Significance of Capacitative Ca^{2+} Entry in the Regulation of Phosphatidylserine Expression at the Surface of Stimulated Cells†

M. Carmen Martínez,‡,§ Sophie Martin,‡,§ Florence Toti,‡,§ Edith Fressinaud,|| Jeanne Dachary-Prigent,⊥ Dominique Meyer,§ and Jean-Marie Freyssinet*,‡,§

Institut d'Hématologie et d'Immunologie, Faculté de Médecine, Université Louis Pasteur, 4, rue Kirschleger, 67085, Strasbourg, France, Unité 143 INSERM, Hôpital de Bicêtre, 94275, Le Kremlin-Bicêtre, France, CHU Hôtel Dieu, Institut de Biologie, Laboratoire d'Hématologie, 44035, Nantes, France, and UMR 5533 CNRS, Hôpital Cardiologique du Haut Lévèque, 33604, Pessac, France

Received January 20, 1999; Revised Manuscript Received May 26, 1999

ABSTRACT: The transverse redistribution of plasma membrane phosphatidylserine is one of the hallmarks of cells undergoing apoptosis and also occurs in cells fulfilling a more specialized function, such as platelets after appropriate activation. Although an increase in intracellular Ca^{2+} is required to trigger the remodeling of the plasma membrane, little information regarding intracellular signals leading to phosphatidylserine externalization has been provided. Scott syndrome is an extremely rare inherited disorder of the migration of phosphatidylserine toward the exoplasmic leaflet of the plasma membrane of stimulated blood cells. We have studied here the intracellular Ca^{2+} mobilization and Ca^{2+} entry involved in tyrosine phosphorylation in Epstein Barr virus (EBV)-infected B cells derived from a patient with Scott syndrome, her daugther, and control subjects. An alteration of Ca^{2+} entry through the plasma membrane and subsequent tyrosine phosphorylation induced by Ca^{2+} were observed in Scott EBV-B cells, but the release of Ca^{2+} from intracellular stores was normal. Furthermore, phosphatidylserine externalization at the surface of stimulated cells does not depend on tyrosine kinases. These results suggest that the defect of phosphatidylserine exposure in Scott syndrome cells is related to the alteration of a particular way of Ca^{2+} entry, referred to as capacitative Ca^{2+} entry, although some differences may be related to the cell type. Hence, this genetic mutant testifies to the prime significance of Ca^{2+} signaling in the regulation of phosphatidylserine expression at the surface of stimulated cells.

The occurrence of phosphatidylserine (PS)[1] in the exoplasmic leaflet of the plasma membrane is considered one of the hallmarks of cells undergoing apoptosis and more generally constitutes one of the determinants for the phagocytosis of stimulated cells to be rapidly cleared (*1*−*3*). PS is also an essential element of the hemostatic response owing to its ability to promote the assembly of the characteristic enzyme complexes of the blood coagulation cascade at the surface of activated platelets (*4*).

In resting, i.e., nonstimulated cells, the maintenance of the asymmetric distribution of phospholipids between the two leaflets of the plasma membrane involves a specific inward transporter of aminophospholipids, phosphatidylethanolamine, and PS, a flippase termed aminophospholipid translocase (*5*). After stimulation, the outward movement of PS, may result from the activation of a nonspecific lipid "scramblase" and/or a floppase having reverse aminophospholipid translocase activity (*3, 6*). Other transporters have been proposed to participate in the transmembrane migration of phospholipids. In the yeast *Saccharomyces cerevisiae*, the vectorial movement of fluorescent-labeled phosphatidylethanolamine is controlled by transcription regulators of some ATP-binding cassette transporters (*7*). The multidrug resistance protein MRP has been shown to be responsible for the outward translocation of NBD-labeled lipids in erythrocyte membranes (*8*), but its action seems limited to modified lipid analogues (*9*). However, the mechanism(s) triggering the outward migration of PS remain(s) mostly unknown. The recent isolation and molecular cloning of a human plasma membrane phospholipid scramblase suggest that this protein of ∼37 kDa mediates the Ca^{2+}-dependent transbilayer movement of phospholipids (*10, 11*). A murine counterpart of ∼34 kDa has also been cloned, and both human and mouse scramblase exhibited a similar activity and affinity for Ca^{2+} (*12, 13*). These data are in agreement with the requirement from an increase of intracellular calcium concentration ($[Ca^{2+}]_i$) for PS exposure in apoptotic cells (*14*) and activated platelets (*15*). Moreover, it has been shown that Ca^{2+}-mobilizing agents [such as Ca^{2+} ionophores,

† This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Université Louis Pasteur, and the Fondation pour la Recherche Médicale.

* To whom correspondence should be addressed. Phone: 33 3 88 24 33 39. Fax: 33 3 88 25 58 83. E-mail: Jean-Marie.Freyssinet@hemato-ulp.u-strasbg.fr.

‡ Institut d'Hématologie et d'Immunologie.

§ Hôpital de Bicêtre.

|| CHU Hôtel Dieu.

⊥ Hôpital Cardiologique du Haut Lévèque.

[1] Abbreviations: PS, phosphatidylserine; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; TG, thapsigargin; EBV, Epstein−Barr virus; Fluo-3, fluo-3/acetoxymethyl ester; BAPTA/AM, 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester.

10.1021/bi990129p CCC: $18.00
Published on Web 07/10/1999

thapsigargin (TG) or thrombin], which act mainly by releasing Ca^{2+} from intracellular stores and allowing Ca^{2+} entry through the plasma membrane channels (*16, 17*), elicit PS exposure and the shedding of membrane microparticles (*18, 19*). It has also been demonstred that exposure of PS in erythrocytes and platelets (*20*) as well as during apoptosis (*21*) is dependent on the presence of extracellular Ca^{2+} and that a continuously elevated $[Ca^{2+}]_i$ may be necessary to maintain this externalization process (*22*). Hence, Ca^{2+} influx across the plasma membrane and maximal exposure of aminophospholipids seem necessary for microparticle formation in platelets (*19*). Nevertheless, little information regarding the upstream and downtream intracellular signals leading to PS externalization or phospholipid scrambling has been provided. Recently, several groups have studied the intracellular signaling that leads to exposure of PS in platelets from normal and Scott subjects. It has been suggested that the development of the procoagulant response, bleb formation, and exposure of PS in platelets need a sustained increase in $[Ca^{2+}]_i$ as a result of activation of tyrosine kinases (*23*) which would control the procoagulant response.

