FM1-43 reports plasma membrane phospholipid scrambling in T-lymphocytes

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We have found using imaging techniques that stimulating Jurkat human leukaemic T-cells with ionomycin in the presence of FM1-43, a dye used to monitor exocytosis and endocytosis, causes large (6–10-fold) increases in FM1-43 fluorescence. These responses are too large to be caused by exocytosis. Instead, three lines of evidence suggest that FM1-43 is responding to phospholipid scrambling. First, ionomycin also stimulates increases in the fluorescence of annexin V, a phosphatidylserine-specific probe, while thapsigargin does not stimulate fluorescence increases of either probe. Secondly, cells that exhibit FM1-43 fluorescence increases after ionomycin stimulation stain with annexin V once FM1-43 is washed out. Thirdly, ionomycin stimulates uptake of

7-nitrobenz-2-oxa-1,3-diazole-labelled phosphatidylcholine, a specific assay for scramblase activity, whereas thapsigargin does not. We find that FM1-43 reports phospholipid scrambling with ‘better’ kinetics than annexin V, and does require extracellular Ca^{2+} to report phospholipid scrambling. We suggest that FM1-43 may be a useful probe to study the dynamics of phospholipid scrambling. The results are the first demonstration that FM1-43 can respond significantly to a biological process other than vesicular trafficking.

Key words: annexin V, necrosis, phosphatidylserine exposure.

INTRODUCTION

Plasma membrane lipid composition is asymmetric in most healthy mammalian cells [1]. An aminophospholipid translocase ensures that essentially all membrane phosphatidylserine (PS) and phosphatidylethanolamine (PE) reside in the inner leaflet of the plasma membrane, while phosphatidylcholine (PC) and sphingomyelin (SM) are found in both leaflets. One of the earliest detectable events in cells undergoing apoptosis or necrosis is a disruption of normal plasma membrane lipid asymmetry, resulting in exposure of PS on the outer leaflet [2]. Exposed PS can activate the alternative pathway of complement [3,4] and act as a macrophage recognition signal [5], effects that contribute to the destruction and removal of dying cells. Phospholipid scrambling is also a key event in the normal activation of platelets [6]. Because phospholipid scrambling is an early event in apoptotic or necrotic cell death [2], the development of a specific probe, annexin V, which identifies cells exposing PS [7], has contributed enormously to our understanding of the sequence of events leading to cell death. Annexin V, a recombinant protein that can be coupled with a fluorophore, binds to PS in the presence of extracellular Ca^{2+} ($\text{Ca}^{2+}_{\text{out}}$) [8], making it possible to identify cells in the early stages of cell death and to follow the course of their progression through the process.

FM1-43 is a styryl dye that has been used widely to monitor exocytosis and endocytosis [9]. It is essentially non-fluorescent in aqueous solution. Upon binding to membranes, its quantum yield increases several-hundredfold, and it is not able to cross from the outer to the inner leaflet of intact membranes [10]. In the present report, we provide evidence that FM1-43 fluorescence is sensitive to changes in plasma membrane lipid composition that accompany phospholipid scrambling stimulated by ionomycin in Jurkat human leukaemic T-cells. FM1-43 is likely to be

a useful probe for monitoring phospholipid scrambling, as it reports scrambling with ‘better’ kinetics than annexin V, and does not require extracellular Ca^{2+} to bind to scrambled membranes. These results are the first demonstration that FM1-43 can respond significantly to a biological process other than vesicular trafficking.

MATERIALS AND METHODS

Chemicals, reagents and cells

Salts for physiological solutions and poly-L-lysine were from Sigma–Aldrich (St. Louis, MO, U.S.A.). Fura-2 AM and FM1-43 were from Molecular Probes (Eugene, OR, U.S.A.). FITC-labelled annexin V was from R&D Systems (San Diego, CA, U.S.A.). Thapsigargin (TG) and ionomycin were purchased from Alexis Biochemicals (San Diego, CA, U.S.A.). C_6 NBD-PC was from Avanti Polar Lipids (Birmingham, AL, U.S.A.). (NBD is 7-nitrobenz-2-oxa-1,3-diazole). RPMI 1640 medium and tissue-culture plastic were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Fetal-calf serum, glutamine and antibiotics were purchased from Gemini Bioproducts (Calabasas, CA, U.S.A.). Jurkat E6-1 cells were provided by Dr Michael Cahalan (University of California, Irvine, CA, U.S.A.), and were maintained in RPMI medium supplemented with 2 mM glutamine, 10% (v/v) heat-inactivated fetal-calf serum and antibiotics at 37 °C in a humidified incubator. For experiments in which Fura-2 was used to assess membrane integrity, cells were loaded by incubating them with 1 μM Fura-2 acetoxymethyl ester (Fura-2 AM) in cell-culture medium for 30 min at room temperature. Cells were washed twice with fresh medium before use. Ringer’s solution contained (in mM): 145 NaCl, 4.5 KCl, 1 MgCl_2 , 2 CaCl_2 , 5 Hepes and 10 glucose (adjusted to pH 7.4 with NaOH). Ca^{2+} -free

