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Ca^{2+} ionophores trigger membrane remodeling without a need for store-operated Ca^{2+} entry

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Abstract

Calcium (Ca^{2+}) ionophores are the most effective agents able to elicit rapid membrane remodeling in vitro. This process exposes aminophospholipids at the surface of platelets and blood cells, thus providing a catalytic surface for coagulation. To explore the underlying mechanism, we examined if cytosolic Ca^{2+} ($[Ca^{2+}]_i$) increase through store-operated Ca^{2+} entry (SOCE) was necessary for the potent effect of ionophores. Recent studies have demonstrated that the Ca^{2+}-ATPase inhibitor thapsigargin, although able to elevate $[Ca^{2+}]_i$ through SOCE, does not trigger the rapid membrane remodeling. However, it was not known if the additional effect of ionophores to promote the process required SOCE or could it occur independently. We took advantage of two mutant B lymphoblast cell lines, characterized either by defective SOCE or altered membrane remodeling, to simultaneously assess $[Ca^{2+}]_i$ increase and membrane remodeling in the presence of ionophores or thapsigargin. Results imply that ionophores trigger membrane remodeling without the requirement for a functional SOCE.

Keywords: Primary immunodeficiency; Scott syndrome; Capacitative calcium entry; Ionophores; FM1-43; Phosphatidylserine

Phosphatidylserine (PS) is a constitutive aminophospholipid of the plasma membrane, mostly sequestered in the inner leaflet of resting cells [1]. When accessible at the outer surface of apoptotic or activated cells and derived-membrane microparticles, PS triggers at least two key functions, as catalyst for the assembly of the prothrombinase and other coagulation complexes [2,3] and determinant for phagocytic clearance [4,5]. It should however be emphasized that PS transmembrane migration (PS scrambling) occurs within minutes in cells participating in procoagulant reactions, but within hours in apoptotic cells, suggesting dependence on different mechanisms. The identification and characterization of the mechanism(s) underlying the rapid membrane remodeling is crucial to the study of how blood coagulation is modulated. PS scrambling was first detected and studied in platelets [6], and also occurs in erythrocytes [7,8] and immortalized B or T lymphocytes [9–12].

PS scrambling is dependent on an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) following cell stimulation or triggered using some Ca^{2+} mobilizing agents [2]. Inhibition of Ca^{2+} influx also abolishes agonist-induced PS externalization in a pluripotent lineage with megakaryoblastic properties [13]. Comparison of different agents revealed that calcium ionophores, ionomycin and A23187, are very effective in inducing PS exposure in platelets in suspension compared to the various agonists also able to

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promote this process, such as thrombin, collagen or their combination [14,15]. The still unsolved mode of action of ionophores remains therefore important to explore, based on the hypothesis that it mimics a physiological process essential for coagulation.

The essential role of the membrane remodeling process in hemostasis has been illustrated by the existence of Scott syndrome, a rare hemorrhagic hereditary disorder of swift PS egress to the cell surface of stimulated platelets and other blood cells, as seen in vitro when challenged by addition of Ca^{2+} ionophores A23187 or ionomycin [10,16–18]. Other platelet functions, such as secretion, granule content or aggregation, were shown to be normal. The Scott cell phenotype has also been observed in Epstein–Barr virus (EBV)-immortalized B lymphocytes (Scott B lymphoblasts) [9,10,17,19]. Functional studies performed with Scott B lymphoblasts derived from the three patients as yet identified demonstrate the same deficiency of rapid membrane response and suggest alteration of Ca^{2+}-dependent transduction pathway(s) [10,17,20].

Whether $[Ca^{2+}]_i$ increase by itself is sufficient to trigger PS scrambling remains a matter of controversy. Ca^{2+} influx from the extracellular medium and release from intracellular stores are the mechanisms of $[Ca^{2+}]_i$ increase within the cell. Store-operated Ca^{2+} entry (SOCE), activated following depletion of the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores, is one way of inducing sustained $[Ca^{2+}]_i$ increase [21,22]. In vitro, the pharmacological agent thapsigargin inhibits the sarco-endoplasmic reticulum Ca^{2+} ATPases responsible for Ca^{2+} uptake into intracellular stores, thus depleting Ca^{2+} stores and activating Ca^{2+} influx through SOCE channels. Although it has been published that ionophores can mediate SOCE at submicromolar concentrations [23,24], it has not yet been established whether these agents provoke membrane remodeling through SOCE or if defective PS exposure in response to ionophores in Scott cells is related to a diminished SOCE [25]. Using fluorescently labeled annexin V, the styryl dye FM1-43, and prothrombinase activity assays, recent studies suggested that thapsigargin, even in conditions allowing high $[Ca^{2+}]_i$ threshold, does not promote membrane remodeling of lymphocytic cells [12,17,26].