Scott syndrome is an extremely rare inherited disorder of PS exposure affecting multiple hematologic lineages with an associated clinical phenotype restricted to severe to moderate bleeding episodes (*24, 25*). In this respect, the lack of PS externalization and microparticle formation in Scott syndrome platelets and erythrocytes seems to be related to a defect of tyrosine phosphorylation (*26, 27*), but not directly causal (*27*). In addition, Scott B lymphocytes (*28, 29*) and erythrocytes (*30*) contain a phospholipid scramblase capable of accelerating transbilayer phospholipid movements, suggesting that the Scott phenotype is caused by the impairment of a regulatory element. Thus, it has been hypothesized that a diminished activation of scramblase by Ca^{2+} may be responsible for the PS exposure defect (*30*). Taking advantage of normal Ca^{2+} response and tyrosine phosphorylation in EBV-transformed B cells from control subjects with respect to the nontransformed counterpart cells (*31, 32*), Ca^{2+} signaling was studied in Scott EBV-B cells.

We report here the defective Ca^{2+} entry across the plasma membrane and subsequent tyrosine phosphorylation induced by Ca^{2+} and a normal Ca^{2+} release from intracellular stores in EBV-transformed B cells presenting the Scott phenotype. It is suggested that the alteration of Ca^{2+} entry, referred to as capacitative Ca^{2+} entry (*33*), is related to the defective exposure of PS in Scott syndrome cells.

EXPERIMENTAL PROCEDURES

Materials. X-VIVO 15 culture medium was purchased from BioWhittaker (Walkersville, MD), RPMI, cyclosporin, and FCS from Life Technologies (Paisley, UK). Fluo-3/acetoxymethyl ester (Fluo-3) was obtained from Molecular Probes (Eugene, Oregon) and Ca^{2+} ionophore A23187 from Calbiochem (La Jolla, California). Genistein, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA/AM) and thapsigargin were from Alexis Corporation (San Diego, CA), and aprotinin, leupeptin, pepstatin, phenylmethanesulfonyl fluoride (PMSF), and Tween-20 from Boehringer Mannheim (Germany). Anti-phosphotyrosine antibody (PY20) was purchased from Transduction Laboratories (Lexington, KY) and horseradish peroxidase-conjugated anti-mouse antibody from Leinco Technologies (Ballwin, Missouri). Enhanced chemiluminescence reagents and CL-XPosure films were obtained from Pierce (Rockford, IL). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Patients, Preparation, and Culture of B Lymphoblasts. Peripheral blood was collected from the propositus, a 74 year-old woman, who has suffered from severe to moderate hemorrhagic episodes for a long time, her daughter who denied any bleeding tendency, and three control subjects. The cases of the propositus and her daughter have been detailed in another study (*25*) where homozygous and heterozygous status have been respectively suggested. In the cases of the propositus and her daughter, only two blood collections were possible for lymphocyte isolation. Lymphocytes were isolated under sterile conditions and stored in liquid nitrogen. Infection of B lymphocytes by EBV (EBV B958, Marmouset) was performed in RPMI culture medium containing 20% FCS in the presence of 50 ng/mL cyclosporin and was achieved within 3 weeks (*25*). Three independent EBV infections could be performed. EBV-infected B cells were expanded in X-VIVO 15 culture medium (free Ca^{2+} concentration = 1.8 mM). Cell mortality was estimated by Trypan blue staining (0.1% final concentration).

Measurement of $[Ca^{2+}]_i$ by Flow Cytometry. EBV-infected B lymphocytes were studied at 10^6 cells/mL. B lymphoblasts were loaded with 3 μM Fluo-3/AM for 30 min at room temperature. Cells were then washed twice in X-VIVO 15 medium, left for 10 min, and resuspended at the same concentration (10^6 cells/mL, 1 mL/analysis). Fluo-3 fluorescence was monitored using a FACScan Becton-Dickinson flow cytometer and the CELLQuest software. Viable B lymphoblasts were gated based on forward and side light scatter to exclude other cell populations and fragments. A baseline value was obtained for each sample by fluorescence measurement for 30 s before addition of pharmacologic agents (1 or 5 μM A23187 and 1 μM TG in the absence and in the presence of 2 mM EGTA). Collection was immediately resumed, then terminated after an additional 5 min ($\sim$50000 events). Fluo-3 fluorescence was measured in arbitrary fluorescence intensity units and plotted as FL-1 versus time, basal fluorescence values being 147 $\pm$ 22, 153 $\pm$ 34, and 154 $\pm$ 36 for control, Scott patient, and daughter's B cells, respectively. To convert these data into absolute $[Ca^{2+}]_i$, calibration was performed at the end of each experiment (*34*). $[Ca^{2+}]_i$ was calculated using the equation: $[Ca^{2+}]_i = K_d[(F - F_{min})/(F_{max} - F)]$, where K_d is the dissociation constant of the Ca^{2+}/Fluo-3 complex (400 nM). F_{max} represents the maximum fluorescence (obtained by treating cells with 10 μM A23187), and F_{min} represents the minimum fluorescence (obtained by addition of 2 mM $MnCl_2$ to ionophore-treated cells). F represents the actual sample fluorescence. Fluorescence intensities were expressed as the increase in fluorescence with respect to baseline fluorescence intensity before stimulation.

