Biogenesis of endoplasmic reticulum proteins involved in Ca^{2+} signalling during megakaryocytic differentiation: an *in vitro* study

Christine LACABARATZ-PORRET, Sophie LAUNAY, Elisabeth CORVAZIER, Raymonde BREDOUX, Béla PAPP and Jocelyne ENOUF¹

Institut National de la Santé et de la Recherche Médicale U 348, IFR 6 Circulation Lariboisière, Hôpital Lariboisière, 8 rue Guy Patin, 75475 Paris Cedex 10, France

The endoplasmic reticulum (ER) plays a key role in Ca^{2+} signalling through Ca^{2+} release via inositol 1,4,5-trisphosphate receptors (InsP_3 -Rs) and Ca^{2+} uptake by sarco/endoplasmic reticulum Ca^{2+} -ATPases (SERCAs). Here, we investigated the organization of platelet ER and its biogenesis during megakaryocytopoiesis. First, erythro/megakaryoblastic MEG 01, UT7, M-O7e and CHRF 288-11 cell lines, platelets and thrombopoietin-induced UT7-Mpl cells were selected for the study of SERCA2b and SERCA3 proteins by Western blotting using the antibodies IID8 and PL/IM430, respectively. As judged by platelet glycoprotein IIIa (GPIIIa) expression, an increase in SERCA3 proteins was observed while that of SERCA2b remained unchanged throughout maturation. Second, these studies were extended to the newly described alternatively spliced SERCA3a–c RNAs and InsP_3 -Rs using the *in vitro* model of PMA-induced differentiation of MEG 01 cells. Time-course and dose–response studies showed a maximal approx. 4-fold up-regulation of SERCA3 proteins using 10^{-8} M PMA for 3 days, which paralleled induction of GPIIIa expression. SERCA3 induction was found to occur at the level of mRNA. The modulation of the different SERCA3 species (i.e. 3a, 3b and 3c) was isoform-specific: while

SERCA3a was slightly increased, an approx. 3-fold induction of SERCA3b, and a 4-fold induction of SERCA3c, was observed after 24 h of PMA treatment. Isoform-specific Western blotting and/or reverse transcriptase PCR studies showed that InsP_3 -R types I, II and III are expressed in MEG 01 cells, as well as in platelets. Study of the expression of these InsP_3 -R types in PMA-induced MEG 01 cells revealed that: (i) InsP_3 -RI protein and mRNA showed no changes; (ii) InsP_3 -RII mRNA was up-regulated and peaked at hour 48 and (iii) InsP_3 -RIII mRNA and protein showed a transitory maximal 3- and 2.3-fold increase at hours 6 and 30, respectively. Upon PMA treatment of CHRF 288-11 cells, in which GPIIIa is not induced upon treatment, a similar pattern of regulation of InsP_3 -R types II and III was seen, but a distinct pattern of SERCA3 regulation was observed. These results suggest a profound reorganization of ER-protein patterns during megakaryocytopoiesis and underline the role of SERCA3 gene regulation in the control of Ca^{2+} -dependent platelet functions.

Key words: calcium, InsP_3 receptor, megakaryocytic maturation, SERCA.

INTRODUCTION

‘One of the most versatile and universal signalling agents in the human body is the calcium ion, Ca^{2+} . How does this simple ion act during cell birth, life and death, and how does it regulate so many different cellular processes?’ [1]. Recent progress reveals that Ca^{2+} signalling acts to channel many ubiquitous receptor-mediated signal-transduction processes into specific responses via selective regulation of enzymes or transcription factors [2–4]. This occurs through the ability of these proteins to decode complex and dynamic changes of cellular Ca^{2+} levels.

Several different proteins, located in the plasma membrane and the endoplasmic reticulum (ER), are involved in the regulation of cell Ca^{2+} signalling, but their precise functions in non-muscle cells have not been fully elucidated to date. Among the better-known structures are Ca^{2+} channels, namely inositol 1,4,5-trisphosphate receptors (InsP_3 -Rs) [5,6] and Ca^{2+} pumps or sarco/endoplasmic reticulum Ca^{2+} -ATPases (SERCAs) [7,8], which co-ordinate opposite Ca^{2+} ion fluxes. InsP_3 -Rs induce an increase in cytosolic Ca^{2+} concentration as a result of Ca^{2+} release from ER through the binding of InsP_3 on InsP_3 -Rs, whereas SERCAs are responsible for a decrease in cytosolic Ca^{2+} concentration due to Ca^{2+} resequestration in the ER Ca^{2+} stores.

Three types of InsP_3 -R, types I–III, are encoded by three distinct genes, which share 60–70% amino acid sequence identity and are expressed in a tissue-specific manner. The existence of two additional InsP_3 -R types, types IV and V, has been proposed based on partial sequence information. Further heterogeneity is generated through alternative splicing of InsP_3 -R types I and II. More recently, a truncated form of InsP_3 -R type II has been described [6]. Similarly, SERCA isoforms are encoded by three different genes, SERCA1–3. The tissue-specific expression and alternative splicing in the 3' end of SERCA1 and SERCA2 have been well described. SERCA1a and SERCA1b isoforms are expressed in adult and neonatal skeletal muscle, respectively. SERCA2a is expressed in cardiac muscle, whereas SERCA2b is expressed ubiquitously. A third non-muscle SERCA cDNA, SERCA3a, has also been described, and the very recent cloning of the human gene provided evidence for at least three distinct mRNAs, SERCA3a, 3b and 3c [8], that differ in their 3' ends due to alternative splicing.

The observation that several InsP_3 -R and SERCA species are co-expressed in the ER suggests that the calcium homeostasis and signalling of this organelle is highly sophisticated, and may also involve functionally distinct ER subcompartments. This would permit fine and complex spatiotemporal variations in Ca^{2+}

Abbreviations used: ER, endoplasmic reticulum; GPIIIa, glycoprotein IIIa; InsP_3 -R, inositol 1,4,5-trisphosphate receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+} -ATPase; TPO, thrombopoietin; RT, reverse transcriptase; MuLV-RT, murine leukaemia virus-RT; GM-CSF, granulocyte macrophage-colony-stimulating factor.

¹ To whom correspondence should be addressed (e-mail jocelyne.enouf@inserm.lrb.ap-hop-paris.fr).

signals, converting the activation of a tremendous variety of receptor-dependent signals into highly specific biological responses.