In this investigation, we examined whether Ca^{2+} ionophore-induced membrane remodeling depends on SOCE activation, without making use of inhibitors of signaling pathways linked to SOCE. We determined the $[Ca^{2+}]_i$ increases and membrane remodeling in the presence of Ca^{2+} ionophores in a mutant B lymphoblast cell line derived from a patient with a very rare primary T-cell immunodeficiency (primary immunodeficiency B lymphoblasts), characterized as having a defective SOCE pathway [27,28]. This SOCE defect had been also observed in T lymphocytes, platelets, granulocytes, and fibroblasts of the patient.

The styryl dye FM1-43, which has been recently demonstrated to monitor rapid membrane remodeling associating PS exposure as an alternative to annexin V [11,12,29] was used in combination with Fura2 in video imaging experiments to simultaneously monitor membrane remodeling and Ca^{2+} signals at single cell levels. Ca^{2+} influx was activated by challenging the primary deficiency, the Scott, and control B lymphoblasts, with either Ca^{2+} ionophores (A23187 or ionomycin were alternatively used) or with thapsigargin. As shown in previous studies, although thapsigargin is able to promote calcium influx through SOCE pathway, it does not directly elicit the membrane remodeling. Ca^{2+} ionophores triggered the rapid membrane remodeling in the primary immunodeficiency B lymphoblasts, suggesting that the mechanism does not need a functional SOCE. In contrast, there was a defective membrane reactivity in response to ionophores in Scott cells as previously shown. These data therefore clearly suggest for the first time that Ca^{2+} ionophores activate a signaling additional or independent of SOCE, which underlies the plasma membrane remodeling and would be deficient in Scott cells.

Materials and methods

Materials. The sources of materials were as follows: RPMI 1640 glutamax-1 culture medium, fetal calf serum (FCS), non-essential amino acids, and gentamicin were from Life Technologies (Paisley, UK), X-VIVO 15 culture medium was from Cambrex (Walkersville, MD, USA), Ca^{2+} ionophore A23187 was from Calbiochem (La Jolla, CA, USA), thapsigargin was from Alomone Labs (Jerusalem, Israël), and FM1-43 and Fura-2/AM (Fura-2) were from Molecular Probes Europe (Leiden, The Netherlands). All other reagents were from Sigma Chemical (St. Louis, MO, USA). 2-aminoethyl diphenylborate (2-APB) was a gift from Yves Chapleur (CNRS, UMR 7565, Nancy, France).

Cell lines and culture conditions. The cases of Scott syndrome or primary T-cell immunodeficiency have been described in other reports [10,27]. B lymphocytes from the patient, presumably homozygous for the mutation, with Scott syndrome (Scott B lymphoblasts) and from an unrelated healthy donor (control B lymphoblasts) were infected by EBV to establish cell lines and characterized as previously detailed [10]. EBV-transformed B lymphocytes from the T-cell immunodeficiency patient (primary immunodeficiency B lymphoblasts) were previously described [28]. All patients and other donors gave fully informed consent. B lymphoblasts were routinely seeded at 3×10^5 cells/ml in humidified 5% CO_2 atmosphere at 37 °C in the respective media traditionally used after their immortalization. Scott B lymphoblasts were expanded in X-VIVO 15 culture medium (extracellular Ca^{2+} concentration $[Ca^{2+}]_O = 1.8$ mM) and primary immunodeficiency B lymphoblasts in RPMI 1640 glutamax-1 medium ($[Ca^{2+}]_O = 0.4$ mM) supplemented with 1% (v/v) non-essential amino acids, sodium pyruvate (1 mM), gentamicin (20 μg/ml), and 10% (v/v) heat-inactivated FCS. Control B lymphoblasts from the unrelated healthy donor were cultured either in X-VIVO 15 medium or in supplemented RPMI 1640 medium. The results were compared between the Scott or primary immunodeficiency B lymphoblasts and the control cells grown in their respective culture medium.

