Evidence that cytosolic calcium increases are not sufficient to stimulate phospholipid scrambling in human T-lymphocytes

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Phospholipid scrambling, the disruption of normal plasma-membrane asymmetry, occurs during apoptotic and necrotic cell death and during the activation of platelets and neutrophils. It is currently believed that phospholipid scrambling is triggered simply by increases in bulk cytosolic $[Ca^{2+}]_i$. We have presented evidence previously that the styryl dye FM1-43 is sensitive to phospholipid scrambling in Jurkat human leukaemic T-lymphocytes. Here we have used FM1-43, in combination with fura 2 and the Ca^{2+} -elevating agents ionomycin and thapsigargin, in imaging experiments to test the idea that increases in bulk cytosolic $[Ca^{2+}]_i$ stimulate scrambling. Intracellular Ca^{2+} increases of $\approx 2 \mu M$ accompanied ionomycin-stimulated scrambling in $\approx 50\%$ of cells, and scrambling occurred in $> 99\%$ of cells in which intracellular Ca^{2+} rose to $4 \mu M$. Chelating intracellular

Ca^{2+} with bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid or EGTA suppressed both ionomycin-stimulated intracellular Ca^{2+} increases and scrambling, demonstrating that intracellular Ca^{2+} increases are necessary for ionomycin-stimulated scrambling. However, elevating intracellular Ca^{2+} to $2\text{--}4 \mu M$ with thapsigargin, a drug that depletes intracellular Ca^{2+} stores and triggers Ca^{2+} entry via Ca^{2+} -release-activated Ca^{2+} channels, did not trigger scrambling, as assessed with either FM1-43 or FITC-labelled annexin V. These results suggest that increases in intracellular $[Ca^{2+}]_i$ are necessary but not sufficient to stimulate scrambling in lymphocytes, and indicate that ionomycin has an additional effect that is required to stimulate scrambling.

Key words: apoptosis, intracellular calcium, plasma membrane.

INTRODUCTION

Plasma-membrane lipid composition is asymmetric in most healthy mammalian cells [1]. An ATP-dependent aminophospholipid translocase ensures that phosphatidylserine (PS) and phosphatidylethanolamine reside in the inner leaflet of the plasma membrane, whereas phosphatidylcholine and sphingomyelin are found in both leaflets. One of the earliest detectable events in cells undergoing apoptosis is phospholipid scrambling, a disruption of plasma-membrane lipid asymmetry that results in exposure of PS on the outer leaflet [2]. Exposed PS can activate the alternative complement pathway [3] and acts as a signal triggering engulfment by phagocytes [4], effects that contribute to the destruction and removal of dying cells. Phospholipid scrambling is also a key event in the normal activation of platelets and neutrophils [3].

Phospholipid scrambling is thought to occur as a result of the inhibition of the aminophospholipid translocase and concomitant activation of a phospholipid scramblase [5], an enzyme that catalyses non-specific transbilayer movement of phospholipids [3,6]. Inhibition of the aminophospholipid translocase alone cannot cause scrambling [5]. Stimulation of the scramblase and inhibition of the aminophospholipid translocase are both believed to be triggered simply by increases in bulk cytosolic Ca^{2+} ($[Ca^{2+}]_i$) [3]. One of the key pieces of evidence supporting this idea is the finding that phospholipid scrambling can be triggered by calcium ionophores such as ionomycin in many cell types [3], including Jurkat cells [7]. Consistent with the idea that scrambling is

stimulated by increases in $[Ca^{2+}]_i$, a scramblase cloned from erythrocytes that is also expressed in Jurkat cells has an EF-hand domain [6], and mediates scrambling when reconstituted into liposomes; half-maximal scrambling is observed at $[Ca^{2+}]_i$ between 20 and $60 \mu M$ [8]. However, the $[Ca^{2+}]_i$ dependence of scrambling in intact cells has proven difficult to determine, and may differ in different cell types. In red-cell ghosts, one study indicated that a $[Ca^{2+}]_i$ value of $5\text{--}10 \mu M$ was sufficient to abolish phospholipid asymmetry [9], while a more recent study found that $75 \mu M$ $[Ca^{2+}]_i$ activated the scramblase half-maximally [10]. In platelets, $[Ca^{2+}]_i$ and scrambling were monitored in cell populations stimulated with a variety of agents that elevate $[Ca^{2+}]_i$ [11], but a clear $[Ca^{2+}]_i$ dependence could not be demonstrated. The Ca^{2+} ionophore A23187 and the Ca^{2+} -ATPase inhibitors thapsigargin (TG) and cyclopiazonic acid elevated $[Ca^{2+}]_i$ to $10\text{--}20 \mu M$ and stimulated phospholipid scrambling, but a structurally unrelated Ca^{2+} -ATPase inhibitor, *t*-butyl hydroquinone, was able to stimulate scrambling, although it elevated $[Ca^{2+}]_i$ to only 600 nM. Previous work has shown that treating Jurkat human leukaemic T-lymphocytes with TG does not cause rapid phospholipid scrambling in Jurkat cells [12,13].

