Platelet Ca\textsuperscript{2+} ATPases
A Plural, Species-Specific, and Multiple Hypertension-Regulated Expression System

Virginie Martin, Raymonde Bredoux, Elisabeth Corvazier, Béla Papp, Jocelyne Enouf

Abstract—Gaining insight into nonmuscle Ca\textsuperscript{2+} signaling requires basic knowledge of the major structures involved. We investigated the expression of platelet Ca\textsuperscript{2+} ATPases in normal and hypertension-associated abnormal Ca\textsuperscript{2+} signaling. First, overall identification of normotensive Wistar-Kyoto rat Ca\textsuperscript{2+} ATPases was attempted by looking for newly described human platelet 3'-end alternatively spliced sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPases (SERCA) 3b mRNA and plasma membrane Ca\textsuperscript{2+} ATPase (PMCA) 1b and 4b proteins, in addition to SERCA2b and SERCA3a isoforms. For SERCAs, comparative analyses of human and Wistar-Kyoto rat SERCA3 platelet mRNA by reverse transcription–polymerase chain reaction (RT-PCR) followed by sequencing established that human platelets coexpressed SERCA3b and a third SERCA3c, while rat cells were devoid of them but expressed a still unknown splice variant that we termed rSERCA3b/3c. Its identification using 3'-end SERCA3 gene and rapid amplification of cDNA ends (RACE)–PCR studies showed that it results from an additional SERCA3 alternative splicing process, which uses a second alternative polyadenylation site located in the last intron. For PMCAs, with the use of gene-specific RT-PCR followed by sequencing and Western blotting using 5F10 monoclonal antibody, expression of human and rat platelet PMCA1b and PMCA4b was similar. Second, comparative analysis of these newly identified Ca\textsuperscript{2+} ATPases and SERCA3a in age-matched spontaneously hypertensive rat platelets demonstrated (1) a marked downregulation of rSERCA3b/3c, which became null, and a 1.71-fold increase in SERCA3a and (2) an opposite regulation of the 2 PMCAs, namely, a 3.3-fold decrease in PMCA1b mRNA and a 3.7-fold increase in PMCA4b mRNA. Hence, platelets coexpress multiple, diverse, and species-specific Ca\textsuperscript{2+} ATPases, including a novel fourth SERCA3. Moreover, expression of PMCA (1b and 4b), SERCA3a, and rSERCA3b/3c was modulated in rat hypertension. Hence, Ca\textsuperscript{2+} ATPases should be regarded as constituting a new rational basis for the understanding of nonmuscle cell Ca\textsuperscript{2+} signaling. (Hypertension. 2000;35:91-102.)

Key Words: platelets \(\text{calcium} \bullet \text{Ca}^{2+} \text{ATPases} \bullet \text{genes}\)

Cell function is accompanied by upward and downward cytosolic Ca\textsuperscript{2+} signaling. In this context, 2 major questions are currently investigated, namely, the understanding of the biological significance of Ca\textsuperscript{2+} signaling and the basic knowledge of the mechanisms involved. Recent progress reveals that (1) the biological significance of Ca\textsuperscript{2+} signaling may reside in the fine tuning of the activities of enzyme or transcription factors, ie, toward cell-specific function and/or gene regulation and (2) Ca\textsuperscript{2+} signaling possesses a very high complexity composed of elementary and global events involving endoplasmic reticulum (ER) and plasma membrane structures. Among these are Ca\textsuperscript{2+} ATPases, enzymes that deplete the cytosol from Ca\textsuperscript{2+} ions by either extruding Ca\textsuperscript{2+} outside the cells, in the case of plasma membrane Ca\textsuperscript{2+} ATPases (PMCAs), or transporting Ca\textsuperscript{2+} toward the ER, in the case of sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPases (SERCAs). Consistent with this fact, overexpression of members of the SERCA family was found to cause an increase in the frequency of Ca\textsuperscript{2+} waves.\textsuperscript{1} Cloning of Ca\textsuperscript{2+} ATPases has revealed that they constitute a growing multigenic family. Four different PMCA genes have been described (PMCA1 to PMCA4),\textsuperscript{2} and alternative splices at 2 different sites of these genes give >20 isoforms. PMCA1 and PMCA4 are widely expressed, whereas PMCA2 and PMCA3 are more specialized isogenes that are only found in tissues such as brain, skeletal muscle, and heart. Similarly, SERCAs were found to be encoded by 3 genes (SERCA1 to SERCA3),\textsuperscript{3–5} and an alternative splice site at the 3' end of each gene gives rise to 2 or 3 isoforms for SERCA1, SERCA2, and SERCA3, respectively. SERCA1 and SERCA2 are the more specialized genes expressed in skeletal and heart muscles, while the less known SERCA3 is the nonmuscle cell counterpart. This Ca\textsuperscript{2+} ATPase multiplicity raises the question of their functional specificities. They may play major roles through...
regulation of their expression in pathological situations associated with abnormal cytosolic Ca\textsuperscript{2+} concentrations. Muscle Ca\textsuperscript{2+} ATPase expression has therefore been particularly explored in cardiovascular diseases, such as the heart SERCA2a isoform in cardiac hypertrophy or heart failure and vascular smooth muscle cell SERCA2a and SERCA2b isoforms in hypertension.\textsuperscript{6} More recently, regulation of the expression of PMCA\textsubscript{s} was also described in vascular smooth muscle cells (SMCs) of hypertensive animals.\textsuperscript{7}

