Store-mediated calcium entry in the regulation of phosphatidylserine exposure in blood cells from Scott patients

Imke C.A. Munnix¹, Marjan Harmsma¹, John C. Giddings², Peter W. Collins², Marion A.H. Feijge¹, Paul Comfurius¹, Johan W. M. Heemskerk¹, Edouard M. Bevers¹

¹Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), University of Maastricht, The Netherlands
²Department of Haematology, Welsh National School of Medicine, Heath Park, Cardiff, United Kingdom

Summary

Scott syndrome is a bleeding disorder, characterized by impaired surface exposure of procoagulant phosphatidylserine (PS) on platelets and other blood cells, following activation with Ca²⁺-elevating agents. Since store-mediated Ca²⁺ entry (SMCE) forms an important part of the Ca²⁺ response in various blood cells, it has been proposed that deficiencies in Ca²⁺ entry may relate to the impaired PS exposure in the Scott syndrome. Here, we have tested this hypothesis by investigating the relationship between Ca²⁺ fluxes and PS exposure in platelets as well as B-lymphoblasts derived from the original Scott patient (M.S.), a newly identified Welsh patient (V.W.) with similar bleeding symptoms, and two control subjects. Procoagulant activity of V.W. platelets in suspension, measured after stimulation with collagen/thrombin or Ca²⁺-ionophore, ionomycin, resulted in 52% or 17%, respectively, compared to that of correspondingly activated control platelets. Procoagulant activity of V.W. erythrocytes treated with Ca²⁺-ionophore resulted in less than 6% of the activity of control erythrocytes. Single-cell Ca²⁺ responses of M.S. and V.W. platelets, adhering to collagen, were similar to those of platelets from control subjects, while PS exposure was reduced to 7% and 15%, respectively, compared to controls. Stimulation of non-apoptotic B-lymphoblasts derived from both patients and controls with Ca²⁺-ionophore or agents causing Ca²⁺ mobilization and SMCE, resulted in similar Ca²⁺ responses. However, in lymphoblasts from M.S. and V.W. Ca²⁺-induced PS exposure was reduced to 7% and 13% compared to controls. We conclude that i. patient V.W. is a new case of Scott syndrome, ii. Ca²⁺ entry in the platelets and lymphoblasts from both Scott patients is normal, and iii. elevated [Ca²⁺]ᵢ as caused by SMCE is not sufficient to trigger PS exposure.

Keywords
Procoagulant activity, Scott syndrome, platelets, B-lymphoblasts, intracellular calcium

Introduction

Cell surfaces containing anionic phospholipids, in particular phosphatidylserine (PS), play a pivotal role in blood coagulation. These procoagulant lipid membranes form a catalytic surface for the assembly and proper conformation and juxtaposition of the factor X and prothrombin activating enzyme complexes. Formation of these complexes strongly accelerates the rate of thrombin formation, which is responsible for the production of a stabile fibrin clot. Cellular procoagulant lipid surfaces are mainly...
provided by platelets. Upon appropriate stimulation, platelets lose their asymmetric transbilayer phospholipid distribution, resulting in exposure of PS at the exterior cell surface.

The Scott syndrome is a rare, hereditary bleeding disorder characterized by impaired surface exposure of PS at the plasma membrane of activated platelets and decreased shedding of procoagulant platelet-derived microvesicles (1). The Scott phenotype, as apparent in platelets, has also been observed in erythrocytes and Epstein-Bar virus (EBV)-transformed B-lymphocytes (2-4), indicating a cell type-independent trait. Weiss et al., in 1979, were the first to identify a patient (M.S.) with this disorder (1). One year later, a report was published on ten individuals from three unrelated Welsh families, who also suffered from an unusual bleeding disorder (5). The latter patients have not been studied extensively since then, but the original data suggest that their bleeding abnormality might be similar or possibly identical to the Scott syndrome. Later, also a French family has been found of which the propositus had platelets deficient in PS exposure and microvesiculation, but not in secretion and aggregation (6-8). Recently, a colony of German shepherd dogs was discovered with a hereditary bleeding disorder and deficient PS exposure in platelets, characteristic of Scott syndrome (9). The inability of platelets and other blood cells to scramble phospholipids, and thus expose PS, has been assigned to abnormal activity of a Ca\(^{2+}\)-dependent phospholipid scramblase activity in the plasma membrane of these cells.

