Intracellular Ca\textsuperscript{2+} Release Triggers Translocation of Membrane Marker FM1–43 from the Extracellular Leaflet of Plasma Membrane into Endoplasmic Reticulum in T Lymphocytes* 

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Stimulation of T cell receptor in lymphocytes enhances Ca\textsuperscript{2+} signaling and accelerates membrane trafficking. The relationships between these processes are not well understood. We employed membrane-impermeable lipid marker FM1–43 to explore membrane trafficking upon mobilization of intracellular Ca\textsuperscript{2+} in Jurkat T cells. We established that liberation of intracellular Ca\textsuperscript{2+} with T-cell receptor agonist phytohemagglutinin P or with Ca\textsuperscript{2+}-mobilizing agents ionomycin or thapsigargin induced accumulation of FM1–43 within the lumen of the endoplasmic reticulum (ER), nuclear envelope (NE), and Golgi. FM1–43 loading into ER-NE and Golgi was not mediated via the cytosol because other organelles such as mitochondria and multivesicular bodies located in close proximity to the FM1–43-containing ER were free of dye. Intralumenal FM1–43 accumulation was observed even when Ca\textsuperscript{2+} signaling in the cytosol was abolished by the removal of extracellular Ca\textsuperscript{2+}. Our findings strongly suggest that release of intracellular Ca\textsuperscript{2+} may create continuity between the extracellular leaflet of the plasma membrane and the lumenal membrane leaflet of the ER by a mechanism that does not require global cytosolic Ca\textsuperscript{2+} elevation.

Membrane trafficking plays an important role in shaping T cell responses. For example, it is well established that engagement of TCR\textsuperscript{1} with an antigen triggers fast trafficking of the receptor between the PM and endocytic organelles, resulting in serial TCR engagements and an amplification of intracellular signaling (1). TCR stimulation is followed by a fast liberation of Ca\textsuperscript{2+} from Ca\textsuperscript{2+}-storing organelles, such as ER and Golgi (2, 3). Alteration of Ca\textsuperscript{2+} homeostasis induces a variety of cellular responses ranging from changes in cell shape and motility to activation of transcription factors (2). However, it is not clear whether changes in intracellular Ca\textsuperscript{2+} dynamics affect membrane trafficking in T lymphocytes.

Styryl dye FM1–43 is a fluorescent membrane marker that reversibly partitions into but does not penetrate biological membranes (4). We have shown previously that FM1–43 was readily internalized from the PM into the endocytic compartments of human T cells by constitutive endocytosis (5). We also demonstrated that Ca\textsuperscript{2+} ionophore ionomycin (Iono) or thapsigargin (Tg), a sarco-endoplasmic reticulum calcium ATPase pumps blocker, facilitated FM1–43 internalization (5). These findings suggested that elevation of cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) might accelerate the rate of endocytosis in T cells. However, an EM study performed previously in the Xenopus larvae hair cells revealed the presence of FM1–43 within non-endocytotic organelles such as ER, NE, and mitochondria (6). These results led to a suggestion that FM1–43 can penetrate the PM and diffuse through the cytosol. To further investigate the nature of enhanced FM1–43 internalization following liberation of intracellular Ca\textsuperscript{2+} in T cells, we studied subcellular distribution of FM1–43 using correlative fluorescence and electron microscopy.

We found that stimulation of Jurkat T cells with TCR agonist, phytohemagglutinin P (PHA), or with the Ca\textsuperscript{2+}-mobilizing agents, Tg or Iono, produced FM1–43 translocation from the PM into the lumen of peripheral ER followed by the dye spreading into NE and Golgi. This effect did not require global elevation in [Ca\textsuperscript{2+}]\textsubscript{i}, since it was observed in the absence of extracellular Ca\textsuperscript{2+}. The presence of FM1–43 in the lumen of the ER, NE, and Golgi appeared not to be mediated via the cytosol as other organelles (mitochondria and multivesicular bodies (MVB)) remained free of dye. Our findings suggest that intracellular Ca\textsuperscript{2+} release may generate continuity between the ER and the PM, resulting in direct membrane exchange between those organelles.

