Platelet sarco/endoplasmic reticulum Ca^{2+} ATPase isoform 3b and Rap 1b: interrelation and regulation in physiopathology

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Platelet Ca^{2+} signalling involves intracellular Ca^{2+} pools, whose content is controlled by sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCAs). Among these, a key role is played by the inositol trisphosphate-sensitive Ca^{2+} pool, associated with the SERCA 3b isoform. We have investigated the control of this Ca^{2+} pool through the cAMP-dependent phosphorylation of the GTP-binding protein, Rap (Ras-proximate) 1b. We first looked for this Ca^{2+} pool target of regulation by studying the expression of the different SERCA and Rap 1 proteins in human platelets and various cell lines, by Western blotting and reverse transcription-PCR. Since co-expression of Rap 1b and SERCA 3b was obtained, we looked for their protein–protein interaction as a function of the cAMP-dependent phosphorylation of Rap 1b. Co-immunoprecipitations of SERCA 3b and Rap 1b proteins were found in the absence of phosphorylation, induced by the

catalytic subunit of the cAMP-dependent protein kinase (csPKA). In contrast, upon pre-treatment of platelet membranes with csPKA, the SERCA 3b dissociated from the Rap 1b protein, in agreement with a role of its phosphorylated state in their interaction. Finally, we looked for adaptation of this complex in a platelet pathological model of hypertension. We investigated the expression of both proteins, as well as the cAMP-dependent phosphorylation of Rap 1b and SERCA 3b activity in platelets from control normotensive Wistar-Kyoto rats and from spontaneously hypertensive rats (SHRs). A decrease in SERCA 3b activity was associated with a decrease in Rap 1b endogenous phosphorylation in SHR platelets, consistent with a functional role in the regulation of the SERCA 3b-associated Ca^{2+} pool.

INTRODUCTION

Although the involvement of intracellular Ca^{2+} pools in platelet Ca^{2+} signalling is well established, much less is known about their identification and respective roles during cell life [1–3]. At least two types of intracellular Ca^{2+} pool are described: inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} pools and IP_3 -insensitive Ca^{2+} pools [4–8]. A new rationale for these distinct roles of multiple Ca^{2+} pools has come from evidence for a plurality of sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCAs), which take up Ca^{2+} into Ca^{2+} pools. First, human platelets co-express the 100 kDa ubiquitous SERCA 2b [9], the polyclonal antibody N 89-recognizable 97 kDa SERCA 3a [10,11] and the monoclonal antibody PL/IM 430-recognizable 97 kDa SERCA 3b isoform [12–15]. Secondly, this latter isoform was found to be associated precisely with the IP_3 -sensitive Ca^{2+} pool. This assignment was made because IP_3 -induced Ca^{2+} release depends upon the Ca^{2+} filling state of the SERCA 3b-associated Ca^{2+} pool [6]. This newly defined function would mean that it accounts for the molecules and structures involved in signal-transduction machinery and would place the SERCA 3b isoform in a multiplex of proteins, including the phospholipase C responsible for IP_3 formation and the cytoskeleton. Accordingly, immunolocalization of the above multi-SERCA system revealed that SERCA 3b was in intracellular membranes linked with the plasma membrane in resting platelets and associated with cytoskeleton upon platelet activation [16].

The next question concerns the functional aspect of this Ca^{2+} pool in platelets and, in particular, the control of its content, both to insure and limit spatio-temporal IP_3 -induced Ca^{2+} release as a function of platelet activation and/or inhibition. Some light may be thrown on the matter through a possible role in this process of the intriguing highly expressed platelet GTP-binding Rap (Ras-proximate) 1b protein. Indeed, as this protein is a member of the ras family, it probably controls the switching of signal transduction, and it has the potential to turn cell functions on or off through its GTP-binding and cAMP-dependent phosphorylation capacities [17,18]. Moreover, this role may converge towards a Ca^{2+} -dependent mechanism involving the above multiplex. Although confirmation is required, Rap 1b has been suggested independently to: (i) be associated with the cytoskeleton during cell activation [19,20]; (ii) be linked to phospholipase C activation; and (iii) regulate Ca^{2+} ATPase function [21], which is of particular interest in the present purpose. Indeed, Rap 1b was reported to affect Ca^{2+} transport mediated by mixed Ca^{2+} ATPases, either in intact platelets or into isolated membrane vesicles [22,23]. Moreover, the regulation was found to involve the functional state of the Rap 1b protein, as it either inhibited Ca^{2+} transport upon GTP γ S binding or stimulated Ca^{2+} transport upon its cAMP-induced phosphorylation [24]. The SERCA target of this regulation was recently suggested to be the SERCA 3b isoform, based on apparent co-expression and co-localization of this isoform with the Rap 1b protein [16,25,26].

The aim of the present study was therefore to improve

Abbreviations used: IP_3 , inositol 1,4,5-trisphosphate; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; Rap, Ras-proximate; RT-PCR, reverse transcription-PCR; DTT, dithiothreitol; SBTI, soya bean trypsin inhibitor; WKY, Wistar Kyoto; SHR, spontaneously hypertensive rat; PKA, cAMP-dependent protein kinase; csPKA, catalytic subunit of the cAMP-dependent protein kinase; ECL, enhanced chemiluminescence; pAb anti-SERCA 1, polyclonal anti-SERCA 1 antibody.

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understanding of the role of Rap 1b. We first identified the intracellular Ca^{2+} pool involved by further looking for a relationship between the expression of the SERCA 3b and Rap 1b proteins in human platelets and different cell lines, by means of Western blots using specific antibodies and Southern blots of reverse transcription (RT)-PCR products using specific *rap 1a* and *rap 1b* probes, respectively. As a prerequisite for the potential interrelation between the SERCA 3b and Rap 1b proteins, we looked for an association between the two proteins by co-immunoprecipitation using the PL/IM 430 antibody. Then we tested the effect of the phosphorylation of Rap 1b using the catalytic subunit of the cAMP-dependent protein kinase (csPKA) on the association of Rap 1b with the SERCA 3b protein. Finally, because of the increase in phospholipase C [27], IP_3 formation and cytosolic Ca^{2+} concentration in hypertension, we looked for a functional interaction between the SERCA 3b and the Rap 1b candidate proteins by investigating their expression, as well as the activity of this SERCA target as a function of phosphorylation state of Rap 1b protein in this pathological model of signal transduction.