Cytosolic [Ca\(^{2+}\)], serves as an intracellular mediator for many extracellular signals in platelets and other cells, regulating processes such as cellular shape, secretion, growth and differentiation (10-12). In earlier work with platelets, it appeared that a prolonged, high increase in [Ca\(^{2+}\)] is required for PS exposure, e.g. as achieved by Ca\(^{2+}\) ionophore, collagen receptor (glycoprotein VI) stimulation (13-15) or the combination of thrombin and thapsigargin (16). Release of Ca\(^{2+}\) from intracellular stores is insufficient for full platelet activation. However, following depletion of the intracellular Ca\(^{2+}\) stores, a mechanism is activated which allows Ca\(^{2+}\) entry across the plasma membrane. This store-mediated Ca\(^{2+}\) entry (SMCE) from the extracellular medium is a prerequisite to reach the high Ca\(^{2+}\) signals required for full platelet activation (17). In platelets and related cells at least a substantial part of the SMCE is mediated by plasma membrane ion channels of the Trp family (18). Blocking of this Ca\(^{2+}\) entry process abolishes agonist-induced PS exposure (15, 19) and, conversely, increased Ca\(^{2+}\) influx in platelets is correlated with higher PS exposure (20, 21). Recently, Martinez et al. reported of a reduced SMCE in B-lymphoblasts derived from the propositus of the French Scott family, whereas the release of Ca\(^{2+}\) from intracellular stores in these cells was normal (4, 22). Together, these observations suggest that defective store-mediated (capacitative) Ca\(^{2+}\) entry may be part of the Scott phenotype. However, studies on SMCE have thus far only been performed with the French Scott patient.

In this paper, we examine the relationship between Ca\(^{2+}\) fluxes and exposure of procoagulant PS in the plasma membrane of both platelets and immortalized, EBV-transfected B-cells from two control subjects and two different Scott patients. We simultaneously measured Ca\(^{2+}\) fluxes and PS exposure in individual platelets from these patients using fluorescence imaging microscopy. In the viable, non-apoptotic population of the B-cell lines, we determined the Ca\(^{2+}\) responses evoked by store-depleting agonists, as well as the PS exposure by dual-label flow cytometry.

### Materials and methods

#### Materials

- Fibrillar type-I collagen from equine tendon (Collagen reagent Horm®) was purchased from Nycomed (Munich, Germany).
- Fluorescein isothiocyanate (FITC)-labeled annexin V (Fl-annexin V) was a product from Nexins Research (Hoeven, The Netherlands).
- Fura-2 and Flu-3 acetoxymethyl esters, Pluronic F-127 and Alexa Fluor 647-labeled annexin V (AF-annexin V) were purchased from Molecular Probes (Leiden, The Netherlands).
- Bovine serum albumin (BSA), apyrase (grade V), ionomycin, caffeine and thapsigargin were from Sigma (St. Louis, MO, USA).
- RPMI-glutamax I was obtained from Bio-Whittaker (Walkersville, MD, USA).
- Fetal calf serum and antibiotic antimycotic solution were obtained from Life Technologies (Paisly, UK), and Lymphoprep came from Nycomed Pharma AS (Oslo, Norway).
- Coagulation factors thrombin, prothrombin, factor Xa and factor Va were purified as described before (23). Thrombin-specific chromogenic substrate, H-D-Phe-Pip-Arg-para-nitroaniline (S2238) was from Chromogenix (Mölndal, Sweden).

#### Patients

Clinical features of patient M.S. have been described extensively before (24). Patient V.W. had presented initially at the age of 14 years with a history of excessive bleeding after dental extractions on five occasions. Dental surgery at age 22 again resulted in excessive blood loss, with a fall in haemoglobin concentration from 14.6 to 9.3 g/dl. At 34 years of age she suffered life-threatening post partum haemorrhage after an uncomplicated forceps delivery. Studies at that time revealed an isolated severe abnormality of prothrombin conversion consistent with a defective interaction between plasma and platelets or phospholipid. All other tests of haemostasis were normal (5).

