aminophospholipid translocase inhibitor but also a reagent that induces the release of Ca^{2+} from internal stores in K562 cells. Following treatment with Ca^{2+}/ionophore, amphiphiles and/or NEM as described above, the erythrocytes (1.8 x 10^7 cells/ml) were labeled with annexin V-FITC (50 ng/ml, 20 min, room temperature) in buffer C, which contains the Ca^{2+} needed for annexin V-FITC binding to PS, or with FM1-43 (100 ng/ml, 3 min, room temperature) in buffer A or B. The analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson).

Kinetic analyses of phospholipid scrambling in the erythrocyte membranes were performed. Erythrocytes were preincubated for 5 min at RT with annexin V-FITC or FM1-43, then treated with ionomycin (3 μM), and thereafter immediately transferred to a flow cytometer for analysis. Following NEM or ionophore treatment, K562 cells (1 x 10^6 cells/ml) were labeled with annexin V-FITC (50 ng/ml, 20 min, room temperature, buffer C) or FM1-43 (100 ng/ml, 3 min, room temperature, buffer A or B) with a subsequent analysis on the flow cytometer. For intracellular Ca^{2+} measurements, erythrocytes (3.3 x 10^8 cells/ml) were loaded with Fluo3-AM (3 μM) at 37ºC for 30 min, washed twice in buffer A or B containing 0.5% BSA, washed twice in the same buffers without BSA, subsequently incubated with ionophores or amphiphiles, and analyzed by flow cytometry.

In the flow cytometer, 3 x 10^4 erythrocytes/sample and 5 x 10^3 K562 cells/sample were measured for fluorescence emission intensity. They were excited at 488 nm and collected with a 530 nm ± 30 nm (FITC and Fluo3) or 585 nm ± 42 nm (FM1-43) band-pass filter on a logarithmic scale. Size was also assessed (forward and side light scattering in FSC and SSC channels, respectively). The data were analyzed using the CellQuest software (Becton Dickinson). The percentage of cells labeled with annexin V-FITC (annexin V-positive cells) was taken as a measure of the PS exposure [37], while the

GeoMean value of the cells labeled with FM1-43 and Fluo3-AM was taken as a measure of the fluorescence intensity of the sample (fluorescence in arbitrary units). Typical flow cytometric histograms for erythrocytes and K562 cells labeled with annexin V-FITC and FM1-43, respectively, are presented in supplementary data (supplementary material at http://dx.doi.org/10.2478/s11658-014-0195-3) to indicate the necessity of using different parameters for presenting the results for the two dyes. The final results are presented as the means ± SE of n separate experiments. Differences between the means were evaluated using Students' *t* test. The values were taken as significantly different when $p \leq 0.05$.