The regulation of scrambling during apoptotic cell death has also been investigated. Bratton et al. [5] found that scrambling stimulated by a number of apoptotic stimuli, including anti-Fas antibodies and UV irradiation, required extracellular Ca^{2+} (Ca^{2+}_o). Hampton et al. [14] showed that chelating Ca^{2+}_o with EGTA inhibited PS exposure during apoptosis triggered by TG, anti-Fas antibodies and tumour necrosis factor/cycloheximide.

Abbreviations used: PS, phosphatidylserine; $[Ca^{2+}]_i$, bulk cytosolic $[Ca^{2+}]$; Ca^{2+}_o , extracellular Ca^{2+} ; $[Ca^{2+}]_o$, extracellular $[Ca^{2+}]$; TG, thapsigargin; fura 2/AM, fura 2 acetoxymethyl ester; EGTA/AM, EGTA acetoxymethyl ester; BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase.

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Results such as these are taken as evidence that scrambling during apoptosis occurs as a result of increases in $[Ca^{2+}]_i$, and, consistent with this idea, increases in $[Ca^{2+}]_i$ have been associated with apoptosis [12].

We recently reported that the styryl dye FM1-43, best known as a probe of exo- and endo-cytosis, is sensitive to phospholipid scrambling [13]. FM1-43 identifies cells that scramble more rapidly than annexin V, the most commonly used probe of scrambling, and does so without a requirement for Ca_0^{2+} . Here we have exploited this property of FM1-43 to test the idea that scrambling is stimulated solely by increases in $[Ca^{2+}]_i$. Surprisingly, our results suggest that cytosolic $[Ca^{2+}]_i$ increases by themselves are not sufficient to trigger scrambling, a finding that challenges the current idea that scrambling is regulated simply by increases in cytosolic $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Fura 2 acetoxymethyl ester (fura 2/AM), FM1-43, EGTA acetoxymethyl ester (EGTA/AM) and bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM) were from Molecular Probes (Eugene, OR, U.S.A.). TG and ionomycin were from Alexis Biochemicals (San Diego, CA, U.S.A.). FITC-labelled annexin V was from R&D Systems (San Diego, CA, U.S.A.). RPMI 1640 and tissue-culture plastic was from Fisher Scientific (Pittsburgh, PA, U.S.A.). Fetal calf serum, glutamine and antibiotics were from Gemini Bio-products (Calabasas, CA, U.S.A.).

Jurkat E6-1 human leukaemic T-cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, antibiotics and glutamine. Ringer's solution contained 145 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM Hepes and 10 mM glucose (made up to pH 7.4 with NaOH). Zero- Ca^{2+} Ringer's was identical in composition, except that $CaCl_2$ was replaced with $MgCl_2$. Zero- Ca^{2+} Ringer's plus EGTA was identical with zero- Ca^{2+} Ringer's, but was supplemented with 1 mM EGTA. For experiments that required $Ca_0^{2+} < 2$ mM, we mixed appropriate volumes of normal Ringer's and zero- Ca^{2+} Ringer's. For experiments that required $Ca_0^{2+} > 2$ mM, we added $CaCl_2$ from a concentrated stock solution to normal Ringer's.

Jurkat cells were loaded with fura 2 by incubating them with 1 μ M fura 2/AM in cell culture medium for 30 min at room temperature. Cells were washed twice with fresh medium before use, then allowed to settle on to poly-L-lysine-coated coverslips. Experiments were performed at room temperature with an imaging system described previously [15], built around a Nikon Diaphot 300 inverted microscope (Nikon, Melville, NY, U.S.A.) equipped for epifluorescence. A Nikon PlanFluor 40 $\times$ oil objective (numerical aperture 1.4) was used for all experiments. Excitation light was provided by a 150 W xenon arc lamp (Optiquip, Highland Mills, NY, U.S.A.), and was attenuated to 12% using neutral-density filters. Excitation wavelength was selected using appropriate excitation filters in a Sutter Lambda-10 filter wheel (Sutter Instruments, Novato, CA, U.S.A.). A fura-BCECF [2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein] dichroic mirror was used in the filter cube, and emission light was filtered with a second Sutter filter wheel. Filters and the dichroic mirror were obtained from Chroma (Brattleboro, VT, U.S.A.). Excitation light was filtered with a 485 nm bandpass filter for FM1-43 and FITC-labelled annexin V, and 340 and 380 nm bandpass filters for fura 2. Emitted light was collected with a 595 nm bandpass filter for FM1-43, a 515 nm bandpass filter for FITC-labelled annexin V, and a 510 nm bandpass filter for fura 2. Images were acquired with a Cooke Sensicam peltier-cooled

interline CCD camera (PCO Computer Optics GmbH, Kelheim, Germany) binned 8 $\times$ 8 at a rate of 1 frame every 15 s. Hardware was controlled and images were acquired and analysed with SlideBook software from Intelligent Imaging Innovations (Denver, CO, U.S.A.) running on a Macintosh G3 computer. Images were analysed by drawing regions of interest over cells, and also over a cell-free region that was used as a background for subtraction. The mean intensity for all the regions of interest on 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.

