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

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The endoplasmic reticulum (ER) plays a key role in Ca\(^{2+}\) signalling through Ca\(^{2+}\) release via inositol 1,4,5-trisphosphate receptors (Ins\(P_2\)-Rs) and Ca\(^{2+}\) uptake by sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA). 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 M-O7e and CHRF 288-11 cells, in which GPIIIa is not induced upon treatment, a similar pattern of regulation of Ins\(P_2\)-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, Ins\(P_2\) 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 (Ins\(P_2\)-Rs) [5,6] and Ca\(^{2+}\) pumps or sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA) [7,8], which co-ordinate opposite Ca\(^{2+}\) ion fluxes. Ins\(P_2\)-Rs induce an increase in cytosolic Ca\(^{2+}\) concentration as a result of Ca\(^{2+}\) release from ER through the binding of Ins\(P_2\) on Ins\(P_2\)-Rs, whereas SERCAs are responsible for a decrease in cytosolic Ca\(^{2+}\) concentration due to Ca\(^{2+}\) resequstration in the ER Ca\(^{2+}\) stores.

Three types of Ins\(P_2\)-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 Ins\(P_2\)-R types, types IV and V, has been proposed based on partial sequence information. Further heterogeneity is generated through alternative splicing of Ins\(P_2\)-R types I and II. More recently, a truncated form of Ins\(P_2\)-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 Ins\(P_2\)-R and SERCA species are co-expressed in the ER suggests that the calcium homoeostasis 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\(^{2+}\).
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$_2$-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$_2$-Rs [13–16] and SERCAs [17]. Although some controversy exists concerning the expression of InsP$_2$-Rs, human platelets were reported to express either InsP$_2$-RI and II or InsP$_2$-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 erythroid/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 vitro 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$_2$-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 levels of these cells shown to simultaneously express the ubiquitous SERCA2b [18] and SERCA3a proteins [19–21] as well as SERCA3b [22] and SERCA3c [23] mRNAs.

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-O7e [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 mg/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 lyase 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% (v/v) SDS, 10% (v/v) glycerol, 5 mM EDTA, 2% 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
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/I430 antibody (a monoclonal anti-Ca<sup>2+</sup>-ATPase antibody raised against highly purified platelet intracellular membranes as immunogen) [32], 1:1000 dilution of the monoclonal anti-SERCA2 IID8 [33] and anti-Insp<sub>R</sub>-type-I 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<sup>-8</sup> M PMA for 3 days was performed on acetone-fixed cells using a 1:200 dilution of anti-GPIIIa, PL/I430 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 MgCl<sub>2</sub>, 2.5 μM oligo d(T)<sub>18</sub>, 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 MgCl<sub>2</sub> and 0.15 μM of the primers. The primer sequences used to amplify specific SERCA2b [19], SERCA3α [39], SERCA3b [40], SERCA3c [23] and Inps<sub>R</sub>-type I [41], II [42] or III [43] and predicted sizes of each amplification product are summarized in Table 1. PCR was 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 SERCA3α–3c. PCR was then conducted for 18 (for SERCA2b), 20 (for SERCA3α), 24 (for SERCA3b and c) or 30 cycles (for Inps<sub>R</sub>-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<sup>+</sup> 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-label 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 GPIIIα 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 erythroid/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/I430 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<sub>IIb</sub>β<sub>3</sub>, GPIIIα and calreticulin were visualized using specific polyclonal antibodies. Figure 1A shows that expression of the 105-kDa platelet GPIIIα 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/I430.
To confirm this observation, the effect of the physiological growth factor of megakaryocytogenesis, 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 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 αIIbβ3 [48].

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

The 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 $10^{-7}$ 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 $10^{-6}$ 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 $10^{-6}$ 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...
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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 fluorescence 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 SERCA3 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 1. The values obtained for control MEG 01 cells were then arbitrarily taken as 1 and results are shown in Figure 4B. 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
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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; △, 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 electrophoretically transferred 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-modulated 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
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 lumino grams (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, ▲), 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.
Molecular identification of InsP$_3$-Rs 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 IIDS 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 Ins\(_{P2}\)-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 Ins\(_{P2}\)-Rs (IP3-Rs) at protein (A) or RNA (B) levels. (A) Whole-cell lysates (30 \(\mu\)g of each sample) were analysed by SDS/PAGE (8% gels), electroblotted and immunodetection of Ins\(_{P2}\)-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 Ins\(_{P2}\)-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 \(\times\) 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 Ins\(_{P2}\)-Rs are shown in Figures 10(A) and 10(B) respectively. Figure 10(A) demonstrated that Ins\(_{P2}\)-RI was expressed in CHRF 288-11 cells and that PMA did not significantly affect its expression, whereas a similar comparative analysis of Ins\(_{P2}\)-RIII showed no detectable Ins\(_{P2}\)-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 Ins\(_{P2}\)-RII and III respectively, compared with Ins\(_{P2}\)-RI. In addition, it explained the apparent absence of the Ins\(_{P2}\)-RIII protein in CHRF 288-11 cells by demonstrating a lower expression of Ins\(_{P2}\)-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 Ins\(_{P2}\)-R but distinct SERCA modulation of expression, pointing to the specific importance of SERCA3 in the organization of ER proteins during megakaryocytogenesis.