Platelet pathophysiology, which is associated with particularly complex and dynamic Ca^{2+} signalling, may be viewed as a paradigm [9]. First, a large body of evidence suggests that human platelet Ca^{2+} signalling is controlled by the content of ER intracellular Ca^{2+} pools. Secondly, in platelets InsP_3 -sensitive and -insensitive Ca^{2+} pools co-exist [10–12]. Thirdly, the identification of platelet ER proteins showed co-expression of multiple and diverse InsP_3 -Rs [13–16] and SERCAs [17]. Although some controversy exists concerning the expression of InsP_3 -Rs, human platelets were reported to express either InsP_3 -RI and II or InsP_3 -RI and III. About SERCAs, platelets account for the first cells shown to simultaneously express the ubiquitous SERCA2b [18] and SERCA3a proteins [19–21] as well as SERCA3b [22] and SERCA3c [23] mRNAs.

Here, in an attempt to address the issue of whether all these ER proteins are needed to achieve platelet function, we investigated the biogenesis of these ER membrane proteins during megakaryocytic differentiation. SERCA expression was first studied at the protein level by using two different experimental models of megakaryocytic differentiation. For this, SERCA2b and SERCA3 were analysed by Western blotting using the IID8 and PL/IM430 monoclonal antibodies respectively. On one hand, different human erythro/megakaryocytic cell lines, which are currently used to study the proliferation, differentiation and maturation of megakaryocytes (reviewed in [24]), were selected and compared with human platelets. On the other hand, we addressed the *in vivo* situation by studying UT7-Mpl cells treated with thrombopoietin (TPO), the endogenous regulator of megakaryocyte and platelet production. Then, we extended these protein studies to SERCA3 mRNAs as well as to InsP_3 -Rs. These studies were facilitated by using a model of the *in vitro* mechanisms governing megakaryocyte maturation, that of MEG 01 cells upon PMA treatment. Dose responses and time courses of PMA effects were analysed for overall protein and mRNA studies by Western blotting using the above antibodies added to specific InsP_3 -R antibodies and reverse transcriptase (RT)-PCR respectively. Last, CHRF 288-11 cells, which did not differentiate upon PMA treatment, were studied in the same way to look for some specificity of ER-protein expression upon megakaryocytic maturation.

Taken together, our results suggest a profound reorganization of the expression of major ER proteins involved in Ca^{2+} signalling during megakaryocytic differentiation. This suggests a process of specialization of the ER and thus a remodelling of intracellular Ca^{2+} stores during megakaryocytopoiesis, required for platelet-specific Ca^{2+} -dependent signalling.

EXPERIMENTAL

Materials

RPMI 1640 medium with Glutamax-I was from Gibco-BRL (Paisley, Scotland, U.K.). Penicillin and streptomycin were from Boehringer Mannheim (Mannheim, Germany). PMA, human recombinant granulocyte macrophage-colony-stimulating factor (GM-CSF) and DMSO were from Sigma (St. Louis, MO, U.S.A.). Monoclonal anti-SERCA 2 (IID8), polyclonal anti-calreticulin and anti- InsP_3 -R type-I antibodies were from Affinity BioReagents (Neshanic Station, NJ, U.S.A.). Anti- InsP_3 -R type III and control HeLa lysate were from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-rabbit and anti-mouse horse-radish peroxidase-conjugated antibodies, Texas Red-conjugated anti-rabbit and FITC-conjugated anti-mouse antibodies were

from Jackson ImmunoResearch (West Grove, PA, U.S.A.). Nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). Coloured protein molecular-mass markers, the 3'-oligolabelling system, enhanced chemiluminescence (ECL[®]) Western/Southern blotting reagents and Hybond-N⁺ nylon membranes were from Amersham International (Little Chalfont, Bucks., U.K.). Electrophoresis reagents were from Bio-Rad (Richmond, CA, U.S.A.). HeLa RNAs were from Clontech (Palo Alto, CA, U.S.A.). RNA Plus solution used for total RNA isolation was from Bioprobe (Montreuil sous Bois, France). Murine leukaemia virus-RT (MuLV-RT), RNase inhibitor and AmpliTaq DNA polymerase (from *Thermus aquaticus*) were from Perkin-Elmer (Branchburg, NJ, U.S.A.). Primers were from Eurogentec (Leuven, Belgium).

Preparation of human platelets

Blood from different samples was used for the isolation of human platelets. Platelet-rich plasma was obtained by centrifugation for 15 min at room temperature at 120 g. Platelets were then pelleted by centrifugation for 15 min at room temperature at 1500 g and washed three times with Tyrode buffer, pH 7.4, containing 5 mM EDTA [21].

Cell culture

The human erythro-megakaryoblastoid MEG 01 [25] and CHRF 288-11 [26] cell lines were grown in RPMI 1640 medium with Glutamax-I supplemented with 10% heat-inactivated fetal calf serum, 50 units/ml penicillin and 0.05 mg/ml streptomycin. The M-07e [27] and UT7 [28] cells, and the UT7-Mpl [29] stable transfectant expressing the murine TPO receptor, are dependent on the presence of the GM-CSF for their growth and survival. These cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and 1 ng/ml recombinant GM-CSF. All cell lines were incubated and cultured at 37 °C with 5% CO_2 in a humidified atmosphere.

PMA-induced differentiation of MEG 01 and CHRF 288-11 cells

Prior to experiments, cells were harvested, resuspended in the above medium at a density of 3×10^5 cells/ml and placed in 9-cm-diameter tissue-culture dishes. Different concentrations of PMA were added for time periods to the cells from concentrated stock solutions in DMSO, which did not exceed 0.1%. The cells, treated for the indicated times, were harvested by centrifugation at 400 g for 10 min, washed in PBS and used for lysate or RNA preparations, and for immunofluorescence experiments.

Preparation of cell lysates

Platelet and human erythro/megakaryocytic cell proteins were concentrated by precipitation of the cells with ice-cold 6% trichloroacetic acid. After centrifugation at 12000 g for 15 min at 4 °C, the pellets were resuspended in electrophoresis sample buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5 mM EDTA, 2 M urea, 100 mM dithiothreitol and 0.02% Bromophenol Blue [30] and homogenized by aspiration (20–30 strokes) using a Hamilton syringe. The protein concentration of the different lysates was determined using BSA as a standard and all samples were stored at -20 °C.