RESULTS

$[Ca^{2+}]_i$ dependence of ionomycin-stimulated phospholipid scrambling

We stimulated Jurkat cells with different concentrations of ionomycin in the presence of various levels of Ca_0^{2+} to elicit $[Ca^{2+}]_i$ increases, and assessed phospholipid scrambling with FM1-43 in imaging experiments. In one set of experiments, we treated Jurkat cells with different concentrations of ionomycin in the presence of 1 mM Ca_0^{2+} . Increases in FM1-43 fluorescence (brightening) associated with phospholipid scrambling were observed in < 1% of cells when 0.1 μ M ionomycin was applied. Raising the concentration of ionomycin to 0.5, 1 or 2 μ M increased the magnitude of the average rise in $[Ca^{2+}]_i$, and caused increasing numbers of cells to scramble. We also varied Ca_0^{2+} at a constant ionomycin concentration (Figure 1). Cells stimulated with 2 μ M ionomycin in the absence of Ca_0^{2+} did not scramble (results not shown). As extracellular $[Ca^{2+}]_o$ ($[Ca^{2+}]_o$) was increased to 0.5, 1 or 2 mM, the number of cells that scrambled increased. Consistent with these results, we found that scrambling could be triggered in cells stimulated with 0.1 μ M ionomycin if $[Ca^{2+}]_o$ was increased to 20 mM (results not shown). The images shown in Figure 1(A) indicate that cells that scrambled typically exhibited sustained $[Ca^{2+}]_i$ increases that were higher than in cells that did not scramble, as the fluorescence of fura 2 excited at 380 nm was inversely correlated with $[Ca^{2+}]_i$, and was lower in cells that scrambled. Consistent with this idea, mean $[Ca^{2+}]_i$ increased to higher levels when Ca_0^{2+} was higher (Figure 1B), and more cells scrambled (Figure 1C). The histograms shown in Figure 1(C) suggest that cells in which FM1-43 fluorescence increased by more than 2.75-fold (Figure 1C, dotted line) can unambiguously be considered to have brightened. We applied this criterion in the analysis presented below in Figures 3 and 4.

To confirm that the Ca_0^{2+} and ionomycin dependence of scrambling reflects a requirement for an increase in $[Ca^{2+}]_i$, we loaded cells with the membrane-permeant Ca^{2+} chelators EGTA/AM and BAPTA/AM (both present in the medium at 100 μ M for 30 min at room temperature). Cells were then stimulated with 2 μ M ionomycin in the presence of 1 mM Ca_0^{2+} . Both chelators were able to prevent ionomycin-stimulated $[Ca^{2+}]_i$ increases and scrambling (data for BAPTA are shown in Figure 2).

To determine the $[Ca^{2+}]_i$ dependence of ionomycin-stimulated phospholipid scrambling, we analysed single-cell recordings of $[Ca^{2+}]_i$ and FM1-43 fluorescence from the experiments described above (Figure 3). Single-cell $[Ca^{2+}]_i$ responses were heterogeneous under any given stimulation condition (Figure 3A). Some cells displayed transient increases in $[Ca^{2+}]_i$ to levels up to 2–5 μ M (Figure 3A, panel 1); these cells did not scramble. Other cells underwent sustained $[Ca^{2+}]_i$ increases to levels of 2–5 μ M (Figure 3A, panel 2). Some of these cells scrambled. A third population of cells displayed a complex response in which $[Ca^{2+}]_i$ first

Figure 1 Dependence of ionomycin-stimulated FM1-43 fluorescence increases on Ca_o^{2+}

(A) Fluorescence images of cells stimulated with $2 \mu M$ ionomycin (iono) in the presence of 0.5, 1 or 2 mM Ca_o^{2+} . FM1-43 fluorescence is displayed in on the right, and the fluorescence of fura 2 excited at 380 nm (Fura 380) is shown on the left. (B) Mean $[Ca^{2+}]_i$ for the experiments shown in (A). $[Ca^{2+}]_i$ increased with increased Ca_o^{2+} . (C) Histograms of fold FM1-43 increases measured from the experiments shown in (A). In this experiment, 106 cells were analysed at 0.5 mM Ca_o^{2+} , 105 cells at 1 mM Ca_o^{2+} and 110 cells at 2 mM Ca_o^{2+} .