MATERIALS AND METHODS

Cell Culture and Chemicals—Human T-cell line, Jurkat E6-1 (ATCC, Manassas, VA), was cultured in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Omega Scientific Inc., Tarzana, CA). Cells were maintained in suspension in 5% CO\textsubscript{2} at 37 °C and passaged every 2 days. Unless indicated, all chemicals were from Sigma. Ionomycin and thapsigargin were from Calbiochem. FM1–43 and Fura-2/AM were from Molecular Probes (Eugene, OR). Modified Tyrode’s solution contained (in mM): 130 NaCl, 5.6 KCl, 1 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 10 HEPES, 10 H-bisulfate; pH 7.3. In Ca\textsuperscript{2+}-free solution, Ca\textsuperscript{2+} was omitted, and 5 mM EGTA was added.

FM1–43 Fluorescence—Cells were plated on poly-L-lysine-coated 35-mm glass bottom microwell dishes (MatTek, Ashland, MA) and incubated with 5 μM FM1–43 as specified under “Results.” Cells were stimulated with 10 μg/ml PHA, 1 μM Tg, or 1 μM Iono. All experiments were done at 37 °C. After incubation, cells were washed five times with Tyrode’s solution and fixed with 4% paraformaldehyde for 30 min at 21 °C. After washing, cells were placed onto the stage of an Axiovert 100 microscope (Carl Zeiss, Gottingen, Germany), and fluorescence images were acquired with LSM 510 laser scanning confocal imaging system (Carl Zeiss) using a ×63/1.4 or ×100/1.4 oil immersion Zeiss objectives. For FM1–43 fluorescence, 488 nm excitation and 505–550 nm emission

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† To whom correspondence should be addressed: Dept. of Physiology and Membrane Biology, University of California, Davis, One Shields Ave., Davis, CA 95616. Tel.: 530-754-4454; Fax: 530-752-5423; E-mail: affomina@ucdavis.edu. The abbreviations used are: TCR, T cell receptor; Iono, ionomycin; MVB, multivesicular bodies; ER, endoplasmic reticulum; NE, nuclear envelope; PCRF, photoconversion reaction product; PHA, phytohemagglutinin P; FM, plasma membrane; Tg, thapsigargin; EM, electron microscopy.
filters were used. A pinhole was opened to collect fluorescence from 2-μm optical slice. MetaMorph v6.1 software (Universal Imaging, Downingtown, PA) was used to determine the pixel densities of FM1–43 fluorescence as shown below (see Fig. 1).

Photoconversion and EM Imaging—Photoconversion experiments were performed as described previously (5). Briefly, cells were incubated with FM1–43 for different times and conditions. Cells were fixed in 2% glutaraldehyde and then placed into oxygenated sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA) containing 1 mg/ml diaminobenzidine on the stage of Axiovert 100 microscope (Carl Zeiss). Cells were illuminated by a 100-watt mercury lamp through a Zeiss/40/1.3 objective for 10–15 min. Samples were processed for EM, and thin sections were examined with a Phillips 400 electron microscope. The total number of cells and number of cells containing the photoconversion reaction product were counted within 6–8 randomly selected sections. Each experiment was repeated 1–3 times.

Cytosolic Ca²⁺ Measurements—Changes in [Ca²⁺]i were recorded as described earlier (7) using Fura-2 acetoxymethylester (Fura-2/AM). Briefly, cells were loaded with 1 μM Fura-2 acetoxymethylester (Fura-2/AM) for 5 min in Tyrode’s solution, washed, and incubated for an additional 30 min. Fluorescence images were acquired from adherent cells with SenSys CCD camera (Roper Scientific, Tucson, AZ) using a ×40/1.15 water immersion Olympus objective. Lambda DG-4 filter changer (Sutter Instrument, Novato, CA) was used for switching between 340 and 380 nm excitation filters. Experiments were done at room temperature. Solution exchange was performed via a gravity-driven perfusion system. Data acquisition and analysis were performed using MetaFluor v6.1 software (Universal Imaging). Cytosolic concentrations of free Ca²⁺ ([Ca²⁺]i) were estimated from Fura-2 calibration as described previously (8).

Data Analysis—Data analysis and statistics were performed with Microcal Origin 7.0 software (Microcal Software, Inc.). All statistical data are presented as mean ± S.E.