Electrophoresis and Western blotting

Samples were subjected to Laemmli-type SDS/PAGE on 8% gels [31]. Electrophoresis was performed at 140 V for 1 h and

Table 1 Primers used and sizes of mRNA amplification products

Oligonucleotide	Sequence and localization	Fragment size (bp)	Reference
SERCA2 (5')	²⁸⁶¹ TCA TCT TCC AGA TCA CAC CGC T ²⁸⁸²	269	[19]
SERCA2b (3')	³¹²⁹ TCA AGA CCA GAA CAT ATC GC ³¹¹⁰		
SERCA3 (5')	²⁶⁷⁴ GAG TCA CGC TTC CCC ACC ACC ²⁶⁹⁴	319	[39,40]
SERCA3a (3')	²⁹⁹² GGC TCA TTT CTT CGT GCA TGT GGT TC ²⁹⁶⁷		
SERCA3 (5')	As above	407	[22,39]
SERCA3b (3')	³⁰⁸⁰ GGC TCA TTT CTT CCG GTG TGG TC ³⁰⁵⁸		
SERCA3 (5')	As above	420	[23,39]
SERCA3c (3')	³⁰⁹³ GGC TCA TTT CTT CAA AGA GGC CAA C ³⁰⁶⁹		
InsP ₃ -R type I (5')	⁷⁹³⁴ TTG GAA AGA GAC AAG TTT GAC ⁷⁹⁵⁷	390	[41]
InsP ₃ -R type I (3')	⁸³²³ GAC ATT CAT GTG AGG AGG ATG ⁸³⁰³		
InsP ₃ -R type II (5')	⁸¹¹⁴ GGA CTT GAG AGA GAC AAG TTT GAT ⁸¹³⁷	408	[42]
InsP ₃ -R type II (3')	⁸⁵²¹ GTG TGG TGG CAT GTG ATG ATT ⁸⁵⁰¹		
InsP ₃ -R type III (5')	⁷⁶⁶⁰ GGT CTG GAG AGG GAC AAC TTT GAT ⁷⁶⁸³	390	[43]
InsP ₃ -R type III (3')	⁸⁰⁴⁹ GCG GCT AAT GCA GTT CTG GAC ATC ⁸⁰²⁶		

proteins were electrotransferred on to nitrocellulose membranes. The membranes were then treated for 2 h at room temperature with 1 µg/ml of the monoclonal anti-SERCA3 PL/IM430 antibody (a monoclonal anti-Ca²⁺-ATPase antibody raised against highly purified platelet intracellular membranes as immunogen) [32], 1:1000 dilution of the monoclonal anti-SERCA2 IID8 [33] and anti-InsP₃-R type-III [34] antibodies, 1:1000 dilution of the polyclonal anti-InsP₃-R type-I [35] antibody, or 1:5000 dilution of the polyclonal anti-glycoprotein IIIa (GPIIIa) [36] and anti-calreticulin [37] antibodies. Thereafter, the nitrocellulose sheets were washed extensively and further incubated with a 1:2000 dilution of the anti-mouse IgG horseradish peroxidase conjugate for immunostaining of the monoclonal antibodies and a 1:10000 dilution of the anti-rabbit IgG horseradish peroxidase conjugate for immunostaining of polyclonal antibodies, for 1.5 h. After washing, antibody binding was detected using ECL Western-blotting reagents according to the manufacturer's instructions. Luminograms were scanned and quantified using an LKB Ultrascan XL Laser Densitometer.

Immunofluorescence

Immunofluorescent staining of MEG 01 cells treated with 10⁻⁸ M PMA for 3 days was performed on acetone-fixed cells using a 1:200 dilution of anti-GPIIIa, PL/IM430 or IID8 antibody, and a 1:200 dilution of either Texas Red-conjugated anti-rabbit or FITC-conjugated anti-mouse antibody.

RNA extraction and RT-PCR

Total RNA was isolated from human platelets and various cell lines according to Chomczynski and Sacchi [38] using the RNA Plus solution. For first strand cDNA synthesis, 250 ng of total RNA sample was used in a total volume of 10 µl containing PCR buffer (10 mM Tris/HCl, pH 8.3/50 mM KCl) and 5 mM MgCl₂, 2.5 µM oligo d(T)₁₆, 1 unit/µl RNase inhibitor, 1.25 mM of each dNTP and 2.5 units/µl MuLV-RT. The reaction mixture was incubated for 30 min at 42 °C and then heated for 10 min at 95 °C to inactivate the RT. The volume was adjusted to 50 µl with the same PCR buffer and 2 mM MgCl₂ and 0.15 µM of the primers. The primer sequences used to amplify specific SERCA2b [19], SERCA3a [39], SERCA3b [40], SERCA3c [23] and InsP₃-R types I [41], II [42] or III [43] and predicted sizes of each amplification product are summarized in Table 1. PCR was

Table 2 Sequences and localizations of 3'-labelled oligonucleotide probes

Oligonucleotide	Sequence and localization
SERCA2b	²⁹⁵⁶ GTG GCC CGC AAC TAC CTG GAA CCT GGT AAA GAG TG ²⁹⁹⁰
SERCA3	²⁹⁶⁰ TGT CCC GGA ACC ACA TGC AC ²⁹⁷⁹
SERCA3b/c	³⁰¹⁷ GGC CTG GAG TAG GCA GCC GCT GAC CAC CTC TTG GAC CCC AG ³⁰⁵⁷
InsP ₃ -R type I	⁸¹⁵² TTC TGA AGG AGA ACA GAA TGA GCT GAG AAA ⁸¹⁸¹
InsP ₃ -R type II	⁸²⁷² TGT GGA GAA GAA TTT GGA TTG GTT TCC TC ⁸³⁰⁰
InsP ₃ -R type III	⁷⁷⁶¹ CCG CGT GAA GAA CAA GAC CGA C ⁷⁷⁸²

initiated by adding 1.25 units of AmpliTaq DNA polymerase and touch-down PCR was performed for 10 cycles with an annealing temperature decrement from 65 to 56 °C for SERCA3a–3c. PCR was then conducted for 18 (for SERCA2b), 20 (for SERCA3a), 24 (for SERCA3b and c) or 30 cycles (for InsP₃-R), each consisting of successive periods of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min. A negative control was run in the absence of RT, in order to check the specificity of the amplification.

Southern blotting

Amplification products were submitted to electrophoresis on 1.5 % ethidium bromide-stained agarose gel, transferred on to Hybond-N⁺ membranes [44] and visualized by Southern blotting using the ECL 3'-oligolabelling system according to the manufacturer's instructions. Prehybridization (1 h) and hybridization were performed at 42 °C. Hybridization was carried out overnight in the same solution containing 5 ng/ml of the 3'-fluorescein-labelled oligonucleotide probes (Table 2). Membranes were washed twice at room temperature in 5 × SSC/0.1 % SDS (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) for 5 min and twice either at 42 °C (for SERCA3 probe) or 55 °C (for other probes) in 0.5 × SSC/0.1 % SDS for 15 min. Membranes were then incubated with a 1:1000 dilution of the anti-fluorescein horseradish peroxidase-conjugated antibody at room temperature for 30 min. After washing the chemiluminescent signal was detected with Hyperfilm ECL. Luminograms were scanned and quantified using an LKB Ultrascan XL Laser Densitometer.