Abbreviations used: $\text{Ca}^{2+}_{\text{out}}$, extracellular calcium; MC 540, merocyanine 540; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; ROI, region of interest; SM, sphingomyelin; TG, thapsigargin; Fura-2 AM, Fura-2 acetoxymethyl ester; NBD, 7-nitrobenz-2-oxa-1,3-diazole.

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Ringer's solution was identical, except that CaCl_2 was replaced with MgCl_2 .

Imaging

Experiments were performed at room temperature with an imaging system described previously [11]. A Nikon PlanFluor 40 $\times$ oil objective (N.A. 1.4) was used for all experiments. Excitation light was filtered with a 485 nm bandpass filter for FM1-43 and FITC-annexin V, and a 360 nm bandpass filter for Fura-2. Emitted light was collected with a 595 nm bandpass filter for FM1-43, a 515 nm bandpass filter for FITC-annexin V, and a 510 nm bandpass filter for Fura-2. A Fura-BCECF [2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein] dichroic mirror was used. Images were acquired with 8 $\times$ 8 binning at a rate of one frame every 15 s. Images were analysed by drawing regions of interest (ROIs) over cells, and also over a cell-free region that was used as a background for FM1-43 or FITC-annexin. The mean intensity for all the ROIs in an image was computed, and exported as a text file to Igor Pro (Wavemetrics, Lake Oswego, OR, U.S.A.) for further processing. After background subtraction, single-cell fluorescence data were normalized to each cell's pre-stimulation fluorescence intensity.

NBD-PC uptake experiments

Cells were incubated in normal Ringer's solution containing 1 $\mu\text{g}/\text{ml}$ NBD-PC with or without either 1 μM ionomycin or TG for 20 min at room temperature, and then centrifuged and resuspended in Ca^{2+} -free Ringer's solution 5% fatty-acid-free BSA for 5 min to extract outer leaflet NBD-PC. They were then washed twice with normal Ringer's solution, and examined on the imaging system using FITC filters.

RESULTS

Ionomycin, but not TG, stimulates FM1-43 fluorescence increases in Jurkat cells

Exposure of Jurkat cells to ionomycin in the continuous presence of 3.2 μM FM1-43 at room temperature caused large increases in FM1-43 fluorescence (Figure 1). On average, fluorescence increased to levels $\approx$ 6-fold above control levels, although in some

cells fluorescence increased as much as 15-fold. There was considerable variability in the delay until cells began to respond. TG, a drug that activates Ca^{2+} influx through Ca^{2+} -release-activated channels in Jurkat cells [12], did not cause FM1-43 brightening, and brightening was not observed when cells were stimulated with ionomycin in the absence of Ca^{2+} . When the fluorescence of cells loaded with Fura-2 AM was monitored at an excitation wavelength of 360 nm (the Ca^{2+} -insensitive isosbestic wavelength) to assess plasma membrane integrity, a small number of cells demonstrated abrupt decreases in Fura-2 fluorescence, consistent with plasma membrane disruption, but the majority (> 99%) did not. Ionomycin-induced fluorescence increases were also observed with two other FM analogues [10]: FM2-10, a less hydrophobic FM dye, and FM4-64, which has a red-shifted excitation spectrum (results not shown).

We investigated whether FM1-43 fluorescence increases could be reversed by removing FM1-43 from the extracellular solution. When FM1-43 was washed out of the extracellular solution 800–1000 s after ionomycin stimulation, fluorescence was reduced by 60–70%, to levels $\approx$ 2-fold above unstimulated values. The $\approx$ 30% of fluorescence that remains might reflect dye trapped in cells as a result of endocytosis. We found that the fluorescence of cells that did not brighten in response to ionomycin was also reduced by 60–70% upon washout of FM1-43 (results not shown). Thus a similar fraction of FM1-43 cannot be washed out of cells, whether they brighten or not. These results are consistent with the idea that dye internalization occurs at similar rates whether cells brighten or not, and that the internalized dye in cells that brighten is brighter because the dye in the membrane that was internalized was brighter.