The overall results suggest that the Rap 1b protein is involved in the control of the SERCA 3b-associated IP_3 -sensitive Ca^{2+} pool through its cAMP-dependent phosphorylation, and that it plays a key role in the subtle balancing effects between platelet activation and inhibition.

EXPERIMENTAL

Materials

RPMI 1640 medium with Glutamax I, Eagle's minimal essential medium, Earle's balanced salt solution and fetal calf serum were from Gibco-BRL (Paisley, U.K.). Streptomycin was from Boehringer (Mannheim, Germany). Protein G-Sepharose, trypsin type XIII, csPKA from bovine heart, deoxycholate acid, Nonidet P-40, aprotinin, leupeptin, PMSF, soya bean trypsin inhibitor (SBTI) and Bowman-Birk trypsin-chymotrypsin inhibitor were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monoclonal anti-SERCA 2 antibody (IID8) was from Affinity BioReagents (Neshanic Station, NJ, U.S.A.). Peroxidase-conjugated anti-rabbit, anti-guinea pig and anti-mouse IgGs were from Jackson ImmunoResearch (West Grove, PA, U.S.A.). Anti-Rap 1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Nitrocellulose membranes were from Schleicher and Schuell (Dassel, Germany). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (110 TBq/mmol, 370 MBq/ml), Enhanced chemiluminescence (ECL), Western-blotting and Southern-blotting reagents and Rainbow-coloured protein-molecular-mass markers were from Amersham International (Little Chalfont, Bucks., U.K.). Electrophoresis reagents were from Bio-Rad (Richmond, CA, U.S.A.). The megakaryocytic MEG 01 cell line was kindly provided by Professor J. Peries, CNRS, Hôpital Saint-Louis, Paris, France. The epithelial HeLa, T lymphoblastoid JUR E 6-1 and monocytic THP-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). RNA Plus kit used for total RNA isolation was from Bioprobe (Montreuil sous bois, France). MuLV reverse transcriptase, RNase inhibitor and *Taq* polymerase were from Perkin Elmer (Branchburg, NJ, U.S.A.). Primers were from Eurogentec (Leuven, Belgium).

Animals

Sixteen-week-old spontaneously hypertensive rats (SHRs) and normotensive Wistar Kyoto (WKY) rats were supplied by the Centre d'Élevage R. Janvier (Le Genest, France). Animals were subjected to ether anaesthesia and blood samples were collected

from the abdominal aorta using ACD-C (130 mM citric acid/170 mM sodium citrate/111 mM glucose) as an anticoagulant.

Methods

Cell culture

The MEG 01, JUR E 6-1 and THP-1 cells were grown in RPMI 1640 medium with Glutamax I, supplemented with 10% heat-inactivated fetal calf serum/50 units/ml of penicillin/0.05 mg/ml of streptomycin. For HeLa cells, RPMI medium was replaced with Eagle's minimal essential medium with non-essential amino acids and Earle's balanced salt solution. All cell lines were incubated and cultured at 37 °C with 5% CO_2 in a humidified atmosphere.

Membrane preparation

Human cell lines

The cells grown in suspension were harvested by centrifugation at 400 g for 10 min and lysed in an ice-cold buffer containing 10 mM Hepes (pH 7.0), 10 mM KCl, 0.05 mM EGTA, 0.5 mM dithiothreitol (DTT) and a mixture of the following protease inhibitors: 0.05 mg/ml of aprotinin, 0.01 mg/ml of leupeptin, 0.025 mM PMSF and 0.1 mg/ml of SBTI. Adherent cells grown in monolayers (HeLa) were scraped into lysis buffer in the culture flasks at 4 °C. Membranes of the different cell lines were prepared as described previously [25].

Platelets

Blood from several donors was used for the isolation of either human or rat platelets. Platelet-rich plasma was obtained by centrifugation for 15 min, at room temperature, at 120 and 150 g for human and rat blood, respectively. Platelets were then pelleted by centrifugation for 15 min at room temperature at 1500 g and platelet membranes were prepared as described in [12]. The protein concentration of the different membrane preparations was determined by the Bradford method [28] using γ -globulin as a standard. All samples were stored at -80 °C.

SDS/PAGE

Membrane proteins were solubilized in Laemmli's buffer, reduced with 10% (v/v) 2-mercaptoethanol at room temperature and analysed by 7.5% or 11% SDS/PAGE [29]. Electrophoresis was performed overnight at 40 V. Proteins were then electro-transferred on to nitrocellulose membranes.

Antibody preparation and characterization

IID8 is a mouse monoclonal antibody raised against dog cardiac muscle SERCA [30]. This antibody recognizes a shared epitope present on SERCA 2 splice variants from cardiac muscle (2a) and non-muscle (2b), i.e. on the first 993 amino acids common to both SERCA 2 isoforms.

The PL/IM 430 monoclonal antibody was raised using highly purified platelet intracellular-membrane vesicles as an immunogen [31] and purified from culture supernatant by Protein A affinity chromatography at high ionic strength, as described in [12]. This antibody specifically recognized the SERCA 3b protein in platelets and in different human cell lines [6,32].

The polyclonal anti-SERCA 3 antibody (N 89) was elicited in rabbits against a synthetic peptide that was specific for SERCA 3. This peptide was selected to be distinct from the similar stretches of amino acids in SERCA 1 or SERCA 2. This anti-

SERCA 3 antibody recognized rat SERCA 3 protein expressed in COS-1 cells and platelets, but neither reacted with SERCA 1a, 2a or 2b fusion proteins [11].

The polyclonal anti-SERCA 1 antibody (pAb anti-SERCA 1) was elicited in guinea pig against rabbit skeletal-muscle Ca^{2+} -ATPase (SERCA 1) [33] and was purified by immunoaffinity chromatography on an antigen (SERCA 1) column from SERBIO (Genevilliers, France). pAb anti-SERCA 1 reacts with SERCA 1 in rabbit-skeletal sarcoplasmic-reticulum-membrane preparation and does not show any cross-reactivity with the erythrocyte or cardiac sarcolemmal Ca^{2+} -ATPases. It recognizes both of the platelet SERCA 3a and 3b proteins [12,16].