#### Platelet preparation and culturing of B-lymphoblasts

Freshly obtained citrated blood from patients M.S., V.W. and two control subjects was shipped at 4°C by airmail to Maastricht (The Netherlands). Immediately upon arrival (24-48 hours later), platelets were washed and used for the experiments.
Loading of platelets with Fura-2 and washing procedures have been described elsewhere (25, 26). Washed platelets were resuspended in Hepes buffer (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 0.1% BSA and 0.1% glucose, adjusted at pH 7.45) at a final concentration of 1.0 x 10⁶ platelets/ml. Erythrocytes were washed 3 times in the same Hepes buffer (pH 7.45) and resuspended at 1 x 10⁸ cells/ml.

Data of platelets from M.S. presented in this study were obtained in 1996 on the last occasion that we received blood from this patient. Since M.S. deceased that same year, there was no opportunity to study the platelets from this patient with those from patient V.W. simultaneously. Experiments with blood cells from patient V.W. were performed in 2002.

EBV-transformed B-cells from M.S. and a control subject were available from previous studies (27). EBV-transformed B-cells from patient V.W. were prepared by Professor E.G.D. Tuddenham (Imperial College School of Medicine, London). Before use, the EBV-infected cells were expanded in RPMI-glutamax I culture medium containing 10% (v/v) fetal calf serum and antibiotic antimitotic solution. Cells were kept at 37°C in a humidified atmosphere with 5% CO₂, and were sub-cultured in 3 volumes fresh medium every 2 or 3 days. Data of EBV-transformed B-lymphocytes from both patients and two corresponding controls were collected from experiments performed in 2002.

Procoagulant activity of platelets and erythrocytes in suspension

Prothrombinase activity of washed platelets in suspension, either activated for 15 min with collagen (10 μg/ml) plus thrombin (4 nM) or for 5 min with ionomycin (1 μM), was determined by measuring the rate of conversion of prothrombin to thrombin by the enzyme complex factor Xa-factor Va, as described before (26). The assay conditions were: 3 x 10⁶ platelets/ml, 3 mM CaCl₂, 3 nM factor Xa, 6 nM factor Va and 4 μM prothrombin (all final concentrations). Chromogenic thrombin substrate S2238 (250 μM) was used to determine the amount of thrombin formed. To measure procoagulant activity of erythrocytes, washed cells at a concentration of 2 x 10⁶/ml were incubated with 5 μM ionomycin in the presence of 1 mM CaCl₂ for 60 min. Samples from this incubation are diluted to a cell count of 10⁷/ml, and prothrombinase activity was measured using the conditions described above.

Dual measurement of [Ca²⁺], and phosphatidylserine exposure by fluorescence imaging microscopy

Intracellular Ca²⁺ and PS exposure were determined by a quasi-simultaneous measurement of fluorescence from fura-2 and FI-annexin V using a combined fluorescence imaging and microphotometric system as described previously (15). Changes in fluorescence were measured in single platelets after 25 minutes adhering to a collagen-coated coverslip. The incubation chamber contained 5 x 10⁷ Fura-2-loaded platelets in 300 μl Hepes buffer with 2 mM CaCl₂ and 0.5 μg/ml FI-annexin V. Fluorescence of about 100 cells in three or more microscopic fields was measured. Conversion of Fura-2 fluorescence ratio to levels of [Ca²⁺], was made as described before (17). During the measurements in 1996 (patient M.S. and control 1) the optical pathway was slightly different from that used in 2002 (patient V.W. and control 2), resulting in slightly different calibration values of [Ca²⁺]. Platelets were considered to be elevated in [Ca²⁺], when their Fura-2 ratio was increased with 0.2 compared to basal, corresponding to a rise of about 85 nM; annexin V positivity was defined as all localized fluorescence in the fluorescein channel of >30 pixels.