Dual measurement of $[Ca^{2+}]_i$ and PS exposure by fluorescence imaging videomicroscopy. B lymphoblasts (1×10^6 cells/ml in X-VIVO 15 cell medium) were allowed to settle onto poly-L-lysine-coated dishes

(Bioptechs, Butler, PA, USA) for 40 min at 37 °C and were meanwhile loaded with 4 μM of the Ca^{2+} probe Fura-2/AM. Cells were washed three times with saline solution containing 116 mM NaCl, 5.6 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM $NaHCO_3$, 1 mM NaH_2PO_4, 20 mM Hepes, pH 7.3, or without $CaCl_2$ in the presence of 100 μM EGTA depending on the experimental procedure. FM1-43 (5 μM) was added to the solution and cells were kept at 37 °C in a temperature-controlled dish fixed above a warmed epifluorescence 40× oil objective (Bioptechs, Butler, PA, USA). FM1-43 fluorescence increased up to a steady state reached after approximately 5 min followed by a slow increase of about 10% over a period of 10 min. The excitation light was supplied by a high pressure 100 W xenon arc lamp, and the 340, 380, and 470 nm wavelengths were selected by a monochromator (Cairn Research, Faversham, Kent, UK). Emitted light was collected with a 515 nm dichroic mirror and a 520 nm longpass filter. Fluorescence images were collected every 2 s by a Sensicam QE CCD camera (PCO Computer Optics GmbH, Kelheim, Germany), digitized, and integrated in real time by an image processor (Metafluor, Princeton, NJ, USA). Background fluorescence signals at 340, 380, and 470 nm were collected at the same rate from cell-free regions and subsequently subtracted from respective fluorescent images. Increases in FM1-43 fluorescence were measured and analyzed from drawing regions of interest over single cells. Elevation in FM1-43 fluorescence was expressed as ratio ($\Delta F = (F - F_O)/F_O$, where F and F_O are the values during a response and at rest, respectively). Increases in $[Ca^{2+}]_i$ were expressed as ratios between 340 and 380 nm fluorescence signals of Fura-2 measured during a response divided by the ratio measured in resting conditions, i.e., before the addition of an agent (ΔRatio $= (R - R_O)/R_O$, where R and R_O are the values during a response and at rest, respectively). Thapsigargin, A23187, ionomycin or $CaCl_2$ were added in the dish using a micropipette and recording medium was gently stirred to achieve final dilution. Addition of A23187 evoked an increase of about 3% in the fluorescence signals at both excitation wavelengths and therefore no change in the ratio. Image recording was paused meanwhile.

Statistical analysis. Results are expressed as means ± standard error (SE). Cells similarly treated in experiments performed in different cell plates and on different days were considered to average. Unpaired Student's t test was used for statistical analysis. A value of $P < 0.05$ was considered significant.