The polyclonal anti-Rap 1 antibody was raised in rabbit against a peptide encompassing Rap 1 protein residues 121–137, which are common to the Rap 1a and 1b proteins.

Western blotting

Nitrocellulose membranes were treated overnight at room temperature with quenching solution containing 150 mM NaCl, 10 mM Tris/HCl (pH 7.4), 0.1 % Tween 20 and 5 % non-fat dry milk. The membranes were then incubated for 2 h at room temperature with a 1:1000 dilution of the PL/IM 430, IID8, anti-SERCA 1, anti-SERCA 3 or anti-Rap 1 antibodies. Thereafter, the membranes were extensively washed and further incubated for 1 h 30 min at room temperature with a 1:1000 dilution of the anti-mouse IgG horseradish-peroxidase conjugate for immunostaining of the monoclonal antibodies, a 1:100000 dilution of the anti-guinea pig IgG horseradish-peroxidase conjugate for immunostaining of the pAb anti-SERCA 1, a 1:100000 dilution of the anti-rabbit IgG horseradish-peroxidase conjugate for immunostaining of the pAb anti-SERCA 3 and a 1:10000 dilution of the anti-rabbit IgG horseradish-peroxidase conjugate for immunostaining of the anti-Rap 1 antibody. After washing, antibody binding was detected using ECL Western blotting reagents according to the instructions of the manufacturer.

Protein phosphorylation induced by cAMP

cAMP-induced phosphorylation was studied with human and rat platelet membranes. Platelet membranes were incubated at 30 °C for 10 min in a reaction mixture containing 10 mM sodium phosphate buffer (pH 7.0), 15 mM MgSO_4 , 100 mM NaF, 6 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (460 MBq/mmol) and 0.1 % Triton X-100 in the presence or absence of the csPKA (enzyme/substrate ratio 0.02). Reactions were started by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and stopped by adding either an equal volume of solubilization buffer or 5 ml of 1 mM ATP. After a 100000 g centrifugation, human membrane proteins were treated for immunoprecipitation experiments.

Co-immunoprecipitation

Human platelet membranes, either untreated or pre-treated with csPKA, were solubilized for 2 h at 4 °C by stirring in a modified RIPA's buffer; 50 mM Tris/HCl (pH 8.0)/150 mM NaCl/1 % Nonidet-P40/1 % sodium deoxycholate/0.1 % SDS/0.1 mM DTT and the following inhibitors; 0.002 mg/ml of aprotinin/0.002 mg/ml of leupeptin/0.05 mM PMSF/0.1 mg/ml of SBTI/0.05 mg/ml of Bowman-Birk trypsin-chymotrypsin inhibitor. Binding of the monoclonal PL/IM 430 antibody to Protein G-Sepharose (50 mg/ml) was achieved after 1 h of incubation with shaking at 4 °C. The solubilized membranes were then added to this mixture for 1 h 30 min. Immunoprecipitates were isolated by centrifugation at 10000 g for 10 min and a second round of immunoprecipitation was performed by adding fresh Protein G-

Sepharose, coupled with the PL/IM 430 antibody, to the supernatant. The pooled immunoprecipitates were then washed once with 10 mM Tris/HCl (pH 5)/5 mM EDTA/1 % Triton X-100/1 % sodium deoxycholate/0.5 M NaCl, and four times with the same buffer, but without NaCl.

The immunoprecipitates and their respective supernatants were submitted to 11 % SDS/PAGE, electro-transferred on to nitrocellulose membranes and autoradiographed at -80 °C using Kodak X-Omat AR film and intensifying screens.

Controlled trypsin proteolysis of membrane vesicles

Human or rat platelet membrane vesicles were trypsinolysed in proteolysis medium containing 160 mM KCl, 17 mM Hepes (pH 7.0), 0.1 mM DTT, 0.05 mM CaCl_2 and 0.1 or 0.2 mg of membrane proteins/ml. Trypsin digestion was conducted under different proteolytic conditions: 10 μg /ml of trypsin at 4 °C for human platelets and 50 μg /ml of trypsin at 4 °C for rat platelets. After various times, the reaction was stopped with a 10-fold excess (w/w) of SBTI. Digested samples were then either used immediately for membrane autophosphorylation of Ca^{2+} -ATPases or precipitated by 6 % trichloroacetic acid and, after centrifugation, solubilized in Laemmli's buffer, submitted to 11 % SDS/PAGE and electroblotted on to nitrocellulose membranes.

Phosphoenzyme-intermediate formation of intact and proteolysed Ca^{2+} -ATPases

Ca^{2+} ATPases were phosphorylated with 0.05 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (460 MBq/mmol) for 1 min at 4 °C in the proteolysis medium, immediately after proteolytic digestion, as described above. The reaction was stopped by addition of a 6 % trichloroacetic acid solution containing 10 mM phosphoric acid and 1 mM ATP. Precipitates were washed three times with the same solution and finally dissolved in the electrophoresis sample buffer [34]. Phosphorylated membrane proteins (100 μg /well) were submitted to 7.5 % acidic SDS/PAGE, electroblotted on to nitrocellulose membranes, and autoradiographed for 24–48 h using Kodak X-Omat AR film at -80 °C.

RNA extraction

The isolation of platelets was slightly modified by blood dilution in 0.9 % NaCl prior to centrifugation steps to isolate platelet-rich plasma (PRP), and PRP was withdrawn until a depth of 1 cm remained over the buffy coat. This avoided lymphocyte contamination. Under these conditions, we verified that platelet contamination by other blood cells was less than 1 leukocyte in 4000 platelets. Total RNA was isolated from human and rat platelets and various cell lines, according to Chomczynski and Sacchi [35], using the RNA Plus solution.