In contrast, little is known about nonmuscle cells such as platelets. Early studies revealed abnormalities in both their cytosolic Ca\textsuperscript{2+} concentration\textsuperscript{8–11} and Ca\textsuperscript{2+}-ATPase activities\textsuperscript{12–17} in hypertension, but understanding of the relevance of these abnormalities to the expression of defined entities depended on their identification, which was long a matter of conjecture. Recent progress\textsuperscript{18} has shown that platelets coexpress a number of Ca\textsuperscript{2+} ATPases, including both SERCA\textsubscript{a} and PMCA\textsubscript{s}, all of them presenting particularities either in their original and concerted expression or in their protein forms. Soon after the identification of the ubiquitous SERCA2b isoform,\textsuperscript{19} a second SERCA was detected and shown to correspond to the first and unique SERCA3 gene product described, now called SERCA3a.\textsuperscript{20–23} Further exploration of human platelets revealed the expression of a third distinct 97-kDa SERCA,\textsuperscript{24} as well as a new SERCA3b gene product,\textsuperscript{25} while this gene yields 3 SERCA3a-3c species.\textsuperscript{5,26,27}

In addition, protein studies revealed the expression of the ubiquitous PMCA1 and PMCA4, which are reported to correspond to the PMCA1b and PMCA4b isoforms, but in an abnormal smaller size for the PMCA1b isoform, for which possible proteolytic cleavage was considered a general feature of the megakaryocytic lineage.\textsuperscript{28,29}

This prompted us to investigate expression of these newly identified Ca\textsuperscript{2+} ATPases in the still unexplored normotensive Wistar-Kyoto rat (WKY) platelets to obtain a complete overview of their expression and regulation in the spontaneously hypertensive rat (SHR). This genetic model of hypertension was also described.\textsuperscript{22,25}

The results show that human and rat platelets coexpress at least 6 and 5 Ca\textsuperscript{2+} ATPases isoforms, respectively, including similar SERCA2b, SERCA3a, PMCA1b, and PMCA4b isoforms but distinct SERCA3b/3c gene products. The 2 latter human isoforms are absent in rats, which express a new fourth SERCA3 spliced variant that reveals a previously unknown alternative splicing process in the SERCA3 gene. Striking differences in the expression of PMCA1b, PMCA4b, SERCA3a, and the rSERCA3b/3c forms were found in rat hypertension, suggesting a profound and general redistribution of the major proteins involved in Ca\textsuperscript{2+} signaling in pathological situations.

### Methods

#### Cell Culture

HeLa (epithelial) cells were from the American Type Culture Collection. Cells were grown in Eagle's minimal essential medium (Life Technologies) with nonessential amino acids and Earle's balanced salt solution at 37°C with 5% CO\textsubscript{2} in a humidified atmosphere.

#### Isolation of Human and Rat Platelets

Human blood was obtained from healthy adult volunteers,\textsuperscript{29} and the investigation was performed according to the requirements of the Declaration of Helsinki. Sixteen-week-old Okamoto SHR and normotensive WKY were supplied by the Centre d'élevage R. Janvier (Le Genest, France). Approximately 45 SHR and WKY were used. Animals were anesthetized with ether. Rat blood was diluted in 0.9% NaCl, and the platelet-rich plasma was withdrawn until a depth of 1 cm remained over theuffy coat. This avoided lymphocyte contamination. Isolation of human and rat platelets was performed as described.\textsuperscript{22,25}

#### Isolation of Total RNA

Total RNA was isolated from platelets, HeLa cells, and rat tissues with the RNA-Plus solution according to the manufacturer's instructions (Quantum Bioprobe). Mouse lung total RNA was from Clontech.

#### Reverse Transcription–Polymerase Chain Reaction

We used 250 to 1000 ng of total RNA as template for first strand–specific cDNA synthesis, as previously described.\textsuperscript{30} After heat inactivation of reverse transcriptase for 10 minutes at 95°C, RT reaction was used as template for PCR in a 50-μL total reaction mixture including PCR buffer, 2 mmol/L MgCl\textsubscript{2} (or 1.3 mmol/L for rSERCA3b/3c), 0.15 μmol/L of the forward and reverse primers (Eurogentec) (Tables 1 and 2), and 1.25 U of AmpliTaq Gold polymerase (Thermus aquaticus) (Perkin Elmer) and conducted in an automated thermocycler for different numbers of cycles, according to the PCR products studied. For human and rat PMCA amplification, PCR was performed for 40 cycles. One cycle consisted of 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C. This was followed by a final extension step at 72°C for 7 minutes. For SERCA3 species, touchdown PCR (TD-PCR) was performed for 10 cycles with annealing temperature decrease from 65°C to 56°C. PCR was then conducted for different numbers of cycles. This was followed by a final extension step, as described above. For internal controls, SERCA2b and glycerolaldehyde 3-phosphate dehydrogenase (G3PDH), PCR was performed either with or without touchdown cycles. PCR products were visualized by means of 1.5% (wt/vol) ethidium bromide–stained agarose gels and analyzed by Southern blotting and subcloning followed by sequencing (Eurogentec).

#### Rapid Amplification of cDNA Ends

To obtain the missing 3' end, we performed rapid amplification of cDNA ends (RACE)-PCR using a rat brain Marathon ready cDNA library (Clontech) and the ready amplification kit according to the manufacturer's instructions. The rat primers f3b/c\textsuperscript{a} (nucleotide [nt] 2971) and f3b/c\textsuperscript{a} (nt 2985) were used for the 2 rounds of PCR, respectively. DNA polymerase mix and TaqStart antibody were used in both rounds of PCR reactions. The 450-bp RACE product was identified by subsequent cloning and sequencing.

#### Rat Genomic DNA Amplification

We used 300 ng of genomic DNA as template for slightly modified TD-PCR. In this case, 2.5 U of a DNA polymerase mix (TaqPlus Precision PCR System kit, Stratagene, Ltd) was used, and TD-PCR was performed with annealing temperature decrease from 70°C to 58°C. PCR was then conducted for 40 cycles. One cycle consisted of 1 minute at 95°C, 1 minute at 60°C, and 3 minutes at 72°C. The primers used to amplify the intronic regions linking exons 20 to 21
and exons 21 to 22 of the rat SERCA3 gene were f3a/b/c (nt 2941)–r3b/c (nt 2981) and f3b/c (nt 2981)–r3a (nt 3149), respectively.

