# Spontaneously hypertensive rats and platelet $Ca^{2+}$-ATPases: specific up-regulation of the 97 kDa isoform

Béla PAPP,*† Elisabeth CORVAZIER,* Clarice MAGNIER,* Tünde KOVÀCS,*† Nathalie BOURDEAU,* Sylviane LÉVY-TOLÉDANO,* Raymonde BREDOUX,* Bernard LÉVY,‡ Pierre POITEVIN,‡ Anne Marie LOMPRÉ,§ Frank WUYTACK|| and Jocelyne ENOUF*¶

*U. 348 INSERM, 8, Rue Guy Patin, Hôpital Lariboisière, 75475 Paris Cedex 10, France, †National Institute of Haematology and Blood Transfusion, 1113 Budapest, Hungary, ‡U. 141 INSERM, 41 Boulevard de la Chapelle, Hôpital Lariboisière, 75475 Paris Cedex 10, France, §U. 275 INSERM, Laboratoire d'Optique Appliquée, Ecole Polytechnique-ENSTA, 91120 Palaiseau, France and ||Laboratorium voor Fysiologie, Katholieke Universiteit, Leuven, Belgium

The use of platelets instead of smooth muscle cells (SMC) to study the abnormal $Ca^{2+}$ handling found in hypertension was investigated using spontaneously hypertensive rats (SHR). We studied the regulation of platelet $Ca^{2+}$-ATPases, as we have recently demonstrated that human platelets, like SMC, contain the $Ca^{2+}$-ATPase isoform termed $SERCA_{2-b}$ (sarco-endoplasmic reticulum $Ca^{2+}$-ATPase). In mixed membranes isolated from platelets of normotensive Wistar–Kyoto (WKY) rats and SHR, total $Ca^{2+}$-ATPase activity was found to be 43% higher in SHR than in WKY rats. By the use of autophosphorylation of rat platelet $Ca^{2+}$-ATPases with [$\gamma$-$^{32}$P]ATP, followed by SDS/PAGE and Western blotting, we found that rat platelets express two distinct $Ca^{2+}$-ATPases: a 100 kDa isoform, recognized by a $SERCA_{2-b}$-specific anti-peptide antibody, and a 97 kDa isoform, specifically recognized by a polyclonal anti-SERCA antibody. Comparative analysis of platelet membrane $Ca^{2+}$-ATPases from WKY rats and SHR demonstrated that the expression of the $SERCA_{2-b}$ isoform did not change significantly ($128 \pm 22$%), whereas that of the 97 kDa isoform reached $300 \pm 35$% in SHR when compared with WKY rats. We concluded that the up-regulation of total platelet $Ca^{2+}$-ATPases in SHR is not due to the 100 kDa $SERCA_{2-b}$ isoform found in SMC, but is specific to the 97 kDa $Ca^{2+}$-ATPase isoform which is not present in SMC. Therefore platelets should be used with extreme caution as a surrogate model of vascular smooth muscle $Ca^{2+}$ homoeostasis.

## INTRODUCTION

It is now well established that essential hypertension is associated with abnormal cytosolic $Ca^{2+}$ concentrations in both platelets and smooth muscle cells (SMC) [1]. Because of their accessibility, platelets have been extensively used to replace SMC in investigations of the mechanisms involved in maintaining the cytosolic $Ca^{2+}$ concentration [2]. Among the mechanisms investigated in human platelets is $Ca^{2+}$ transport driven by $Ca^{2+}$-transport ATPases which deplete the cytosol of $Ca^{2+}$ [3,4]. However, these studies were conducted when molecular identification of the $Ca^{2+}$-ATPase system was unknown, so that the major problem of whether platelets and SMC are identical remains unsolved. Since that time, considerable progress has been made in the understanding of the $Ca^{2+}$-ATPase system in different cells, including SMC and, very recently, platelets. It is now known that $Ca^{2+}$-ATPases constitute a large family of proteins which fall into two distinct groups, termed PMCA (plasma membrane $Ca^{2+}$-ATPase) and SERCA (sarcoendoplasmic reticulum $Ca^{2+}$-ATPase). Different isoforms of PMCA and SERCA are expressed in different tissues and are the products of both distinct genes and alternative splicings. The PMCA isoforms are encoded by a multigene family [5]. For the SERCA-type $Ca^{2+}$-ATPases, three distinct genes have been identified: SERCA 1, which is expressed in fast skeletal muscle [6], SERCA 2, which gives rise to $SERCA_{2-a}$ and $SERCA_{2-b}$ isoforms [7], mainly expressed in cardiac and smooth muscle respectively, and SERCA 3, which would be expressed in non-muscle tissues [8].