increased to 2–5 μM , and then underwent a secondary increase to reported levels as high as 15 μM (Figure 3A, panel 3). The fraction of cells displaying this pattern of $[Ca^{2+}]_i$ increase was larger at higher Ca_o^{2+} values, and these cells always scrambled, although scrambling began before the secondary increase in $[Ca^{2+}]_i$. In the cell represented in Figure 3(A), panel 3, for example, scrambling began when $[Ca^{2+}]_i$ was $\approx 2.8 \mu M$. We reasoned that the appropriate measure of $[Ca^{2+}]_i$ associated with scrambling was the $[Ca^{2+}]_i$ at the time that FM1-43 brightening

began, as this corresponds to the earliest detectable change in plasma-membrane outer-leaflet properties. We defined this value as $[Ca^{2+}]_{thresh}$. The beginning of brightening was defined using a threshold level of FM1-43 fluorescence of 2.5 as described above. For cells that did not brighten during the observation period, we set $[Ca^{2+}]_{thresh}$ to the $[Ca^{2+}]_i$ observed at the end of the experiment. This procedure allowed us to distinguish between cells that exhibited sustained versus transient increases in $[Ca^{2+}]_i$, like those shown in Figure 3(A). We plotted fold FM1-43 brightening

Figure 2 Preventing $[Ca^{2+}]_i$ increases with intracellular chelators prevents increases in FM1-43 fluorescence

(A) $[Ca^{2+}]_i$ increases in cells stimulated with 2 μ M ionomycin (dashed arrow) in the presence of 1 mM Ca_0^{2+} . Traces are averages of 64 (control) and 51 (BAPTA) cells. (B) Histograms of FM1-43 brightening from control and BAPTA-loaded cells. Data were compiled from three experiments under each set of conditions. Similar results were obtained when cells were loaded with EGTA.

against $[Ca^{2+}]_{i, \text{thresh}}$ determined for 840 cells from 16 experiments (Figure 3B). As $[Ca^{2+}]_{i, \text{thresh}}$ increased from ≈ 0.7 to 3.5 μ M, increasing numbers of cells scrambled. We analysed these data further by determining the fraction of cells that brightened as a function of $[Ca^{2+}]_{i, \text{thresh}}$, binned in 250 nM increments. Figure 3(C) displays the results of this analysis, which demonstrates a clear dependence of ionomycin-stimulated phospholipid scrambling on $[Ca^{2+}]_i$. Sustained ionomycin-stimulated $[Ca^{2+}]_i$ increases of $\approx 1.5 \mu$ M accompanied scrambling in $\approx 50\%$ of cells. At a $[Ca^{2+}]_{i, \text{thresh}}$ value of $> 4 \mu$ M, $> 99\%$ of cells scrambled.

TG-stimulated $[Ca^{2+}]_i$ rises do not cause scrambling

TG, a drug that elevates $[Ca^{2+}]_i$ in lymphocytes by blocking sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPases (SERCAs), thus activating influx through Ca^{2+} -release-activated Ca^{2+} channels [16], does not cause rapid phospholipid scrambling in Jurkat cells [13,14], a result that is inconsistent with the idea that scrambling is stimulated by $[Ca^{2+}]_i$ increases. However, two possibilities must be eliminated before this conclusion can be made: first, that TG itself prevents scrambling, perhaps by altering the Ca^{2+} dependence of the scramblase, and second, that

Figure 3 $[Ca^{2+}]_i$ dependence of ionomycin-stimulated increases in FM1-43 fluorescence

(A) Simultaneous measurement of phospholipid scrambling and $[Ca^{2+}]_i$ for three cells stimulated with 1 μ M ionomycin (dotted line) in the presence of 1 mM Ca_0^{2+} . Note the heterogeneity in the responses. (B) Plot of fold FM1-43 brightening against $[Ca^{2+}]_{i, \text{thresh}}$ for experiments similar to the one shown in Figure 1. (C) $[Ca^{2+}]_i$ dependence of phospholipid scrambling. $[Ca^{2+}]_{i, \text{thresh}}$ was binned in 250 nM increments, and the fraction of cells that brightened was determined for each bin.

Figure 4 TG does not stimulate FM1-43 fluorescence increases at any level of $[Ca^{2+}]_i$

(A) Simultaneous measurement of FM1-43 fluorescence and $[Ca^{2+}]_i$ in two different TG-stimulated cells. In this experiment, cells were stimulated with $1 \mu M$ TG in zero- Ca^{2+} Ringer's, and data collection was started 600 s after TG treatment. Application of 20 mM Ca^{2+}_o (dotted line) caused robust $[Ca^{2+}]_i$ increases to levels that accompany ionomycin-stimulated scrambling, but cells did not brighten. (B) Plot of fold FM1-43 brightening against $[Ca^{2+}]_{i,thresh}$. (C) Analysis of data from (B).