Southern Blotting
RT-PCR products were analyzed by Southern blotting, as previously described.25 The probes were labeled with the use of the enhanced chemiluminescence (ECL) 9-oligolabeling and detection system kit (Amersham). RNAs were quantified by densitometric measurements of the bands with an LKB Ultroscan XL laser densitometer. For each experiment, the control values (normotensive WKY) were arbitrarily taken as 100%. In SHR platelets, the expression of Ca2+ATPases was given as percentages of the control value (mean±SD of at least 3 different experiments).

Western Blotting
HeLa cell and platelet lysates were isolated as previously described. 29 Protein concentrations were estimated; samples were submitted to 7.5% SDS-PAGE and electrotransferred on nitrocellulose membranes. The membranes were then incubated with a 1:5000 dilution of the anti-platelet glycoprotein IIIa antibody (anti-b3 antibody) or a 1:1000 dilution of the polyclonal anti-SERCA2b and monoclonal anti-PMCA 5F10 (Affinity BioReagents) antibodies. Thereafter, the membranes were incubated with a 1:10 000 dilution

### TABLE 1. Primer Sequences for the Various Human Ca2+ ATPases

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer</th>
<th>5’ Position†</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERCA2b</td>
<td>f2b</td>
<td>2861</td>
<td>5’-TCATCTTCAGCTCACTACCGT-3’</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>r2b</td>
<td>3110</td>
<td>5’-CAGAGCAGAGACATATCGC-3’</td>
<td>22</td>
</tr>
<tr>
<td>SERCA3a</td>
<td>f3a/b/c</td>
<td>2674</td>
<td>5’-GGGTCAATTCTGGAGATGTGCT-3’</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>r3a</td>
<td>2967</td>
<td>5’-CCTTCCAGGAGCTGCTCG-3’</td>
<td>5</td>
</tr>
<tr>
<td>SERCA3b</td>
<td>r3b/c</td>
<td>3000</td>
<td>5’-GGCTCTCACTCGGAAAG-3’</td>
<td>5, 25</td>
</tr>
<tr>
<td></td>
<td>f3b/c</td>
<td>3000</td>
<td>5’-CCCTTCAGGAGCTGCTCG-3’</td>
<td>5, 25</td>
</tr>
<tr>
<td></td>
<td>r3a/b/c</td>
<td>3113</td>
<td>5’-GGCTGCTTATTTAGCGGCC-3’</td>
<td>5</td>
</tr>
<tr>
<td>SERCA3c</td>
<td>r3c</td>
<td>3069</td>
<td>5’-GGGTCAATTCTGGAGATGTGCT-3’</td>
<td>5</td>
</tr>
<tr>
<td>PMCA1</td>
<td>f1</td>
<td>3423</td>
<td>5’-TCATCTTCAGCTCACTACCGT-3’</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>r1</td>
<td>3794</td>
<td>5’-GGGTCAATTCTGGAGATGTGCT-3’</td>
<td>31</td>
</tr>
<tr>
<td>PMCA4</td>
<td>f4</td>
<td>3662</td>
<td>5’-GGGTCAATTCTGGAGATGTGCT-3’</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>r4</td>
<td>3983</td>
<td>5’-GGGTCAATTCTGGAGATGTGCT-3’</td>
<td>31</td>
</tr>
</tbody>
</table>

f indicates forward primer; r, reverse primer.
†5’ position of the primer sequence on the cDNA.

### TABLE 2. Primer Sequences for the Various Rat Ca2+ ATPases

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer</th>
<th>5’ Position†</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERCA2b</td>
<td>f2b</td>
<td>2861</td>
<td>As in Table 1</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>r2b</td>
<td>3110</td>
<td>As in Table 1</td>
<td>22</td>
</tr>
<tr>
<td>SERCA3a</td>
<td>f3a/b/c</td>
<td>2674</td>
<td>As in Table 1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>r3a</td>
<td>2967</td>
<td>5’-GGGTCAATTCTGGAGATGTGCT-3’</td>
<td>36</td>
</tr>
<tr>
<td>SERCA3b</td>
<td>f3a/b/c</td>
<td>2605</td>
<td>5’-CAGAGCAGAGACATATCGC-3’</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>r3b/c*</td>
<td>2985</td>
<td>5’-CCCTTCAGGAGCTGCTCG-3’</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>r3a</td>
<td>3149</td>
<td>5’-CAGAGCAGAGACATATCGC-3’</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>f3b/c*</td>
<td>2971</td>
<td>5’-CAGAGCAGAGACATATCGC-3’</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>f3a/b/c</td>
<td>2941</td>
<td>5’-CAGAGCAGAGACATATCGC-3’</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>r3b/c*</td>
<td>3241</td>
<td>5’-CAGAGCAGAGACATATCGC-3’</td>
<td>36</td>
</tr>
<tr>
<td>SERCA3c</td>
<td>f3a/b/c</td>
<td>2674</td>
<td>As in Table 1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>r3c</td>
<td>3050</td>
<td>5’-GGGTCAATTCTGGAGATGTGCT-3’</td>
<td>5</td>
</tr>
<tr>
<td>PMCA1</td>
<td>f1</td>
<td>3040</td>
<td>5’-TAGGCAGTCTGGAGATGTGCT-3’</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>r1</td>
<td>3870</td>
<td>5’-CCCTTCAGGAGCTGCTCG-3’</td>
<td>32</td>
</tr>
<tr>
<td>PMCA4</td>
<td>f4</td>
<td>3409</td>
<td>5’-ATGCCAGATGAGCTGCTCG-3’</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>r4</td>
<td>3825</td>
<td>5’-ATGCCAGATGAGCTGCTCG-3’</td>
<td>32</td>
</tr>
<tr>
<td>G3PDH</td>
<td>fG3</td>
<td>214</td>
<td>5’-ATGCGCAGATGAGCTGCTCG-3’</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>rG3</td>
<td>961</td>
<td>5’-ATGCGCAGATGAGCTGCTCG-3’</td>
<td>33</td>
</tr>
</tbody>
</table>