In SMC, the $Ca^{2+}$-ATPase system consists of a PMCA isoform and the $SERCA_{2-a}$ and $SERCA_{2-b}$ isoforms. However, large differences exist in their relative expression: firstly, the PMCA is much less abundant than the SERCAs; secondly, the $SERCA_{2-a}$ protein constitutes 10% of the $SERCA_2$ proteins, the remainder consisting of the $SERCA_{2-b}$ protein. Consequently, the $SERCA_{2-b}$ isoform is the predominant isoform in SMC [9–11].

Although less is known about the $Ca^{2+}$-ATPase system in platelets, because of the complexity of this cell type, our most recent studies established that human platelets express two distinct SERCA-type $Ca^{2+}$-transport ATPases of molecular mass about 100 and 97 kDa [12,13]. Moreover, the 100 kDa platelet protein has definitely been identified as the $SERCA_{2-b}$ isoform [14].

Consequently, the $SERCA_{2-b}$ protein is a $Ca^{2+}$-ATPase isoform common to platelets and SMC, which argues in favour of the hypothetical similarities between the two cell types. However, as mentioned above, in addition to this $SERCA_{2-b}$ isoform, human platelets also express a second, 97 kDa, SERCA isoform which is distinct from all isoforms, as it gives rise to a 80 kDa fragment on trypsin proteolysis, forms a phosphorylated intermediate during its catalytic cycle which is enhanced by lanthanum, and (an essential difference in relation to the present investigation) is not found in SMC [13].

The above findings provided the first direct opportunity of testing the similarities between platelets and SMC in essential hypertension. We investigated here a well-established model of hypertension, i.e. spontaneously hypertensive rats (SHR), the

Abbreviations used: SMC, smooth muscle cells; WKY, Wistar–Kyoto; SHR, spontaneously hypertensive rats; $Ca^{2+}$-ATPase, $Ca^{2+}$-activated $Mg^{2+}$-dependent ATPase; PMCA, plasma membrane $Ca^{2+}$-ATPase; SERCA, sarco-endoplasmic reticulum $Ca^{2+}$-ATPase; DTT, dithiothreitol; TBS, Tris-buffered saline.

¶ To whom correspondence should be addressed.

$Ca^{2+}$-ATPases of which have not so far been studied. We therefore began by comparing the regulation of total platelet $Ca^{2+}$-ATPase activities in SHR and in control platelets from Wistar–Kyoto (WKY) rats. Then, using the highly specific technique of autophosphorylation followed by SDS/PAGE and immunoblotting, we demonstrated that the total $Ca^{2+}$-ATPase activities in rat platelets involved the same two SERCA-type $Ca^{2+}$-ATPases as in human platelets. Lastly, the expression of these two SERCA-type $Ca^{2+}$-ATPases was compared in isolated membranes from control WKY rat platelets and SHR platelets.

## EXPERIMENTAL

### Animals

Spontaneously hypertensive Okamoto rats and normotensive WKY rats were supplied by the Centre d'élevage R. Janvier, Le Genest, France. About 50 SHR and 50 WKY rats were used. Most were 16 weeks old but a few were 24 weeks old. Animals were submitted to ether anaesthesia, and blood samples were collected from the abdominal aorta using ACD-C (130 mM citric acid, 170 mM trisodium citrate, 111 mM glucose) as anticoagulant.

### Preparation of platelet membrane fractions

Blood from different samples was pooled for the isolation of either human or rat platelets, and the membrane preparations specified below were used for both platelet species. Platelet-rich plasma was obtained by centrifugation at 150 *g* for 15 min at room temperature. Platelets were pelleted by centrifugation at 3000 *g* for 15 min at room temperature, washed in Tyrode's buffer, pH 7.4, containing 130 mM NaCl, 5 mM KCl, 1 mM $NaH_2PO_4$, 24 mM $NaHCO_3$, 2 mM disodium EDTA, 10 mM glucose, 12.5 mM saccharose and 0.35% BSA, centrifuged (3000 *g*) and resuspended in a homogenizing medium containing 25 mM Hepes, pH 7, 100 mM KCl, 2 mM $MgSO_4$, 25 mM NaCl, 12 mM trisodium citrate, 10 mM glucose, 5 mM ATP, 0.35% BSA and the following protease inhibitor mixture: 5 mM benzamidine, 0.1 unit/ml aprotinin, 0.01 mM leupeptin and 1 mM dithiothreitol (DTT). After this resuspension step, all the procedures were carried out at 4 °C. Platelets were lysed by ultrasonication and the resulting lysate was centrifuged at 19000 *g* for 25 min to eliminate unlysed platelets, mitochondria and granules. The supernatant was centrifuged at 100000 *g* for 60 min and the pellet was used as a source of membrane proteins. The protein concentration of the membrane fraction was determined by the Bradford method using $\gamma$-globulins as standard. Pooled blood from four SHR and four WKY rats produced about 0.7 mg of protein.