Western Blots. After incubation with the pharmacologic agents for different periods of time at 37 °C, the reaction was stopped by addition of 500 μL/mL of ice-cold buffer A (50 mM Tris, 250 mM NaCl, 8 mM $MgCl_2$, 10 μg/mL of aprotinin, leupeptin and pepstatin, 1 mM PMSF, 5 mM EDTA, 0.5 mM EGTA, and 2 mM sodium orthovanadate). Cells were spun down and lysed for 30 min in 1 mL of ice-

cold lysis buffer [buffer A plus 1% (vol/vol) Triton X-100]. Approximately 10 μg of protein was resuspended in Laemmli sample buffer (*35*) and separated on 10% SDS−PAGE and western blotted. Blots were probed with the monoclonal antibody anti-phosphotyrosine (PY20) and developed with horseradish peroxidase-conjugated anti-mouse antibody. Blots were visualized with enhanced chemiluminescence reagents for 10 min and then exposed to CL-XPosure films.

Functional Detection of Procoagulant Phospholipids. Procoagulant phospholipid exposure in stimulated cells and derived microparticles was detected using a human prothrombinase assay in which PS promotes the activation of prothrombin by factor Xa in the presence of factor Va (*36*). Thrombin generated by assembled prothrombinase complex was measured using a chromogenic assay as described elsewhere (*25*). EBV-infected lymphocytes were studied at 2×10^5 cells/mL, and the ability to expose procoagulant PS was examined after stimulation by 1 μM A23187 (10 min at 37 °C, in the presence of 1.8 mM external $CaCl_2$) in the absence and in the presence of 10 and 100 μM genistein (preincubation of 30 min at 37 °C). B cells were separated from derived microparticles by centrifugation at 12000*g* for 1 min before measurements. For each protocol, results were compared with the prothrombinase activities developed in samples from healthy volunteers.

Statistical Analysis. Results are expressed as means ± SEM of at least six separate experiments performed at different culture stages. Unpaired Student's *t*-test was used for the statistical analysis. A $p < 0.05$ value was considered significant.

RESULTS

Changes of $[Ca^{2+}]_i$. To investigate the changes of $[Ca^{2+}]_i$, B lymphoblasts were loaded with the Ca^{2+}-binding fluorophore Fluo-3 and emission fluorescence shifts were assessed by flow cytometry. No significant difference was detected in the basal fluorescence intensity of normal, daughter, and Scott B lymphoblasts ($[Ca^{2+}]_i$ basal values were 37 ± 10, 44 ± 10, and 44 ± 13 nM, respectively). To study Ca^{2+} responses, drastic activation with Ca^{2+} ionophore A23187 was performed, since the Scott phenotype was observed in B lymphoblasts from the patient after this treatment (*25*). A23187 at 1 μM induced a time-dependent increase in Fluo-3 fluorescence in both normal and Scott EBV-transformed B cells (Figure 1). This increase in fluorescence intensity was significantly lower ($p < 0.05$) in Scott than in normal cells. Ionophore-stimulated cells from Scott patient's daughter exhibited an increase in the fluorescence intensity which was not significantly different from values corresponding to normal cells (Figure 1). The difference in the response to A23187 of Scott versus normal B lymphoblasts was also observed at higher concentration of ionophore (5 μM) (not shown).

Normally, depletion of intracellular Ca^{2+} stores by TG treatment is responsible for Ca^{2+} currents through the plasma membrane which results in an elevation of $[Ca^{2+}]_i$ and refilling of stores. This mechanism is referred to as capacitative Ca^{2+} entry (*33*). To establish whether the reduced response of the Scott cells was due to decreased Ca^{2+} release from intracellular stores or to decreased Ca^{2+} capacitative entry, we used TG (1 μM), an inhibitor of reticulum

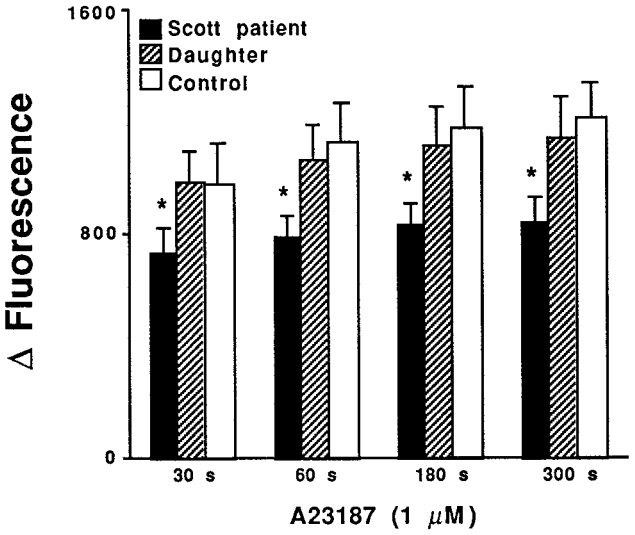

FIGURE 1: Histograms showing the increase in Fluo-3 fluorescence in B lymphoblasts from the patient with Scott syndrome, from her daughter, and from control donors. Cells were activated with 1 μM A23187. Results are expressed as the mean of the increase in fluorescence ± SEM as a function of time. Data are representative of eight experiments. *$p < 0.05$ responses of Scott versus normal B lymphoblasts.

endoplasmic Ca^{2+}-ATPases, in the absence and in the presence of EGTA (2 mM), and compared the response of the Scott versus normal B lymphoblasts. In the absence of EGTA, TG produced an increase of Fluo-3 fluorescence intensity, which was lower than the increase induced by A23187 at the same concentration. Again, we observed that this increase in fluorescence intensity was higher in normal than in Scott B cells ($p < 0.01$) (Figure 2A). Interestingly, at the outset of the measurements, cells from daughter showed a lower increase in fluorescence which was similar to the response of Scott cells, but rapidly increased to reach a maximal intensity not significantly different from that induced by TG in normal B lymphoblasts (Figure 2A). In the presence of EGTA (2 mM), TG-induced response was weak and similar in normal, daughter's, and Scott cells, suggesting that Ca^{2+} release from intracellular Ca^{2+} stores is not altered in Scott cells. However, when $CaCl_2$ (1.8 mM) was added to raise the extracellular Ca^{2+} concentration and to allow the capacitative Ca^{2+} entry into the cytosol, the increase of Fluo-3 fluorescence intensity was significantly higher in normal and daughter's than in Scott lymphoblasts ($p < 0.01$), suggesting that capacitative entry is reduced in Scott cells (Figure 2B). It should be noted that the increase in Fluo-3 fluorescence induced by A23187 or by TG in the continuous presence of EGTA and the subsequent addition of $CaCl_2$ was not affected by treatment with either of the voltage-dependent Ca^{2+} channel blocker verapamil (10 μM) (not shown) or the tyrosine kinase inhibitor genistein (100 μM) (not shown), suggesting that influx of Ca^{2+} involves voltage-independent Ca^{2+} channels and is not modulated by tyrosine kinase activity.