FM1-43 brightening is due to phospholipid scrambling

The amount of FM1-43 brightening that was observed is too large to be due to exocytosis. Jurkat cells are not known to be secretory cells, although it is possible that ionomycin stimulates some exocytosis. For example, Chinese hamster ovary cells, considered, like Jurkat cells, to be non-secretory cells, increase their surface area by $\approx$ 20–30% by exocytosis when intracellular calcium levels are raised to micromolar levels [13]. Membrane capacitance, a measure of cell-surface area, increases by a similar

Figure 1 Ionomycin causes FM1-43 fluorescence increases

(A) FM1-43 fluorescence images taken before and 840 s after ionomycin stimulation. 2 $\times$ 2 binning was used for display purposes. (B) Representative single-cell fluorescence traces. Arrows indicate addition of ionomycin.

Figure 2 Ionomycin causes FITC-annexin V fluorescence increases

(A) Annexin V fluorescence images taken before and 1440 s after ionomycin stimulation. 2×2 binning was used for display purposes. (B) Representative single-cell fluorescence traces. Arrows indicate addition of ionomycin.

amount (15–20%) in ionomycin-stimulated Jurkat cells (A. Zweifach and G. Burgett, unpublished work). We might therefore expect exocytosis to cause a 15–30% increase in fluorescence, as increases in FM1-43 fluorescence are thought to correlate 1:1 with exocytosis when FM1-43 is present in the extracellular solution [14], except in cases in which secretory-granule contents are stained with FM1-43, causing enhanced fluorescence signals and visible single exocytic events [15,16]. Brightening of Jurkat cells in response to ionomycin was uniform, with no bright spots (Figure 1). Therefore, for exocytosis to cause 10-fold increases in FM1-43 fluorescence, Jurkat cells would have to undergo more exocytosis than is observed upon massive stimulation of secretory cells such as mast cells [17] and chromaffin cells [18], in which surface area increases 2–5-fold.

We conjectured, for the following three reasons, that the FM1-43 fluorescence increases we observed might be due to phospholipid scrambling. First, previous work using flow cytometry has demonstrated that treatment of Jurkat human leukaemic T-cells with the calcium ionophore ionomycin results in rapid phospholipid scrambling, as detected by annexin V labelling [19], consistent with the observation with Jurkat cells express high levels of the phospholipid scramblase [20]. Secondly, merocyanine 540 (or MC 540), a styryl dye that is structurally related to FM1-43, has been used to identify PS-exposing membranes [5]. MC 540 is believed to respond to decreases in lipid-packing density that accompany phospholipid scrambling. Thirdly, a recent study that examined the effect of lipid head-group charge on the apparent affinity of FM1-43 for lipid vesicles found that FM1-43 displayed increased affinity for vesicles containing PG, a phospholipid with a negatively charged head group [21]. PS exposure might cause similar effects.

To test the hypothesis that FM1-43 is responding to phospholipid scrambling, we performed the following three experiments. First, we tested whether the conditions that cause FM1-43 brightening also cause phospholipid scrambling, as assessed by the appearance of PS on the outer leaflet. We bathed cells in Ringer's solution containing 100 ng/ml FITC-annexin V, and stimulated them with $1 \mu\text{M}$ ionomycin (Figure 2). Initially, most cells exhibited little or no annexin staining, indicating normal plasma membrane asymmetry. Approx. 100 s after exposure to ionomycin, some cells began to brighten. There was considerable

heterogeneity in the delay until cells began to brighten. FITC-annexin V fluorescence increased slowly and continuously in the cells that responded, with no sign of saturation over ≈ 40 min (Figure 2). As with the results described above for FM1-43, when the fluorescence of Fura-2 was monitored at an excitation wavelength of 360 nm to assess plasma membrane integrity, only a small number of cells demonstrated abrupt decreases in Fura-2 fluorescence. TG treatment did not cause increased staining with FITC-annexin over the ≈ 40 -min observation time, consistent with a previous report [22], and with our results with FM1-43.

Secondly, we tested whether individual cells that display FM1-43 brightening had exposed PS (Figure 3). We stimulated cells with ionomycin in the presence of FM1-43. The extracellular solution contained 1 mM Ca^{2+} to reduce the number of responding cells. Figure 3 shows images of a cluster of five cells using this protocol. In another experiment, in which an entire field of cells was monitored, 58 cells had brightened after 840 s. We then washed off FM1-43. The chamber was perfused with solution containing FITC-annexin V, and images were acquired ≈ 1000 s later. Three FM-brightened cells washed away, but the remaining 55 were all FITC-annexin V-positive. Note that two cells that were not stained with FM1-43 were positive for FITC-annexin V. These cells may have exposed PS after FM1-43 was removed. Thus there is a good correlation between FM1-43 brightening and staining with FITC-annexin. Note that we could not perform double-labelling experiments using FM1-43 and FITC- or rhodamine-conjugated annexin V to determine whether cells that exhibited FM1-43 brightening had exposed PS, since FM1-43 exhibits a broad emission spectrum that spans FITC and rhodamine emission wavelengths (≈ 515 – 595 nm).