### Measurement of $Ca^{2+}$-ATPase activity

$Ca^{2+}$-ATPase activity was measured using Malachite Green, as previously described [15]. Briefly, rat membrane proteins were incubated at 37 °C in 0.2 ml of reaction mixture containing 20 mM Hepes, pH 8, 100 mM KCl, 0.5 mM EGTA and 2 mM $MgCl_2$ with or without $CaCl_2$ (0.5 mM). The final concentration of free $Ca^{2+}$ was 1.6 $\mu$M, as calculated using a computer program kindly supplied by Dr. L. Brass (University of Pennsylvania, PA, U.S.A.). The reaction was started by adding 0.5 mM ATP (final concentration) and stopped with 2 ml of Malachite Green, used for colorimetric determination of $P_i$. The ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity was calculated by subtracting the values obtained in the presence of $Mg^{2+}$ but without $Ca^{2+}$ from those obtained in the presence of both $Mg^{2+}$ and $Ca^{2+}$.

### Detection of the platelet $Ca^{2+}$-ATPase phosphorylated intermediates

Human and rat platelet membranes were labelled with [$\gamma$-$^{32}$P]ATP by the method of Sarkadi et al. [16]. For these experiments, the membrane preparation described above was slightly modified: platelets were disrupted in lysis buffer without $MgSO_4$ and ATP. Membrane proteins were phosphorylated for 1 min at 4 °C with [$\gamma$-$^{32}$P]ATP (0.05 $\mu$M final concentration) in a buffer containing 17 mM Hepes, pH 7, 160 mM KCl, 0.05 mM $CaCl_2$ and 1 mM DTT. The reaction was stopped by adding a mixture of 10 mM phosphoric acid, 1 mM ATP and 6% trichloroacetic acid, and the preparation was kept on ice. The precipitate was washed twice with the same solution and dissolved in 150 mM Tris/HCl, pH 7.4, 2% SDS, 8 mM sodium EDTA, 20% sucrose, 0.014% Bromophenol Blue and 1% DDT. Membrane proteins were then submitted to acidic PAGE (7.5% gels) and electroblotted on to nitrocellulose membranes overnight at 4 °C in a buffer containing 25 mM Tris/HCl, pH 8.3, 192 mM glycine, 20% methanol and 0.1% SDS. Autoradiography of the dried blots was performed with Kodak X-Omat films using intensifying screens at −80 °C [12].

### Antibody preparations and characteristics

The anti-SERCA polyclonal antibody was elicited in guinea pig against rabbit skeletal muscle $Ca^{2+}$-ATPase ($SERCA_1$). It was prepared and characterized as already described [17]. This antibody was shown to react with a 100 kDa single band of rabbit skeletal sarcoplasmic reticulum corresponding to purified $Ca^{2+}$-ATPase. Although it could react with both cardiac ($SERCA_{2\text{-}a}$) and smooth muscle ($SERCA_{2\text{-}b}$), the main reaction was with skeletal sarcoplasmic reticulum ($SERCA_1$). In contrast, no cross-reactivity was observed between this antibody and erythrocyte or cardiac sarcolemmal $Ca^{2+}$-ATPases (PMCA).

The $SERCA_{2\text{-}b}$ isoform-specific polyclonal antibody was elicited in rabbit against a synthetic peptide specific for $SERCA_{2\text{-}b}$. Although the first 993 amino acids are common to the $SERCA_{2\text{-}a}$ and $SERCA_{2\text{-}b}$ isoforms, their difference is that the last four C-terminal amino acids in the $SERCA_{2\text{-}a}$ isoform are replaced in $SERCA_{2\text{-}b}$ by an extended sequence of 49 amino acids. The peptide used to prepare the antibody was specific for $SERCA_{2\text{-}b}$ as it encompassed the last 12 C-terminal amino acids of this extended sequence. The preparation and specificity of this antibody have both previously been described [18].

### SERCA immunodetection by Western blot

Membrane proteins were solubilized and reduced for 30 min at room temperature in 50 mM Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 0.01% (v/v) Bromophenol Blue, 25% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol. Samples were analysed by SDS/PAGE (8% gels) and blotted on to nitrocellulose membranes.

For the immunodetection of the 97 kDa protein, the nitrocellulose membranes were left to stand for 1 h at 37 °C in Tris-buffered saline (TBS) containing 5% (w/v) fat-free dried milk, and then incubated for 1 h at room temperature with a 1:1000 dilution of guinea-pig anti-rabbit sarcoplasmic reticulum $Ca^{2+}$-ATPase immune serum in TBS containing 0.5% (w/v) fat-free dried milk. After washing steps, the nitrocellulose membranes were incubated for 1 h at room temperature with $^{125}$I-protein A

(300000 c.p.m./ml), washed five times and subjected to autoradiography using Kodak X-Omat AR films and intensifying screens at −80 °C. The radioactive bands were quantified by densitometric measurement, using an LKB Ultroscan XL laser densitometer.