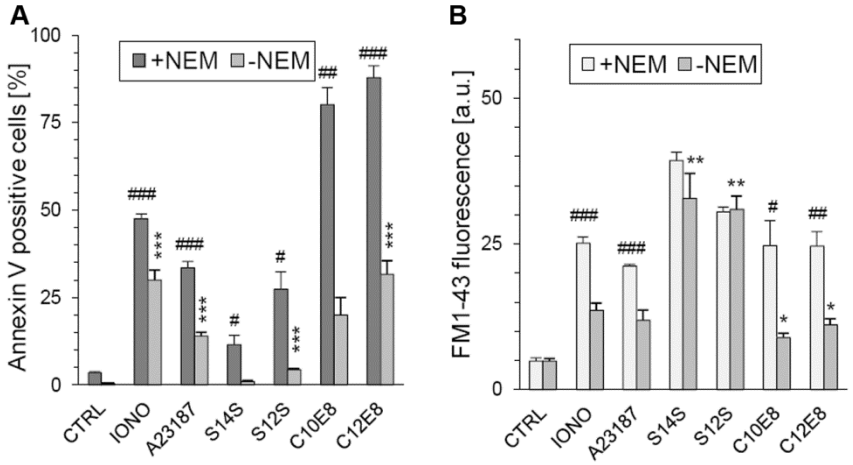

Fig. 1. The effect of NEM on phospholipid scrambling in human erythrocytes. Human erythrocytes that were untreated or pretreated with NEM were incubated with ionophores or amphiphilic compounds in the presence of Ca^{2+} (1.8 mM) and then labeled with annexin V-FITC (A) or FM1-43 (B). The data are the means ± SE (n = 3–5). Statistical differences are indicated between ionophore- or amphiphile-treated erythrocytes and the respective control for NEM free samples (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) or between samples in pairs preincubated with and without NEM (# $p < 0.05$; # # $p < 0.01$; # # # $p < 0.001$).

RESULTS

FM1-43 reports erythrocyte scrambling induced by Ca^{2+}/ionophores and amphiphilic compounds

The effect of NEM-pretreatment on Ca^{2+}- and amphiphile-induced scrambling reported by annexin V-FITC and FM1-43 in erythrocytes is shown in Fig. 1. The results reveal that pretreatment with an inhibitor of aminophospholipid translocase (NEM) significantly increases the PS exposure reported by annexin V-FITC and the phospholipid scrambling reported by FM1-43. However, in the case of the anionic amphiphiles S12S and S14S, there was no difference between NEM-pretreated and non-pretreated samples when reported by FM1-43 (compare Fig. 1A and B).

Incubation of erythrocytes with ionophores in the presence of 1.8 mM Ca^{2+} lead to phospholipid scrambling manifested by PS exposure in the outer leaflet of the membrane. After incubation of erythrocytes with 0.3 μM ionomycin or 1 μM A23187, the numbers of annexin V-FITC-positive cells were about 50% and 30%, respectively (Fig. 2A). These treatments also caused a significant increase in FM1-43 fluorescence (~fourfold), which was approximately the same for both ionophores (Fig. 2B).

Next, lipid scrambling caused by membrane intercalation of structurally different uncharged and charged water-soluble amphiphiles and PMA was examined. The profile of FM1-43 fluorescence (Fig. 2B) for various amphiphile treatments resembles the profile of PS exposure reported by annexin V-FITC, with the exception of the anionic amphiphiles S12S and S14S, which gave extremely high FM1-43 fluorescence values (seven- to ninefold; Fig. 2A). While the numbers of PS-positive cells detected with annexin V-FITC differed significantly between the samples, the increase in FM1-43 fluorescence was more equal for the different treatments. The positively charged amphiphiles C12T and CPZ, the zwittergent amphiphile C12Z, and the protein kinase C activator PMA did not cause significant PS exposure when monitored with annexin V-FITC (≤10 %). A similar analysis performed with FM1-43 resulted in a moderate (two- to threefold) increase in fluorescence.