Finally, we monitored phospholipid scramblase activity directly by measuring uptake of NBD-labelled PC [6,23,24] (Figure 4). Unstimulated control cells exhibited a low level of NBD-PC fluorescence, NBD-PC stained the plasma membrane, as well as intracellular organelles, consistent with previous reports that exogenous lipids label intracellular organelles [25]. Cells stimulated with ionomycin exhibited ≈ 10 -fold higher levels of NBD-PC fluorescence than controls, indicating that ionomycin treatment stimulated uptake of NBD-PC. As in the case of unstimulated cells, both the plasma membrane and intracellular

Figure 3 Cells that display FM1-43 brightening stain with annexin V

(A) Cells 840 s after stimulation with 2 μ M ionomycin in the presence of 1 mM Ca^{2+}_o . Fura-2 fluorescence excited at 360 nm (red) and FM1-43 fluorescence (excitation at 485 nm, emission at 565 nm; green) are shown. (B) Cells after the washing out of FM1-43. As in (A), fluorescence of Fura-2 excited at 360 nm is shown, as well as FITC fluorescence (excitation at 485 nm, emission collected at 535 nm; green). (C) Cells after perfusion of the chamber with FITC-annexin. As in (B), fluorescence of Fura-2 excited at 360 nm (red), as well as FITC fluorescence (green), are shown. Cells that were FM1-43-bright were stained with FITC-annexin V. 2×2 binning was used for display.

Figure 4 Ionomycin, but not TG, stimulates uptake of NBD-PC

(A) Fluorescence images of control cells incubated with NBD-PC in normal Ringer's solution for 20 min, followed by 5 min of albumin extraction. (B) Fluorescence images of cells incubated with NBD-PC in normal Ringer's solution + 1 μ M ionomycin for 20 min followed by 5 min of albumin extraction. (C) Fluorescence images of cells incubated with NBD-PC in normal Ringer's + 1 μ M TG for 20 min, followed by 5 min of albumin extraction. All images are scaled identically. 4×4 binning was used.

membranes were stained with NBD-PC. Consistent with the results obtained with FITC-annexin and FM1-43, TG-stimulated cells were indistinguishable from control ones, indicating that NBD-PC uptake was not stimulated by TG treatment.

FM1-43 reports the kinetics of phospholipid scrambling with higher fidelity than annexin V

A striking difference between annexin V and FM1-43 brightening is that annexin V fluorescence increases continuously, whereas FM1-43 fluorescence begins earlier and reaches a steady state after 15–20 min (compare Figures 1 and 2). Saturation of FM1-43 brightening could reflect saturation of the ability of FM1-43 to bind to scrambled membranes, or could indicate that phospholipid scrambling has resulted in a stable new phospholipid composition of the outer leaflet. We performed two experiments to distinguish between these possibilities (Figure 5). First, we stimulated cells with ionomycin in the presence of 3.2 μ M FM1-43. After 1245 s, the chamber was perfused with solution containing 6.4 μ M FM1-43 (Figure 5A). Fluorescence approximately doubled, indicating that the ability of FM1-43 to bind to the membrane was not saturated. Secondly, cells were stimulated with ionomycin in normal Ringer's solution containing 3.2 μ M FM1-43 (Figure 5B). After 1185 s, the chamber was perfused with EGTA/Ringer's solution lacking Ca^{2+}_o , but containing FM1-43. Fluorescence approximately doubled following perfusion of the chamber with Ca^{2+}_o -free solution. The brightening observed following removal of Ca^{2+}_o is consistent with the idea FM1-43 responds to phospholipid scrambling, in part due to increased

negative surface charge (see above). Note that when the identical experiment was performed with annexin V, fluorescence decreased to the baseline level within 30 s (results not shown), consistent with the known Ca^{2+} -dependence of annexin V binding to PS [7]. Importantly, these results suggest that saturation of FM1-43 brightening does not reflect saturation of FM1-43 binding, but reflects rather the fact that membrane composition has reached a steady state.