Dual measurement of [Ca²⁺], and phosphatidylserine exposure by flow cytometry

Cultured EBV-transformed B-lymphocytes derived from patients and control subjects were centrifuged at 700 g for 10 min, and resuspended in Hepes buffer pH 7.45 and the cell count was adjusted to 10⁶ cells/ml. Washed B-lymphoblasts were loaded with 3 μM Fluo-3 acetoxyethyl ester in the presence of 1 mg/ml Pluronic F-127 at room temperature for 30 min. Loaded cells were activated with 10 μM ionomycin (5 min), 1 μM thapsigargin (5 min), or 10 mM caffeine (10 min). Activations were carried out in the presence of either CaCl₂ (2 mM) or EGTA (2 mM) to study total Ca²⁺ responses and Ca²⁺ mobilization separately. Where indicated, the loaded cells were pre-incubated with AF-annexin V, as described by the manufacturer, to monitor PS exposure. After activation, Fluo-3 and Alexa Fluor 647 fluorescence were simultaneously measured using a FACScan flow cytometer, and Cell-Quest software (Becton-Dickinson, CA, USA). For analysis, viable B-cells were gated based on their forward and side scatter, to exclude apoptotic cells and cellular fragments. Per assay, a minimum of 5000 events was counted. Control measurements were always run with unlabeled/stimulated as well as labeled/unstimulated cells. Green (Fluo-3) and red (Alexa Fluor 647) fluorescence intensities were expressed as arbitrary units. Dot-plots of fluorescence versus forward scatter of control cells in the absence of fluorescent annexin V were used to define the region containing 99% of the annexin V-negative cells. Events outside this region were considered to be positive. List-mode data were analyzed using WinMDI 2.8 software (http://facs.scripps.edu).

Results

Procoagulant activity of platelets and erythrocytes in suspension

First we established the Scott syndrome phenotype of the Welsh patient, V.W. As shown in Table 1, washed platelets of this patient in suspension activated by collagen plus thrombin result-
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ed in a prothrombinase activity of 52% of that achieved with similarly activated platelets from the control subject, whereas stimulation with ionomycin induced a procoagulant response of only 17% of that of ionomycin-treated control platelets. Considering the normal levels of coagulation factors and normal platelet aggregation (5), these findings confirm the phenotype of a Scott syndrome of patient V.W. This was further substantiated by the observation that washed erythrocytes of V.W., treated with ionomycin and Ca\(^{2+}\), were deficient in forming a procoagulant surface: prothrombinase activity was 5.6 ± 1% (mean ± SD, n = 5) of that of control erythrocytes. When the prothrombinase activity of completely lysed cell preparations (obtained by sonication) of either platelets or erythrocytes was measured, there was no appreciable difference between patients and control subjects.

### Table 1: Prothrombinase activity of suspensions of platelets from control subjects and Scott patients.

<table>
<thead>
<tr>
<th>Activation</th>
<th>control 1</th>
<th>patient M.S.</th>
<th>control 2</th>
<th>patient V.W.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>136 ± 11</td>
<td>54 ± 3 (40%)</td>
<td>130 ± 22</td>
<td>84 ± 16 (65%)</td>
</tr>
<tr>
<td>Collagen + thrombin</td>
<td>358 ± 37</td>
<td>119 ± 44 (33%)</td>
<td>336 ± 10</td>
<td>176 ± 30 (52%)</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>1159 ± 247</td>
<td>247 ± 71 (18%)</td>
<td>1172 ± 6</td>
<td>200 ± 6 (17%)</td>
</tr>
</tbody>
</table>

Values presented are means ± SD of three independent measurements performed in 1996 for patient M.S and in 2002 for patient V.W., each with their corresponding control. In brackets is given the activity expressed as a percentage of the correspondingly treated control platelets. It should be emphasized that due to the time between collection of the blood and the prothrombinase measurement (24-48 hrs), the prothrombinase activity of the non-stimulated platelets is slightly increased compared to that of freshly prepared platelet suspensions.