A23187 and TG Treatment of B Lymphoblasts Triggers Tyrosine Phosphorylation. Comparison of phosphorylation patterns induced by A23187 (1 μM) in normal, daughter's, and Scott cells showed that basal levels of tyrosine phosphorylation in the three types of cells were different. Contrary to observations in normal B lymphoblasts, in daughter's cells A23187 induced a weak increase in tyrosine phosphorylation

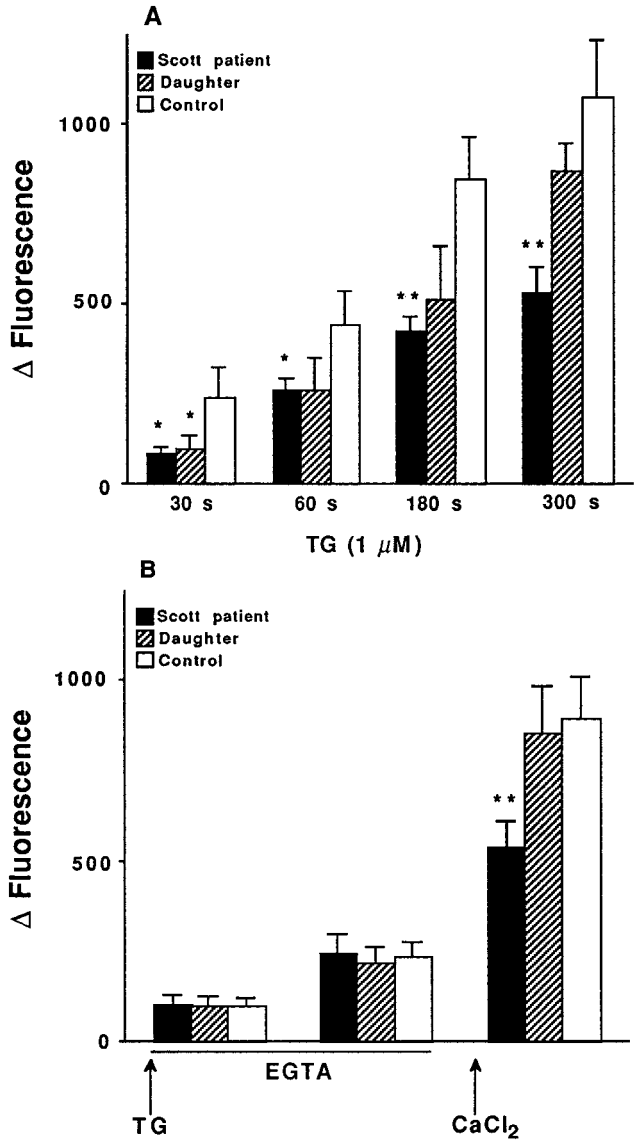

FIGURE 2: Histograms showing the increase in Fluo-3 fluorescence in B lymphoblasts from the patient with Scott syndrome, from her daughter, and from control donors. Cells were activated with the endoplasmic reticulum Ca^{2+}-ATPases inhibitor, thapsigargin (TG), 1 μM, in the presence of 1.8 mM extracellular Ca^{2+} (A), and in the presence of 2 mM EGTA and the subsequent addition of 1.8 mM $CaCl_2$ in the continuous presence of TG (B). Results are expressed as the mean of the increase in fluorescence ± SEM as a function of time. Data are representative of eight experiments. *$p < 0.05$, **$p < 0.01$, responses of Scott B cells versus control cells.

after 30 s of treatment, and this level slightly enhanced at 3 min. However, A23187 failed to promote any increase of phosphorylation in Scott cells (Figure 3). These results suggest an alteration of tyrosine phosphorylation in such cells, probably due to a defective Ca^{2+} ionophore-induced signal. In normal B lymphoblasts, genistein, an inhibitor of tyrosine kinases, produced a concentration-dependent inhibition of tyrosine phosphorylation induced by A23187. At 1 μM, genistein inhibited tyrosine phosphorylation only weakly, but, at 100 μM, it considerably reduced this phosphorylation (not shown).

In the presence of EGTA (2 mM), TG produced an increase in tyrosine phosphorylation in B lymphoblasts from control subjects (Figure 4). This response was similar at 30 s and at 3 min of stimulation with TG. After 3 min, the addition of $CaCl_2$ (1.8 mM) induced an increase in tyrosine phosphorylation of several proteins (~38, 75, and 100 kDa). In daughter's and in Scott cells, in the presence of EGTA, TG induced an increase in tyrosine phosphorylation similar to that observed in control cells at 30 s. However, while in daughter's cells, this level of phosphorylation was stable at 3 min, it declined in Scott cells. Moreover, the subsequent addition of $CaCl_2$ weakly and strongly diminished tyrosine phosphorylation in daughter's and Scott cells, respectively (Figure 4).

In the presence of BAPTA/AM (30 μM), an intracellular Ca^{2+} chelator, TG did not affect the level of tyrosine phosphorylation (after 30 s and 3 min of stimulation) in all of the cell types. Addition of $CaCl_2$ (1.8 mM) induced a weak increase of phosphorylation in cells from normal subjects, but failed to produce any significant effect in Scott and daughter's cells (Figure 5). The fact that BAPTA/AM inhibited tyrosine phosphorylation induced by TG and by subsequent Ca^{2+} addition suggests that Ca^{2+} release from intracellular stores and Ca^{2+} influx from the 1.8 mM extracellular Ca^{2+} are saturated at 30 μM of BAPTA/AM. These findings are in accordance with the above results, and taken together, they indicate that the capacitative Ca^{2+} entry and the associated tyrosine phosphorylation induced by Ca^{2+} are impaired in Scott B lymphoblasts.