Reverse transcription-PCR

The different primers for *rap 1a*, *rap 1b* and *SERCA 2b* mRNA amplifications were the same as those used in [36]. Control experiments showed that the *rap 1a*-amplified products were not recognized by the specific *rap 1b* probe and the *rap 1b*-amplified products were not recognized by the specific *rap 1a* probe (results not shown).

Total RNA (500 ng) was overlaid with mineral oil. First-strand cDNA was synthesized by adding 2.5 μM oligo d(T)₁₆/1 unit/ μl RNase inhibitor/1 mM of each dNTP/2.5 units/ μl MuLV reverse transcriptase for 30 min at 42 °C in PCR buffer [10 mM Tris/HCl (pH 8.3)/50 mM KCl] and 5 mM MgCl_2 in a

total volume of 10 μ l. After heat inactivation of reverse transcriptase for 5 min at 99 °C, the volume was adjusted to 50 μ l with the same PCR buffer/2 mM $MgCl_2$ /0.15 μ M of the primers. PCR was initiated by adding 1.25 units of *AmpliTaq* DNA polymerase (from *Thermus aquaticus*) and conducted in an automated thermocycler (Techne OSI; Cambridge, U.K.) for 30 cycles, each cycle consisting of successive periods of denaturation at 94 °C for 30 s, annealing at 55 °C for 2 min and extension at 72 °C for 2 min. Negative controls were run in the absence of reverse transcriptase, to check the specificity of the amplifications.

Southern blotting

RT-PCR products (10 μ l) were electrophoresed on 1.5% ethidium bromide-stained agarose gel and transferred on to Biohylon Z+ membranes, according to Sambrook et al. [37]. The membranes were prehybridized for 1 h at 42 °C in the gold hybridization buffer from the direct nucleic acid-labelling kit containing 0.5% blocking reagent/0.5 M NaCl. Hybridization was carried out overnight at 42 °C in the same solution containing 1 ng/ml of the direct-labelled cDNA probes. The following three probes (*SERCA 2b*, *rap 1a* and *rap 1b*) were prepared using the direct nucleic acid-labelling kit as described in [36]. Membranes were washed, peroxidase-conjugated probe staining was revealed using a chemiluminescence kit and autoradiography was carried out with X-Omat AR Kodak film.

RESULTS

Identification of the 97 kDa SERCA target of Rap 1b

We observed previously a correlated expression of the SERCA 3b and Rap 1b proteins in platelets and different cell lines [25]. As we showed recently that another 97 kDa SERCA 3a protein, which is recognized by the N 89 antibody, exists [10], we first investigated the expression of the different SERCA proteins and of the Rap 1a and 1b proteins in different cell lines.

Comparative expression of SERCA proteins in human cells

We selected cell lines based on the previous observation that SERCA 3b was suggested to be specific to haemopoietic cells. As controls, we used the T lymphoblastoid JUR E 6-1 and non-haemopoietic epithelial HeLa cell lines for their expression of the PL/IM 430-recognizable 97 kDa SERCA 3b and N 89-recognizable SERCA 3a, respectively, as well as human platelets, which express both proteins, in addition to the ubiquitous SERCA 2b isoform [13]. We isolated membrane fractions and studied SERCA expression by Western blotting (Figure 1). The monoclonal antibody IID8 (Figure 1A), the polyclonal antibody N 89 (Figure 1B) and the monoclonal antibody PL/IM 430 (Figure 1C) were used to visualize SERCAs 2b, 3a and 3b, respectively. Figure 1 shows the results of the comparative studies of the human platelets (lanes 1), the megakaryocytic MEG 01 (lanes 2), the T lymphoblastoid JUR E 6-1 (lanes 3), the monocytic THP-1 (lanes 4) and the epithelial HeLa (lanes 5) cell lines. The SERCA 2b isoform appeared equally expressed in all cell lines, as demonstrated by the similar recognition of the 100 kDa protein band, thus verifying that the same amount of membrane protein was used. A clear difference was found between the expression levels of the N 89-recognizable SERCA 3a and PL/IM 430-recognizable 97 kDa SERCA 3b isoforms. Indeed, the comparison of the N 89 immunostainings (Figure 1B) of the different cell lines with HeLa cells, which expressed exclusively this SERCA 3a protein (compare Figures 1B and 1C, lanes 5), showed that the two new haemopoietic cell lines, MEG

Figure 1 Comparative expressions of SERCA

Mixed membrane fractions (50 μ g of protein) isolated from platelets and various cell lines were reduced, analysed by SDS/PAGE (11%) and electroblotted on to nitrocellulose membranes. SERCAs 2b, 3a and 3b were immunodetected using the monoclonal IID8 (A), the polyclonal N 89 (B) and the monoclonal PL/IM 430 (C) antibodies, respectively. Lanes 1–5 were loaded with membrane proteins from the following cells; platelets, MEG 01, JUR E6-1, THP-1 and HeLa, respectively. The molecular masses on the left indicate the size of the Ca^{2+} ATPases, estimated with standard rainbow-coloured markers. This figure is representative of six experiments.

01 (lanes 2) and THP-1 (lanes 4), did not express this protein. These newly explored cell lines (lanes 2 and 4) were immunostained by the PL/IM 430 antibody (Figure 1C) in a similar manner to the JUR E6-1 cells (lane 3), i.e. to a significantly lesser extent than human platelets (lane 1), thus confirming the specific expression of this protein in haemopoietic cells.