RESULTS

FM1–43 Stains the Nuclear Membrane after Mobilization of Intracellular Ca²⁺—Incubation of Jurkat T cells with FM1–43 resulted in spontaneous dye accumulation within the cytosol (Fig. 1, A–C) similarly to what was described previously for human T lymphocytes (5). Stimulation of TCR with PHA enhanced FM1–43 accumulation (Fig. 1, D–F) in parallel with
elevation in \([\text{Ca}^{2+}]_i\) (Fig. 1G). High resolution imaging revealed the existence of a bright ring of FM1–43 fluorescence originating from the perinuclear area in PHA-treated cells (Fig. 1, E and F). Direct stimulation of intracellular \(\text{Ca}^{2+}\) release with Iono or Tg (not shown) produced similar results. Removal of extracellular \(\text{Ca}^{2+}\) significantly reduced \([\text{Ca}^{2+}]_i\) elevation in response to \(\text{Ca}^{2+}\)-mobilizing agents (Fig. 1G) but had little effect on the enhancement of perinuclear fluorescence (Fig. 1H). Overall, stimulation of \(\text{Ca}^{2+}\) release in the presence or absence of extracellular \(\text{Ca}^{2+}\) resulted in an ~2-fold increase in average amplitude of perinuclear FM1–43 fluorescence. These data suggested that stimulation of \(\text{Ca}^{2+}\) release triggered FM1–43 accumulation at the perinuclear area by a mechanism that did not require global \([\text{Ca}^{2+}]_i\) elevation.
Staining of the intracellular organelles including NE with FM1–43 may occur as a result of the “flip-flop” of FM1–43 through the PM and partitioning into the cytosolic leaflet of organelle membranes. To determine an origin of the intracellular FM1–43 fluorescence, we subjected FM1–43-loaded cells to a photoconversion procedure that has been shown to result in a formation of an electron-dense photoconversion reaction product (PCRP) in place of a fluorophore, such as FM1–43 (9, 10). This allowed EM investigation of the subcellular FM1–43 distribution.

Precipitation of PCRP within the ER, NE, and Golgi Lumen after Ca²⁺ Liberation—EM study of non-stimulated Jurkat T cells incubated with FM1–43 (Control) revealed the presence of PCRP within endocytic organelles (early and late endosomes and MVB, Fig. 2A), which closely resembled endocytic compartments in activated human T lymphocytes (5). The ER (Fig. 2Ai) and Golgi (Fig. 2Aii) remained free of dye, indicating that they were not directly connected with the constitutive endocytic pathway. These results demonstrated that FM1–43 did not penetrate the PM of unstimulated Jurkat T cells but spread within the lumen of the endocytic organelles due to constitutive endocytosis.

Consistent with the appearance of FM1–43 fluorescence at the perinuclear area (Fig. 1E), EM study revealed an accumulation of PCRP within the ER, NE, and Golgi of PHA-stimulated cells in the presence or absence of extracellular Ca²⁺ (Fig. 2, B and C). Similar effects were observed after stimulation with Tg (Fig. 3A) or Iono (Fig. 3B). The percentage of cells displaying strong PCRP precipitation within the ER lumen increased from 5% at unstimulated conditions to 15–30% after stimulation of Ca²⁺ release in the presence or absence of extracellular Ca²⁺ (Fig. 3C). Thus, EM study suggested that stimulation of Ca²⁺ release triggered translocation of FM1–43 from the PM into the lumen of the ER, NE, and Golgi.

Accumulation of FM1–43 within the ER, NE, and Golgi Is Not Mediated via the Cytosol—In unstimulated cells, the PCRP was frequently observed within the mitochondria (Fig. 2A). Previously, we reported that in human T cells, some mitochondria precipitated PCRP even in the absence of FM1–43 (5). The high oxidative potential of the mitochondria might account for
FIG. 5. FM1–43 spreads from the PM via the peripheral ER toward NE and Golgi. A, cell with partial staining of ER and NE. i–iii, enlargements of the corresponding boxed areas in the whole cell image. PRCP (black staining) is present in the peripheral ER (arrowheads) and adjacent NE in Ai. PCRP is absent in perinuclear ER, Golgi (G) in Ai, and NE (arrows) in Ai.ii. E, endosome; N, nucleus. B and C, ER fragments located in close apposition to the PM. Note the bound ribosomes (arrows) in B and PCRP within the ER in C. Bars, 2 μm in A; 500 nm in Ai–Aiii; 200 nm in B and C.