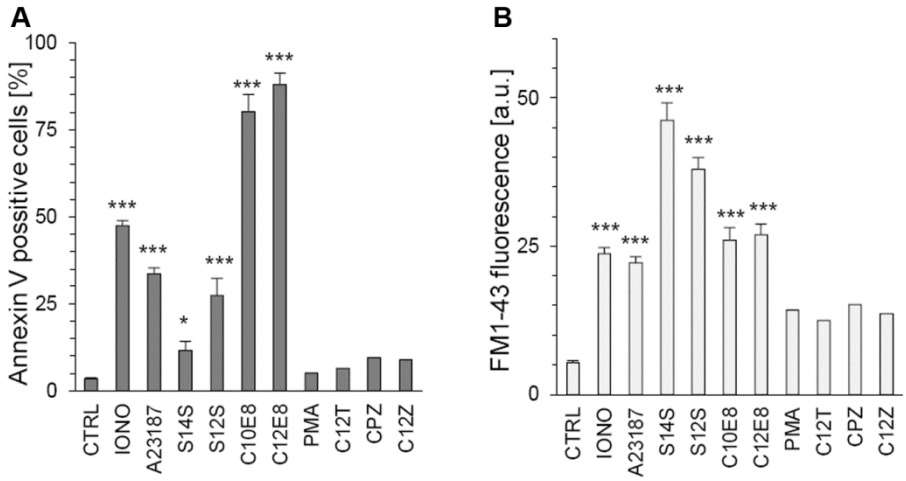

Fig. 2. Phospholipid scrambling in human erythrocytes monitored by annexin V-FITC and FM1-43. Human erythrocytes pretreated with NEM were incubated with ionophores or amphiphilic compounds in the presence of Ca^{2+} (1.8 mM). Cells labeled with annexin V-FITC (A) or FM1-43 (B) were analyzed with flow cytometry. The data are presented as the means ± SE (n = 5–12). For PMA, C12T, CPZ, and C12Z, the bars represent the means of two experiments. Statistical differences between ionophore- and amphiphile-treated erythrocytes and the control are indicated (*$p < 0.05$; ***$p < 0.001$). See the Materials and Methods section for details.

Extracellular Ca^{2+} has no impact on amphiphilic compound-induced scrambling in erythrocytes

To clarify the role of Ca^{2+} in the induction of phospholipid scrambling caused by amphiphiles, we compared PS exposure and the corresponding increase in FM1-43 fluorescence. Human erythrocytes were treated with amphiphiles in the absence or presence of 1.8 mM Ca^{2+}. Erythrocytes incubated with ionomycin or A23187 served as positive controls in these experiments. As shown in Fig. 3A and B, there was no statistically significant difference in PS exposure and FM1-43 fluorescence between samples treated with amphiphiles in the absence or presence of Ca^{2+}.