Comparative expression of *rap 1a* and *rap 1b* mRNAs

We then looked for the expression of Rap 1 proteins in the same cells (Figure 2). As the now-available specific anti-Rap 1a and anti-Rap 1b antibodies did not appear specific under our conditions, we investigated *rap 1* mRNA expression. We designed primers corresponding to specific regions in the *rap 1a* and *rap 1b* nucleotide sequences that permitted the selective amplification of the two mRNAs. We performed reverse transcription followed by PCR experiments on RNAs obtained from the different cell lines. *SERCA 2b* mRNA expression [25] was used to verify that the same amounts of RNAs were loaded (Figure 2A). Figures 2(B) and 2(C) show the Southern blots resulting from hybridization at high stringency of the amplified products of *rap 1a* and *rap 1b* (362 bp), respectively, with the respective labelled probes. The results showed that the different cell lines expressed the *rap 1* mRNAs, and were consistent with those of Culine et al., who suggested differential expression of *rap 1a* and *rap 1b* in human tumours [38]. Indeed, significant differences were observed: the platelets (Figure 2, lanes 1), MEG 01 (lanes 2), JUR E6-1 (lanes 3) and THP-1 (lanes 4) cell lines expressed more *rap 1b* than *rap 1a*, whereas the opposite was seen with HeLa cells (lanes 5). Hence, comparison of Figures 1 and 2 shows that cells expressing the SERCA 3b isoform expressed more *rap 1b* mRNA (lanes 2–4), whereas cells expressing only the SERCA 3a protein expressed more *rap 1a* mRNA (lanes 5), so that the mRNA level of *rap 1b* corroborated the expression for the

Figure 2 Comparative mRNA expressions of *rap 1a*, *rap 1b* and *SERCA 2b*

Total RNAs were extracted from platelets and various cell lines as described in the Experimental section. Each RNA species (500 ng) underwent reverse transcription using oligo d(T)₁₆ and MuLV reverse transcriptase. PCR was then performed and one fifth of the amplification products were analysed by Southern blotting using *SERCA 2b* (A), *rap 1a* (B) or *rap 1b* (C) labelled cDNA probes. Lanes 1–5 show the amplification products from the RNAs of platelets, MEG 01 cells, JUR E6-1 cells, THP-1 cells and HeLa cells, respectively. Numbers on the left indicate the size of the amplification products in bp. This figure is representative of three experiments.

SERCA 3b protein (compare Figures 1C and 2C). Consequently, the combination of these results eliminated the SERCA 3a protein as a target of the regulation by the Rap 1b protein, thus

Figure 3 cAMP-dependent association of SERCA 3b and Rap 1b

Human platelet-membrane proteins were phosphorylated for 10 min at 30 °C by cAMP-dependent protein kinase, using 10 µg/ml of csPKA (C. Sub. in the Figure; +) or not (–). Reactions were started by the addition of [γ -³²P]ATP and stopped by adding either an equal volume of solubilization buffer, for control membranes (A), or 1 mM ATP (B). After a 100 000 g centrifugation step, membrane proteins phosphorylated by csPKA or not were treated for immunoprecipitation experiments using the PL/IM 430 monoclonal antibody, as described in the Experimental section. The different samples were then analysed by 11% SDS/PAGE, electrophoreted on to nitrocellulose membranes and either autoradiographed (lane 1), or treated for immunoblotting using the polyclonal anti-Rap 1 (lanes 2 and 4) or the monoclonal PL/IM 430 (lanes 3 and 5) antibody. The approximate molecular masses of the Rap 1b and the SERCA 3b proteins are estimated with standard rainbow-coloured markers. This figure is representative of six experiments.

confirming previous observations pointing to SERCA 3b as the preferred target of Rap 1b protein regulation.

cAMP-dependent association of Rap 1b and SERCA 3b

As the hypothesis explored was based on an effect of cAMP on the regulation of Ca²⁺ATPase, we further investigated the potent effect of cAMP-induced phosphorylation of the Rap 1b protein by looking for a cAMP-dependent association of Rap 1b with the SERCA 3b isoform. To this end, we performed PL/IM 430 immunoprecipitation experiments (Figure 3) in the presence or absence of prior cAMP-dependent Rap 1b phosphorylation, through the use of the csPKA. As controls, human platelet-membrane proteins (100 000 g fractions) were phosphorylated and analysed by autoradiography (Figure 3A, lane 1) and Western blotting using the anti-Rap 1 (lane 2) or the PL/IM 430 antibodies (lane 3). The autoradiography showed that, under our experimental conditions, the 24 kDa Rap 1b is the major target of cAMP-dependent protein kinase (PKA) activity, as demonstrated previously [24], and that the conditions used did not interfere with recognition of Rap 1b (Figure 3A, lane 2) and SERCA 3b (lane 3) proteins by their respective antibodies. These platelet-membrane proteins, pre-phosphorylated (lanes +) or not (lanes –), were then solubilized and immunoprecipitated using the PL/IM 430 antibody (Figure 3B), as described in the Experimental section. Control experiments were performed in the absence of the PL/IM 430 antibody to check the specificity of the immunoprecipitation. Neither the SERCA 3b nor the Rap 1 proteins were detectable (results not shown). The immunoprecipitates were tested for their immunoreactivities by Western blotting using the anti-Rap 1 (Figure 3B, lanes 4) or the PL/IM 430 (lanes 5) antibodies. The immunoprecipitations were complete, since neither residual Rap 1b nor SERCA 3b were detected in the supernatants (results not shown). In the absence of phosphorylation, SERCA 3b (lane 5, –) and Rap 1b (lane 4, –) were present in the PL/IM 430 immunoprecipitates, thus demonstrating their co-immunoprecipitation. When membrane proteins were previously phosphorylated and then immunoprecipitated, Rap 1b was not found in the immunoprecipitates (lane 4, +) and was recovered in the supernatants (results not shown). This showed that the phosphorylation of Rap 1b eliminated its interaction with the SERCA 3b protein (lane 5, +).

Inter-relation and regulation of Rap 1b and SERCA 3b in hypertension

We investigated the adaptation of the complex of SERCA 3b and Rap 1b proteins in a platelet-disease state, in which cytosolic Ca²⁺ concentrations are elevated. We used the model of rat hypertension and studied the expression of SERCA 3b and Rap 1b proteins, as well as the functional interaction between these two proteins, i.e. the activity of SERCA 3b depending upon the phosphorylation state of Rap 1b protein.