Effects of Tyrosine Kinase Inhibitor on Phospholipid Externalization. In the absence of stimulation, normal B lymphoblasts and their corresponding supernatants exhibited low prothrombinase activities (0.77 ± 0.23 and 0.4 ± 0.05 NIH units of thrombin generated per mL/min/2 × 10^5 cells, respectively). Genistein (10 and 100 μM) did not affect the basal procoagulant phospholipid activity in these cells (not shown). After Ca^{2+} ionophore treatment, prothrombinase activity was enhanced in both cells and supernatants, being 1.78 ± 0.47 and 0.73 ± 0.22 NIH units of thrombin generated per mL/min/2 × 10^5 cells, respectively. Genistein did not affect the generation of prothrombinase activity induced by Ca^{2+} ionophore in normal B lymphoblasts (1.77 ± 0.49 and 2.00 ± 0.49 NIH units of thrombin generated per mL/min/2 × 10^5 cells, in the presence of 10 and 100 μM of genistein, respectively) or the corresponding supernatants (0.78 ± 0.27 and 0.94 ± 0.28 NIH units of thrombin generated per mL/min/2 × 10^5 cells, in the presence of 10 and 100 μM of genistein, respectively). As previously reported (*25*), B lymphoblasts from the Scott patient and her daugther displayed, respectively, a 87 and 53% reduction of prothrombinase activity when compared with control cells after stimulation by Ca^{2+} ionophore A23187 (1 μM).

DISCUSSION

The present study shows that Scott B lymphoblasts, which present a defect of PS externalization, have decreased capacitative Ca^{2+} entry without any detectable alteration in the mobilization of Ca^{2+} from intracellular stores. This defective Ca^{2+} influx across the membrane results in an alteration of tyrosine kinase activity. However, tyrosine kinases are not involved in PS exposure.

Augmentation of $[Ca^{2+}]_i$ is due to Ca^{2+} release from intracellular stores, mainly endoplasmic reticulum, and Ca^{2+} transmembrane influx (*37*). In the present study, the Ca^{2+} responses induced by A23187 and TG in the presence of

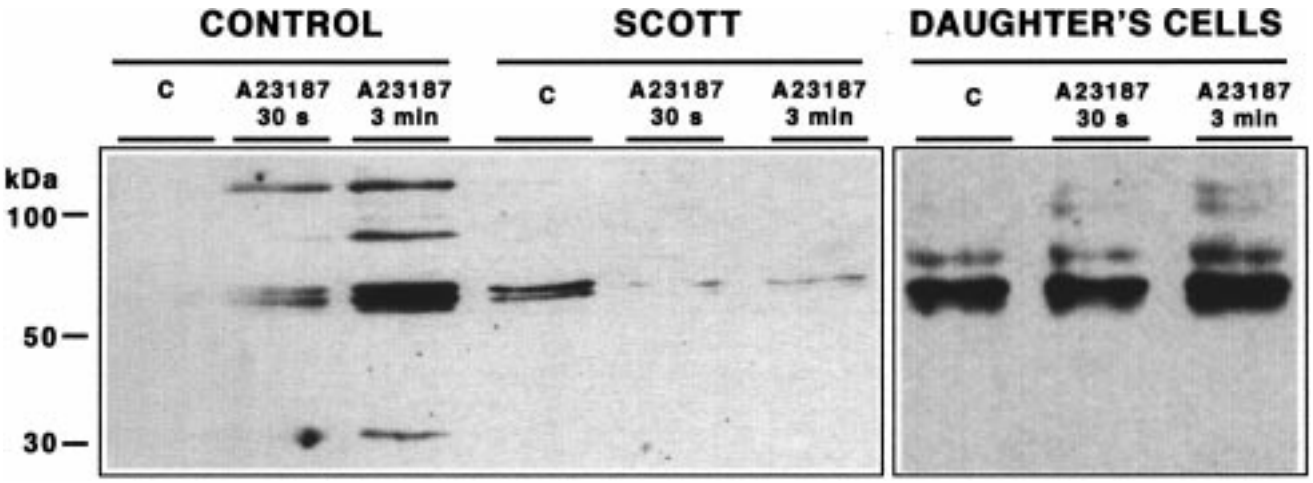

FIGURE 3: Western blotting of tyrosine phosphorylated proteins from B lymphoblasts from a control donor, from the patient with Scott syndrome, and from her daughter. Cells were stimulated by 1 μM A23187 during 30 s and 3 min in the presence of 1.8 mM extracellular Ca^{2+}. Blots are representative of four separate experiments.