For the immunodetection of the $SERCA_{2\text{-}b}$-type isoform, the nitrocellulose membranes were blocked for 1 h at room temperature in TBS containing 0.1 % Tween 20 and then incubated for 1 h at room temperature with a 1:100 dilution of anti-$SERCA_{2\text{-}b}$ serum. After three washes in TBS/Tween, the membranes were further incubated for 1 h at room temperature with a $^{125}I$-labelled anti-rabbit IgG (1:1000 dilution), washed three times and the immunolabelling was revealed by autoradiography using Kodak X-Omat AR films and intensifying screens at −80 °C.

## RESULTS

### Characterization of the SHR strain

Table 1 summarizes the characteristics of the SHR strain used. Most of the rats were 16 weeks old.

**Table 1 Characterization of the SHR strain**

Results are means ± S.D.

| | Body weight (g) | Mean blood pressure (mmHg) | Diastolic pressure (mmHg) | Systolic pressure (mmHg) |
|---|---|---|---|---|
| WKY | 347 ± 42 | 116 ± 10 | 102 ± 9 | 129 ± 11 |
| SHR | 334 ± 26 | 196 ± 15 | 175 ± 15 | 221 ± 19 |

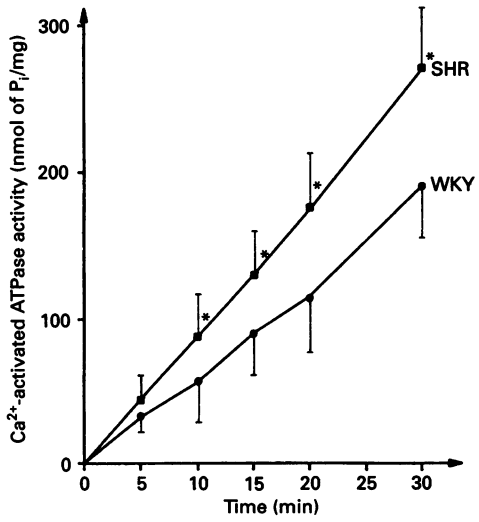


**Figure 1 Comparison of $Ca^{2+}$-ATPase activities in platelet membranes from WKY rats and SHR**

Mixed membrane fractions (100000 *g*) were isolated from platelets of 16-week-old WKY rats and SHR as described in the Experimental section, and used for the determination of $P_i$. $Ca^{2+}$-ATPase activities were measured by subtracting the values obtained in the presence of $Mg^{2+}$ but without $Ca^{2+}$ from those obtained in the presence of both $Mg^{2+}$ and $Ca^{2+}$. Results are expressed in nmol of $P_i$ hydrolysed/mg of protein and are the means ± S.D. of six experiments. *$P < 0.03$, compared with WKY rats (Student's *t*-paired test). ●, Time course of $Ca^{2+}$-ATPase activities of membranes isolated from WKY rat platelets; ■, time course of $Ca^{2+}$-ATPase activities of membranes isolated from SHR platelets.

### Comparison of time courses of total $Ca^{2+}$-ATPase activity in platelets from WKY rats and SHR

The time course of $Ca^{2+}$-ATPase activity was measured in mixed membrane vesicles isolated from platelets of 16-week-old SHR and compared with the corresponding time course in vesicles isolated from WKY rats. $Ca^{2+}$-ATPase activities were determined by testing the $Ca^{2+}$ activation of $Mg^{2+}$-ATPases, i.e. those corresponding to the ($Ca^{2+} + Mg^{2+}$)-ATPases. As shown in Figure 1, the reaction rates of $Ca^{2+}$-ATPase activities were linear up to 30 min, for concentrations of 0.5 mg of protein/ml and 1.6 $\mu$M free $Ca^{2+}$ concentration, in membrane vesicles isolated from platelets of both WKY rats and SHR. However, a statistically significant 43 % increase was found in SHR membranes, resulting in mean specific activities of 9.2 nmol of $P_i$/min per mg of protein and 6.4 nmol of $P_i$/min per mg of protein in SHR and WKY rats respectively.

These results for 16-week-old rats were compared with those obtained for 24-week-old rats, as similarities have been described between the effects of aging and hypertension. However, no significant difference could be detected as a function of age (results not shown), thus eliminating the possibility that the regulation of $Ca^{2+}$-ATPases is age-related.