RESULTS

Relationship between SERCA3 expression and megakaryocytic maturation

Co-expression of SERCA3 and platelet GPIIIa in different megakaryocytic cell lines

As a first approach to study megakaryocytic differentiation, lysates from the MEG 01, UT7, M-O7e and CHRF 288-11 human erythro/megakaryocytic cell lines, as well as human platelets, which represent the final step of megakaryocytic maturation, were tested for their SERCA-protein expression by Western blotting (Figure 1A). The monoclonal PL/IM430 and IID8 antibodies were used to visualize SERCA3 and SERCA2b isoforms, respectively [20–22,32]. As controls of platelet maturation and ER proteins, the β subunit of integrin α_{IIb}β₃, GPIIIa and calreticulin were visualized using specific polyclonal antibodies. Figure 1(A) shows that expression of the 105-kDa platelet GPIIIa clearly differed from one cell line to another, and appeared to be greater in platelets. The same profile was obtained for the expression of SERCA3 proteins recognized by PL/IM430. In

Figure 1 SERCA expression and megakaryocytic maturation

(A) Total lysates (30 μ g) from either human platelets or erythro/megakaryocytic cell lines, MEG 01, UT7, M-07e and CHRF 288-11, were submitted to SDS/PAGE (8% gels) and electroblotted on to nitrocellulose sheets. Immunodetection of GPIIIa, SERCA3, SERCA2b and calreticulin was performed using the polyclonal anti-GPIIIa, the monoclonal PL/IM430 and IID8, and the polyclonal anti-calreticulin antibodies, respectively. The estimated molecular masses of the proteins estimated using standard rainbow-coloured markers are indicated on the left in kDa. This Figure is representative of three experiments. (B) UT7-Mpl cells, cultured in the presence of GM-CSF (–) or induced with 50 ng/ml TPO (+), were a generous gift from Dr G. Uzan (INSERM U506, Villejuif, France). Whole-cell lysate (30 μ g) was analysed as above. This Figure is representative of two experiments.

contrast, in the same samples, the housekeeping SERCA2b isoform as well as calreticulin appeared equally expressed in all cell lines and platelets, as demonstrated by the similar recognition of the 100- and 60-kDa bands, respectively. These results suggested that the expression of SERCA3 proteins accompanies megakaryocytic maturation, in contrast with that of SERCA2b and calreticulin.

Co-regulation of the expression of SERCA3 and GPIIIa upon TPO treatment of UT7-Mpl cell line

To confirm this observation, the effect of the physiological growth factor of megakaryocytopoiesis, TPO, was investigated for its effect on the expression of SERCA proteins. Because of the restricted expression of the TPO receptor c-Mpl in megakaryoblastic cells [45], experiments were carried out using the UT7-Mpl cell line expressing the murine c-Mpl. TPO treatment of these cells has been shown to induce increased levels of specific megakaryocytic differentiation antigens and to reduce those of erythroid markers [46,47]. Control and TPO-treated UT7-Mpl cell lysates were tested for their expression of GPIIIa, SERCAs and calreticulin as in Figure 1(A). The comparative results of UT7-Mpl cells treated with GM-CSF (lane –) or TPO (lane +) are shown in Figure 1(B) and reveal a clear difference in GPIIIa and SERCA3 proteins as their expression level dramatically increased in TPO-induced UT7-Mpl cells. Conversely, UT7-Mpl cells incubated with either GM-CSF or TPO exhibited the same level of SERCA2b and calreticulin, thus demonstrating again the absence of regulation of these two proteins. Thereby, this (i) confirmed the differentiation of UT7-Mpl cells upon TPO treatment and (ii) showed that the correlation between the

Figure 2 Dose response of PMA-induced MEG 01 cell differentiation

MEG 01 cells were incubated for 3 days with various PMA concentrations ranging from 10^{-9} to 10^{-7} M as indicated. Control (lanes 0) and treated cells were used for lysate preparation and 30 μ g of each sample was analysed as in Figure 1. The estimated molecular masses of the proteins are indicated on the left. This Figure is representative of three independent experiments.

expression level of SERCA3 was also observed under physiologically induced megakaryocytic differentiation.

This prompted us to try to gain further insight into the regulation of the overall ER proteins. For this, we selected a well-characterized model of megakaryocytic maturation, the treatment of MEG 01 cells with a phorbol ester, PMA, which enhances the expression of surface megakaryocytic lineage-specific differentiation antigens, such as integrin $\alpha_{IIb}\beta_3$ [48].

Regulation of SERCA proteins upon PMA-induced differentiation of MEG 01 cells

Dose–response effects of PMA-induced MEG 01 cell differentiation and SERCA expression

To look for PMA as a co-inducer of GPIIIa and SERCA3 expression in this model, MEG 01 cells were treated with PMA concentrations ranging from 10^{-9} to 10^{-7} M for 3 days (Figure 2). The cell lysates were then tested for their expression of SERCAs and calreticulin and compared with that of GPIIIa. As expected, expression of GPIIIa increased in PMA-treated MEG 01 cells as compared with control, and was maximal from 10^{-9} M PMA, as shown by the similar expression level up to 10^{-7} M, whereas no significant change in SERCA2b and calreticulin expression was detected. Up-regulation of the expression of SERCA3 proteins was observed in PMA-treated cells, which peaked at 10^{-8} M PMA. These experimental conditions have been used in the following experiments.

Immunofluorescent staining

To see if the overexpression of GPIIIa and SERCA3 proteins in MEG 01 cells treated with 10^{-8} M PMA occurred at the single-cell level, immunofluorescent studies were performed (Figure 3). Although PMA-treated MEG 01 cells (Figures 3B, 3D and 3F) were less numerous than control cells (Figures 3A, 3C and 3E), due to inhibition of proliferation during differentiation, the

Figure 3 Immunofluorescent staining of PMA-treated MEG 01 cells

MEG 01 cells were cultured for 3 days in the absence (A, C and E) or in the presence (B, D and F) of 10^{-8} M PMA, and immunofluorescent staining was performed using the anti-GPIIIa (A and B), PL/IM430 (C and D) or IID8 (E and F) antibodies, as described in the Experimental section. This Figure is representative of three experiments.

number of cells positive for GPIIIa (Figures 3A and 3B) and SERCA3 (Figures 3C and 3D) was enhanced in PMA-treated cells, when compared with control, whereas the signal fluor-

escence for SERCA2b was not modified significantly (Figures 3E and 3F).