![Figure 1: Collagen-induced Ca\(^{2+}\) responses of single platelets from control subjects and Scott patients.](image)

Fura-2-loaded platelets were allowed to adhere to collagen fibers in the presence of CaCl\(_2\) (2 mM). Changes in Fura-2 fluorescence ratio in single platelets were measured by fluorescence video imaging microscopy. (A) Experiments were performed in 1996 with platelets from control 1 and patient M.S. (B) Experiments with platelets from control 2 and patient V.W. were carried out in 2002. Changes in Fura-2 fluorescence ratio (340/380 nm excitation) were recorded during adhesion and activation, as described in Materials and Methods. Traces are averaged overlays of single traces obtained from at least twenty platelets per subject (n≥3 incubations/subject).
controls. This excludes that the decreased procoagulant activity in the patient’s platelets and erythrocytes is due to alterations in the overall phospholipid composition, i.e. a reduced content of PS.

Finally, similar to previous findings on the morphology of the erythrocytes of propositus M.S. (2), the red cells of V.W. did not change into echinocytes after treatment with ionomycin and were unable to produce microvesicles.

Relation between calcium responses and phosphatidylserine exposure of single platelets
Fluorescence video microscopic imaging was used to examine whether the Ca$^{2+}$ fluxes and PS exposure of the platelets from the patients differ from those of control subjects. In one set of experiments, Fura-2-loaded platelets from M.S. were compared with the simultaneously collected platelets from control 1. In a later set of experiments, platelets from V.W. were compared with those of control 2. The platelets were allowed to interact with collagen fibers, during which glycoprotein VI-induced changes in [Ca$^{2+}$], were measured at a single-cell level by capturing fluorescence ratio images. Patient and control platelets showed prominent increases in [Ca$^{2+}$]$_i$ shortly after adhesion to collagen (Fig. 1). It appeared that the collagen-induced rises in [Ca$^{2+}$]$_i$ in the platelets from either patient were similar in magnitude to those of the platelets from the two controls.

As the glycoprotein VI-induced increase in [Ca$^{2+}$]$_i$ is a major signal in the procoagulant response, i.e. PS exposure (19), we determined in the same experiment also the binding to platelets of FL-labeled annexin V, which binds to surface-exposed PS (28, 29). After 25 min adhesion to collagen, 40% of the activated platelets of control 1 were annexin V positive, in comparison to less than 3% of the platelets of Scott patient M.S. (Fig. 2A). Similarly, the collagen-activated platelets from patient V.W. showed a strongly reduced PS exposure in comparison to those of control 2, although still 8% of the patient platelets did bind FL-annexin V, i.e. slightly more than observed for patient M.S. (Fig. 2B). These observations confirm the Scott syndrome phenotype in patient V.W.

Calcium responses of activated B-lymphoblasts
Immortalized B-cells from patient M.S. and control subject 1 and from patient V.W. and control subject 2 were compared simultaneously. To analyze the Ca$^{2+}$ responses of these four lymphoblast cell lines, the cells were loaded with the fluorescent probe Fluo-3 and then stimulated with agonists, reported to cause reduced SMCE in B-lymphoblasts derived from the French Scott patient (22). Activation was carried out in the presence of EGTA to measure Ca$^{2+}$ mobilization from intracellular stores only, and in the presence of CaCl$_2$ to allow complementary SMCE. Agonist-evoked changes in Fluo-3 fluorescence were measured in single cells by flow cytometry, using a gating profile so that only fluorescence from non-apoptotic, viable cells was analyzed. Treatment of lymphoblasts from patients and control subjects with a high dose of ionomycin (10 µM) in the presence of EGTA caused the same increase in Fluo-3 fluorescence, indicative for a similar, maximal degree of Ca$^{2+}$ store

![Figure 2: Phosphatidylserine exposure of activated, collagen-adherent single platelets from control subjects and Scott patients. Fura-2-loaded platelets were allowed to adhere to collagen fibers in the presence of FL-annexin V and CaCl$_2$, as described for Fig. 1. Results given are percentages of annexin V-positive platelets which exhibit an increased Fura-2 fluorescence ratio, i.e. elevated [Ca$^{2+}$]. Values are means ± SD from at least three independent incubations. (A) Experiments were performed in 1996 with platelets from control 1 and patient M.S. (B) Experiments with platelets from control 2 and patient V.W. were carried out in 2002.](image-url)
depletion (Table 2). Activation with the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (1 µM) in the presence of EGTA to deplete SERCA-related store compartments, induced similar [Ca^{2+}]i rises in control and patient cell lines, but the levels were slightly lower than obtained with ionomycin (Table 2). Addition of caffeine (10 mM) in the presence of EGTA, which causes depletion of the ryanodine-sensitive Ca^{2+} stores (in which Ca^{2+} is released through ryanodine-rather than inositol-trisphosphate-activated receptors) (22), had an effect on all cell lines comparable to that of thapsigargin. Together, these data indicate that the Ca^{2+} release from the intracellular store compartments is similar for the various control and patient B-cell lines.