f indicates forward primer; r, reverse primer.
*Mouse primers.
†5’ position of the primer sequence on the cDNA.
of the horseradish peroxidase–conjugated anti-rabbit IgG (Jackson ImmunoResearch) for immunostaining of β, as well as SERCA2b and a 1:2000 dilution of the horseradish peroxidase–conjugated anti-mouse IgG (Jackson ImmunoResearch) for immunostaining of PMCs. After they were washed, antibody binding was detected with the use of ECL Western blotting reagents according to the instructions of the manufacturer. Chemiluminescent bands were quantified as described above.

Results
SERCA3 Alternative Spliced Transcripts in Human and Normotensive WKY Platelets: Evidence for a New Rat SERCA3b/3c Alternative Transcript
The human and mouse SERCA3 genes were recently found to give three 3'-end alternative spliced transcripts termed SERCA3a to SERCA3c (Figure 1A, I), as a result of exclusion or partial or complete insertion of a new penultimate exon 21.5,25,26 If we found SERCA3a in human and rat platelets and SERCA3b in human cells,25 rat SERCA3b and both human and rat SERCA3c were lacking. We first compared human and rat platelets to identify (1) SERCA3 gene products and (2) any differences between the 2 species as reported for SERCA3b in human and mouse. For this, because of the apparent low expression of the SERCA3b and SERCA3c RNAs, we used TD-PCR followed by sequencing of the PCR products. Specific sets of human and rat SERCA3a, SERCA3b, and SERCA3c primers were designed on the basis of the human SERCA3 gene or on a postulated high homology of unknown rat SERCA3 gene with that of mouse.5,26

Figure 1A, II shows SERCA3 mRNAs in human and WKY platelets. Control amplifications in the absence of RT (lanes 1) did not result in PCR products, in agreement with the absence of DNA in RNA preparations. SERCA2b RNAs (lanes 2) were used as internal controls.22,25 In human platelets, lanes 3 to 6 show the expected 2 SERCA3a (lane 3), SERCA3b (lanes 4 and 5), and SERCA3c mRNAs (lane 6), thus adding SERCA3c in these cells. Subsequent sequencing (data not shown) established that the 3 human products presented 100% homology with human SERCA3a, SERCA3b, and SERCA3c RNAs (Figure 1A, III).5 In rat platelets, similar SERCA3a mRNA (lane 3) was confirmed,22,25 but striking differences were found for SERCA3b (lanes 4 and 5) and SERCA3c (lane 6). Similar upstream SERCA3b/3c RNAs (lane 4) were present when we used rat forward primers located in exon 18 with mouse reverse primers located in the 5' end of the putative rat exon 21. Sequencing of these products (III) showed 100% homology with the mouse sequence, ie, excluding the ACLYP peptide sequence found in human SERCA3b/3c mRNAs. In contrast, the use of inverse complement of mouse primers used in lane 4 as forward primers with rat reverse primers located in coding (not shown) or untranslated regions of exon 22 used by SERCA3a allowed neither SERCA3b (lane 5) nor SERCA3c RNA amplification (lane 6). This suggested that while SERCA3b/3c mRNA was expressed in rat platelets, it did not seem to have the same 3' end as humans and mice, thus pointing to a new species-specific SERCA3 mRNA. On the basis of PMCA nomenclature, we temporarily termed it the rSERCA3b/3c variant.
Reexamination of available SERCA3 mRNAs and gene sequences revealed the absence of SERCA3b stop codon at position nt 62 of rat exon 22 compared with mice and humans. This could explain the absence of SERCA3b/3c in rats. To verify this, we studied other rat cells (Figure 1B, II) for rSERCA3b/3c mRNAs. The left part of the figure shows that the mouse primers detected mouse lung SERCA3a (lane 1) and SERCA3b mRNAs, irrespective of the primers used for upstream and downstream SERCA3b/3c RNA (lanes 2 and 3), as described.26 The right part of the figure shows that rat tissues (lung, liver, and brain) coexpressed the same rSERCA3b/3c species as platelets (lanes 2 and 3) with SERCA3a (lane 1). The first part of exon 21 was present (lane 2), while no amplifications could be obtained with primers located downstream in exon 21 (not shown) or in exon 22 (lane 3). Alternatively, significant differences appeared as a function of the tissues in terms of their rSERCA3b/3c expression levels, the highest being found in brain. These results strengthened the data of Figure 1A and argued for a distinct 3'-end rSERCA3b/3c species, thus pointing to a new SERCA3 messenger.

Identification of rSERCA3b/3c mRNA
To isolate this SERCA3 RNA, we selected a rat brain Marathon ready cDNA library because of the highest expression of rSERCA3b/3c species in this tissue (Figure 1B) and used a 3'-end RACE-PCR approach. PCR products of ~0.5 kb were obtained by using exons 20 and 21 overlapping forward primers for the first round of RACE-PCR and f3b/c (nt 2985) primers for the second round of nested PCR. Sequencing of this RACE product is shown in Figure 2A. It revealed that the 3'-end SERCA3 mRNA isolated was 357 nt long and 1004 nt shorter than that of SERCA3a mRNA. It contained the complete 3'-end SERCA3b/3c region and part of the poly(A') tract. Comparison of its sequence with mouse exon 21 showed that it expressed the same first 26 nt, then largely diverged downstream in the sequence, with the substitution of 7 nucleotides (thus explaining the apparent absence of entire exon 21 using mouse primers) and the deletion of 7 others and was followed by an additional unknown sequence. This novel sequence contained a potential consensus polyadenylation signal AATAAA beginning at nt 64 upstream of the poly(A') tract. Moreover, the predicted amino acid residues from the longest reading frame of this mRNA species located a stop codon at position nt 204, suggesting that this mRNA species contained a 3183-nt coding region and a short 151-nt untranslated region. These findings showed that this SERCA3 mRNA results from an unknown alternative splicing process, using a second alternative polyadenylation site in the last intron(s), a new exon located downstream of exon 21 in the SERCA3 gene, or both.