Evidence that rat platelets express the same SERCA isoforms as human platelets

Because it was still unknown whether rat platelets express the SERCA 3b isoform, we looked for the expression of this protein. We used the combination of the two detection methods for SERCAs: the formation of phosphorylated intermediates of the

Figure 4 Evidence that rat platelets express the same SERCA 3 isoforms as human platelets

Human or rat platelet-membrane proteins (100 μ g) were proteolysed using trypsin at 4 °C for 2 and 10 min. Trypsin/protein ratios of 0.05 and 0.25 were used for human (parts 1) and rat (parts 2) platelet membranes, respectively. Intact and trypsin-proteolysed SERCAs were then labelled with 0.05 μ M [γ - 32 P]ATP, resolved by electrophoresis on 7.5% acidic SDS/polyacrylamide gels, blotted on to nitrocellulose membranes, and either autoradiographed (**A**) or immunostained using the pAb anti-SERCA 1 (**B**) as described in the Experimental section. **A**, Human (part 1) and rat (part 2) platelet-membrane autoradiographs; **B**, human (part 1) and rat (part 2) Western blots. Lanes marked 0, membranes from intact untreated platelets; lanes marked 2 and 10, membranes from platelets trypsinolysed for 2 and 10 min, respectively. The apparent molecular masses of the intact proteins and their tryptic fragments are estimated using standard rainbow-coloured markers. This figure is representative of three experiments.

Ca^{2+} ATPases (**A**); and Western blotting using the pAb anti-SERCA 1 (**B**) to replace the PL/IM 430 antibody, which did not react with rat antigens [11,12]. Even if the pAb anti-SERCA 1 recognized both 97 kDa SERCA 3a and 3b proteins [12,16], one could circumvent this problem by performing trypsinolysis of Ca^{2+} ATPases (Figure 4). Indeed, they each present distinct tryptic patterns: the formation of (i) a 73/68 kDa doublet giving rise to a 40 kDa fragment for the SERCA 3b isoform [12]; and (ii) 80 and 25 kDa fragments for the SERCA 3a isoform [11]. Tryptic digestions were performed both on human (Figures 4A and 4B, parts 1) and rat (parts 2) platelets for 2 and 10 min.

Figure 5 Comparative expression of SERCA 3b in platelet membranes of normotensive WKY rats and SHR

Tryptic digestion of the Ca^{2+} ATPases in normotensive WKY-rat or SHR platelet membranes was conducted using 50 μ g/ml of trypsin (trypsin/protein ratio 0.25) at 4 °C for 2 min. Intact and trypsin-proteolysed Ca^{2+} ATPases were then resolved by electrophoresis on 11% SDS/PAGE (50 μ g of membrane proteins/lane), blotted on to nitrocellulose membranes and immunostained using the pAb anti-SERCA 1, as described in the Experimental section. **A**, Platelet-membrane proteins from WKY rats; **B**, platelet-membrane proteins from SHR. Lanes marked 0, membranes from intact untreated platelets; lanes marked 2, membranes from platelets trypsinolysed for 2 min. The apparent molecular masses of the intact proteins and their tryptic fragments are estimated using standard rainbow-coloured markers. This figure is representative of three experiments.

SERCA 2b and 3a proteins could be visualized in both species by their autophosphorylated tryptic fragments of 55 and 80 kDa, respectively (Figure 4A) [12]. Figure 4(B) confirmed the presence of the SERCA 3a protein through the detection of its 25 kDa tryptic fragment, and demonstrated the presence of tryptic fragments of 73/68 kDa (2 min lanes), further degraded to the 40 kDa fragment (10 min lanes) relative to SERCA 3b, thus establishing the expression of this isoform in rat platelets.

Comparative expression of the SERCA 3b isoform in platelets from normotensive and hypertensive rats

The effect of hypertension on this expression of the SERCA 3b isoform was then investigated (Figure 5). We isolated membranes from normotensive WKY-rat and SHR platelets. Following mild trypsinolysis of the SERCA isoforms for 2 min, using a trypsin/protein ratio of 0.25, proteins were electrophoresed and immunostained using the pAb anti-SERCA 1, as above. WKY-rat (Figure 5A) and SHR (Figure 5B) platelet membranes showed similar apparent expressions of the total 97 kDa SERCA proteins recognized by this antibody (Figure 5, 0 min lanes), thus already suggesting a down-regulation of the SERCA 3b, since an up-regulation of SERCA 3a has been reported in hypertension [10]. Comparison of the tryptic fragments obtained revealed the lower expression of the 73/68 kDa doublet and 40 kDa fragment (Figure 5, 2 min lanes) relative to the SERCA 3b in SHR platelets compared with WKY rat platelets.

Determination of *rap 1a* and *rap 1b* mRNA expression in hypertension

We compared *rap 1* expression in hypertension (Figure 6) by RT-PCR experiments using primers and probes corresponding to each *rap 1a* and *rap 1b* mRNA, as in Figure 2. We performed reverse transcription followed by PCR of the *rap 1a* and *rap 1b* RNAs of WKY-rat (Figure 6, lanes 1) and SHR (lanes 2) platelets in the absence or presence of reverse transcriptase. As expected, no amplification products were obtained without

Figure 6 Comparative expression of *rap 1a* and *rap 1b* mRNA in platelets of normotensive WKY rats and SHRs

Total RNAs were extracted from 16-week-old WKY-rat or SHR platelets. Each RNA (500 ng) underwent reverse transcription using oligo d(T)₁₆ and MuLV reverse transcriptase. PCR was then performed and one fifth of the amplification products were analysed by Southern blotting using the labelled *SERCA 2b* (A), *rap 1a* (B) or *rap 1b* (C) cDNA probes. Lanes 1, amplification products from the RNAs of WKY-rat platelets; lanes 2, amplification products from the RNAs of SHR platelets. Numbers on the left indicate the size of the amplification products in bp. This figure is representative of three experiments.

reverse transcriptase (results not shown). To verify the amounts of *SERCA 2b* found previously to be unchanged in hypertension [10], we performed RT-PCR using *SERCA 2b* primers followed by Southern blotting of the amplified products (270 bp) using the ECL-labelled *SERCA 2b* probe (Figure 6A). Figures 6(B) and 6(C) show the Southern blots of the *rap 1a* and *rap 1b* amplified products (362 bp), respectively, revealed with the respective labelled probes. The results showed that rat platelets exhibited both *rap 1a* and *rap 1b* mRNAs, which were expressed equally, whereas human platelets expressed preferentially *rap 1b* mRNA species. Differential regulation of the two mRNAs appeared as the expression of *rap 1a* mRNA was increased in SHR platelets (compare Figure 6B, lanes 1 and 2), whereas that of *rap 1b* was unchanged (compare Figure 6C, lanes 1 and 2). Densitometric measurements of the chemiluminescent bands from the autoradiographs were performed and the values obtained, expressed as the ratio of SHR/WKY rats (mean $\pm$ S.D.), were 1.98 ± 0.64 and 1.06 ± 0.15 for *rap 1a* and *rap 1b* mRNA, respectively. The results established that expression of *rap 1a* was about two-fold greater in SHR than in normotensive WKY-rat platelets, whereas no significant variation in the expression of *rap 1b* was detected.