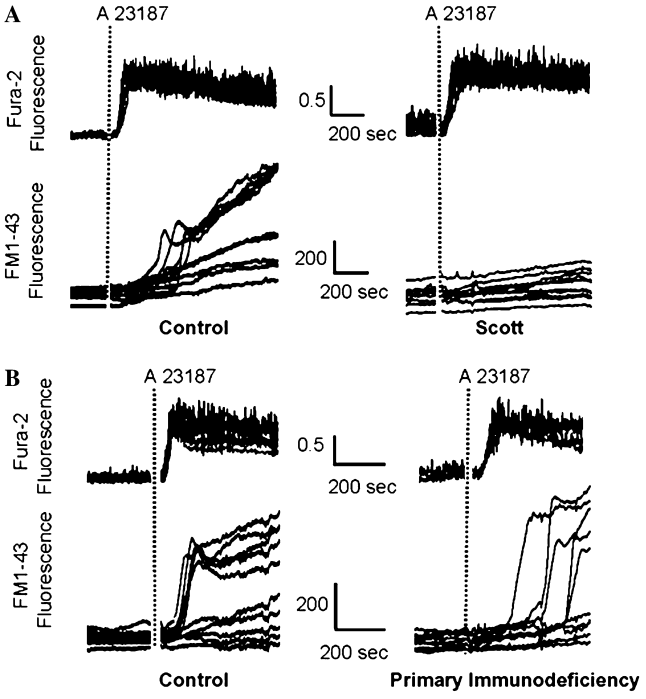

Fig. 1. Effect of Ca^{2+} ionophore A23187 on Ca^{2+} signal and membrane remodeling in B lymphoblasts. Representative single-cell traces showing the effect of A23187 (2 μM) in the presence of 1.8 mM $[Ca^{2+}]_O$ on Fura2 (ratios between values at 340 and 380 nm, upper traces) and FM1-43 (arbitrary units, lower traces) fluorescence signals, as a function of time. The control cell line was cultured in either one of the media respectively used for the mutant cells. (A) Responses of Scott and control B lymphoblasts grown in X-VIVO 15 cell, (B) Responses of primary immunodeficiency and control B lymphoblasts grown in supplemented RPMI 1640 medium. Although the existence of bursts for FM1-43 traces was reproducible, the overall signals reflected individual recordings for a given experiment rather than specific differences between cultures (see the control cells signals in A and B).

Results

A23187 elicits membrane remodeling in primary immunodeficiency B lymphoblasts

The fluorescent styryl dye FM1-43, used as a probe of exo- and endo-cytosis [30], has also been shown to report membrane remodeling, including phospholipid scrambling, more rapidly than the most commonly used annexin V probe [11,12,29]. The effect of ionophore A23187 on Fura-2 and FM1-43 fluorescence in Scott and primary immunodeficiency B lymphoblasts was analyzed in the presence of 1.8 mM extracellular Ca^{2+} concentration ($[Ca^{2+}]_O$) in comparison with the control B lymphoblast cell line (Fig. 1). In all cell lines, 2 μM A23187 provoked a rapid and sustained increase in Fura-2 signals, which was simultaneous in all cells on an individual plate. FM1-43 fluorescence responses, however, exhibited different signals. In control B lymphoblasts, A23187 triggered a response constituted of a slow increase in FM1-43 fluorescence followed for 43% of the cell traces (over a period of 10 min, $n = 28$) by a sharp burst of large amplitude ($\Delta F = 4.03 \pm 0.43$ at the peak), indicating rapid membrane remodeling individually at different times (Figs. 1A and B and figure in Supplementary Material). In comparison, no burst in FM1-43 fluorescence could be detected in Scott cells ($n = 18$) during the time of measurement (up to 18 min), correlating with the known defect of membrane phospholipid reorganization in these cells and validating the use of FM1-43 to monitor membrane remodeling (Fig. 1A). When considering the nonbursting traces, a similar increase in FM1-43 fluorescence was observed in Scott and control B lymphoblasts, reaching respectively ΔF values of 0.47 ± 0.08 and 0.64 ± 0.1 at 5 min after the addition of A23187. For similar threshold value of Fura-2 fluorescence increase at the influx onset, control B lymphoblasts individually demonstrated or not the membrane remodeling 10 min after A23187 addition. Wurth and Zweifach also reported for Jurkat T cells that only a fraction of cells exhibited a ionophore-stimulated membrane remodeling in the presence

of 1 or 2 mM $[Ca^{2+}]_O$ [12]. These authors further showed that increasing ionophore concentration or the $[Ca^{2+}]_O$ augmented the proportion of responding cells. Rapid membrane remodeling was never observed in individual Scott B lymphoblasts even for Fura-2 fluorescence increases correlating with occurrence of this process in control cells.

FM1-43 fluorescence signals of primary immunodeficiency ($n = 64$) and control ($n = 63$) B lymphoblasts showed comparable patterns in response to A23187 (Fig. 1B). Again, the observed membrane remodeling was an "all or none" mechanism, appearing soon after but not concomitantly with Ca^{2+} entry. The burst peak occurred on average at 296 ± 25 and 230 ± 14 s ($P < 0.05$) after A23187 addition, for primary immunodeficiency and control cells, respectively. The averaged intensities of FM1-43 fluorescence rises, at the bursts, were equivalent ($\Delta F = 3.21 \pm 0.25$ and 3.14 ± 0.15, respectively). Upon further addition of 2 μM ionomycin, all the primary immunodeficiency and control B lymphoblasts traces exhibited a burst in FM1-43 fluorescence (data not shown), demonstrating that all cells were potentially able to undergo the rapid membrane remodeling.