### Evidence that rat platelets express two $Ca^{2+}$-ATPase isoforms: the 100 kDa $SERCA_{2\text{-}b}$ and a 97 kDa one

As we very recently succeeded in dissociating two similar but distinct $Ca^{2+}$-ATPases in human platelets, we investigated WKY rat platelet $Ca^{2+}$-ATPases to see whether the above results in fact involved the same two isoforms. The method used to detect $Ca^{2+}$-ATPase was based on its capacity to form a phosphorylated intermediate complex corresponding to a transient step in the catalytic cycle. Platelet-membrane $Ca^{2+}$-ATPase(s) was therefore phosphorylated by [$\gamma$-$^{32}P$]ATP in the presence of $Ca^{2+}$ and then submitted to acidic SDS/PAGE under the conditions previously established in human platelets. Figure 2(a) shows the comparative electrophoretic migration of the human (lane 1) and rat (lane 2) platelet $Ca^{2+}$-ATPases, determined by this highly specific method. As a further control of the $Ca^{2+}$ specificity of the formation of these phosphorylated intermediates, rat platelet membranes were phosphorylated in the presence of 0.5 mM EGTA (lane 3); no phosphorylation was detected. The electrophoretic profiles for human and rat platelet $Ca^{2+}$-ATPases were exactly the same, and consisted of two distinct bands of about 100 and 97 kDa. There was a slight difference between the two platelet species in the apparent relative proportions of the two $Ca^{2+}$-ATPases: whereas the 100 and 97 kDa isoforms formed equal amounts of phosphorylated intermediates in human platelets (lane 1), the 97 kDa isoform was formed in markedly larger amounts in rat platelets than in human platelets (lane 2).

In addition, as one of the main biochemical characteristics of the human 97 kDa isoform is the production of a proteolytic fragment of 80 kDa on trypsin treatment [12], we compared the effect of trypsin on both human and rat platelets, and this typical fragment again appeared in the rat platelets (results not shown). Consequently, the human and rat platelet $Ca^{2+}$-ATPase systems displayed similar electrophoretic migration and trypsin fragmentation.

Then, to establish this identity, we looked for their recognition by different antibodies. The 100 kDa protein was previously demonstrated to correspond to the $SERCA_{2\text{-}b}$ isoform in human platelets, by autophosphorylation, transfer on to nitrocellulose and Western blotting on the same membrane nitrocellulose [12]. Whereas the two distinct phosphorylated bands of molecular