the nonspecific PCRP precipitation. Although at unstimulated conditions, PCRP was not found within the ER, NE, and Golgi, a nonspecific PCRP precipitation could be induced by changes in the intralumenal environment within those organelles upon Ca\(^{2+}\) mobilization. To eliminate this possibility, we performed prolonged photoconversion (20–40 min) after cells were incubated with Iono in the absence of FM1–43. This resulted in an enhanced precipitation of PCRP within mitochondria and at the PM but not at the ER, NE, and Golgi (Fig. 4A). Only a small fraction of cells (Fig. 3C) displayed nonspecific precipitation of PCRP within the ER. Thus, changes in intralumenal environment after Ca\(^{2+}\) liberation could not account for the appearance of PCRP within the ER, NE, and Golgi. We concluded that the presence of PCRP indicated the presence of FM1–43 within the lumen of the ER, NE, and Golgi.

The remaining question was how FM1–43 appeared within non-endocytic organelles. We demonstrated that mitochondria precipitate PCRP in the absence of FM1–43. Consistent with functional heterogeneity of the mitochondria within a cell (11), typical photoconversion produced PCRP precipitation in only some mitochondria (Figs. 2 and 3) or did not produce it at all (Fig. 4B). In those cells, regions of the ER and Golgi containing PCRP were frequently located at close proximity to PCRP-free mitochondria (Fig. 4Bi). In addition, Jurkat T cells usually contained large MVB that did not accumulate FM1–43. Those organelles were often located in close proximity to the PCRP-containing ER and Golgi (Fig. 4C). These observations were difficult to reconcile with the hypothesis that FM1–43 “flip-flopped” through the PM and spread via the cytosol as in that case, PCRP should be present at the cytosolic leaflet of the membranes of all organelles. We concluded that upon Ca\(^{2+}\) liberation FM1–43 was translocated from the PM directly into the lumen ER-NE and Golgi bypassing the cytosol.

**FM1–43 Spreads from the Peripheral ER toward NE and Golgi**—The ER is connected with the NE lumenally (12) and with Golgi by vesicular trafficking (13). Therefore, FM1–43 should spread within the lumen of ER, NE, and Golgi even if only one of those organelles establishes continuity with the PM. To determine the direction of the FM1–43 spreading within intracellular organelles, we investigated cells with partial staining of the intracellular compartments (Fig. 5). In those cells, PCRP was always located within the peripheral ER (Fig. 5Ai) and neighboring NE, whereas regions of the ER, Golgi (Fig. 5Aii), and NE (Fig. 5Aiii) located in the middle of the cell remained free of dye. Because a number of cells displayed FM1–43 loading into the peripheral ER but not into the Golgi and all cells with stained Golgi displayed PCRP within the ER, we concluded that FM1–43 spread from the peripheral ER toward the NE and Golgi.

Our EM study revealed the existence of tubular membrane compartments adjacent to the PM (Fig. 5B). The presence of bound ribosomes at some regions of those compartments indicated that they were parts of the ER. Smooth areas of the same compartment were docked to the PM. At membrane contact sites, the distances between ER and PM were ~10 nm, implying that interactions at the molecular level could take place. Docking of the ER-like compartments to the PM remained unaffected after stimulation of Ca\(^{2+}\) release (Fig. 5C), implying that FM1–43 transfer may be mediated via ER-PM contact sites.

**DISCUSSION**

Our study demonstrated that in Jurkat T lymphocytes, activation of intracellular Ca\(^{2+}\) release by stimulation of membrane receptors or by Ca\(^{2+}\)-mobilizing agents produced a fast loading of membrane-bound lipid marker FM1–43 from the extracellular leaflet of the PM into the lumen of intracellular organelles: ER, NE, and Golgi. The presence of FM1–43 within those compartments was detected with fluorescent microscopy as the FM1–43 fluorescence from the NE and with EM as
the PCRP precipitation within the lumen of ER, NE, and Golgi. These observations are consistent with previously observed FM1–43 loading into the ER lumen of Xenopus larvae hair cells (6). Our EM study demonstrated that cells with FM1–43 loading into ER, NE, and Golgi lumen did not display any ultrastructural changes such as chromatin condensation, nuclear fragmentation, cell shrinkage, mitochondrial swelling, or inflammation of the ER and NE that were found to be associated with different stages of T cell apoptosis or necrosis (14–16). These observations strongly suggest that the ability to accumulate FM1–43 within ER lumen upon stimulation is the property of normal viable cells.