Thapsigargin does not promote the rapid membrane remodeling in B lymphoblasts

To further explore the role of Ca^{2+} influx related to SOCE on Fura-2 and FM1-43 fluorescence, the effect of thapsigargin was analyzed. Addition of 1 μM thapsigargin in the presence of 1.8 mM $[Ca^{2+}]_O$ provoked a rapid increase in Fura-2 signals in Scott and control B lymphoblasts, followed by a plateau phase (Fig. 2). Different profiles were observed for primary immunodeficiency B lymphoblasts demonstrating a transient $[Ca^{2+}]_i$ increase indicating a normal intracellular store release followed by a diminished Ca^{2+}influx characteristic of the defective SOCE [28]. In these cells, the defect leading to the absence of Ca^{2+} influx is most probably located in the sequence of events which link the release of stores to the opening of the SOCE channels of the plasma membrane, including a dysfunction of the channels themselves. The amplitude of the $[Ca^{2+}]_i$ increase at 5 min after the addition of thapsigargin was reduced by 64% in primary immunodeficiency B lymphoblasts when compared to control cells. Contrasting with bursting traces observed in the presence of A23187 for control or primary immunodeficiency B lymphoblasts, no burst in FM1-43 fluorescence could be observed with thapsigargin treatment.

To examine whether the cells stimulated with thapsigargin in the presence of external Ca^{2+} still demonstrated the rapid membrane remodeling ability, Ca^{2+} ionophore was added to the thapsigargin-treated cells. As shown in Fig. 2, 2 μM ionomycin evoked a burst in

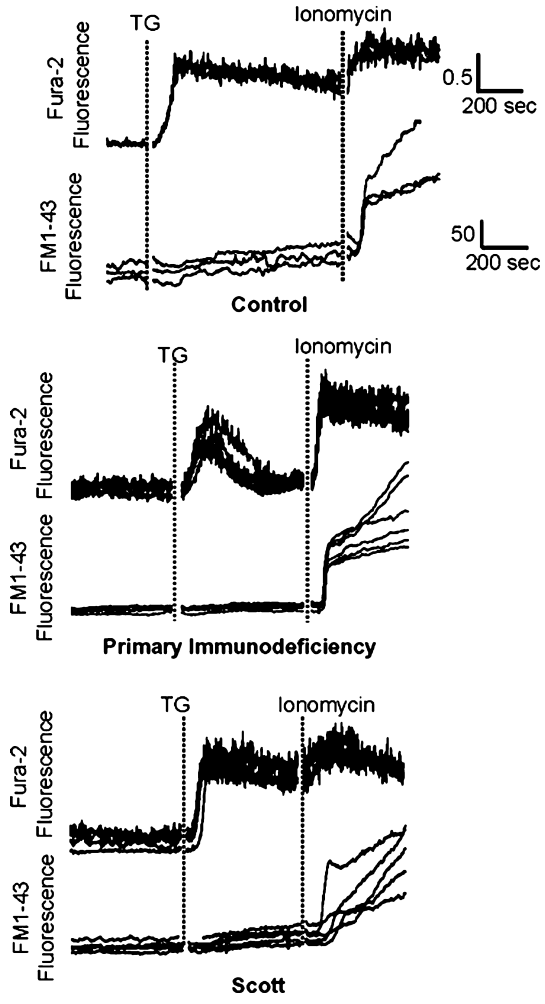

Fig. 2. Analysis of Fura-2 and FM1-43 fluorescence in B lymphoblasts following thapsigargin and ionomycin treatments. Representative single-cell signals as a function of time for control, primary immunodeficiency, and Scott B lymphoblasts show the effects of thapsigargin (TG, 1 μM) followed by ionomycin (2 μM) on Fura2 (upper traces) and FM1-43 (lower traces) fluorescence signals in the presence of 1.8 mM $[Ca^{2+}]_O$.