To allow capacitative Ca^{2+} entry, the Fluo-3-loaded B-lymphoblasts were activated using the same agonists with extracellular CaCl_2 present. Ionomycin/CaCl_2 caused a high increase in [Ca^{2+}]i, which was similar for the control and patient B-lymphoblasts (Table 2). Thapsigargin/CaCl_2 resulted in a smaller increase in [Ca^{2+}]i due to SMCE. This response was somewhat lower in the V.W. cells in comparison to the control, but the difference was not statistically significant (Table 2). When capacitative Ca^{2+} entry was induced with caffeine/CaCl_2, the Ca^{2+} responses were lower than with thapsigargin/CaCl_2, but no differences in responsiveness were observed between the various cell lines. Combined treatment with thapsigargin/caffeine/CaCl_2 did not further increase the thapsigargin-induced Ca^{2+} response (fold fluorescence increase: 4.6 ± 0.2 for control 1, 4.7 ± 0.4 for M.S. (mean ± SD, n = 3)), indicating efficient coupling of the thapsigargin-sensitive stores to the SMCE Ca^{2+} influx channels. These results typically differ from those obtained with EBV-transfected B-cells from the French Scott patient, in which case both Ca^{2+}-ionophore/CaCl_2 and thapsigargin/CaCl_2 induced substantially lower Ca^{2+} responses (22). Thus, the present observations show that Ca^{2+} release from intracellular stores and Ca^{2+} entry are not significantly altered in viable, non-apoptotic B-lymphoblasts cells from these two different Scott patients.

Relation between calcium responses and phosphatidylserine exposure in B-lymphoblasts from patients
To address the question whether the B-lymphoblasts from the patients have retained the Scott-type characteristics, we used a double-labelling procedure to directly compare Ca^{2+} responses (Fluo-3 loading) and PS exposure (AF-annexin V addition) by flow cytometric analysis. Knowing that apoptosis of B-lymphoblasts, even from Scott patients, is accompanied by PS exposure (27), all analysis were performed exclusively on the non-apoptotic cell population. In the absence of stimulation, both the control B-cells and the cells derived from patients M.S. and V.W. exhibited comparably low PS exposure (Table 3). When treated with ionomycin (10 µM) in the presence of CaCl_2, 85-90% of the B-lymphoblasts from the controls became PS-positive, versus only 6% and 11% of the B-lymphoblasts of patient M.S. and V.W., respectively.

When SMCE was induced by CaCl_2 after emptying the Ca^{2+} stores with thapsigargin, no PS exposure was observed in either control or patient B-lymphoblasts (Table 3), despite the fact that

### Table 2: Total Ca^{2+} responses and Ca^{2+} mobilization in activated EVB-B lymphoblasts from two controls and two Scott patients. Flow cytometric results are expressed as fold increase in fluorescence intensity (arbitrary units) relative to the control (1.00). Under unstimulated conditions, averaged fluorescence intensities of the populations of Fluo-3-loaded cells were similar for all four cell lines. Values shown are means ± SD from at least four independent experiments.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Fluo-3 fluorescence (fold increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control 1</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>1.00</td>
</tr>
<tr>
<td>Ionomycin, EGTA</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Thapsigargin, EGTA</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Caffeine, EGTA</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Ionomycin, CaCl_2</td>
<td>7.5 ± 1.4</td>
</tr>
<tr>
<td>Thapsigargin, CaCl_2</td>
<td>4.8 ± 0.8</td>
</tr>
<tr>
<td>Caffeine, CaCl_2</td>
<td>2.9 ± 1.1</td>
</tr>
</tbody>
</table>

### Table 3: Phosphatidylserine exposure in non-apoptotic Fluo-3-loaded B-lymphoblasts from two controls and two Scott patients. Data represent percentages of AF-annexin V positive cells (means ± SD, n = 4-6). *P<0.05 compared to value of corresponding control cells.