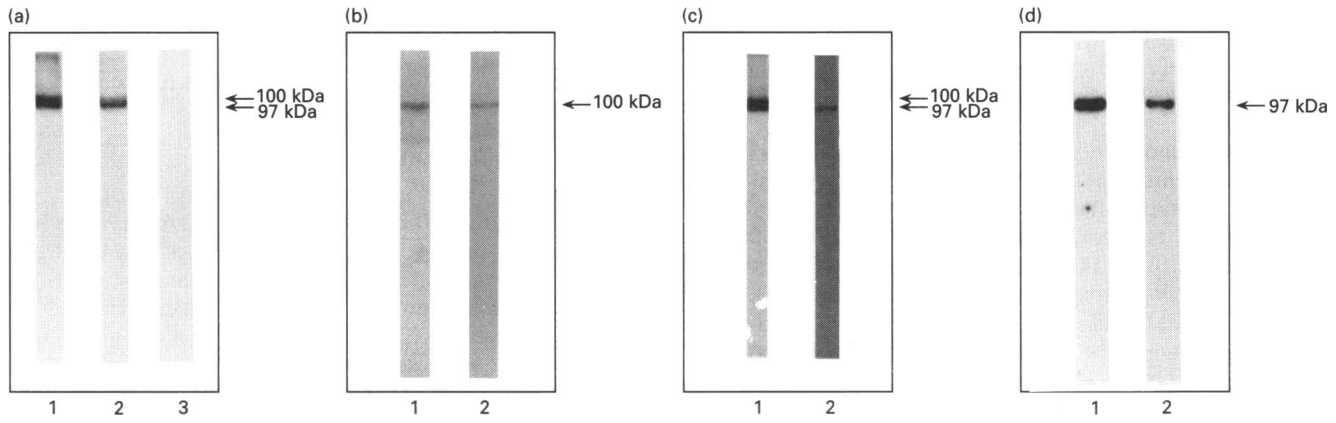


**Figure 2 Evidence that rat platelets express the two $SERCA_{2-b}$ and 97 kDa $Ca^{2+}$-ATPase isoforms**

(**a**) Demonstration of two distinct $Ca^{2+}$-ATPases in rat platelets. The 100000 **g** membrane proteins (100 $\mu$g) were isolated from either human platelets or platelets from 16-week-old WKY rats. They were phosphorylated on ice by 0.05 $\mu$M [$\gamma$-$^{32}$P]ATP in the absence (0.5 mM EGTA) or presence of 0.05 mM $CaCl_2$ as described in the Experimental section. After trichloroacetic acid precipitation, the phosphorylated proteins were subjected to acidic SDS/PAGE (7.5% gels), blotted on to nitrocellulose, and autoradiographed for 48 h at −80 °C. Lane 1, phosphoenzyme formation by human platelet $Ca^{2+}$-ATPases in the presence of $Ca^{2+}$; lane 2, phosphoenzyme formation by WKY rat $Ca^{2+}$-ATPases in the presence of $Ca^{2+}$; lane 3, phosphoenzyme formation by WKY rat $Ca^{2+}$-ATPases in the absence of $Ca^{2+}$ (0.5 mM EGTA). Numbers on the right indicate the approximate molecular masses of the $Ca^{2+}$-ATPases, estimated using standard markers. The Figure is representative of four different experiments. (**b**) Immunodetection of the $SERCA_{2-b}$ isoform. Membrane proteins were isolated from human and 16-week-old WKY rat platelets; 50 $\mu$g of human platelet membranes and 100 $\mu$g of rat platelet membranes were submitted to SDS/PAGE (8% gels) and electrotransferred on to nitrocellulose membranes. The proteins were further treated for Western blotting with 1:100 dilution of the anti-$SERCA_{2-b}$ serum under the conditions described in the Experimental section. Immunodetection was performed using anti-rabbit $^{125}$I-labelled IgG and revealed by autoradiography. Lane 1, human platelet $Ca^{2+}$-ATPase; lane 2, WKY rat platelet $Ca^{2+}$-ATPase. The molecular mass of the $Ca^{2+}$-ATPase was estimated using standard markers. The Figure is typical of three experiments. (**c**) Specific immunodetection of the human 97 kDa platelet $Ca^{2+}$-ATPase isoform. Human membrane platelet proteins (12.5 $\mu$g) were isolated and phosphorylated as described in the Experimental section. The phosphorylated proteins were subjected to acidic SDS/PAGE (7.5% gels), blotted on to nitrocellulose, and autoradiographed for 48 h at −80 °C. After the membranes had been left for a month to allow the $^{32}$P radioactivity to decay (verifying the absence of radioactive bands by autoradiography), they were subjected to Western blotting with a 1:1000 dilution of the anti-SERCA antibody. Immunodetection using $^{125}$I-protein A was performed as described in the Experimental section. Lane 1, phosphoenzyme formation by human platelet $Ca^{2+}$-ATPases; lane 2, Western blot. Numbers on the right indicate the approximate molecular masses of the $Ca^{2+}$-ATPases, estimated using standard markers. The Figure is representative of four experiments. (**d**) Comparative Western-blot analysis of human and rat 97 kDa platelet $Ca^{2+}$-ATPases. Membranes were isolated from human and 16-week-old WKY rat platelets; 25 $\mu$g of human proteins and 100 $\mu$g of rat proteins were submitted to electrophoresis and electrotransferred on to nitrocellulose membranes under the conditions described in the Experimental section. The membranes were then immunoblotted, with the anti-SERCA antibody, as in (**c**). The molecular mass of the $Ca^{2+}$-ATPase was estimated using standard markers. Lane 1, human platelet membranes; lane 2, rat platelet membranes. The Figure is representative of three experiments.

mass 100 and 97 kDa were obtained, only the 100 kDa band was stained by the $SERCA_{2-b}$ isoform-specific antibody. We therefore first compared the reactivity of this anti-$SERCA_{2-b}$ antibody with human and rat platelet $Ca^{2+}$-ATPases. As shown in Figure 2(b), similar immunoreactions were detected in human platelets (lane 1) and rat platelets (lane 2). However, the rat platelet $Ca^{2+}$-ATPase was less reactive, and this reactivity level correlated with the comparatively smaller amount of phosphorylated intermediate formed for this isoform (Figure 2a).

As regards the 97 kDa isoform, by using the same approach as the one described above (autophosphorylation of the $Ca^{2+}$-ATPases, transfer on to nitrocellulose and Western blotting), we tested different anti-SERCA antibodies on the two distinct $Ca^{2+}$-ATPase isoforms. Among the antibodies tested was one previously shown to react with the $Ca^{2+}$-ATPase(s) in human platelets. As shown in Figure 2(c), the comparison of the migration of the 100 and 97 kDa phosphorylated $Ca^{2+}$-ATPases (lane 1) with the pattern of Western blotting performed using this anti-SERCA antibody (lane 2) clearly shows that only the 97 kDa $Ca^{2+}$-ATPase isoform is recognized by the antibody.

This pertinent finding to the present study allowed us to look for the presence of this isoform in rat platelets. The comparative Western-blot analysis of the human (Figure 2d, lane 1) and rat (Figure 2d, lane 2) $Ca^{2+}$-ATPases demonstrates that, again in a similar manner for human and rat platelets, a clear immunoreaction was obtained with this antibody. However, the rat platelets showed a lower immunoreactivity than the human platelets.