FM1-43 fluorescence for control and for primary immunodeficiency B lymphoblasts which was enhanced 6 times at 5 min as compared with that measured in the presence of 1 μM thapsigargin ($\Delta F = 3.66 \pm 0.35$ compared to 0.58 ± 0.06 without ionomycin). These results with primary immunodeficiency B lymphoblasts indicate again that rapid membrane remodeling induced by ionophores does not require a functional SOCE. Some traces for Scott B lymphoblasts also demonstrated an augmentation in FM1-43 fluorescence, testifying a membrane remodeling in these cells when ionophore is added after preincubation with thapsigargin.

To verify that our experimental conditions specifically corresponded to SOCE analysis, control cells were simultaneously treated with thapsigargin in the presence or absence of 2-APB, an inhibitor of Ca^{2+} and SOCE channels, in the presence of 1.8 mM

Table 1
Fura-2 and FM1-43 fluorescence measurements in control B lymphoblasts treated with thapsigargin[a]

$[Ca^{2+}]_O$ in the medium (mM)	Treatment	Fura-2 (ΔR)	FM1-43 (ΔF)
1.8	TG	1.56 ± 0.12	0.58 ± 0.06
1.8	TG + 2-APB	-0.21 ± 0.07 ***	0.29 ± 0.06*
1.8	DMSO	0.01 ± 0.01	0.07 ± 0.02
—	TG	-0.05 ± 0.01	0.16 ± 0.03
—	TG + $CaCl_2$[b]	2.06 ± 0.1###	0.57 ± 0.08###
—	$CaCl_2$	0.08 ± 0.02	0.09 ± 0.01

[a] Simultaneous measurements of Fura-2 and FM1-43 fluorescences for single control B lymphoblasts cultured in X-VIVO 15 medium determined at 5 min after reagents' addition. Fluorescence increases were normalized to the basal fluorescence. The cells were stimulated in the presence or absence of 1.8 mM $[Ca^{2+}]_O$ with thapsigargin (TG,1 μM), or with 1 μM TG and 2-APB (50 μM).

[b] For experiments initiated in Ca^{2+}-free medium, 1.8 mM $[Ca^{2+}]_O$ was added after thapsigargin. Results are expressed as means of fluorescence ± SE of at least 11–31 traces.

* $P < 0.05$.

*** $P < 0.0001$ versus the corresponding values in the absence of 2-APB.

$P < 0.0001$ versus the corresponding values before the addition of 1.8 mM $[Ca^{2+}]_O$.

$[Ca^{2+}]_O$ [31]. Table 1 gives the fluorescence increases at 5 min after treatments. A transient increase in $[Ca^{2+}]_i$ was seen in the presence of 2-APB, corresponding to the release of Ca^{2+} from stores followed by a decrease due to SOCE inhibition (Ratio = 0.45 ± 0.08, compared to 1.95 ± 0.15 for thapsigargin-treated cell, at the peaks occurring before the 5 min time points data given in Table 1). A FM1-43 fluorescence of low range value observed for thapsigargin-treated cells was also decreased by 49% after 5 min of treatment in the presence of 2APB (Table 1). Second, FM1-43 and Fura-2 fluorescences were further examined in a Ca^{2+}-free medium after the addition of thapsigargin. A transient increase in $[Ca^{2+}]_i$ was again observed due to the stores' release (ΔRatio = 0.35 ± 0.05 at the peak) and the FM1-43 fluorescence was not significantly different from that measured in the presence of 2-APB (Table 1). Subsequent addition of 1.8 mM Ca^{2+} in the external solution, allowing Ca^{2+} entry via SOCE, enhanced Fura-2 and, very slightly, FM1-43 fluorescence. This study validated the SOCE analyses and confirmed the absence of bursting traces for FM1-43 fluorescence in these conditions.

Discussion

Since the rapid membrane remodeling occurs in primary immunodeficiency B lymphoblasts upon treatment with ionophores, it seems reasonable to assume that a functional SOCE is not needed for this process. This observation correlates with the fact that the patient did not demonstrate any bleeding tendency [27,28]. These remarks and the fact that the Scott B lymphoblasts are not rapidly labeled by FM1-43 upon ionophore treatments corroborate with previous studies demonstrating by comparison with annexin V that the styryl dye probe reports phospholipid scrambling in correlation with PS exposure [11,29].