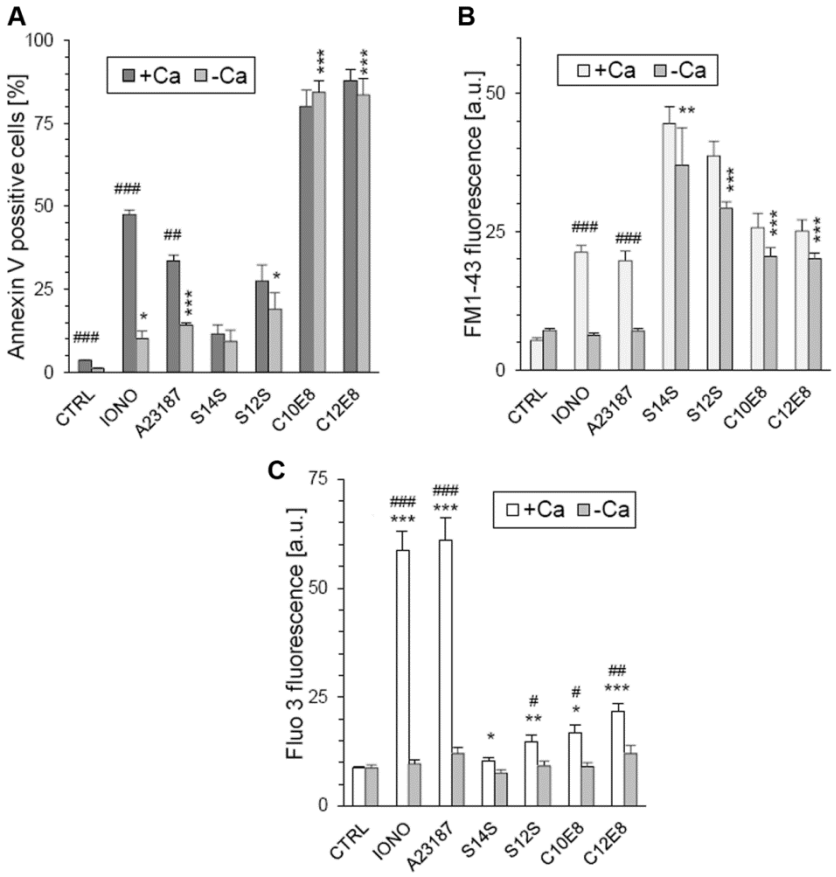

Fig. 3. The effects of extracellular Ca^{2+} on phospholipid scrambling induced by ionophores and amphiphilic compounds in human erythrocytes. Human erythrocytes pretreated with NEM were incubated with ionophores or amphiphilic compounds in the absence or presence of Ca^{2+} (1.8 mM). Cells labeled with annexin V-FITC (A) or FM1-43 (B) or loaded with Fluo3-AM (C; loading before treatment) were analyzed using flow cytometry. The data are the means ± SE (n = 4–6). Statistical differences are indicated between ionophore- or amphiphile-treated erythrocytes and the respective controls (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) or between samples in pairs incubated with and without Ca^{2+} (# $p < 0.05$; # # $p < 0.01$; # # # $p < 0.001$).

In order to check if incorporation of amphiphilic compounds into erythrocyte membranes leads to an increase in cytoplasmic Ca^{2+} levels due to unspecific leakage, we utilized the fluorescent calcium indicator Fluo3. As shown in Fig. 3, the small but statistically significant increase in Fluo3 fluorescence observed for S14S, S12S, C10E8, and C12E8 (Fig. 3C) does not lead to significant differences in PS exposure (Fig. 3A) or FM1-43 fluorescence (Fig. 3B). As expected, PS exposure and the fluorescence of FM1-43 were significantly lower in erythrocytes incubated with ionomycin and A23187 in a nominally Ca^{2+}-free buffer than in a buffer containing Ca^{2+}. FM1-43 fluorescence in erythrocytes incubated with ionophores without Ca^{2+} was the same as the fluorescence obtained in control erythrocytes (Fig. 3B). By contrast, PS exposure in erythrocytes incubated with ionophores without Ca^{2+} was higher than in the control (Fig. 3A). Higher PS exposure in these samples was probably caused by Ca^{2+} entry during the final labeling step with annexin V-FITC, which was performed for all samples in buffer C, which contained Ca^{2+}.

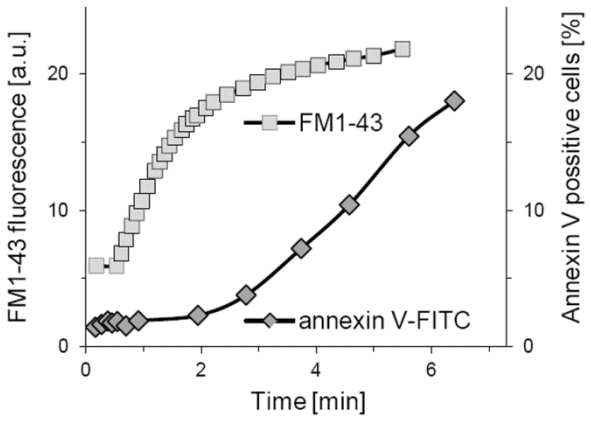

Fig. 4. Time course of phospholipid scrambling in human erythrocytes monitored by annexin V-FITC and FM1-43. NEM-pretreated human erythrocytes were preincubated with FM1-43 or annexin V-FITC and after the addition of ionomycin (3 μM), were immediately transferred to a flow cytometer for analysis. Data from one representative experiment out of 4 performed with blood from different donors are shown.

FM1-43 reports the onset of scrambling

In order to compare the usefulness of annexin V-FITC and FM1-43 in kinetic measurements of phospholipid scrambling, two kinds of experiment were performed with human erythrocytes. First, the time course of annexin V-FITC and FM1-43 binding to Ca^{2+}/ionophore-treated erythrocytes was analyzed using a flow cytometer. Here, both annexin V-FITC and FM1-43 binding to already scrambled erythrocytes begins immediately and was similar, reaching a plateau after 100 s (data not shown). In the second type of kinetic experiments, Ca^{2+}/ionophore was added to erythrocytes preincubated with annexin V-FITC or

FM1-43, after which the incubation mixture was immediately analyzed. The results presented in Fig. 4 show a difference in the time course of phospholipid scrambling monitored by these two probes. The FM1-43 fluorescence increased in a rapid fashion, with saturation after 5 min, whereas the number of annexin V-FITC positive cells increased slowly without saturation even after 30 min (data not shown). These results show that substitution of annexin V-FITC with FM1-43 in a continuous flow cytometry-based assay allows kinetic measurements of phospholipid scrambling stimulated by ionophores in the presence of Ca^{2+}.