Phosphorylation state of Rap 1b in hypertension

The above results led us to perform a comparative study of the phosphorylated state of Rap 1b in WKY-rat platelets and SHR platelets (Figure 7). On the one hand, the similar expression of Rap 1b in both strains allowed this comparative study in terms of phosphorylation itself. On the other hand, although Rap 1a was present in significant amounts in rat, compared with human, platelets, Siess et al. [39] clearly demonstrated that Rap 1b was the preferred substrate for protein kinase A using Rap 1a- and Rap 1b-specific peptides. We carried out cAMP-induced phosphorylation of membranes isolated from normotensive

Figure 7 cAMP-induced phosphorylation of Rap 1 protein in hypertension

Platelet membrane proteins (100 μ g) were isolated from WKY rats and SHRs. They were phosphorylated for 10 min at 30 $^{\circ}$ C by cAMP-dependent protein kinase in the absence (–) or presence (+) of 10 μ g/ml of csPKA (C. Sub. in the Figure), reduced with 2-mercaptoethanol, separated by SDS/PAGE (11%), transferred on to nitrocellulose membranes and either autoradiographed (A) or immunostained using the anti-Rap 1 antibody (B), as described in the Experimental section. Lanes 1, membranes isolated from WKY-rat platelets; lanes 2, membranes isolated from SHR platelets. The approx. molecular masses of the Rap 1 proteins, estimated with standard rainbow-coloured markers, are indicated on the right. This figure is representative of three experiments.

WKY rat (Figure 7A, lane 1, + and –) and SHR (Figure 7A, lane 2, + and –) platelets. The same experiments were conducted in the absence of PKA (Figure 7A, lanes –) to ensure the specificity of the phosphorylation by the cAMP in this model. An increase was seen (Figure 7A) in the exogenous phosphorylation of Rap 1 in the membranes isolated from SHR platelets (Figure 7A, lane 2, +) compared with those from control WKY-rat platelets (Figure 7A, lane 1, +), thus revealing a decrease in endogenous phosphorylation of Rap 1b. As a control, the expression of total Rap 1 protein was analysed using the anti-Rap 1 antibody (Figure 7B), which confirmed the total increase of rap 1 messengers in SHR platelets (Figure 6).

Functional relationship between Rap 1b and SERCA 3b in this pathological model

The above findings allowed us to look for the functional relationship between the two proteins, as a function of the phosphorylation state of Rap 1b by studying the *SERCA 3b* activity in WKY-rat and SHR platelets. For this purpose, we studied the autophosphorylation of the 97 kDa band. Although the phosphorylated intermediate of the 97 kDa band covered the bands of both the *SERCA 3b* and *3a* proteins, the latter could be specifically dissociated by the formation of its related typical 80 kDa tryptic fragment. We thus studied autophosphorylation of the 97 kDa *SERCAs* in the absence and presence of trypsinolysis, as shown in Figure 4. Figure 8 shows the comparative results for the phosphorylated intermediate of the two 97 kDa *SERCA* proteins (Figure 8A) and for the *SERCA 3a* protein (Figure 8B). Figure 8(A) shows a net decrease in the intact phosphorylated 97 kDa bands in SHR (lane 2) compared with WKY-rat platelets (lane 1). Densitometric measurements of the autoradiographs showed that this decrease was by a factor of 3.26 ± 1.57 . Figure 8(B) shows that *SERCA 3a* exhibited an increase in the autophosphorylation of the 80 kDa related tryptic fragment (compare Figure 8B, lanes 1 and 2). This was in full agreement with the overexpression of this *SERCA* protein in hypertension [10], and allowed us to discount its involvement in the decrease in the 97 kDa autophosphorylated intermediate. Moreover, this decrease in the autophosphorylated intermediate formation also differed from the summed expression of 97 kDa *SERCAs*, which was invariable (compare lanes 1 of Figures 5A and 5B). This also eliminated its relationship to *SERCA* ex-

Figure 8 SERCA 3b activity in hypertension

Rat platelet-membrane proteins (100 μ g) were proteolysed using 50 μ g/ml of trypsin at 4 $^{\circ}$ C for 5 min (trypsin/protein ratio of 0.25). Intact (**A**) and trypsin-proteolysed (**B**) SERCA were then labelled with 0.05 μ M [γ - 32 P]ATP, resolved by electrophoresis on 7.5% acidic SDS/polyacrylamide gels, blotted on to nitrocellulose membranes and autoradiographed. Lanes 1, membranes isolated from WKY-rat platelets; lanes 2, membranes isolated from SHR platelets. The apparent molecular masses of the intact proteins and their tryptic fragments are estimated using standard rainbow-coloured markers. This figure is representative of three experiments.

pression and argued for another effect on the SERCA 3b activity itself.

The most likely interpretation of these results is therefore that the decrease in the SERCA 3b activity is due to the lower endogenous phosphorylation of Rap 1b.