To obtain the genetic basis for rSERCA3b/3c mRNA, we reconstructed the 3' end of the rat SERCA3 gene by performing rat genomic DNA amplifications by PCR using rat-specific sets of primers covering exons 20 to 22 (data not shown) and analyzed the PCR products by sequencing (Figure 2B). In the region covering exons 20 to 21, a 0.39-kb
Figure 1. Evidence for similar but distinct human and rat platelet SERCA3 mRNAs. A, SERCA3 expression in human and normotensive WKY platelets. I, Schematic representation of the 3'-end human and mouse SERCA3 gene products. Boxes represent the exons, and lines represent introns. Broken lines show SERCA3a, SERCA3b, and SERCA3c mRNAs. The black box represents the human ACLYP sequence absent in mouse. Sc, Sa, Sb, and pA locate stop codons and polyadenylation signal, respectively. Arrows under exon 21 refer to upstream and downstream SERCA3b/3c species. II, Human and WKY platelet SERCA3 mRNAs; 250 ng of total RNA or 1000 ng (rSERCA3b/3c) was submitted to RT-PCR for 26, 25, or 35 cycles to amplify SERCA2b, SERCA3a, and human (h) and rat (r) SERCA3b/3c mRNAs, respectively, using human (Table 1) and mouse or rat (Table 2) primers. PCR products were analyzed on agarose gels. Lanes 1, RNA controls (f2b–r2b); lanes 2, SERCA2b (f2b–r2b); lanes 3, SERCA3a (f3a/b/c nt 2674–r3a nt 2967); lanes 4, upstream SERCA3b/3c (f3a/b/c nt 2674–r3b/c nt 3000 and f3a/b/c nt 2605–r3b/c* nt 2985); lanes 5, downstream SERCA3b/3c (f3b/c nt 3000–r3a/b/c nt 3113 and f3b/c* nt 2985–r3a nt 3149); lanes 6, SERCA3c (f3a/b/c nt 2674–r3c nt 3069 and f3a/b/c nt 2674–r3c nt 3050). III, Human (top) and rat (bottom) SERCA3a-3c amino acid similarity comparison. Identical amino acids are connected by vertical lines. B, Species specificity of rSERCA3b/3c mRNA. I, Schematic representation of putative rat SERCA3 gene. The dotted box represents unidentified exon 21. Broken lines show SERCA3a and putative SERCA3b/3c mRNAs. II, Mouse and rat SERCA3 mRNAs; 250 ng (SERCA3a) or 1000 ng (SERCA3b/3c) of total RNA isolated from mouse (m) lung and the WKY (r) tissues indicated was submitted to RT-PCR to amplify SERCA3 RNAs, as in panel A. PCR products were analyzed as above. Lanes 1, SERCA3a; lanes 2 and 3, upstream and downstream SERCA3b/3c. Numbers indicate the sizes of the PCR products in base pairs. The figure is typical of at least 3 independent experiments.
Figure 2. Identification of rSERCA3b/3c as a new SERCA3 mRNA. A, Isolation of rSERCA3b/3c. Coding and noncoding sequences are shown in uppercase and lowercase letters, respectively. The bold letters denote the stop codon used in rSERCA3b/3c. The polyadenylation signal is underlined. B, Sequence and organization of the rat 3'-end SERCA3 gene. Exons and introns are shown in uppercase and lowercase letters, respectively. Nucleotides are numbered according to Dode et al.5 The sizes of the penultimate and ultimate introns are indicated. The bold letters and stars denote the stop codons used in rSERCA3b/3c and SERCA3a. The polyadenylation signals are underlined. C, Expression of rSERCA3b/3c mRNAs. I, Schematic representation of the rat SERCA3 gene and of its SERCA3 alternative splicing processes. The black part of exon 21 represents its extension by the 3'-end part of the last intron. Broken lines show rSERCA3a and rSERCA3b/3c mRNAs. Sa, Sb/c, and pA locate stop codons and polyadenylation signals, respectively. II, Comparative expression of rSERCA3b/3c in WKY brain and platelets; 1000 ng of total RNA was submitted to RT-PCR for 35 cycles, and PCR products were analyzed as in Figure 1. Lanes 1, exons 18 to 21 (f3a/b/c nt 2605–r3b/c nt 2981); lanes 2, exons 20 to 21 (f3a/b/c nt 2941–r3b/c nt 3241); lanes 3, exons 21 to 21 (f3b/c nt 2981–r3b/c nt 3241); lanes 4, exons 21 to 22 (f3b/c nt 2981–r3a nt 3149). Numbers indicate the sizes of the PCR products in base pairs. The figure is typical of at least 3 independent experiments.
intronic sequence was identified that presented 82% identity with the mouse sequence. The rat 3' acceptor splice site of SERCA3b/3c was found 15 nt downstream of the one used in humans, thus establishing the absence of ACLYP peptide sequence in this species (Figure 1A). A similar study of the region covering exon 21 to 22 revealed a second 3-kb intronic sequence. Comparative sequence analysis of its 5'-end region with rSERCA3b/3c RNA showed 100% homology with the additional unknown part of the RACE product sequence. The polyadenylation signal was recovered at position nt 208 of this intronic sequence. This demonstrated that the new exonic sequence expressed in rSERCA3b/3c corresponds to a first part of the 5'-end sequence of the last intron, thus extending exon 21 by 278 nt, and is due to the use of a second polyadenylation site in this last intron of the SERCA3 gene.