The slower time course of FITC-annexin V compared with FM1-43 fluorescence increases, and the observation that FM1-43 apparently reports a stable phospholipid composition after 15–20 min while FITC-annexin V does not, can be explained if FITC-annexin V binds more slowly to scrambled membranes than FM1-43. If this is the case, the slow binding of FITC-annexin V would distort the apparent kinetics of phospholipid scrambling. To test this, we performed the experiments illustrated in Figure 5(C). Cells were stimulated with 1 μ M ionomycin in normal Ringer's solution. After FM1-43 fluorescence had increased to a stable elevated value, the chamber was perfused with FM1-43-free solution. As described above, fluorescence was reduced by $\approx 70\%$ by the washing procedure. The chamber was then perfused with normal Ringer's solution containing either FM1-43 or FITC-annexin V, and the time course of fluorescence increases was analysed. FM1-43 fluorescence increased rapidly after re-addition, reaching a steady-state level equivalent to the pre-washout level within two frames (30 s). By contrast, when FITC-annexin V was added following washout of FM1-43, fluorescence increased extremely slowly. We conclude that FITC-annexin V binding to PS-exposing membranes is extremely

Figure 5 FM1-43 fluorescence can increase further after apparent saturation, and stains cells that have already scrambled phospholipids faster than FITC–annexin V

(A) Cells were stimulated with 1 μM ionomycin in normal Ringer's solution + 3.2 μM FM1-43. After stimulation (1245 s), the chamber was perfused with Ringer's containing 6.4 μM FM1-43. Fluorescence increased by $\approx 100\%$. (B) Cells were stimulated with 1 μM ionomycin in normal Ringer's + 3.2 μM FM1-43. After stimulation (1140 s), the chamber was perfused with Ca^{2+} -free Ringer's containing 3.2 μM FM1-43. Fluorescence increased by $\approx 75\%$. (C) After cells were exposed to 1 μM ionomycin in the presence of FM1-43 (1140 s) the chamber was perfused with normal Ringer's solution that did not contain FM1-43. The chamber was then perfused with Ringer's solution containing FM1-43 or annexin V. Traces are normalized to the fluorescence remaining after washout of FM1-43. Note that FM1-43 fluorescence increased rapidly, attaining a steady-state level after ≈ 30 s, while annexin V fluorescence increases more slowly.

slow compared with FM1-43, and that the kinetics of phospholipid scrambling are faster than those reported with annexin V.

DISCUSSION

In the present paper, we provide evidence that FM1-43 is sensitive to plasma membrane phospholipid scrambling. Note that, on the basis of analogy to MC 540, we might also expect FM1-43 to respond to changes in lipid-packing density unrelated to scrambling. It seems most likely that the effects we observed were due primarily to scrambling-induced changes in packing, since conditions that cause FM1-43 fluorescence increases also cause scrambling, as assessed by either annexin staining or NBD-PC fluorescence increases. FM1-43 offers significant advantages over annexin V for the study of phospholipid scrambling. FM1-43, unlike annexin V, does not require Ca^{2+} to report phospholipid scrambling (Figure 5B), so studies designed to investigate the Ca^{2+} -dependence of phospholipid scrambling or apoptosis will be simpler to conduct with FM1-43 than with annexin V. FM1-43 binds more quickly to scrambled membranes than annexin V (Figure 5C), and is thus a more accurate reporter of the kinetics of phospholipid scrambling. Consistent with our

results, a previous study found that annexin bound slowly to platelets that exposed POS [26]. It is possible to observe ionomycin-induced FM1-43, but not FITC–annexin V, fluorescence increases in Jurkat cells using a spectrofluorimeter (A. Zweifach, unpublished work), because the quantum yield of FM1-43 increases several-hundredfold when it partitions into lipid bilayers. In contrast, total FITC–annexin V fluorescence does not increase, but rather redistributes from the extracellular solution to membrane. FM1-43 is significantly less expensive than annexin V. FM1-43 also brightly stains cells that have been induced to undergo apoptosis by UV-irradiation (A. Zweifach, unpublished work), suggesting that FM1-43 may be used to identify cells in the earliest stages of apoptosis. Note that one disadvantage of the use of FM1-43 as a reporter of phospholipid scrambling is its broad emission spectrum (515–595 nm), which makes it difficult to use a second fluorophore that emits in either the FITC or rhodamine channels.

Our results are the first report that a process other than vesicular trafficking can cause significant increases in FM1-43 fluorescence. That FM1-43 can give large responses to phospholipid scrambling is a significant caveat for its use as an optical probe of exocytosis and endocytosis.

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