Although the underlying mechanism remains mainly unknown, the outward translocation of membrane PS hypothetically results from the Ca^{2+}-dependent activation of a lipid "scramblase" and/or a floppase having a reverse aminophospholipid translocase activity [1,32,33]. However, the various agents provoking $[Ca^{2+}]_i$ increases in vitro are not equally efficient to promote a rapid PS exposure. Although A23187 and thapsigargin provoked an increase of $[Ca^{2+}]_i$ in control B lymphoblasts, only A23187 could trigger a characteristic rapid burst of FM1-43 fluorescence significant of rapid membrane remodeling.

Ca^{2+} ionophores are valuable tools to manipulate intracellular ionic homeostasis. A23187 and ionomycin allow movement of Ca^{2+} between the major membrane-bound compartments of the cell, extracellular space, cytosol, endoplasmic reticulum, and mitochondria, by equilibrating the calcium gradient [34]. Moreover, the character of the $[Ca^{2+}]_i$ increase can be different depending on the type of cells and the ionophore concentration. Low concentrations (submicromolar concentrations) of ionomycin mobilize Ca^{2+} stores and subsequent Ca^{2+} influx via SOCE pathway [23,24]. Our results however correlated with other studies demonstrating that higher concentrations of ionophores allow a sustained $[Ca^{2+}]_i$ increase of higher intensity [24]. Indeed, although a deficient Ca^{2+} influx significant of the SOCE defect was observed by Le Deist et al. [28] in primary immunodeficiency lymphocytes treated with 0.1 μM ionomycin, 0.5 μM ionomycin provoked, in these cells, a high $[Ca^{2+}]_i$ increase of similar intensity as in controls. In our study, we confirmed the defective SOCE in these cells after thapsigargin stimulation whereas $[Ca^{2+}]_i$ increase was comparable to control upon 2 μM A23187 treatment. Altogether, these data and others [12,17] emphasize that although $[Ca^{2+}]_i$ elevation is required, Ca^{2+} ionophore elicit necessary additional (or independent) effect(s) to trigger rapid membrane remodeling.

Out of the three cell types analyzed in this study, only Scott B lymphoblasts did not undergo ionophore-triggered rapid membrane remodeling. However, we have previously shown that $[Ca^{2+}]_i$ elevation in Scott B lymphoblasts is sufficient to mediate signaling pathway(s), such as those promoting the rapid actin cytoskeleton reorganization stimulated by thapsigargin or A23187 in the presence of 1.8 mM $[Ca^{2+}]_O$ [35]. Moreover, $[Ca^{2+}]_i$ threshold values allowing the membrane remodeling process to occur in control cells were observed in

this study for individual Scott B lymphoblasts. These data exclude a defective Ca^{2+} influx as being the only cause for the defective phenotype. The manifestation of membrane remodeling in Scott B lymphoblasts treated with ionophore after thapsigargin remains to be explored. This result, correlating with previous results showing an increase of A23187-induced procoagulant PS externalization ability in Scott cells activated with 1 μM thapsigargin in combination with 5 μM of the ionophore [26], may suggest that the defective response to ionophore can be, at least partially, compensated by a SOCE-dependent effect in these cells. The characterization of the ionophores effects will help understanding the mechanisms governing membrane remodeling. The signaling pathway(s) leading to PS scrambling induced in vitro by ionophores could theoretically be assessed through analysis of protein phosphorylation profiles in platelets and other cells [25,36,37]. However, it is now clear that such studies are rendered difficult because a variety of other Ca^{2+}-dependent processes associated with protein phosphorylations occur simultaneously, including cytoskeleton remodeling [35,38]. Scott syndrome cells remain however interesting models for the identification of aberrant component(s) that could be involved in transmembrane PS redistribution triggered in vitro by ionophores or physiological agonists. Collagen receptor glycoprotein VI-induced increase in $[Ca^{2+}]_i$ has been shown to be an efficient signal for procoagulant PS exposure in platelets adherent to collagen [39,40]. Since the response to collagen is also deficient in adherent Scott platelets [17], it may be assumed that this ligand promotes a signaling of the same kind as that triggered by ionophores. This work orientates future research to examine the role of pathways alternative to SOCE for this process and consequently, to define new candidate genes that could be mutated in Scott syndrome.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2004.12.018.

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