To prove that this 3'-end SERCA3 mRNA was relevant to rSERCA3b/3c (Figure 2C), we performed comparative RT-PCR amplifications of rat brain (bottom left) and platelet (bottom right) SERCA3 products covering the last 3' exons 18 to 22. Lanes 1 and 2, see Figure 1A; lanes 3, PMCA1b (f1-r1); lanes 4, PMCA4b (f4-r4). III, Human and rat PMCA1b and PMCA4b amino acid similarity comparison. B, PMCA proteins; 20 μg of HeLa cells and 50 μg of human and rat platelet lysates were treated for Western blotting (see Methods). The top part of the figure shows the results obtained using the antibodies indicated in the lower table. Lanes 1, 3, and 5, SERCA2b; lanes 2, 4, and 6, PMCA1b and PMCA4b. The numbers indicate the molecular masses of the Ca\textsuperscript{2+} ATPases. The figure is typical of at least 3 independent experiments.
rSERCA3b/3c RNA amplification. Lanes 3 showed total expression of rat RACE product through its amplification by using downstream exon 21–located primers. Lanes 4 established the absence of PCR products using forward exon 21–located primers with reverse exon 22–located primers. These rSERCA3b/3c species were similarly expressed in rat brain and platelets, albeit at lower levels in platelets. These findings provide evidence for distinct human and rat
SERCA3b/3c gene products, resulting from species-specific alternative splicing mechanisms, a still undescribed SERCA3 characteristic.

Evidence for PMCA1b and PMCA4b Species in Human and Rat Platelets

Recent data suggested the unique presence of the PMCA1b and PMCA4b proteins, although at a low level for PMCA4b and at an apparent lower molecular weight for the PMCA1b protein in human platelets.28,29 To address these questions, we looked for unknown human and rat platelet PMCA1b and PMCA4b mRNAs and searched for PMCA proteins in rat platelets by again seeking an analogy with human cells (Figure 3). We used a typical RT-PCR approach and human and rat PMCA1 and PMCA4 gene specific primers to amplify the overall PMCA1 and PMCA4 alternatively spliced transcripts at site C (Figure 3A, I). Preliminary studies of megakaryocytic cell lines showed PMCA1b and PMCA4b mRNAs but no trace of PMCA2 and PMCA3 species (not shown). As controls, HeLa cell RNA was used, on the basis of previous studies showing that SERCA2b,25 as well as hPMCA1b and hPMCA4b, was expressed in these cells.29,30 Part II shows the comparative results obtained in HeLa cells and human and rat platelets. Lanes 1 and 2 show controls (see Figure 1A). Lanes 3 and 4 show PMCA1 and PMCA4 PCR products, which appeared as single bands, in agreement with the unique and typical expression of PMCA1b and PMCA4b mRNA species, respectively. PMCA1b mRNA appeared to be more highly expressed than PMCA4b mRNA in both platelet species. Sequencing of these PCR products (not shown) confirmed these identifications since they were found to present a 100% homology with the human31 and rat32 PMCA sequences (III). Hence, while the presence of PMCA transcripts at site C (Figure 3, I) shows the same analysis of the PCR products as in Figure 1, while part II shows their Southern blotting using specific oligonucleotide probes. Expression of G3PDH and SERCA2b mRNA in WKY and SHR platelets is shown in lanes 1, 3 and 2, 4, respectively. Lanes 5 to 8 correspond to SERCA3a (lanes 5 and 6) and rSERCA3b/3c (lanes 7 and 8) in WKY (lanes 5 and 7) and SHR platelets (lanes 6 and 8). Results revealed an increased expression of SERCA3a species, while that of rSERCA3b/3c became undetectable, as shown in lane 8 and further verified by testing different RT-PCR conditions (not shown). Comparative quantification of SERCA3a mRNA demonstrated that it increased by a factor of 1.71±0.16 in SHR platelets compared with that of the control WKY platelets.

The relative expression of PMCA1b and PMCA4b mRNAs was studied by the same approach (Figure 4B). Control mRNAs are shown in lanes 1, 3 and 2, 4, as in Figure 4A. Lanes 5 to 8 compare the expression of the 2 PMCA1b (lanes 5 and 6) and PMCA4b transcripts (lanes 7 and 8) in WKY (lanes 5 and 7) and SHR platelets (lanes 6 and 8). There was a net decrease in PMCA1b mRNA expression, while that of PMCA4b increased. Comparative quantitation demonstrated that PMCA4b mRNA increased 3.7-fold (±0.2), whereas that of PMCA1b decreased 3.3-fold (±0.1) in SHR platelets compared with that of the control WKY platelets.

The expression of PMCA proteins was studied by testing the immunoreactivity of 5F10 antibody in platelet lysates (Figure 4C). SERCA2b protein (lane 1) and PMCA1b and PMCA4b (lane 2) expressed by HeLa cells were again used as molecular weight controls. Platelet β3 integrin (lanes 3 and 4) and SERCA2b (lanes 5 and 6) were used to verify that protein loads were similar. Comparative Western blotting of both PMCA isoforms in WKY (lane 7) and SHR platelets (lane 8) revealed no significant difference between the net expression of the 2 isoforms. Further comparative quantification of PMCA proteins established that the total expression of PMCA1b and PMCA4b did not differ significantly between SHR platelets and control WKY platelets (data not shown).

Hypertension was therefore found to be associated with striking differences in the expression of the 3 newly identified platelet Ca2+ ATPases, in agreement with their functional roles in this pathology.