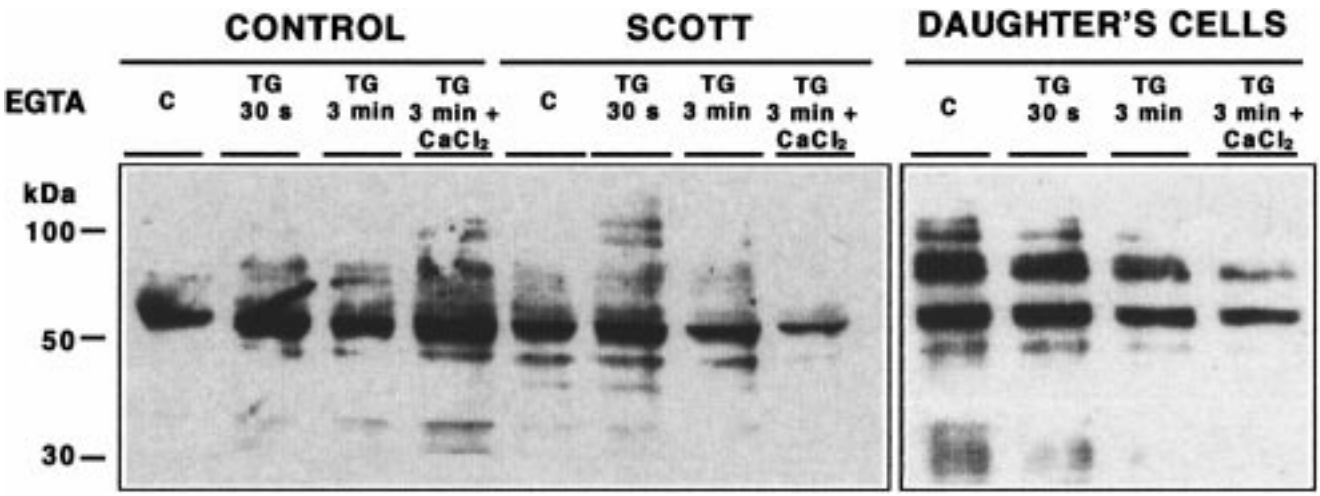

FIGURE 4: Thapsigargin (TG)-induced tyrosine phosphorylation in B lymphoblasts from a control donor, from the patient with Scott syndrome, and from her daughter. Cells were treated with the Ca^{2+} chelator EGTA, 2 mM, for 5 min followed by addition of 1 μM TG during 30 s and 3 min. $CaCl_2$, 1.8 mM, was added 3 min after TG addition and tyrosine phosphorylation was probed 1 min later. Blots are representative of four separate experiments.

extracellular Ca^{2+} are reduced in Scott B lymphoblasts when compared to control B lymphoblasts. Although A23187-induced Ca^{2+} entry may occur through the ionophore itself, the differences of responses clearly suggest the existence of other Ca^{2+} channels. It has also been reported that in platelets from the first Scott patient, stimulation by collagen and thrombin induces an increase of $[Ca^{2+}]_i$, lower but not significantly different from that in control platelets (*15*). Here, a somewhat intermediate pattern was observed in cells from the patient's daugther, with a normal A23187-induced increase in Fluo-3 fluorescence, but a response to TG consistently lower than in controls although significantly different from that of Scott cells, suggesting that this increase in $[Ca^{2+}]_i$ might be sufficient to produce ~50% of PS exposure as already described (*25*). This is additional evidence supporting the view that the daughter has probably a heterozygous status with respect to Scott syndrome. Both A23187 and TG have been reported to induce Ca^{2+} release from intracellular stores, and after sustained depletion, voltage-independent Ca^{2+} influx across the plama membrane (*16*, *17*) becomes activated. To assess which is the affected pathway in Scott cells, experiments were performed in the presence of EGTA, and TG, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPases, was used to empty Ca^{2+} stores, and capacitative Ca^{2+} entry after subsequent addition of $CaCl_2$ was studied. Under these conditions, response to TG was similar in all cell types, suggesting that intracellular Ca^{2+} stores are not altered in either of Scott or daughter's cells. However, when $CaCl_2$ was added in the continuous presence of TG, Ca^{2+} entry was diminished in cells from Scott patient and was normal in daughter's cells. Taken together, these data are in favor of an alteration of capacitative Ca^{2+} entry in Scott cells, but not in daughter's cells. Impaired A23187- and TG-induced Ca^{2+} responses in Scott B cells could also be explained by a multidrug resistance process responsible for a rapid elimination of these compounds out of the cells. Another possibility is that endoplasmic reticulum Ca^{2+}-ATPases of Scott cells are insensitive to TG. Two observations exclude these hypotheses. First, it has been reported that EBV lymphoblasts from this french Scott patient do not express multidrug resistance genes (*38*). Second, intracellular Ca^{2+} release induced by TG in the presence of EGTA is normal in Scott cells (the same results were obtained with A23187, not shown).

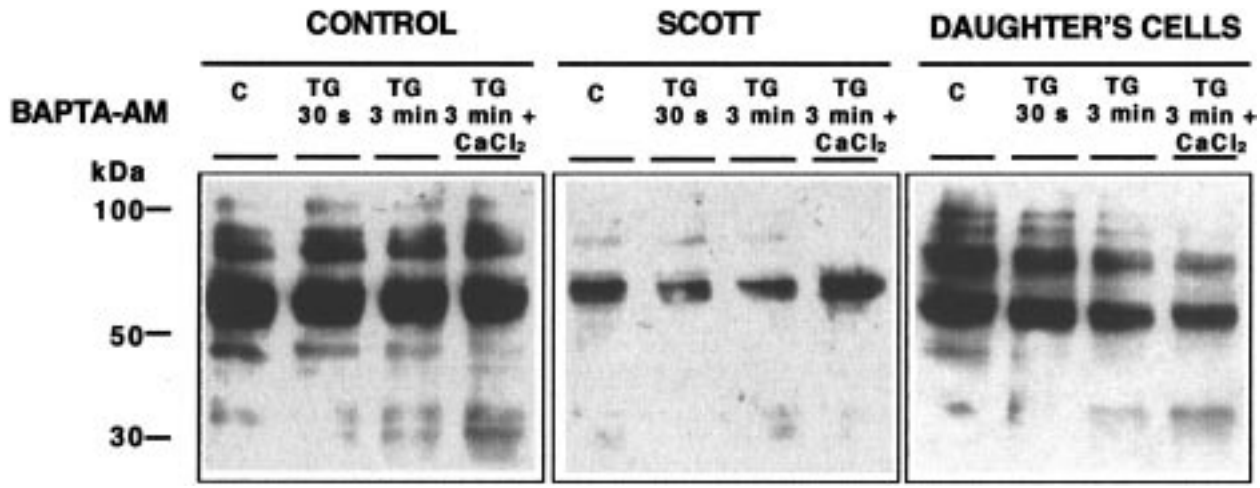

FIGURE 5: Prevention of thapsigargin (TG)-induced tyrosine phosphorylation by the intracellular Ca^{2+} chelator BAPTA/AM in B lymphoblasts from a control donor, from the patient with Scott syndrome, and from her daughter. Cells were treated with BAPTA/AM 30 μM for 30 min followed by addition of 1 μM TG during 30 s and 3 min. $CaCl_2$, 1.8 mM, was added 3 min after TG addition and tyrosine phosphorylation was probed 1 min later. Blots are representative of four separate experiments.