<table>
<thead>
<tr>
<th>Condition</th>
<th>% AF-annexin V binding cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control 1</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>4.0 ± 1.8</td>
</tr>
<tr>
<td>Ionomycin, CaCl_2</td>
<td>90.0 ± 2.7</td>
</tr>
<tr>
<td>Thapsigargin, CaCl_2</td>
<td>5.0 ± 2.5</td>
</tr>
<tr>
<td>Caffeine, CaCl_2</td>
<td>3.0 ± 0.1</td>
</tr>
</tbody>
</table>

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Calcium entry and procoagulant activity in Scott syndrome blood cells

Figure 3: Relation between [Ca²⁺]i and phosphatidylserine exposure in stimulated non-apoptotic EBV-B lymphoblasts from controls and patients. Fluo-3-loaded B-lymphoblasts derived from controls 1 and 2 (closed symbols) and Scott patients M.S. and V.W. (open symbols) were activated with ionomycin, thapsigargin or caffeine in the presence of CaCl₂, as described in Materials and Methods. After staining with AF-annexin V, levels of Fluo-3 fluorescence and Alexa Fluor 647 fluorescence were compared in the same population of non-apoptotic cells using flow cytometry. Data are means ± SD (n = 4-6 independent experiments).

the thapsigargin-induced Ca²⁺ responses were high (see Table 2). Similarly, addition of caffeine – either alone or in combination with thapsigargin – did not lead to significant PS exposure in viable cells for any of the cell lines.

Further analysis of the double label flow-cytometric data clearly indicate that the Ca²⁺ signals caused by thapsigargin/CaCl₂ were lower but in the same range as those evoked by ionomycin/CaCl₂, whereas only the latter agonist was capable to induce PS exposure in control lymphoblasts (Fig. 3). Thus, in the control cells, ionomycin appears to be a much better stimulus for PS exposure than thapsigargin or caffeine, despite the fact that the latter agents cause substantial Ca²⁺ influx, suggesting a particular action of ionomycin/Ca²⁺ on the phospholipid scrambling process.

Discussion

The transmembrane phospholipid asymmetry, characteristic of the plasma membrane of most mammalian cells, is maintained by two distinct plasma membrane proteins: the aminophospholipid translocase, which causes a rapid inward transport of aminophospholipids, and –as demonstrated for erythrocytes- the multidrug resistance protein MRP1, which facilitates a slow outward movement of membrane phospholipids, irrespective the composition of the polar headgroup. These proteins assure that procoagulant anionic phospholipids, such as PS, remain localized in the cytoplasmic leaflet of the plasma membrane (reviewed in 30). A third protein is responsible for a rapid bidirectional transbilayer movement of the phospholipids in the plasma membrane. Activation of this protein causes a collapse of the asymmetric lipid distribution in the plasma membrane; hence this protein has been termed ‘phospholipid scramblase’ (30). Activation of the scramblase causes exposure of PS at the cell surface, which serves an important function in the process of blood coagulation. Patients with an impaired scramblase activity suffer from a bleeding disorder, referred to as Scott syndrome, illustrating the importance of this membrane remodelling process. Scrambling activity has been demonstrated in most haematological cells; Sims and coworkers have cloned a protein from erythrocytes with Ca-dependent scrambling activity, a protein which is now referred to as human phospholipid scramblase (hPLSCR1, reviewed in 31). However, there is increasing doubt about the true function of this protein. As was found recently, adult PLSCR1(-/-) mice showed no obvious haematologic or haemostatic abnormality, and blood cells from these animals normally mobilized phosphatidylinerine to the cell surface upon stimulation (32). Thus, the identity of the scramblase remains to be elucidated. The finding that the phenotype of defective PS exposure in the Scott syndrome affects all cells of the haematological lineage as well as the hereditary transmission of this disorder strongly suggest the involvement of a protein in the scrambling process. Although it has been demonstrated that lipid scrambling does not require hydrolysis of ATP and that activity of the scramblase requires a persistent elevation of intracellular Ca²⁺, the mode of action and the regulation of the scramblase remain to be elucidated.