Expression of Rat Platelet Ca2+ ATPases in Hypertension

We isolated platelet RNA and lysates from different pooled blood samples from 4 to 8 WKY and SHR and investigated these newly identified Ca2+ ATPases. As internal controls we used SERCA2b, found to be unchanged in hypertension,22 as well as the constitutively expressed G3PDH.33

The relative expression of SERCA3a and rSERCA3b/3c mRNAs was studied by using comparative RT-PCR. Figure 4A, I shows the same analysis of the PCR products as in Figure 1, while part II shows their Southern blottings using specific oligonucleotide probes. Expression of G3PDH and SERCA2b mRNA in WKY and SHR platelets is shown in lanes 1, 3 and 2, 4, respectively. Lanes 5 to 8 correspond to SERCA3a (lanes 5 and 6) and rSERCA3b/3c (lanes 7 and 8) in WKY (lanes 5 and 7) and SHR platelets (lanes 6 and 8). Results revealed an increased expression of SERCA3a species, while that of rSERCA3b/3c became undetectable, as shown in lane 8 and further verified by testing different RT-PCR conditions (not shown). Comparative quantification of SERCA3a mRNA demonstrated that it increased by a factor of 1.71±0.16 in SHR platelets compared with that of the control WKY platelets.

The relative expression of PMCA1b and PMCA4b mRNAs was studied by the same approach (Figure 4B). Control mRNAs are shown in lanes 1, 3 and 2, 4, as in Figure 4A. Lanes 5 to 8 compare the expression of the 2 PMCA1b (lanes 5 and 6) and PMCA4b transcripts (lanes 7 and 8) in WKY (lanes 5 and 7) and SHR platelets (lanes 6 and 8). There was a net decrease in PMCA1b mRNA expression, while that of PMCA4b increased. Comparative quantitation demonstrated that PMCA4b mRNA increased 3.7-fold (±0.2), whereas that of PMCA1b decreased 3.3-fold (±0.1) in SHR platelets compared with that of the control WKY platelets.

Discussion

This work comes at a time when understanding of Ca2+ signaling calls for basic knowledge of the molecular structure and distribution of the Ca2+ channels and Ca2+ ATPases that govern Ca2+ movements.34 A first essential feature of this work, in which platelets are considered as a possible paradigm of nonmuscle cells, is that it may shed some light on the
coexpression of multiple, diverse, species-specific, and modulable Ca\(^{2+}\) ATPases. The complexity of platelet Ca\(^{2+}\) ATPases has begun to be understood only in the last decade, through well-documented evidence for human and rat 100-kDa SERCA2b and 97-kDa SERCA3a isoforms.\(^9\) An additional, distinct 97-kDa SERCA protein was detected through its recognition by an antibody termed PLIM430 and/or particular tryptic fragmentation in both human and rat platelets.\(^{24}\) Its SERCA3 nature was suggested,\(^{25-27,33}\) but evidence for increasing numbers of SERCA3 isoforms renders reassessment necessary. There is evidence for SERCA3b mRNA\(^{25}\) and for PMCA1b and PMCA4b in human platelets,\(^{28,29}\) although the molecular weight of PMCA1b is undefined or abnormal.\(^{29}\) Here, we show the additional expression of at least 2 distinct SERCA3 mRNAs, namely, SERCA3c and a still undescribed rSERCA3b/3c species in human and rat platelets, respectively, and demonstrate the expression of normal PMCA1b and PMCA4b mRNAs in both cell species.

For human platelet SERCAs, we clearly demonstrated the additional expression of SERCA3c mRNA. Striking differences were found in rat platelets since SERCA3b and SERCA3c species were lacking, while they expressed a new rSERCA3b/3c alternative spliced variant. This finding was initiated by the observation that while SERCA3b species seemed to be expressed when the 5' end of exon 21 was explored, this study did not allow detection of the last SERCA3 exon 22 present in the 3 human and mouse SERCA3a-3c mRNAs. We could argue that this unmasked a novel SERCA3 alternative splicing process, which uses a second polyadenylation site located in the last intron to give rise to expression of an extended exon 21.

Intriguingly, in regard to other Ca\(^{2+}\) ATPase genes, only 1 rat cDNA SERCA3a had been described\(^{36}\) until recent, independent reports of 3 distinct 3'-end mouse and human SERCA3 gene products.\(^{5,25-27}\) Therefore, after inclusion in the SERCA family of the SERCA3 gene, the present study adds a fourth one as a result of an additional alternative splicing mechanism. Hence, the SERCA3 gene may also share features with the other SERCA1 and SERCA2 genes, as well as with PMCA genes such as PMCA3, since such mRNA species, which are distinct because of the use of a second alternative polyadenylation site, were also described in this family.\(^{37}\) Alternatively, the present study largely emphasizes the species specificity of the expression of its different gene products as a feature of the nonmuscle SERCA3 gene, compared with SERCA1 and SERCA2 genes.\(^{5,26}\) This includes the ACLYP peptide sequence divergences between human and mouse SERCA3b/3c, confirmed here together with the demonstration of additional differences in SERCA3 alternatively spliced products, regarding rSERCA3b/3c, between human and mouse on the one hand and rat on the other hand. Taken together, this highlights an apparent higher complexity of the nonmuscle cell SERCA3 gene. A tentative explanation is a possible diversity in Ca\(^{2+}\) requirements of various nonmuscle cells to ensure their functional specificities.

For PMCA, RNA and protein studies showed that PMCA1b and PMCA4b isoforms are expressed in human and rat platelets. Typical PMCA1b and PMCA4b RNAs are present in both platelet species. In agreement with previous findings, the PMCA in the 2 cases have a similar molecular weight, ie, that of the PMCA4b protein,\(^{29}\) thus supporting the hypothesis of some in situ proteolysis occurring at the posttranscriptional level.

Hence, human and rat platelets appear to have Ca\(^{2+}\) ATPase equipment comprising multiple isoforms, including ubiquitous PMCA1b and PMCA4b as well as SERCA2b and SERCA3a isoforms, together with distinct new species-dependent SERCA3 nonmuscle spliced products.