Among the molecular mechanisms proposed for the activation of capacitative Ca^{2+} entry, tyrosine kinases have been studied in platelets where the influx of Ca^{2+} evoked by TG induces tyrosine phosphorylation and tyrosine kinase inhibitors block capacitative Ca^{2+} entry (*39*, *40*). In our study, genistein did not affect the response induced by $CaCl_2$ in the sustained presence of EGTA and TG, indicating that capacitative Ca^{2+} entry is not likely to be regulated by tyrosine kinase activity. At the opposite, the lack of increase in tyrosine phosphorylation in the presence of BAPTA/AM indicates that tyrosine phosphorylation induced by TG depends on elevation of intracellular Ca^{2+}.

Several groups have recently studied the possible relationships between tyrosine kinase activity and the externalization of PS. In platelets, inhibition of tyrosine kinases by genistein diminished the increase in $[Ca^{2+}]_i$, PS exposure, and bleb formation induced by collagen, but did not affect the responses induced by A23187 (*23*). In normal erythrocytes, staurosporine did not prevent the generation of PS-dependent procoagulant, i.e., prothrombinase activity induced by ionomycin suggesting that tyrosine kinase activity is not required for lipid scrambling (*27*). In agreement with these results, we have shown that in normal B lymphoblasts, genistein did not affect the $[Ca^{2+}]_i$ responses or prothrombinase activity induced by A23187. Taken together, these results suggest that tyrosine kinases are not likely to be involved in the regulation of the sustained increase in $[Ca^{2+}]_i$ induced by drastic activation conditions (Ca^{2+} ionophore). However, it cannot be excluded that the increase in $[Ca^{2+}]_i$ induced by other agents could be mediated by tyrosine kinase activity.

In platelets and erythrocytes from the two Scott patients, an abnormal pattern of tyrosine phosphorylation in response to the physiologic agonist combination of collagen and thrombin has been reported (*26*, *27*). Here, in addition to defective Ca^{2+} entry in Scott B lymphoblasts, we have observed an alteration in tyrosine phosphorylation linked to Ca^{2+} entry across the membrane in response to A23187 and TG. Although defective Ca^{2+} entry can result in impaired Ca^{2+}-induced tyrosine phosphorylation, increased tyrosine phosphatase activity or reduced tyrosine kinase activity cannot be excluded. In this respect, a normal tyrosine phosphatase activity has been reported in platelets from the same Scott patient (*26*) and we have observed a normal pattern of A23187-induced tyrosine phosphorylation in her derived B lymphoblasts when sodium orthovanadate (an inhibitor of tyrosine phosphatases) was omitted in the lysis buffer (see Experimental Procedures) (not shown). On the other hand, a reduced tyrosine kinase activity could explain the impaired tyrosine phosphorylation in Scott cells after Ca^{2+} influx. However, the finding that in these cells in the presence of EGTA, TG-induced tyrosine phosphorylation is normal but declined after $CaCl_2$ addition suggests that the impairment of tyrosine kinase activity occurs only after the deficient Ca^{2+} influx. Hence, the alteration of Scott cell tyrosine kinase activity may well result from defective Ca^{2+} influx. When the same protocol was used in daughter's cells, a weak diminution of tyrosine phosphorylation was observed, in agreement with a possible heterozygous status. However, the involvement of tyrosine kinases in phospholipid scrambling remains to be elucidated, according to experimental protocols allowing simultaneous measurements of PS externalization, cytosolic Ca^{2+} variations, and protein tyrosine phosphorylation.

Little is known about the Ca^{2+} channels mediating the capacitative Ca^{2+} entry or the intracellular signals governing this Ca^{2+} influx. Concerning the channels (also referred to as store-operated or Ca^{2+} release-activated Ca^{2+} channels), it is established that they are regulated by the level of Ca^{2+} in the intracellular stores. Regarding the signaling mechanisms, different second messengers have been proposed [for review see Fasolato et al. (*41*)]. Whether the defective Ca^{2+} responses observed in Scott cells are due to a mutation of Ca^{2+} channels or elements involved in the regulation of intracellular signaling remains to be clarified. The absence of intracellular stores in erythrocytes and resealed ghosts might question the results obtained with B cells. However, Ca^{2+} ionophore-induced PS exposure and expression of membrane procoagulant activity in red blood cells occurs much more slowly and to a much lesser extent than in platelets (*20*) and in B cells (in our study, phothrombinase activity was maximal after 10 min of A23187 stimulation). Therefore, it seems reasonable to assume that, although the protein involved in the PS exposure is the same in B cells

and erythrocytes, the intracellular regulatory mechanism(s) may be different.

The present study shows that the capacitative Ca^{2+} entry is reduced but not totally abolished in Scott B cells. It remains, however, to be established whether this occurs in other cell lineages presenting the Scott phenotype. In this respect, an interesting observation is that of the absence of Ca^{2+} entry across the plasma membrane with a normal Ca^{2+} mobilization from the intracellular stores associated with a case of primary T-cell immunodeficiency (*42*). Although the same abnormality was found in other hematopoietic cell lineages and in fibroblasts, the authors showed that B cell proliferation, platelet aggregation, and polymorphonuclear neutrophil functions were not affected. The Scott patient studied here did not present other clinical symptoms than bleeding episodes, and (auto)immune disorders have not been observed (*25*). Platelet aggregation is also normal in this patient (*25*), suggesting that a reduced Ca^{2+} influx is harmless for a normal immune response but not for the induction of rapid PS externalization, necessary for the hemostatic response.