In an earlier study, Nishikawa and Sasaki (6) found PCRP in both the ER and the mitochondria. That was interpreted as a non-selective staining of intercellular organelles and led the authors to the conclusion that FM1–43 penetrated the PM and spread through the cytosol. Two lines of evidence indicated that FM1–43 loading into ER, NE, and Golgi in Jurkat T cells was not mediated via the cytosol. First, our previous results (5) and the present study (Fig. 3A) demonstrated PCRP precipitation by mitochondria in the absence of FM1–43, challenging the assumption that an existence of PCRP within the mitochondria validates the presence of FM1–43 within those organelles. Second, FM1–43-filled ER regions were located in close apposition to other organelles that did not contain FM1–43 (Fig. 4, B and C), indicating that FM1–43 was selectively targeted into the ER, NE, and Golgi lumen. These data strongly suggest that upon Ca^{2+} liberation, continuity may be established between the extracellular leaflet of the PM and the luminal membrane leaflet of the ER, allowing a direct flow of the membrane-bound marker from the PM into the lumen of interconnected intracellular organelles.

Our data demonstrated that FM1–43 first appeared at the peripheral ER and then spread toward the NE and Golgi. ER and PM membrane contact sites were present in unstimulated cells (Fig. 5B) and remained intact after stimulation of Ca^{2+} release and FM1–43 loading into ER lumen (Fig. 5C). These observations suggest that FM1–43 transfer into the ER lumen could occur via ER-PM contact sites. Smooth ER was found in close apposition to the PM in several cell types. There is increasing evidence that non-vesicular transport of small lipids between the cytosolic membrane leaflets of the ER and other organelles is concentrated at the membrane contact sites and is mediated by carrier proteins (17). This transport mechanism, however, appears to be different from the FM1–43 transport mechanism into the ER since the latter does not involve the cytosolic membrane surfaces.

Recently, fusion of the ER with the PM and an exchange of proteins was described in macrophages during phagocytosis (18). It was established that stimulation of macrophage lipopolysaccharide receptors activated phospholipase C, which in turn resulted in the production of inositol 1,4,5-trisphosphate and intracellular Ca^{2+} liberation (19). Therefore, we hypothesize that in T lymphocytes, intracellular Ca^{2+} release could trigger fusion of ER with the PM, and FM1–43 might laterally diffuse from the PM into the ER lumen. Although the exact mechanism underlying molecular exchange between the PM and ER lumen requires further investigation, the structural organization of the ER (20) implies that it may serve as a vehicle for spreading molecular signals from the extracellular space to intracellular organelles.

Quantification of the photoconversion experiments revealed that 15–30% of cells precipitated PCRP within the ER. Although this was a dramatic increase when compared with the control cell population (≪5%), it appeared that only a fraction of cells may have established the continuity between the ER and PM upon stimulation. It should be noted, however, that the photoconversion procedure may not reveal all cells that accumulated FM1–43 within the ER since PCRP precipitation may be affected by the concentration of fluorophore and environmental factors. It is conceivable that only a fraction of cells at the certain physiological state may establish strong ER-PM links and may accumulate a sufficient amount of the FM1–43 within the ER lumen to produce PCRP precipitation, whereas cells with low FM1–43 concentration may fail to precipitate PCRP. Therefore, the exact incidence of the FM1–43 loading into ER lumen in total cell population and the specific characteristics of the cells that display strong dye accumulation upon stimulation remain to be determined.

Weak dependence of FM1–43 loading into ER-NE-Golgi lumen from [Ca^{2+}]_i implied that Ca^{2+} concentration within ER may be the key factor that triggered ER-PM interactions. Our data suggest that ER-PM fusion could take place upon store depletion; therefore, it is conceivable that soluble molecules can follow the same pathway as a membrane-bound dye. Because ER, NE, and Golgi are major constituents of the intracellular Ca^{2+} store (2, 3, 21), the ER-PM fusion sites may allow Ca^{2+} entry from the extracellular space directly into the store lumen. Although the store can be effectively replenished from the cytosol via Ca^{2+}-ATPase (22), a recent study demonstrated that in Jurkat T cells, the store can be refilled in the absence of sarco-endoplasmic reticulum calcium ATPase pump activity (23). Direct Ca^{2+} flow from the extracellular space into the ER might complement the Ca^{2+}-ATPase refilling pathway and might be important for both Ca^{2+} signaling and protein handling within intracellular organelles.

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