To obtain information on the biological significance of this multiplicity of Ca\(^{2+}\) ATPase species, we focused on rat platelets by looking for the regulation of expression of the newly identified species in a pathological model of hypertension. We selected SHR compared with age-matched normotensive WKY because partial data were obtained showing similar SERCA2b and increased total SERCA3 expression.\(^{22}\) The present extensive study reveals a more profound reorganization of platelet Ca\(^{2+}\) ATPases, since it concerns almost all of the SERCA and PMCA species. Indeed, opposing upregulations and downregulations were apparent for the mRNAs of SERCA3a and PMCA4b and of rSERCA3b/3c and PMCA1b, respectively.

Hence, although the rationale for major or specific expression of Ca\(^{2+}\) ATPase isoforms remains to be understood, these results suggest that multiple members can play a role in pathological situations such as hypertension. SERCA transfection has been shown to modify Ca\(^{2+}\) oscillation,\(^1\) which in turn modifies enzyme activity.\(^{28}\) Therefore, the different expression levels of the SERCA3 isoforms in hypertension may mean a perturbation of local cytosolic Ca\(^{2+}\) concentrations and of Ca\(^{2+}\)-dependent enzyme activities in hypertensive platelets. Interestingly, knockout of the SERCA3 gene resulted in abnormal endothelium-dependent relaxation of SMC by decreasing endothelial nitric oxide synthase,\(^{39}\) a gene also found to be positively associated with essential hypertension.\(^{40}\) Since rSERCA3b/3c decreased in hypertensive platelets, one can postulate that Ca\(^{2+}\) controlled by rSERCA3b/3c may be involved in the local Ca\(^{2+}\) concentration required for nitric oxide synthase in this pathology. PMCA regulation may also play a significant role in hypertension, albeit to a lesser degree. It is well known that platelet activation, which is observed in hypertension, is associated with a cascade of intracellular signaling, including protein phosphorylations, such as that by pp60\(^{src}\). Moreover, a recent study showed that PMCA4b can be inhibited by pp60\(^{src}\)-induced phosphorylation,\(^{28}\) suggesting a lower PMCA4b activity in hypertension. Hence, the increase in PMCA4b expression in hypertension may be a compensatory mechanism to limit a potential decrease in PMCA4b activity.

The present work clarifies the role of Ca\(^{2+}\) ATPases in the abnormalities in platelet Ca\(^{2+}\) concentration observed in many cardiovascular pathologies, such as human and rat hypertension. Early studies of platelets, which are easily accessible and constitute a potential model of SMC, indicated the same abnormalities in cytosolic Ca\(^{2+}\) concentration in human and rat hypertension. Subsequent investigation of the role of Ca\(^{2+}\) ATPases in this process showed that Ca\(^{2+}\) ATPase activities are regulated in rat SMC as well as in human and rat
platelets. However, the regulation of Ca\(^{2+}\) ATPase activities differed in human and rat platelets, raising questions about (1) the mixture of membrane preparations, (2) different types of hypertension, (3) similarities between human and rat platelet Ca\(^{2+}\) ATPase systems, and (4) the relative expression and regulation of the different PMCA and SERCA isoforms in the 2 platelet species.

In this study we first established similarities between SMC and platelets, with abnormalities in both SERCA and PMCA expressions in SHR. Although the 2 cell types differ in their expression of SERCA isoforms, both of them regulate the expression of their major related isoforms, SERCA2b in SMC and rat SERCA3a in platelets. Similarly, while an increase in PMCA1b expression was detected in SMC, PMCA4b expression in platelets also increased. Moreover, such PMCA regulation may be a general feature of this model of hypertension. Indeed, if PMCA studies of kidney distal convoluted tubule cells showed no apparent mRNA regulation, this is in contrast to total protein studies that showed a significant modulation.\(^{41}\) Concerning rat mesangial cells, also studied in this model, the apparent absence of PMCA modulation may be due to large differences between cultured and mature cells, as revealed by the absence of variation in Ca\(^{2+}\) concentration.\(^{42}\)

Second, an apparent increase in total Ca\(^{2+}\) ATPase activities in mixed rat platelet membranes can be explained by taking into account the similar expression of PMCA, the increase in major SERCA3a expression, and the decrease in minor rSERCA3b/c3c expression.\(^{21}\) Third, on the basis of similar modulations of the different isoforms in rat and human hypertension, as recently described,\(^{43}\) this study may also explain apparent differences between the 2 species by reference to upregulation and downregulation of a greater mixture of isoforms in humans. While further investigation is required to establish similarities and differences in the modulation of Ca\(^{2+}\) ATPases in rat and human hypertension, our work allows fine analysis, as well as a better understanding of their roles in this pathology, a prerequisite for their use as new potential targets for pharmacological modulation.

Acknowledgments

This work was supported by the Institut National de la Santé et de la Recherche sur le Cancer, the Association pour la Recherche sur le Cancer, and the Association pour la Recherche contre le Cancer. We thank Valérie Drouet for help in blood collection. We acknowledge Dr Jean-Philippe Rosa (U 348 INSERM) for kindly giving us the anti-\(\beta\)-polyclonal antibody. We thank Dr Véronique Briquet-Laugier (U 348 INSERM) for technical advice and Dr Tünde Kovács, National Institute of Hematology and Immunology, Budapest, Hungary, for helpful discussions. We are grateful to Prof Frank Wuytack, Katolieke Universiteit Leuven, Belgium, for generously supplying the anti-SERCA2b antibody.

References


Platelet Ca²⁺-ATPases : A Plural, Species-Specific, and Multiple Hypertension-Regulated Expression System

Virginie Martin, Raymonde Bredoux, Elisabeth Corvazier, Béla Papp and Jocelyne Enouf

Hypertension. 2000;35:91-102
doi: 10.1161/01.HYP.35.1.91

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/35/1/91

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/