FM1-43 reports scrambling induced by NEM and Ca^{2+}/A23187 in K562 cells

K562 cells were selected for three reasons. First, we aimed to ensure that FM1-43 reports phospholipid scrambling in nucleated cells other than lymphocytes [16, 33]. Secondly, we wanted to compare scrambling induced by an ionophore plus extracellular Ca^{2+} treatment in nucleated cells and erythrocytes. Third, we wanted to characterize scrambling induced by Ca^{2+} release from internal stores.

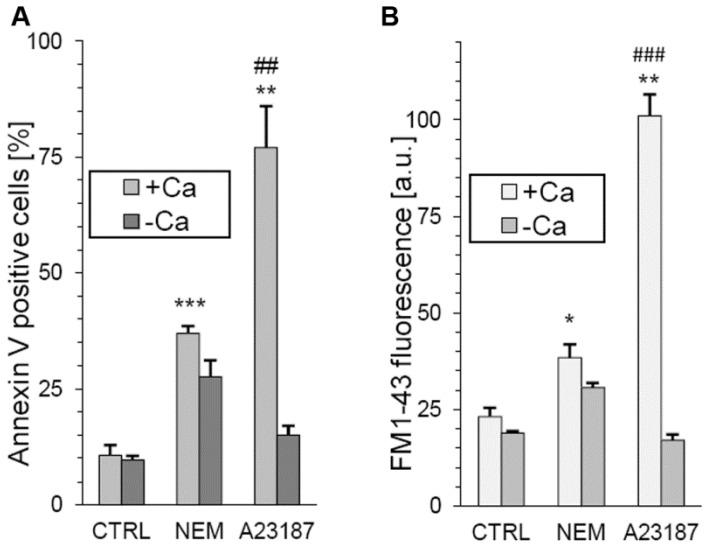

Fig. 5. Phospholipid scrambling in erythroleukemia K562 cells monitored by annexin V-FITC and FM1-43. Cells were stimulated by NEM or ionophore A23187 in the absence or presence of Ca^{2+} (1.8 mM), labeled with annexin V-FITC (A) or FM1-43 (B), and analyzed using flow cytometry. The data are the means ± SE (n = 3). Statistical differences are indicated between ionophore- or NEM-treated cells and the control (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) and between samples in pairs incubated with and without Ca^{2+} (# # $p < 0.01$; # # # $p < 0.001$).

As has been reported previously, unlike erythrocytes, nucleated cells externalize PS after sulfhydryl modifications, e.g., by NEM [40, 41]. The results illustrated in Fig. 5 demonstrate that after NEM treatment, about 30% of K562 cells were PS positive (as detected with annexin V-FITC), while an about 1.5-fold increase in FM1-43 fluorescence occurred. Neither PS exposure (Fig. 5A) nor FM1-43

fluorescence (Fig. 5B) were dependent on extracellular Ca^{2+}. Upon incubation with ionophore A23187 in the presence of Ca^{2+} (1.8 mM), about 80% of K562 cells showed PS exposure (Fig. 5A), while an about 4.5-fold increase in FM1-43 fluorescence was observed (Fig. 5B). Following incubation with A23187 without Ca^{2+} the FM1-43 fluorescence and the number of PS positive K562 cells were similar as in the controls (Fig. 5A and B).