Time-course effects of PMA-induced MEG 01 cell differentiation and SERCA-protein expression

To define whether SERCA3 expression accompanies or follows that of GPIIIa, the modulation of SERCA proteins was investigated in MEG 01 cells induced with 10^{-8} M PMA for 6, 24, 30, 48 or 72 h (Figure 4). The expression levels of GPIIIa, SERCAs and calreticulin were estimated as in Figure 1 (Figure 4A). Results showed that the increase in the expression of GPIIIa started at 6 h after PMA treatment and increased until 72 h, whereas the expression of SERCA proteins started to increase at 24 h, thus indicating that overexpression of SERCA3 proteins follows that of GPIIIa. As above, the expression levels of SERCA2b and calreticulin did not change. Further quantification of these expressions was performed by densitometric measurements of the luminograms and was expressed as a function of SERCA2b, taken as 100 %. Values obtained for control MEG 01 cells were then arbitrarily taken as 1 and results are shown in Figure 4(B). A 9.13 ± 0.86 -fold increase in GPIIIa level (Figure 4B, ■) was found in PMA-treated MEG 01 cells after 3 days, whereas the overexpression of SERCA3 (Figure 4B, ●) was slower and reached a value of 3.87 ± 0.68 -fold in PMA-treated MEG 01 cells after 3 days. These results established a significant PMA-induced overexpression of SERCA3 proteins that followed megakaryocytic maturation.

Time-course effects of PMA-induced MEG 01 cell differentiation and SERCA RNA expression

To both address the questions of the SERCA3 isoforms concerned and the levels of regulation, we studied the three new 3'-end alternatively spliced platelet transcripts, i.e. SERCA3a, 3b

Figure 4 Time course of SERCA3 expression in PMA-treated MEG 01 cells

(A) MEG 01 cells were incubated with 10^{-8} M PMA for various time periods as indicated and treated for lysate preparations. Control (lanes 0) or PMA-treated cell lysates (30 µg) were analysed as in Figure 1. The estimated molecular masses of the proteins are indicated on the left. (B) Densitometric measurements of the luminous bands were calculated as a function of SERCA2b, taken as 1. The values obtained for control MEG 01 cells were then arbitrarily taken as 1 and results are the means $\pm$ S.E.M. from four independent experiments. ■, GPIIIa expression; ●, expression of SERCA3 proteins recognized by the PL/IM430 antibody.

Figure 5 Time course of SERCA3 mRNA expression in PMA-treated MEG 01 cells

(A) Total RNA was extracted from control (lanes 0) or PMA-treated MEG 01 cells for the same time periods as in Figure 4. Each RNA (250 ng) underwent reverse transcription using oligo d(T)₁₆ and MuLV-RT. Touch-down PCR was then performed for 20 or 24 cycles using specific primers for SERCA3a, SERCA3b or SERCA3c (shown in Table 1). SERCA2b amplification was used as an internal control. Amplification products were then analysed by Southern blotting using either a common SERCA3 (for SERCA3a) or a specific SERCA3b/3c probe (see Table 2). The size of each amplification product is indicated on the left in bp. (B) Densitometric measurements of the luminescent bands were calculated as in Figure 4(B) and results are the means ± S.E.M. from three independent experiments. □, SERCA3a mRNA expression; ○, SERCA3b mRNA expression; Δ, SERCA3c mRNA expression.

Figure 6 Investigation of InsP₃-R expression in human platelets and MEG 01 cells

(A) Total lysate (5 µg) from rat cerebellum and 30 µg of total lysates from human platelets, human MEG 01 and HeLa cell lines were analysed by SDS/PAGE (8% gels) and electroblotted on to nitrocellulose sheets. InsP₃-R (IP3-R) types I and III were then immunostained using specific antibodies as described in the Experimental section, and SERCA2b immunoblotting was used as a control. The molecular masses of the proteins estimated using standard rainbow-coloured markers are indicated on the left. This panel is representative of two experiments. (B) Total RNA (250 ng) from HeLa cells, platelets and MEG 01 cells underwent reverse transcription using oligo d(T)₁₆ and MuLV-RT. PCR was then performed for 30 cycles using specific primers for InsP₃-R types I, II or III (Table 1) and amplification products were visualized on ethidium bromide-stained 1.5% agarose gels. SERCA2b amplification was used as an internal control. The size of each amplification product is indicated on the left. The panel is representative of three independent experiments.

and 3c mRNAs [22,23]. We performed reverse transcription followed by comparative specific PCR on total RNA isolated from control and PMA-treated MEG 01 cells (Figure 5). Absence of DNA contamination was verified as no amplification products were obtained without RT (results not shown). As non-modu-

lation of SERCA2b protein was found, SERCA2b mRNA expression was used to verify that the same amounts of RNAs were loaded. For SERCA3 mRNA analyses, we used common forward and specific reverse SERCA3a, 3b and 3c primers (see Table 1), a protocol previously demonstrated to give rise to

Figure 7 Expression of InsP₃-R proteins in PMA-treated MEG 01 cells

(A) MEG 01 cells were treated as in Figure 4. Control (lanes 0) or PMA-treated MEG 01 cell lysates (30 µg) were analysed by SDS/PAGE (8% gels) as in Figure 6(A). The estimated molecular masses of the proteins are indicated on the left. (B) Densitometric measurements of the luminescent bands were calculated as a function of SERCA2b, taken as 1 (Figure 4). The values obtained for control MEG 01 cells were then arbitrarily taken as 1 and results are the means ± S.E.M. from three independent experiments. ▲, Expression of InsP₃-R (IP3-R) type I; ■, expression of InsP₃-R type III.