TG-stimulated $[Ca^{2+}]_i$ increases are of insufficient amplitude to trigger scrambling. We determined that TG does not interfere with scramblase activity by comparing responses of Jurkat cells to $2 \mu M$ ionomycin with or without a 1 min pretreatment with $1 \mu M$ TG. TG pretreatment did not prevent ionomycin-induced scrambling (results not shown), indicating that TG does not shift the $[Ca^{2+}]_i$ dependence of scrambling to higher $[Ca^{2+}]_i$ values. However, we did find that in the presence of $2 mM$ Ca^{2+}_o , $[Ca^{2+}]_i$ increases stimulated by TG reached 0.8 – $1 \mu M$ (results not shown), levels that supported scrambling in only $\approx 20\%$ of cells in the ionomycin experiments described above (Figure 3C). We were able to cause larger $[Ca^{2+}]_i$ increases by treating cells with $1 \mu M$ TG in zero- Ca^{2+} Ringer's, and then perfusing the chamber with Ringer's containing 10 or 20 mM Ca^{2+}_o . Note that FM1-43 responses to stimulation with ionomycin in the presence of 20 mM Ca^{2+}_o were indistinguishable from those observed with $2 mM$ Ca^{2+}_o , indicating that high $[Ca^{2+}]_o$ does not interfere with the ability of FM1-43 to detect scrambling (results not shown). As shown in Figures 4(B) and 4(C), TG-stimulated cells still did not brighten over 20 min following Ca^{2+}_o addition, despite $[Ca^{2+}]_i$ increases to levels that were comparable with those observed under ionomycin stimulation. We estimated the number of TG-treated cells that we would have expected to brighten if scrambling

was stimulated simply by elevated $[Ca^{2+}]_i$ as follows. We determined $[Ca^{2+}]_{i,thresh}$ values as described above for 839 cells from 10 experiments (Figure 4B), binned them (Figure 4C) and multiplied the number of cells in each bin by the fraction of cells that brightened in each bin for ionomycin stimulation. Based on this analysis, we would have expected 316 TG-stimulated cells to brighten if scrambling were caused by high $[Ca^{2+}]_i$; instead, only 15 cells did. These results are inconsistent with the idea that $[Ca^{2+}]_i$ elevation triggers scrambling.

To confirm that elevating cytosolic $[Ca^{2+}]$ with TG does not cause scrambling in Jurkat cells, we performed experiments with FITC-labelled annexin V to monitor exposure of PS (Figure 5). Cells were stimulated with either $1 \mu M$ ionomycin in normal Ringer's or $1 \mu M$ TG in zero- Ca^{2+} Ringer's, followed after 10 min by Ringer's containing 20 mM $CaCl_2$. Images acquired before and 900 s after elevation of $[Ca^{2+}]_i$ confirm that ionomycin causes exposure of PS, but TG does not. The results shown in Figure 5 are representative of four separate experiments. These results are consistent with the results in Figure 4 indicating that TG treatment does not cause scrambling. That TG does not cause scrambling when $[Ca^{2+}]_i$ is increased to levels that support ionomycin-stimulated scrambling (Figures 4 and 5) indicates that ionomycin does not cause scrambling simply by elevating

Figure 5 FITC-labelled annexin V staining confirms that TG does not stimulate phospholipid scrambling

Fluorescence images of cells stimulated with ionomycin (top panels) or TG (bottom panels) before (A) and 900 s after (B) addition of ionomycin or 20 mM CaCl_2 . In each pair of images, the fluorescence of fura 2 excited at 340 nm (F340) is shown on the left and FITC-labelled annexin V is shown on the right. Cells stimulated with ionomycin exposed PS, but cells stimulated with TG did not. All images are scaled identically.

$[\text{Ca}^{2+}]_i$, and therefore suggests that ionomycin has an additional effect that is required for scrambling.

DISCUSSION

It is currently believed that phospholipid scrambling is stimulated by increases in $[\text{Ca}^{2+}]_i$. By analogy with other Ca^{2+} -dependent plasma-membrane proteins, there are several mechanisms by which increases in $[\text{Ca}^{2+}]_i$ could result in scramblase activation. Ca^{2+} could activate the scramblase by binding directly to it, as is believed to be the case for some Ca^{2+} -activated Cl^- channels [17]. Ca^{2+} could bind to calmodulin that is physically associated with the scramblase, as has been shown for Ca^{2+} -stimulatable adenylate cyclases [18], or to calmodulin free in the cytosol, which could then activate Ca^{2+} -calmodulin-dependent protein kinase(s) or phosphatases that could regulate the scramblase via changes in phosphorylation state. Ca^{2+} could activate a protease such as calpain [19], which could activate the scramblase via cleavage. However, based on the observation that scrambling is reversible [7], this mechanism seems unlikely. As an EF-hand domain has been identified in a putative cloned scramblase [6], direct binding of Ca^{2+} to the scramblase is an attractive possible mechanism for activation of scrambling. Given the widespread acceptance of the idea that increases in $[\text{Ca}^{2+}]_i$ regulate scrambling, it is quite surprising that our results comparing the ability of ionomycin and TG to stimulate scrambling (Figures 3–5) suggest that ionomycin does not in fact stimulate scrambling simply by increasing $[\text{Ca}^{2+}]_i$, at least in Jurkat cells, although $[\text{Ca}^{2+}]_i$ increases are required for ionomycin-stimulated scrambling (Figure 2). Taken together, these results suggest that ionomycin must have an additional effect that is required to stimulate scrambling, an effect that TG does not have. As the ability of ionomycin to stimulate scrambling is one of the cornerstones of the idea that scrambling is regulated simply by elevated $[\text{Ca}^{2+}]_i$, these results therefore challenge prevailing notions about the regulation of plasma-membrane phospholipid scrambling.