DISCUSSION

In this study, we provide evidence that FM1-43, previously reported to be sensitive to breakdown in membrane asymmetry in ionomycin-stimulated human T-lymphocytes [16, 33], can substitute for annexin V-FITC in monitoring phospholipid scrambling induced by elevated intracellular Ca^{2+} levels and membrane-intercalated amphiphilic compounds in human erythrocytes. The increase in fluorescence of FM1-43 caused by the disruption of lipid asymmetry (two- to fivefold compared to the control) is high enough to allow measurements of phospholipid scrambling using flow cytometry. We show that all treatments that induced PS exposure reported by annexin V-FITC are also identified by an increase in FM1-43 fluorescence (Figs 1–3), although the profile of FM1-43 fluorescence among the group of compounds studied is not exactly the same (Fig. 2A and B). The number of annexin V-detected PS-positive cells varies significantly among samples, whereas FM1-43 fluorescence is more equal, except for the anionic amphiphiles S12S and S14S.

It is not clear how FM1-43 responds to scrambling, but it has been suggested, based on the structural analogy to merocyanine 540 [40], that FM1-43 is sensitive to changes in lipid packing [33]. The results obtained with FM1-43 indicate that phospholipid scrambling induced by Ca^{2+}/ionophore and amphiphilic compounds lead to approximately the same changes in lipid packing in the outer leaflet of the membrane bilayer (Fig. 2B). The exceptionally high values of FM1-43 fluorescence observed for S12S and S14S are likely due to the electrostatic attraction between the positively charged FM1-43 ($z = +2$) and the negatively charged surface of the erythrocyte after incorporation of anionic compounds (Fig. 2B). It has been reported that FM1-43 binds better to negatively charged lipid vesicles than to neutral ones [36]. The adsorption of FM1-43 to the lipid vesicles is mainly driven by the insertion of the non-polar part of the dye into the hydrophobic part of the membrane [36, 41]. The adsorption equilibrium is further modulated by an electrostatic interaction of the cationic dye with the membrane surface [36], an effect that must be taken into account when using the FM1-43 dye under conditions where the surface membrane potential is altered.

The effect of the aminophospholipid translocase inhibitor NEM on scrambling reported by FM1-43 and annexin V-FITC is shown in Fig. 1. NEM has a much stronger impact on amphiphile-induced PS exposure as measured by annexin

V-FITC than on the increase in FM1-43 fluorescence. The number of PS-positive cells decreased dramatically for erythrocytes not pretreated with NEM. FM1-43 fluorescence is also significantly lower in erythrocytes not pretreated with NEM, but the differences are small in comparison to differences in PS exposure. The aminophospholipid translocase actively transport mainly PS to the inner leaflet and its inhibition by NEM leads to an increased PS exposure that can be measured with annexin V-FITC. On the other hand, FM1-43 responds to the total change in lipid packing caused by lipid scrambling.

It is likely that in spite of the differences in PS exposure induced by various amphiphilic compounds, the changes in lipid packing are comparable. The lack of difference between FM1-43 fluorescence observed for S12S and S14S with and without an NEM pretreatment confirms the previous suggestion that the very high values in fluorescence (a seven- to ninefold increase) observed for anionic amphiphiles are not simply the effect of phospholipid scrambling, but also an effect of an electrostatic interaction between a more negatively charged membrane and a positive dye. Therefore, these results must be treated as a false positive. It is likely that the electrostatic attraction between a cationic dye and erythrocyte membrane with incorporated anionic compounds is big enough to cover the effect of diminished scrambling observed without an NEM pretreatment. It is therefore, in contrast to PS exposure, not possible to see a decrease in FM1-43 fluorescence.