**DISCUSSION**

Recent understanding in the role of Ca\(^{2+}\) 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 Ca\(^{2+}\) oscillations by various and cell-specific key enzymic activities or transcription factors. This raises new fundamental questions, including: (i) how many Ca\(^{2+}\) signals are required to insure a diversity of cell-function specificities; (ii) how many structures are involved in Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) concentration. First, these circulating cells are constantly submitted to a large variety of stimuli, and show striking variability in their agonist-dependent platelet Ca\(^{2+}\) responses. Accordingly, the platelet Ca\(^{2+}\) signal appears particularly complex and dynamic when compared with other cell types. Second, platelets may exhibit a complex organization of intracellular Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ins\(_{P2}\)-R.

Here, we attempted to investigate the involvement of these ER proteins in the Ca\(^{2+}\)-dependent signal-transduction needs of platelets through their biogenesis during megakaryocytogenesis. 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. Gélibart, 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 megakaryocytosis.

Concerning InsP$_2$-R types, their expression was currently not clearly established in platelets. If InsP$_2$-R types I [14–16] and II [15] have been found in human platelet membranes, the presence of InsP$_2$-R type III was either not detected [15] or indirectly demonstrated in plasma membranes using an antibody recognizing both InsP$_2$-RI and III subtypes [16]. First, using InsP$_2$-R type-I and -III specific antibodies, we demonstrated the presence of both InsP$_2$-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$_2$-RI-III species in the same cells. Thirdly, megakaryocytic differentiation studies revealed major modulations of expression of InsP$_2$-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$_2$-R type-I protein upon PMA treatment of MEG 01 cells, [14] InsP$_2$-R type I was found equally expressed in control and PMA-treated MEG 01 cells. In contrast, InsP$_2$-R type-II and -III mRNAs were found here to be differentially up-regulated. Lastly, similar regulation of these InsP$_2$-Rs was observed in the absence of differentiation of CHRF 288-11 cells. Hence, if InsP$_2$-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, megakaryocytosis 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$_2$-R type-I expression and positive remodelling of InsP$_2$-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 megakaryocytogenesis [51].

Secondly, the increasing complexity in the nature of both SERCA isoforms and InsP$_2$-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$_2$-Rs, the SERCA3 proteins and InsP$_2$-R types II and III being close to, or inserted into, plasma membranes, while SERCA2b and InsP$_2$-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$_2$-R type II and III proteins, in contrast with SERCA2b and InsP$_2$-R type I. This might imply a reconstruction of the ER from pre-existing SERCA2b- and InsP$_2$-RII-associated intracellular Ca$^{2+}$ stores towards additional SERCA3- and InsP$_2$-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$_2$-RII appear to be progressively up-regulated, SERCA3c and InsP$_2$-RII present biphasic increments of their expression during megakaryocytosis. Similar findings were observed for InsP$_2$-RII and II upon HL-60 myeloblastic 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 κB (NF-κB) 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$_2$-R subtypes [59]. SERCA2b overexpression shows repetitive InsP$_2$-induced Ca$^{2+}$ oscillations, which can appear as a sustained release of Ca$^{2+}$ upon SERCA2b association with calreticulin. About InsP$_2$-Rs, the properties of InsP$_2$-R types I and II are ideal for supporting Ca$^{2+}$ oscillations, whereas InsP$_2$-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$_2$-RII and III, megakaryocytosis 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$_2$-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$_2$-Rs and SERCAs, to
govern cell-specific Ca\(^{2+}\) functions including megakaryocyteopoiesis. In addition, while the field of subcellular Ca\(^{2+}\) homeostasis is evolving rapidly, this study opens areas of investigation to understand normal and abnormal platelet Ca\(^{2+}\) signalling and the relationship between platelet ER proteins and their multiple signalling pathways.

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