Therefore the rat platelet $Ca^{2+}$-ATPase system consists of the same two distinct SERCA isoforms as those present in human platelets. Moreover, both the 100 kDa $SERCA_{2-b}$ isoform and the 97 kDa SERCA isoform can be specifically immunodetected.

### Up-regulation of the 97 kDa $Ca^{2+}$-ATPase isoform but not of the 100 kDa smooth muscle $Ca^{2+}$-ATPase isoform in SHR

Finally, these results led us to explore the positive regulation of the total $Ca^{2+}$-ATPase activities to see if it involved one or both of the above isoforms. Figure 3 shows the relative expression of the two $Ca^{2+}$-ATPase isoforms in hypertension as detected by Western blotting. Figure 3(a) shows the expression of the 100 kDa $Ca^{2+}$-ATPase isoform in platelet membranes from WKY rats (lane 1) and SHR (lane 2) which was specifically recognized by the anti-$SERCA_{2-b}$ polyclonal antibody. As Figure 3 indicates, there was no significant difference between the expression of this 100 kDa isoform in SHR platelets and control WKY rats platelets. Figure 3(b) shows the results for the 97 kDa isoform. Clearly, this isoform was more strongly expressed in SHR (lane 2) than in WKY rats (lane 1).

Further comparative quantification of the $SERCA_{2-b}$ and 97 kDa isoform expression by densitometric scanning of the radioactive bands is shown in Table 2. The results presented clearly establish that there is no significant differences between the expression of $SERCA_{2-b}$ in WKY rat (100%) and SHR platelet membranes ($128 \pm 22$%), whereas the expression of the 97 kDa isoform was significantly increased in SHR ($300 \pm 35$%). This finding confirms the overall up-regulation of platelet $Ca^{2+}$-ATPase activities in the SHR and can explain its modest increase

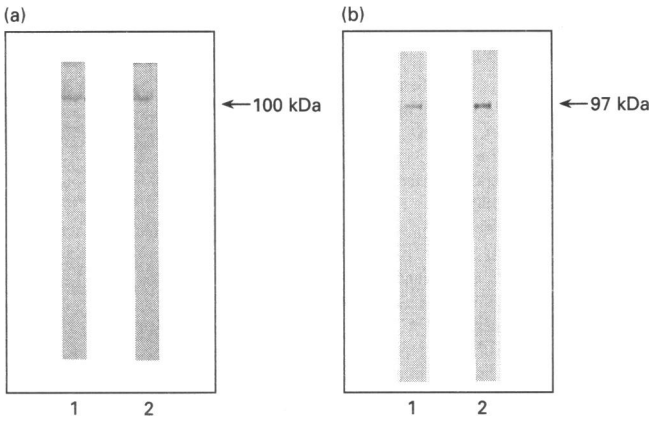


**Figure 3 Specific regulation of the 97 kDa platelet $Ca^{2+}$-ATPase in SHR**

Platelet membranes were isolated from 16-week-old WKY rats and SHR, and 100 $\mu$g of protein was then submitted to electrophoresis and electrotransferred on to nitrocellulose under the conditions described in the Experimental section. The membranes were then treated for immunoblotting with either the anti-$SERCA_{2\text{-}b}$ antibody (**a**) or the anti-SERCA antibody (**b**) and immunodetection was performed as described in the legend for Figure 2(b) and Figure 2(d) respectively. Lanes 1, membranes isolated from WKY rat platelets; lanes 2, membranes isolated from SHR platelets. The approximate molecular masses of the $Ca^{2+}$-ATPases are indicated. The Figure is representative of four experiments.

**Table 2 Comparative expressions of the $SERCA_{2\text{-}b}$ and 97 kDa isoforms in WKY rat and SHR platelet membranes**

Unsaturated autoradiograms were scanned using a laser densitometer. For each experiment, the control value (WKY rats) was arbitrarily taken as 100%. In SHR platelets, the expressions of either $SERCA_{2\text{-}b}$ or the 97 kDa isoform were given as percentage of the control values (means $\pm$ S.D. of four different experiments). $^*P < 0.02$ (Student's *t*-paired test).

| | SERCA 2b (%) | 97 kDa Isoform (%) |
|---|---|---|
| WKY | 100 | 100 |
| SHR | 128 $\pm$ 22 | 300 $\pm$ 35 |

in establishing that this up-regulation only involves the 97 kDa isoform and not the 100 kDa $SERCA_{2\text{-}b}$ isoform.