While we do not yet know how FM1-43 responds to scrambling, we feel that it is appropriate to use FM1-43 to monitor the $[\text{Ca}^{2+}]_i$ dependence of scrambling, as FM1-43 faithfully identifies

cells that expose PS, as assessed by annexin V binding [13]. Thus the alteration(s) in plasma-membrane properties that FM1-43 senses occur only in cells that expose PS, and FM1-43 enables us to monitor very early changes in plasma-membrane properties associated with scrambling. Importantly, our results with FM1-43 were confirmed using annexin V (Figure 5). Although some of the $[\text{Ca}^{2+}]_i$ values (2–4 μM) that we have estimated are quite high for fura 2 (K_d 200–300 nM), we are confident that this does not affect our conclusions. We observe ionomycin-stimulated scrambling at $[\text{Ca}^{2+}]_i$ values as low as 0.7 μM , and 50% of cells scramble when $[\text{Ca}^{2+}]_i$ is 1.75 μM ; these are values that are well within the range of $[\text{Ca}^{2+}]_i$ that is easily measured with fura 2. Thus our conclusions do not depend on data acquired at higher $[\text{Ca}^{2+}]_i$ values. Fura 2 has been used by others to measure $[\text{Ca}^{2+}]_i$ as high as 6 μM in Jurkat cells [20]. Furthermore, as we were comparing the ability of ionomycin and TG to stimulate scrambling, systematic errors would probably similarly affect the data obtained with the two agents. Additionally, while measuring $[\text{Ca}^{2+}]_i$ of 2–4 μM requires the ability to measure accurately changes in fura 2 saturation of only a few per cent, we were able to do this because we were monitoring the emission of two Ca^{2+} -sensitive wavelengths using a very-low-noise 12 bit CCD camera. Consistent with the idea that fura 2 is not completely saturated at reported values of 2–4 μM , we found that, in some cells, $[\text{Ca}^{2+}]_i$ increased further to reported levels of 8–10 μM after brightening began (Figure 3C).

What additional effect could ionomycin have that is involved in stimulating scrambling? We propose five possibilities. Two involve ionomycin-induced $[\text{Ca}^{2+}]$ increases in restricted subcellular domains, whereas the others involve what might be considered side-effects of ionomycin. These are discussed in detail below.

Firstly, ionomycin might stimulate a localized $[\text{Ca}^{2+}]$ increase in microdomains of elevated Ca^{2+} at the endofacial surface of the plasma membrane near scramblase molecules. Microdomains are spatially restricted regions that form within several nm of Ca^{2+} -influx sites, in which $[\text{Ca}^{2+}]$ can increase to levels that are substantially higher than the $[\text{Ca}^{2+}]_i$ reported by indicators such as fura 2 [21]. In Jurkat cells, both ionomycin and thapsigargin

deplete endoplasmic-reticulum Ca^{2+} stores and activate Ca^{2+} -release-activated Ca^{2+} channels [16], but ionomycin also carries Ca^{2+} directly across the plasma membrane. Therefore, microdomains in the vicinity of channels will be similar after stimulation with ionomycin or TG, but it could be argued that if the scramblase is located in regions of the plasma membrane physically segregated from Ca^{2+} -release-activated Ca^{2+} channels, TG treatment might not elevate local $[\text{Ca}^{2+}]$ sufficiently to activate the scramblase. An analogous but opposite situation has been reported by Cooper and colleagues [22], who demonstrated that Ca^{2+} influx via store-operated channels can regulate Ca^{2+} -sensitive adenylate cyclase, but that direct entry via ionomycin cannot. These results have been interpreted as indicating that cyclase and channels are co-localized physically in cholesterol-rich membrane domains, and that ionomycin does not have access to the cholesterol-rich membrane domain that contains channels/cyclases. While this possibility is consistent with the observation that the purified scramblase demonstrates a substantially lower affinity for Ca^{2+} than we observed [8], and in fact our estimates are at the lower end of previous estimates of the $[\text{Ca}^{2+}]$ dependence of scrambling in red-cell ghosts [10] or in platelets stimulated with A23187, TG or cyclopiazonic acid [11], it seems unlikely to us for the following reasons. Firstly, ionomycin is not expected to generate microdomains, because it has a relatively low transport rate of $20\text{--}100\text{ s}^{-1}$ [23,24]. Second, we found that EGTA/AM and BAPTA/AM were both able to suppress ionomycin-stimulated scrambling (Figure 2). If scrambling were due to ionomycin-generated Ca^{2+} microdomains, we would expect EGTA to be ineffective, because its slow on-rate for Ca^{2+} binding renders it unable to suppress $[\text{Ca}^{2+}]$ rises at distances less than $\approx 150\text{ nm}$ from sites of influx, where microdomains form [25].