Figure 8 Expression of the three InsP₃-R mRNAs in PMA-treated MEG 01 cells

(A) Total RNA was extracted from control (lanes 0) or PMA-treated MEG 01 cells as in Figure 5. Each RNA (250 ng) underwent reverse transcription using oligo d(T)₁₆ and MuLV-RT. PCR was then performed for 30 cycles using specific primers for InsP₃-R types I, II or III. Amplification products were analysed by Southern blotting using specific 3'-fluorescein-labelled oligonucleotide probes (Table 2). The size of each amplification product is indicated on the left. (B) Densitometric measurements of the luminescent bands were calculated as a function of SERCA2b, taken as 1 (Figure 5), and results are the means ± S.E.M. from four independent experiments. Δ, Expression of InsP₃-R type I mRNA; ○, expression of InsP₃-R type II mRNA; □, expression of InsP₃-R type III mRNA.

unique and specific SERCA3a, 3b and 3c species by sequencing the PCR products. The PCR products were visualized by Southern blotting (Figure 5A). Results showed first that the three SERCA3 species were expressed in MEG 01 cells, although at different levels. Whereas PMA-treatment induced a modest increase in SERCA3a mRNA level during differentiation, a clear difference was found for SERCA3b and SERCA3c mRNAs. Indeed, their expression appeared strongly increased at hour 24 of PMA treatment and thereafter differed, as that of SERCA3b increased until hour 72, whereas a down-regulation at hour 48

was observed for SERCA3c. Further quantifications of these modulations were performed by densitometric measurements of the luminograms (Figure 5B), and calculated as in Figure 4(B). It established (i) the low level of SERCA3a regulation (Figure 5B, □), with a maximal induction of 1.63-fold at 24 h of PMA treatment, (ii) a maximum 3.97-fold increase in SERCA3b expression (Figure 5B, ○) at 72 h and (iii) a biphasic response for SERCA3c mRNA (Figure 5B, Δ), with a first and maximum 3.93-fold increase at 24–30 h, then a decrease and a second induction at 72 h of PMA treatment of MEG 01 cells.

Figure 9 Comparison of SERCA3 modulation in PMA-treated MEG 01 and CHRF 288-11 cells

MEG 01 and CHRF 288-11 cells were cultured for 3 days without (—) or with (+) 10^{-8} or 5×10^{-9} M PMA, respectively, and treated for lysate (A) or RNA preparations (B). (A) Whole-cell lysates (30 μ g of each sample) were analysed by SDS/PAGE (8% gels), electroblotted and immunodetection of GPIIb/IIIa, SERCA3, SERCA2b and calreticulin was performed as in Figure 1. The estimated molecular masses of the proteins are indicated on the left. This Figure is representative of three experiments. (B) Each RNA (250 ng) underwent reverse transcription using oligo d(T)₁₆ and MuLV-RT. Touch-down PCR was then performed for 20 or 24 cycles using specific primers for SERCA3a, SERCA3b or SERCA3c. SERCA2b amplification was used as an internal control. Amplification products were then visualized on ethidium bromide-stained 1.5% agarose gels. The size of each amplification product is indicated on the left.

Hence, these results indicated that modulation of SERCA3 observed at the protein level in PMA-treated MEG 01 cells is caused by overexpression of the three SERCA3 mRNA species.

Expression and regulation of InsP_3 -Rs upon PMA-induced MEG 01 cell differentiation

Lastly, to obtain an overview of the ER proteins controlling platelet Ca^{2+} signalling, we investigated InsP_3 -R subtypes during megakaryocytopoiesis, using the same experimental model.

Molecular identification of InsP_3 -R(s) in platelets and MEG 01 cells

As a prerequisite, because some controversy exists concerning the expression of the different InsP_3 -Rs in platelets [15,16], we re-investigated InsP_3 -RI and InsP_3 -RIII proteins, looked for still undescribed InsP_3 -R RNAs and performed the same studies in MEG 01 cells (Figure 6). Lysates from MEG 01 cells and human platelets were immunoblotted and detected using commercially selected specific anti- InsP_3 -R type-I and -III antibodies. As controls, the IID8 anti-SERCA2 antibody was used (Figure 6A). InsP_3 -RI was detected in platelets and MEG 01 cells, as expected [14–16]. InsP_3 -RIII was also detected in both MEG 01 and platelets, thus confirming previous suggestions [16]. However, when compared with their positive controls, i.e. lysates from cerebellum for InsP_3 -RI or HeLa cells for InsP_3 -RIII, platelet InsP_3 -RI and III appear to present apparently smaller molecular masses, in contrast to MEG-01 cells. This may be due to the susceptibility of platelet InsP_3 -Rs to proteolytic degradation, as shown also for plasma-membrane Ca^{2+} -ATPases [49].

To address this question, studies of InsP_3 -R (types I, II and III) mRNAs in MEG 01 cells and platelets were performed by RT-PCR using specific oligonucleotides and analysed by ethidium bromide-stained agarose gels as shown in Figure 6(B). As positive controls, HeLa cell mRNA was used. Unique and specific products were obtained and controlled for the three InsP_3 -R types by restriction mapping (results not shown). Results clearly indicated a similar expression of InsP_3 -RI, II and III mRNAs in platelets and MEG 01 cells, although at a very low level for InsP_3 -RIII. These results thus demonstrated that platelets and MEG 01 cells simultaneously express the three types of InsP_3 -R mRNA.

Expression of InsP_3 -Rs in PMA-induced MEG 01 cell differentiation

For this purpose, we studied the same samples as in Figures 4 and 5 for the expression of the different InsP_3 -R types, in terms of proteins (Figure 7) or mRNAs (Figure 8). Immunoblotting of control and PMA-treated MEG 01 cell lysates, using the specific anti- InsP_3 -RI and III antibodies (Figure 7A), showed that InsP_3 -RI protein was equally expressed, indicating that its expression is unchanged upon megakaryocytic differentiation. In contrast, InsP_3 -RIII expression increased until hour 30 of treatment and then decreased to only 1.32 ± 0.2 times the control levels, as shown in Figure 7(B) (■). Figure 8(A) shows the Southern blotting of PCR products using probes specifically designed to detect mRNA of each InsP_3 -R type, and longer exposure times for InsP_3 -RIII. Time courses of each InsP_3 -R type differed during differentiation. Type-I mRNA level was unchanged in control and MEG 01 cells treated for various times, in total agreement with the absence of its regulation at the protein level. InsP_3 -R type-II mRNA appeared first to decrease at hour 24, then to increase from hours 30 to 48 of PMA treatment, so that a 2.59-fold increase (Figure 8B, ○) compared with controls was found in MEG 01 cells treated for 72 h. The mRNA of InsP_3 -R type III peaked at 6 h and then decreased to control levels at 72 h of treatment (0.34 ± 0.1 ; Figure 8B, □). This explained and confirmed the delayed up-regulation of InsP_3 -RIII protein. Taken together, these findings revealed a profound and general remodelling of the major proteins controlling ER Ca^{2+} intracellular pools upon megakaryocytic differentiation.