In the present study, we introduce a new case with characteristics of the Scott syndrome. Patient V.W. has been described in an early study published in 1980 as the propositus of a family with a platelet-related bleeding disorder despite a normal coagulation profile (5), but has not been recognised as having Scott syndrome. The data obtained in this study clearly demonstrate an impaired Ca²⁺-induced PS exposure in the patient’s erythrocytes, platelets and lymphoblasts as judged from decreased prothrombinase activities and annexin V binding, confirming the Scott phenotype. Note that for patient V.W. the procoagulant response of platelets treated with ionophore seems to be more impaired than that of platelets activated with collagen plus thrombin. This has been a consistent finding over several years with the platelets of patients M.S. In both the French propositus with Scott syndrome and the canine model of Scott syndrome, platelet prothrombinase activity was virtually absent, independent of whether the cells were activated with ionophore or with collagen plus thrombin (6, 9). The reason for this difference in phenotype is as yet unclear. Whether the partially impaired procoagulant response after collagen plus
thrombin stimulation of platelets of patients V.W. and M.S. illustrates different pathways of activation or the existence of more than one mechanism for phospholipid scrambling in platelets remains to be investigated. In this respect, it is of interest to mention that the defect in ionomycin-induced PS exposure in the EBV-transformed B cells of the two patients is not observed for the apoptotic cells from these patients (data not shown), in agreement with the suggestion that phospholipid scrambling in apoptotic cells is differently regulated from the scrambling caused by Ca²⁺-elevating agents (27)).

SMCE following Ca²⁺ mobilization from intracellular stores presents the most important Ca²⁺ entry pathway in platelets and lymphocytes. Recently, Martinez and co-workers have described that B-lymphoblasts from a French Scott patient exhibit normal Ca²⁺ release from the thapsigargin- and caffeine-depletable Ca²⁺ stores, but show a considerably reduced subsequent Ca²⁺ entry, along with a reduced PS exposure (4, 22). Interestingly, Ca²⁺ entry following depletion of both the thapsigargin and caffeine-sensitive stores became normalized in these B-lymphoblasts. In the present study, we also measured thapsigargin and caffeine-mediated release of Ca²⁺ from stores as well as subsequent Ca²⁺ entry. In agreement with their observations, we found no indications for disturbances in the release from stores. However, in contrast to the French Scott B-lymphoblasts, the B-cells from both patients M.S. and V.W. appeared to have normal Ca²⁺ influx via SMCE with thapsigargin or caffeine as well as Ca²⁺ ionophore. Furthermore, the combination of thapsigargin and caffeine did not further increase the Ca²⁺ signal or the PS exposure. The reason for this discrepancy is not known, but may reflect a variant of the Scott syndrome.

The finding that Ca²⁺ responses in both the platelets and B-cells from patients M.S. and V.W. are unaltered indicates that aberrant Ca²⁺ signaling - at least in these two patients - is not part of the Scott phenotype. The double-labelling techniques applied with both platelets and non-apoptotic B-cells show that Scott cells of either type are greatly diminished in PS exposure although being high in [Ca²⁺], Even for the control B-cells, there is no simple relationship between elevated [Ca²⁺] and PS exposure (Fig. 3), suggesting that the scrambling process is not dependent on high [Ca²⁺], only, but relies on other intracellular processes as well, in agreement with previous findings (16). This suggestion is supported by recent evidence that also in stimulated human T-lymphocytes the phospholipid scrambling with Ca²⁺ ionophore is mediated by factors other than elevated [Ca²⁺] only (33).

In summary, the present study shows that patient V.W. can be considered as having a Scott syndrome phenotype. The impaired lipid scrambling in platelets and B-cells from both Scott patients presented here, cannot be ascribed to alterations in Ca²⁺ release from intracellular stores or subsequent Ca²⁺ entry.

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