The effect of extracellular Ca^{2+} on scrambling induced by Ca^{2+}/ionophores and amphiphiles in the human erythrocyte membrane is shown in Fig. 3. Comparing Fig. 3A and B, at least two conclusions may be drawn. First, the increase in FM1-43 fluorescence gives the same information as the number of annexin V-FITC-positive cells regarding how the presence of Ca^{2+} in the incubation medium effects the scrambling induced by ionophores and amphiphiles. Second, extracellular Ca^{2+} strongly increases ionomycin and A23187 scrambling, but has no effect on amphiphile-induced scrambling. Amphiphile-induced scrambling is apparently due to phospholipid leak fluxes resulting from the induced membrane perturbation. Furthermore, FM1-43 fluorescence values of erythrocytes incubated with ionophores without Ca^{2+} do not differ from the control (Fig. 3B), whereas PS exposure measured by annexin V-FITC in ionophore-treated cells without Ca^{2+} is higher than in the control cells (Fig. 3C), which is likely due to scrambling taking place during labeling. This comparison clearly demonstrates the advantage of FM1-43 over annexin V, namely that high Ca^{2+} levels are not needed for labeling, which strongly reduces the risk of "false" positive results.

With the kinetic experiments performed by means of flow cytometry, we confirmed the usefulness of FM1-43 as a reporter for measuring the rate of phospholipid scrambling [33]. A preincubation of erythrocytes with FM1-43 before Ca^{2+}/ionophore treatment allows one to follow the process when a normal lipid distribution collapses. FM1-43 apparently intercalates into the membrane during preincubation and therefore can immediately report altered phospholipid packing by the increase in fluorescence. By contrast, since membrane binding of

annexin V-FITC starts upon the onset of scrambling, the initial scrambling rate cannot be efficiently monitored with this label (Fig. 4). An additional advantage of FM1-43 is the fact that it can also be measured with a spectrofluorimeter, since the spectral properties of this dye change according to its environment, i.e., the fluorescence increases upon incorporation into a cell membrane [33]. In the case of annexin V-FITC, a flow cytometer is required because this label is also fluorescent in an extracellular environment.

Erythrocytes that lack intracellular organelles externalize PS but do not undergo classical apoptosis. Because nucleated cells, in contrast to human erythrocytes, demonstrate PS externalization upon sulfhydryl modification, e.g., by NEM [17, 18], it has been suggested that PS exposure in these cells is regulated by a mechanism that is not present in erythrocytes. Despite different intracellular signaling events leading to PS exposure in nucleated cells, our experiments conducted on erythrocyte progenitor K562 cells stimulated by NEM and Ca^{2+}/ionophore A23187 demonstrate that the number of PS-positive cells detected by annexin V-FITC correlate with an increase in the fluorescence of FM1-43 (Fig. 5). Notably, it remains unclear how FM1-43 internalization to the intracellular membrane pool adds to the final successful detection of phospholipid scrambling in K562 cells. Experiments with both annexin V-FITC and FM1-43 demonstrate that the NEM-induced phospholipid scrambling in K562 cells does not depend on extracellular Ca^{2+}. The lack of Ca^{2+} dependence in NEM-induced scrambling, as monitored by two different labels in K562 cells, supports the statement that nucleated cells are able to externalize PS after thiol modification because of their ability to recruit Ca^{2+} from intracellular stores [17, 18]. This also explains why erythrocytes, which lack intracellular organelles, are unable to externalize PS after NEM treatment alone. These results indicate the usefulness of FM1-43 as a marker of phospholipid scrambling also in nucleated cells, particularly in situations when annexin V cannot be used due to its requirement of a high calcium concentration for PS binding.

CONCLUSIONS

In this study, we demonstrate that FM1-43 detects phospholipid scrambling in both nucleated K562 cells and non-nucleated human erythrocytes induced by widely different stimuli. An increase in FM1-43 fluorescence gives qualitatively the same information about phospholipid scrambling as PS exposure reported by annexin V-FITC. The rapid and Ca^{2+}-independent membrane binding of FM1-43, as well as the strong increase in FM1-43 fluorescence upon loss of normal membrane phospholipid distribution, makes FM1-43 a useful probe for exploring kinetic alterations in membrane phospholipid asymmetry.

Acknowledgements. This study was supported by grants from the Research Institute of Åbo Akademi University, Medicinska Understödsföreningen Liv och Hälsa, and the Tor, Joe and Pentti Borg Foundation.

Conflict of interest. We declare that no conflict of interest exists.

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