Specificity of megakaryocytic ER-protein regulation

To determine whether this concerted modulation of both SERCAs and InsP_3 -R proteins was a feature of megakaryocytic

Figure 10 Comparison of InsP₃-R modulation in PMA-treated MEG 01 and CHRF 288-11 cells

The same samples as in Figure 9 were tested for their expression of InsP₃-Rs (IP3-Rs) at protein (A) or RNA (B) levels. (A) Whole-cell lysates (30 µg of each sample) were analysed by SDS/PAGE (8% gels), electroblotted and immunodetection of InsP₃-R types I and III was performed as in Figure 6(A). The molecular masses of the proteins estimated using standard rainbow-coloured markers are indicated on the left. This Figure is representative of three experiments. (B) Each RNA (250 ng) underwent reverse transcription using oligo d(T)₁₆ and MuLV-RT. PCR was then performed for 30 cycles using specific primers for InsP₃-R types I, II or III and amplification products were analysed by Southern blotting as in Figure 8(A). The size of each amplification product is indicated on the left. This Figure is representative of three experiments.

differentiation, we looked for their comparative expression upon similar PMA treatment of CHRF 288-11 cells, which are more mature, as suggested by GPIIIa expression (Figure 1). The cells were treated for 3 days with 5×10^{-9} M PMA (Figures 9 and 10).

Figure 9(A) shows the expression of GPIIIa and SERCA isoforms by Western blotting as in Figure 1. Again, control SERCA2b and calreticulin showed no differences between untreated or PMA-treated MEG 01 and CHRF 288-11 cells, whereas both GPIIIa and SERCA3 proteins increased in PMA-treated MEG 01 cells. In contrast, PMA treatment of CHRF 288-11 cells, which did not induce significant variations in GPIIIa (compare lane + and control lane -), slightly decreased expression of SERCA3 proteins. Further studies of SERCA3a-c RNAs were performed as in Figure 5(A), and analysed on ethidium bromide-stained agarose gels (Figure 9B). While, again, an increase in SERCA3a, 3b and 3c mRNAs was observed in PMA-treated MEG 01 cells, a modest down-regulation in the three SERCA3 mRNA species was observed in PMA-treated CHRF 288-11 cells, in agreement with the slight decrease in SERCA3 proteins.

The comparative protein and RNA studies of InsP₃-Rs are

shown in Figures 10(A) and 10(B) respectively. Figure 10(A) demonstrated that InsP₃-RI was expressed in CHRF 288-11 cells and that PMA did not significantly affect its expression, whereas a similar comparative analysis of InsP₃-RIII showed no detectable InsP₃-RIII protein in CHRF 288-11 cells either in the absence or the presence of PMA. Figure 10(B) shows in both cell types that PMA treatment induced major up- and down-regulation of InsP₃-RII and III respectively, compared with InsP₃-RI. In addition, it explained the apparent absence of the InsP₃-RIII protein in CHRF 288-11 cells by demonstrating a lower expression of InsP₃-RIII RNA in these cells compared with MEG-01 cells. Hence, these studies of CHRF 288-11 cells, which do not differentiate further towards the megakaryocyte lineage upon PMA treatment, in contrast to MEG-01 cells, revealed similar InsP₃-R but distinct SERCA modulation of expression, pointing to the specific importance of SERCA3 in the organization of ER proteins during megakaryocytopoiesis.

DISCUSSION

Recent understanding in the role of Ca²⁺ signalling reveals that it resides in the modulation of generic transduction signals into specific responses [2–4]. This is due to the decoding of information in the frequency, duration and spatial distribution of Ca²⁺ oscillations by various and cell-specific key enzymic activities or transcription factors. This raises new fundamental questions, including: (i) how many Ca²⁺ signals are required to insure a diversity of cell-function specificities; (ii) how many structures are involved in Ca²⁺ signalling, and what are their molecular identities; (iii) how do physiological or pharmacological situations affect their degrees of expression and (iv) what is their functional role in cellular Ca²⁺ responses?

In this context, platelets constitute an interesting model of haematopoietic cells which may help in the understanding of the nature and expression level of the major ER proteins regulating intracellular Ca²⁺ concentration. First, these circulating cells are constantly submitted to a large variety of stimuli, and show striking variability in their agonist-dependent platelet Ca²⁺ responses. Accordingly, the platelet Ca²⁺ signal appears particularly complex and dynamic when compared with other cell types. Second, platelets may exhibit a complex organization of intracellular Ca²⁺ pools, i.e. an ER heterogeneity, a concept now well accepted, but still incompletely understood, which may be sustained in an original system of co-expression of multiple ER proteins to govern Ca²⁺ movements. Of a particular interest, human platelets account for the first cell type suggested to co-express at least four SERCA isoforms, including three very recent non-muscle SERCA3a–c species in combination with at least two types of InsP₃-R.

Here, we attempted to investigate the involvement of these ER proteins in the Ca²⁺-dependent signal-transduction needs of platelets through their biogenesis during megakaryocytopoiesis. To do this, we selected three complementary *in vitro* experimental models of cell differentiation towards megakaryocytic lineage, namely (i) cell lines representative of increasing stages of megakaryocytic differentiation, (ii) TPO-treated UT7-Mpl cells and (iii) treatment of MEG 01 cells with PMA. These models were studied for ER-protein expression in comparison with that of the most consistent marker of platelet maturation, GPIIIa. Whereas the ubiquitous SERCA2b isoform was equally expressed, a concomitant up-regulation of GPIIIa and total SERCA3 protein was observed in the three models. In addition, while studies on isoform-specific antibodies showing the three SERCA3a–c platelet proteins were in progress (T. Kovács, F. Ferenc, B. Papp, K. Paszty, P. Gélébart, R. Bredoux, A. Enyedi

and J. Enouf, unpublished work), RNA studies demonstrated that the three SERCA3a-c species were involved, albeit to a lesser apparent extent for SERCA3a. Lastly, this SERCA3 regulation was not observed in the absence of differentiation of CHRF 288-11 cells. This suggests that the expression of three SERCA3 isoforms is linked to the differentiation process and that the degrees of expression levels of the SERCA3b and SERCA3c species should be particularly involved in megakaryocytopoiesis.

Concerning InsP_3 -R types, their expression was currently not clearly established in platelets. If InsP_3 -R types I [14–16] and II [15] have been found in human platelet membranes, the presence of InsP_3 -R type III was either not detected [15] or indirectly demonstrated in plasma membranes using an antibody recognizing both InsP_3 -RI and III subtypes [16]. First, using InsP_3 -R type-I and -III specific antibodies, we demonstrated the presence of both InsP_3 -R types in MEG 01 cells and human platelets. Secondly, original mRNA studies established the simultaneous, but differential co-expression of the three typical InsP_3 -RI–III species in the same cells. Thirdly, megakaryocytic differentiation studies revealed major modulations of expression of InsP_3 -R types II and III in terms of protein and/or mRNA levels. Indeed, in agreement with a previously reported absence of regulation of InsP_3 -R type-I protein upon PMA treatment of MEG 01 cells, [14] InsP_3 -R type I was found equally expressed in control and PMA-treated MEG 01 cells. In contrast, InsP_3 -R type-II and -III mRNAs were found here to be differentially up-regulated. Lastly, similar regulation of these InsP_3 -Rs was observed in the absence of differentiation of CHRF 288-11 cells. Hence, if InsP_3 -R regulation appears associated with megakaryocytic differentiation, this necessary condition is an insufficient prerequisite that has to be coupled with an up-regulation of SERCA3 to ensure this functional specificity.