## DISCUSSION

The essential feature of this work is that the use of platelets as a model of SMC to study abnormal $Ca^{2+}$ handling under hypertensive conditions now appears questionable, at least for the studies of the regulation of their $Ca^{2+}$-ATPase system. Our data clearly demonstrate that, in the genetic model of SHR, the total platelet $Ca^{2+}$-ATPase activity is positively regulated. However, further analysis showed clear evidence that: (1) these total $Ca^{2+}$-ATPase activities referred to two distinct 100 and 97 kDa SERCA-type $Ca^{2+}$-ATPases; (2) the regulation specifically concerned a newly described 97 kDa $Ca^{2+}$-ATPase isoform; (3) the smooth-muscle-type 100 kDa $SERCA_{2\text{-}b}$ isoform exhibited no change in its expression during hypertension. Therefore, although regulation did occur, it involved the platelet $Ca^{2+}$-ATPase isoform not present in SMC. Also, regulation did not affect the $SERCA_{2\text{-}b}$ isoform, which is common to platelets and SMC.

This finding inspires several comments. (1) Even though these results do not totally exclude the existence of similarities between platelets and SMC with regard to the apparent absence of significant regulation of the $SERCA_{2\text{-}b}$ isoform, the use of platelets instead of SMC to study the regulation of the $Ca^{2+}$-ATPase system must now be more detailed. In particular, the dissociation of the two $Ca^{2+}$-ATPase isoforms will be necessary to explore the precise regulation of the isoform concerned. (2) SMC also express the $SERCA_{2\text{-}a}$ isoform which is not detectable in platelets as no cross-reactivity could be found with this isoform (results not shown). Therefore whether this isoform is regulated or not, it will be different from the 97 kDa platelet $Ca^{2+}$-ATPase. (3) To our knowledge, this is the first work conducted on the identification and regulation of the rat platelet $Ca^{2+}$-ATPase system during hypertension, and it might give rise to new interpretations of the results previously obtained for human platelet $Ca^{2+}$-ATPases. Although large differences may exist between the nature of hypertension in rats and humans, the platelets of both species exhibit increased cytosolic $Ca^{2+}$ concentrations [19,20]. Also, regulation of $Ca^{2+}$-ATPase activities has been demonstrated in human platelets irrespective of the disagreement among different authors as to its nature. Thus an increase in basal $Ca^{2+}$-ATPase activity was described initially [3], whereas in a subsequent study the $V_{max.}$ of hypertensive patients was shown to be significantly lower than that of normotensive subjects [4]. Consequently, it was suggested that the increase in $Ca^{2+}$-ATPase activity results from a secondary process, or conversely that the reduced activity is a primary abnormality. As the approaches used referred to overall $Ca^{2+}$-ATPase activities, it was not known whether the results concerned the $SERCA_{2\text{-}b}$ or 97 kDa $Ca^{2+}$-ATPase isoform, or both, as expressed in human or rat platelets (Figure 2). One possible interpretation of the discrepancies is that the different membrane preparations used contained different amounts of up- or down-regulated $Ca^{2+}$-ATPase isoforms, or non-regulated isoforms.

Another essential feature of the present findings is that they indicate that the 97 kDa platelet $Ca^{2+}$-ATPase isoform might play a significant part in regulating $Ca^{2+}$ concentration in hypertension, as the precise functions of the different $Ca^{2+}$-ATPases are still unknown except for their general role of depleting cells of cytosolic $Ca^{2+}$. An approach recently used to solve this problem was to study the regulation of their expression under different physiological and pathological conditions. Differences were found in their regulation, in particular for the SERCA-type $Ca^{2+}$-transport ATPases. Changes in the amounts of $SERCA_1$ mRNAs have been demonstrated during development [21] or chronic stimulation [22,23], and $SERCA_{2\text{-}a}$ was found to be regulated in response to pressure overload, cardiac hypertrophy and thyroid hormone [24–27]. Lastly, isoform switching of $SERCA_{2\text{-}b}$ towards $SERCA_{2\text{-}a}$ has been shown during differentiation [28] and under platelet-derived growth factor-induced SMC proliferation [29]. The present results show positive regulation of the platelet 97 kDa SERCA isoform in hypertension, but no regulation of the $SERCA_{2\text{-}b}$ isoform. Therefore, unlike the latter isoform, the 97 kDa SERCA isoform might play a significant role in regulating $Ca^{2+}$ concentration such as $SERCA_1$ or $SERCA_{2\text{-}a}$, the most extensively studied members of the SERCA family so far.

Finally, the present work constitutes the first evidence for the regulation of a new $Ca^{2+}$-ATPase isoform, which was recently detected in human platelets and appears to be expressed in different human cells [13]. Although it has not yet been identified, this $Ca^{2+}$-ATPase appeared to be specific in the sense that it is distinct from the known $SERCA_1$, $SERCA_{2\text{-}a}$ and $SERCA_{2\text{-}b}$ isoforms; no information is so far available as to whether it is

identical with the SERCA 3 gene product, as only the cDNA has been described [8]. The present evidence for its specific regulation therefore highlights the need for further investigations, which are now in progress, in order to reveal its identity.

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