A second possibility is that organelles such as the endoplasmic reticulum and the Golgi, which rely on SERCAs for Ca^{2+} loading, are likely to be depleted of Ca^{2+} during TG treatment, but may experience high $[\text{Ca}^{2+}]$ following stimulation with ionomycin, and high $[\text{Ca}^{2+}]$ in such an organelle might be involved in triggering scrambling. If this possibility is correct, then some presently unknown mechanism must couple increases in organellar $[\text{Ca}^{2+}]$ with scramblase activation.

A third possibility is that scrambling is stimulated by a combination of elevated $[\text{Ca}^{2+}]$ and altered intracellular pH caused by ionomycin's obligate $\text{Ca}^{2+}/2\text{H}^{+}$ exchange activity [26]. This possibility seems unlikely, as we have found that treatment of TG-stimulated cells with $20\text{ mM NH}_4\text{Cl}$ followed by washout of NH_4Cl (expected to alkalinize the cytosol and then acidify it [20]) did not trigger scrambling (A. Zweifach and G. A. Wurth, unpublished work).

A fourth possibility is that ionomycin itself might alter the Ca^{2+} dependence of the scramblase, shifting it to a lower $[\text{Ca}^{2+}]$. This possibility is consistent with the observation that the purified scramblase demonstrates a substantially lower affinity for Ca^{2+} than we observed, [8], as does scrambling in red-cell ghosts [10] and platelets [11]. While we cannot exclude this possibility entirely because we only observed scrambling in the presence of ionomycin, one argument against it is that we could stimulate scrambling with concentrations of ionomycin ranging from 0.1 to $2\text{ }\mu\text{M}$, provided that $[\text{Ca}^{2+}]_0$ was high enough to elevate $[\text{Ca}^{2+}]$ sufficiently. However, to definitively test the idea that ionomycin alters the Ca^{2+} dependence of scrambling, it will be necessary to determine the Ca^{2+} dependence of scrambling in the presence and absence of ionomycin in a cell-free system, such as red-cell ghosts or plasma-membrane vesicles.

Finally, ionomycin might trigger scrambling because it raises $[\text{Ca}^{2+}]$ and also acidifies the mitochondrial matrix. Ionomycin is

known to transport Ca^{2+} directly out of mitochondria in Jurkat cells [20], and so is expected to cause a dissipative Ca^{2+} cycling that begins as mitochondrial $[\text{Ca}^{2+}]$ increases subsequent to increases in $[\text{Ca}^{2+}]_i$. Because ionomycin is an obligate $\text{Ca}^{2+}/2\text{H}^{+}$ exchanger, this dissipative cycling is likely to cause acidification of the mitochondrial matrix and collapse of the proton-motive force, conditions that have been shown to lead to cellular ATP depletion ([27], reviewed in [28]), or alternatively could trigger the mitochondrial permeability transition [29]. TG is not expected to produce any of these effects on mitochondria. An attractive feature of this possibility is that it is consistent with the known involvement of mitochondria in apoptotic and necrotic cell death [29] and thus might provide an explanation for the stimulation of scrambling during apoptotic and necrotic cell death.

In summary, we have shown that, while $[\text{Ca}^{2+}]_i$ increases are necessary for ionomycin-stimulated scrambling, $[\text{Ca}^{2+}]_i$ increases by themselves are not sufficient to trigger scrambling in lymphocytes. The current idea that scrambling is triggered simply by rises in $[\text{Ca}^{2+}]_i$ is therefore incomplete. Determining the additional effect of ionomycin that is required for scrambling may provide important information about the regulation of scrambling.