Thus, megakaryocytopoiesis appears to be associated with a major reorganization of ER proteins, the meaning of which may reside in the increasing importance, heterogeneity, organization and dynamic nature of ER Ca^{2+} stores. First, considering the relative importance of ER Ca^{2+} pools in platelets compared with their progenitors, the combination of unaltered SERCA2b and InsP_3 -R type-I expression and positive remodelling of InsP_3 -RII and III subtypes as well as SERCA3 proteins leads to a global up-regulation of ER proteins. As these proteins control Ca^{2+} store content, this should sustain a larger size of ER Ca^{2+} pools in platelets than in cell precursors. Alternatively, this would emphasize an idea that we have already formulated on the basis of the down-regulation of another type of structure involved in Ca^{2+} signalling, that of plasma-membrane Ca^{2+} -ATPases, in platelets compared with MEG 01 cells [49]. Such a positive balance in the expression of ER proteins can be required for platelet effectors that need ER Ca^{2+} stores for their signal transduction. Accordingly, comparative thrombin-induced Ca^{2+} oscillations appeared to be much more dependent on Ca^{2+} influx in MEG 01 cells than in human platelets [50]. Moreover, this agonist was recently reported to induce an up-regulated Ca^{2+} response during megakaryocytopoiesis [51].

Secondly, the increasing complexity in the nature of both SERCA isoforms and InsP_3 -R types may strengthen the previously suggested idea of platelet Ca^{2+} -store heterogeneity. Initial immunological studies suggested different localizations of both SERCA isoforms and InsP_3 -Rs: the SERCA3 proteins and InsP_3 -R types II and III being close to, or inserted into, plasma membranes, while SERCA2b and InsP_3 -R type I appeared to be localized in intracellular membranes [15,16,52]. The present study of their expression shows a concerted up-regulation of SERCA3 and InsP_3 -R type II and III proteins, in contrast with SERCA2b and InsP_3 -R type I. This might imply a reconstruction

of the ER from pre-existing SERCA2b- and InsP_3 -RI-associated intracellular Ca^{2+} stores towards additional SERCA3- and InsP_3 -RII- and III-associated ER subcompartments in connection with plasma membrane. Again, this new ER network may be needed to create links between agonists and their fine spatial tuning of Ca^{2+} -dependent signalling processes, as shown recently for SERCA and insulin-receptor substrate, IRS-1 [53].

Thirdly, this endpoint of platelet ER organization would appear to result from a non-linear dynamic process of periodic modulation of ER Ca^{2+} stores. Indeed, whereas SERCA3a, SERCA3b and InsP_3 -RIII appear to be progressively up-regulated, SERCA3c and InsP_3 -RII present biphasic increments of their expression during megakaryocytopoiesis. Similar findings were observed for InsP_3 -RI and II upon HL-60 myeloblastic leukaemia cell differentiation induced by all-*trans* retinoic acid or PMA [54]. As a possible explanation, it was formulated that these modulations might be associated with the different cascades of Ca^{2+} -dependent gene expression involved in cell differentiation, such as *c-myc* or genes involving nuclear factor of activated T-cells (NF-AT) and nuclear factor κ B (NF- κ B) transcription factors [55].

Lastly, concerning the relevance of the ER-protein specialization in the understanding of megakaryocyte and platelet Ca^{2+} signalling, this remains an open question due to the difficulties in obtaining human megakaryocytes [56]. Moreover, unfortunately, no information can be expected from rat or mouse, because of their striking differences at least in the nature of their platelet SERCA3 [22,23] and levels of SERCA2b-protein expression [57]. However, it was recently found that the temporal patterns of Ca^{2+} signals depend critically on the SERCA2 isoforms [58] or on the set of InsP_3 -R subtypes [59]. SERCA2b overexpression shows repetitive InsP_3 -induced Ca^{2+} oscillations, which can appear as a sustained release of Ca^{2+} upon SERCA2b association with calreticulin. About InsP_3 -Rs, the properties of InsP_3 -R types I and II are ideal for supporting Ca^{2+} oscillations, whereas InsP_3 -R type III is reported to generate monophasic Ca^{2+} transients [60].

Based on the simultaneous expression of SERCA2b and calreticulin, and on the major up-regulations of InsP_3 -RII and III, megakaryocytopoiesis could support a modulation of Ca^{2+} oscillations into the platelet-specific encoding of non-oscillatory up and down Ca^{2+} signalling. However, the importance of various SERCA3 isoforms, which may be submitted to distinct biochemical regulatory mechanisms, although potentially as relevant as that of the InsP_3 -R receptors, remains to be established. In agreement with this hypothesis, a body of evidence suggests an important role for the SERCA3 gene in haematopoietic cells and its control of Ca^{2+} -dependent function. First, SERCA3 has been shown to be expressed in different cells of haematopoietic origin. Secondly, modulations of SERCA3 expression have been found in platelets of spontaneously hypertensive rats [19,23], and upon lymphocyte activation [61] or granulocytic and monocyte/macrophage differentiation [62]. Accordingly, the analysis of the 5'-untranslated promoter region of the human SERCA3 gene revealed multiple sites for transcription factors including specific haematopoietic factors GATA and Ets. Thirdly, SERCA3 gene knock-out showed abnormal endothelium-dependent smooth-muscle relaxation due to abnormal nitric oxide production [63]. This means that the ER compartment expressing SERCA3 may play a role in Ca^{2+} oscillations decoded by the nitric oxide synthase activity.

To conclude, the importance of the present work is that it advances our basic understanding of the ER by showing that it has to be viewed as a major modulable compartment depending upon the relative distribution of InsP_3 -Rs and SERCAs, to

govern cell-specific Ca²⁺ functions including megakaryocytopoiesis. In addition, while the field of subcellular Ca²⁺ homeostasis is evolving rapidly, this study opens areas of investigation to understand normal and abnormal platelet Ca²⁺ signalling and the relationship between platelet ER proteins and their multiple signalling pathways.

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