DISCUSSION

The present findings show that: (i) the SERCA 3b isoform is the sole SERCA target of the Rap 1b protein in the platelet multi-SERCA system, and that both proteins present a protein-protein interaction; (ii) platelet inhibition occurring upon an increase in cAMP concentration leads to Rap 1b phosphorylation and its dissociation from the SERCA 3b protein; and (iii) the controlled but sustained platelet activation mimicked by hypertension results in both the regulation of the SERCA 3b activity and cAMP-dependent phosphorylation of the Rap 1b protein. The first feature of this work is that these observations, taken together, constitute a compelling body of evidence for a role of the GTP-binding Rap 1b protein in platelet Ca^{2+} signalling through its regulation of the SERCA 3b-associated IP_3 -sensitive Ca^{2+} pool. Consequently, it gives new insights into a hypothesis raised many years ago, based on the inter-relation between the two messengers, Ca^{2+} and cAMP, in platelet activation/inhibition. As cAMP-induced platelet inhibition was clearly associated with a decrease in cytosolic Ca^{2+} concentration [40], it was thought that cAMP may affect mechanisms involved in the regulation of cytosolic Ca^{2+} concentration, such as Ca^{2+} ATPases. Accordingly, previous studies demonstrated a relationship between the cAMP-dependent phosphorylation of a 22 kDa protein, now identified as the Rap 1b protein [21,24,39,41], and an increase in Ca^{2+} transport, mediated by Ca^{2+} ATPases, into platelet membrane vesicles, i.e. in total agreement with a cAMP-induced cytosolic Ca^{2+} depletion [23,42–46]. However, evidence was still lacking for a direct effect of the Rap 1b protein on Ca^{2+} ATPases. Subsequently, other studies suggested distinct roles for the Rap 1b protein, but all pointed to a Ca^{2+} -dependent phenomenon.

Hence, we first showed that the SERCA 3b isoform associated with the IP_3 -sensitive Ca^{2+} pool is the sole target of the Rap 1b protein among the platelet multi-SERCAs (Figures 1 and 2). Indeed, haemopoietic cells expressing SERCA 3b were found to co-express the Rap 1b protein. In contrast, cells devoid of the

SERCA target, such as HeLa cells, were not found to express Rap 1b. Secondly, the mechanism of action of the regulatory effect of Rap 1b on Ca^{2+} transport into the SERCA 3b-associated Ca^{2+} pool may involve a switching in the interaction between the two proteins. Indeed, the Rap 1b protein co-immunoprecipitated with its SERCA target using the monoclonal PL/IM 430 antibody, indicating an association between these two proteins. In contrast, the phosphorylation of Rap 1b induced its dissociation from its target protein, as shown by its absence in the SERCA 3b immunoprecipitate when platelet membranes were pre-treated with the cPKA (Figure 3). Finally, the pathological model of rat hypertension enhanced our understanding of the functional interaction between these two proteins, as we found a concomitant decrease in both the phosphorylation state of the Rap 1b protein and SERCA 3b activity. Although Rap 1b expression was unchanged (Figure 6C), cAMP-induced phosphorylation demonstrated an increase in the level of Rap 1b phosphorylation in SHR compared with WKY-rat platelets (Figure 7A). The most plausible explanation was that the decrease in endogenous phosphorylation of this protein, probably due to a reduced cAMP content in hypertension, as already described by Mazeaud et al. [47]. In terms of SERCA 3b activity, the lower global autophosphorylation of the 97 kDa band (Figure 8A) could refer neither to the lower expression of SERCA 3b, which was compensated by overexpression of SERCA 3a (Figures 5A and B, lanes 1), nor to the activity of SERCA 3a (Figure 8B). So, the most likely interpretation was that the decrease in the autophosphorylation of the 97 kDa band referred to a lower activity of SERCA 3b due to a lower phosphorylation of Rap 1b, consistent with a functional relationship between the two proteins.

Hence, the combined available data provide some new insights into the control of the Ca^{2+} content of the IP_3 -sensitive Ca^{2+} pool in normal as well as in abnormal platelets. In normal platelets, which are circulating cells submitted to simultaneous activation and inhibition, a fine control of this Ca^{2+} pool may involve the Rap 1b protein in Ca^{2+} signalling, as follows.

First, upon platelet activation – based on: (i) previous observations showing that GTP inhibits Ca^{2+} transport into platelet intracellular Ca^{2+} pools [24]; (ii) a regulatory role of the Rap 1 protein as a GTP-binding protein; and (iii) the demonstration of a Ca^{2+} -mediated activation of the Rap 1b protein in human platelets (GTP-binding) while this work was in progress [48] – the following cascade of events would occur. An increase in Ca^{2+} concentration would result in the activation (GTP-binding) of the Rap 1b protein, association with SERCA 3b and inhibition of its activity, thus diminishing the filling state of the associated Ca^{2+} pool. Secondly, upon platelet inhibition – based on: (i) the previously observed cAMP-induced increase in Ca^{2+} transport into isolated membrane vesicles from normal platelets [23]; (ii) the similar increase in endogenous cAMP-dependent phosphorylation of Rap 1b in Gray Syndrome platelets, which exhibit a decrease in cytosolic Ca^{2+} concentration [49]; and (iii) studies showing that an increase of phosphorylation of Rap 1b by PKA or its analogues was concomitant either with a decrease in the phospholipase C-induced formation of inositol phospholipids [50] or with inhibition of IP_3 receptors [51] – the following cascade of events would occur. An increase in cAMP concentration would result in the phosphorylation of the Rap 1b protein, its dissociation from SERCA 3b and the stimulation of its activity, thus increasing the filling state of the associated Ca^{2+} pool. Thirdly, in the pathological model of hypertension, a further intervention of the complex of SERCA 3b and Rap 1b proteins can be expected to counteract the permanent increase in phospholipase C activity, IP_3 formation and elevated cytosolic

Ca^{2+} concentration, observed in this disease. So, reduced cAMP concentration would result in the decrease of both endogenous phosphorylation of Rap 1b and SERCA 3b activity, resulting in a smaller Ca^{2+} pool, thus limiting IP_3 -induced Ca^{2+} release. Hence, both the Rap 1b-regulatory and SERCA 3b-regulated proteins may be involved in the adaptation of platelets in hypertension. This phenomenon could be essential for platelet survival by avoiding intracellular Ca^{2+} overloading.

Hence, the Rap 1b protein may regulate the Ca^{2+} content of the IP_3 -sensitive Ca^{2+} pool and constitute a reflection of the activation/inhibition state of platelets, in total agreement with the suggestion of the use of this protein as a marker of the functional state of platelets [52].

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