REFERENCES

1. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992) *J. Immunol. 148*, 2207−2216.
2. Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J., Schie, R. v., LaFace, D., and Green, D. (1995) *J. Exp. Med. 182*, 1545−1556.
3. Verhoven, B., Schlegel, R. A., and Williamson, P. (1995) *J. Exp. Med. 182*, 1597−1601.
4. Zwaal, R. F. A., and Schroit, A. J. (1997) *Blood 89*, 1121−1132.
5. Devaux, P. (1991) *Biochemistry 30*, 1163−1173.
6. Gaffet, P., Bettache, N., and Bienvenue, A. (1995) *Biochemistry 34*, 6762−6769.
7. Kean, L. S., Grant, A. M., Angeletti, C., Mahé, Y., Kuchler, K., Fuller, R. S., and Nichols, J. W. (1997) *J. Cell Biol. 138*, 255−270.
8. Kamp, D., and Haest, C. W. (1998) *Biochim. Biophys. Acta 1372*, 91−101.
9. Dekkers, D. W. C., Comfurius, P., Schroit, A. J., Bevers, E. M., and Zwaal, R. F. A. (1998) *Biochemistry 37*, 14833−18437.
10. Bassé, F., Stout, J. G., Sims, P. J., and Wiedmer, T. (1996) *J. Biol. Chem. 271*, 17205−17210.
11. Zhou, Q., Zhao, J., Stout, J. G., Luhm, R. A., Wiedmer, T., and Sims, P. J. (1997) *J. Biol. Chem. 272*, 18240−18244.
12. Stout, J. G., Zhou, Q., Wiedmer, T., and Sims, P. J. (1998) *Biochemistry 37*, 14860−14866.
13. Zhou, Q., Sims, P. J., and Wiedmer, T. (1998) *Biochemistry 37*, 2356−2360.
14. Bratton, D. L., Fadok, V. A., Richter, D. A., Kailey, J. M., Guthrie, L. A., and Henson, P. M. (1997) *J. Biol. Chem. 272*, 26159−26165.
15. Weiss, H. J., and Lages, B. (1997) *Blood 89*, 1599−1611.
16. Thastrup, O., Cullen, P. J., Drobak, B. J., Hanley, M. R., and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2466−2470.
17. Pasquet, J. M., Dachary-Prigent, J., and Nurden, A. T. (1996) *Eur. J. Biochem. 239*, 647−654.
18. Bevers, E. M., Wiedmer, T., Comfurius, P., Shattil, S. J., Weiss, H. J., Zwaal, R. F. A., and Sims, P. J. (1992) *Blood 79*, 380−388.
19. Dachary-Prigent, J., Pasquet, J.-M., Freyssinet, J.-M., and Nurden, A. T. (1995) *Biochemistry 34*, 11625−11634.
20. Comfurius, P., Senden, J. M. G., Tilly, R. H. J., Schroit, A. J., Bevers, E. M., and Zwaal, R. F. A. (1990) *Biochim. Biophys. Acta 1026*, 153−160.
21. Hampton, M. B., Vanags, D. M., Pörn-Ares, M. I., and Orrenius, S. (1996) *FEBS Lett. 399*, 277−282.
22. Williamson, P., Bevers, E. M., Smeets, E. F., Comfurius, P., Schlegel, R. A., and Zwaal, R. F. A. (1995) *Biochemistry 34*, 10448−10455.
23. Heemskerk, J. W., Vuist, W. M., Feijge, M. A., Reutelingsperger, C. P. M., and Lindhout, T. (1997) *Blood 90*, 2615−2625.
24. Weiss, H. J. (1994) *Semin. Hematol. 31*, 312−319.
25. Toti, F., Satta, N., Fressinaud, E., Meyer, D., and Freyssinet, J. M. (1996) *Blood 87*, 1409−1415.
26. Dachary-Prigent, J., Pasquet, J. M., Fressinaud, E., Toti, F., Freyssinet, J. M., and Nurden, A. T. (1997) *Br. J. Haematol. 99*, 959−967.
27. Dekkers, D. W. C., Comfurius, P., Vuist, W. M. J., Billheimer, J. T., Dicker, I., Weiss, H. J., Zwaal, R. F. A., and Bevers, E. M. (1998) *Blood 91*, 2133−2138.
28. Zhou, Q., Sims, P. J., and Wiedmer, T. (1998) *Blood 92*, 1707−1712.
29. Janel, N., Leroy, C., Laude, I., Toti, F., Fressinaud, E., Meyer, D., Freyssinet, J. M., and Kerbiriou-Nabias, D. (1999) *Thromb. Haemost. 81*, 322−323.
30. Stout, J. G., Bassé, F., Luhm, R. A., Weiss, H. J., Wiedmer, T., and Sims, P. J. (1997) *J. Clin. Invest. 99*, 2232−2238.
31. Simon, H. U., Mills, G. B., Hashimoto, S., and Siminovitch, K. A. (1992) *J. Clin. Invest. 90*, 1396−1405.
32. Kitanaka, A., Ito, C., Coustan-Smith, E., and Campana, D. (1997) *J. Immunol. 159*, 184−192.
33. Putney, J. W., Jr. (1991) *Adv. Pharmacol. 22*, 251−269.
34. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem. 260*, 3440−3450.
35. Laemmli, U. K. (1970) *Nature 227*, 680−685.
36. Connor, J., Bucana, C., Fidler, I. J., and Schroit, A. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3184−3188.
37. Berridge, M. J. (1993) *Nature 361*, 315−325.
38. Toti, F., Schindler, V., Riou, J. F., Lombard-Platet, G., Fressinaud, E., Meyer, D., Uzan, A., LePecq, J. B., Mandel, J. L., and Freyssinet, J. M. (1997) *Biochem. Biophys. Res. Commun. 241*, 548−552.
39. Sargeant, P., Farndale, R. W., and Sage, S. O. (1993) *FEBS Lett. 315*, 242−246.
40. Vostal, J. G., and Shafer, B. (1996) *J. Biol. Chem. 271*, 19524−19529.
41. Fasolato, C., Innocenti, B., and Pozzan, T. (1994) *Trends Pharmacol. Sci. 15*, 77−83.
42. Le Deist, F., Hivroz, C., Partiseti, M., Thomas, C., Buc, H. A., Oleastro, M., Belohradsky, B., Choquet, D., and Fischer, A. (1995) *Blood 85*, 1053−1062.

BI990129P