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REFERENCES

- Bruckheimer, E. and Schroit, A. (1996) Membrane phospholipid asymmetry: host responses to the externalization of phosphatidylserine. *J. Leukocyte Biol.* **59**, 784–788
- Martin, S., Reutelingsperger, C., McGahon, A., Rader, J., Van Schie, R., LaFace, D. and Green, D. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* **182**, 1545–1556
- Beyers, E., Comfurius, P., Dekkers, D. and Zwaal, R. (1999) Lipid translocation across the plasma membrane of mammalian cells. *Biochim. Biophys. Acta* **1439**, 317–330
- Fadok, V., Voelker, D., Cambell, P., Cohen, J., Bratton, D. and Henson, P. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* **148**, 2207–2216
- Bratton, D., Fadok, V., Richter, D., Kailey, J., Guthrie, L. and Henson, P. (1997) Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *J. Biol. Chem.* **272**, 26159–26165
- Zhou, Q., Zhao, J., Stout, J., Luhm, R., Wiedmer, T. and Sims, P. (1997) Molecular cloning of human plasma membrane phospholipid scramblase. *J. Biol. Chem.* **272**, 18240–18244
- Li, W. and Tait, J. (1998) Regulatory effect of CD9 on calcium-stimulated phosphatidylserine exposure in Jurkat T lymphocytes. *Arch. Biochem. Biophys.* **351**, 89–95
- Basse, F., Stout, J., Sims, P. and Wiedmer, T. (1996) Isolation of an erythrocyte membrane protein that mediates calcium-dependent transbilayer movement of phospholipid. *J. Biol. Chem.* **271**, 17205–17210
- Williamson, P., Algarin, L., Bateman, J., Choe, H.-R. and Schlegel, R. (1985) Phospholipid asymmetry in human erythrocyte ghosts. *J. Cell. Physiol.* **123**, 209–214
- Woon, L., Holland, J., Kable, E. and Roufogalis, B. (1999) Calcium sensitivity of phospholipid scrambling in human red cell ghosts. *Cell Calcium* **25**, 313–320
- Dachary-Prigent, J., Pasquet, J.-M., Freyssinet, J.-M. and Nurdin, A. (1995) Calcium involvement in aminophospholipid exposure and microparticle formation during platelet activation: a study using Ca^{2+} ATPase inhibitors. *Biochemistry* **34**, 11625–11634
- McConkey, D. and Orrenius, S. (1996) The role of calcium in the regulation of apoptosis. *J. Leukocyte Biol.* **59**, 775–783
- Zweifach, A. (2000) FM1-F43 reports plasma membrane phospholipid scrambling in T lymphocytes. *Biochem. J.* **349**, 255–260
- Hampton, M., Vanags, D., Porn-Ares, I. and Orrenius, S. (1996) Involvement of extracellular calcium in phosphatidylserine exposure during apoptosis. *FEBS Lett.* **399**, 277–282
- Zweifach, A. (2000) Target cell contact activates a highly selective capacitative calcium entry pathway in cytotoxic T lymphocytes. *J. Cell Biol.* **148**, 603–614

- 16 Zweifach, A. and Lewis, R. S. (1993) Mitogen-regulated Ca^{2+} current of T lymphocytes is activated by depletion of intracellular Ca^{2+} stores. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6295–6299
- 17 Kidd, J. F. and Thorn, P. (2000) Intracellular Ca^{2+} and Cl-channel activation in secretory cells. *Annu. Rev. Physiol.* **62**, 492–513
- 18 Gu, C. and Cooper, D. M. (1999) Calmodulin-binding sites on adenylyl cyclase type VIII. *J. Biol. Chem.* **274**, 8012–8021
- 19 Sato, K. and Kawashima, S. (2001) Calpain function in the modulation of signal transduction molecules. *Biol. Chem.* **382**, 743–745
- 20 Hoth, M., Fanger, C. M. and Lewis, R. S. (1997) Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. *J. Cell Biol.* **137**, 633–648
- 21 Smith, S. J. and Augustine, G. J. (1988) Calcium ions, active zones and synaptic transmitter release. *Trends Neurosci.* **11**, 458–464
- 22 Fagan, K., Mons, N. and Cooper, D. (1998) Dependence of the calcium inhibitable adenylyl cyclase of C6-2B glioma cells on capacitative calcium entry. *J. Biol. Chem.* **273**, 9297–9305
- 23 Verhage, M., McMahon, H., Ghijsen, W., Boomsma, F., Scholten, G., Wiegant, V. and Nicholls, D. (1991) Differential release of amino acids, neuropeptides, and catecholamines from isolated nerve terminals. *Neuron* **6**, 517–524
- 24 Fagan, K., Mahey, R. and Cooper, D. (1996) Functional colocalization of transfected calcium-stimulable adenylyl cyclases with capacitative calcium entry sites. *J. Biol. Chem.* **271**, 12438–12444
- 25 Zweifach, A. and Lewis, R. S. (1995) Rapid inactivation of depletion-activated calcium current (I_{CRAC}) due to local calcium feedback. *J. Gen. Physiol.* **105**, 209–226
- 26 Grinstein, S. and Cohen, S. (1987) Cytoplasmic $[\text{Ca}^{2+}]$ and intracellular pH in lymphocytes. Role of membrane potential and volume-activated Na^+/H^+ exchange. *J. Gen. Physiol.* **89**, 185–213
- 27 Reed, P. and Lardy, H. (1972) A23187: a divalent cation ionophore. *J. Biol. Chem.* **247**, 6970–6977
- 28 Nicholls, D. and Budd, S. (2000) Mitochondria and neuronal survival. *Physiol. Rev.* **80**, 316–360
- 29 Green, D. and Reed, J. (1998) Mitochondria and apoptosis. *Science* **281**, 1309–1312

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