Cardiac sarcoplasmic reticulum characteristics in hypertrophic hearts from spontaneously hypertensive rats

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Summary: Sarcoplasmic reticulum (SR) from left ventricles of rats that developed spontaneous hypertension was studied in vitro. Similar increases of left ventricular mass were found when grouping the animals into mild and severe hypertensives (average systolic arterial pressure of 168 ± 4 and 202 ± 6 mm Hg, respectively). The amount of SR protein (mg/g of left ventricle) was higher when obtained from hypertrophic ventricles of both hypertensive groups than from ventricles of the control group. The result agreed with the enhanced Ca$^{2+}$ uptake exhibited by left ventricular homogenates of the hypertensive groups. Consequently, Ca$^{2+}$ uptake in SR microsomes isolated per gram of left ventricle (nmol Ca$^{2+}$/g muscle) was 51.62 ± 10.06 and 64.99 ± 12.84 in mildly and severely hypertensive groups vs. 17.37 ± 5.79 in the control group (P<0.05). The SR microsomes obtained from ventricles of hypertensive rats showed an enhanced Ca$^{2+}$ activated ATPase activity that was not accompanied by increased Ca$^{2+}$ uptake at saturating calcium concentrations, but by increased affinity for calcium (K$_{app}$ of 1.09 ± 0.28 and 2.67 ± 0.16 μM in SR microsomes of hypertrophic and control ventricles respectively; P < 0.05). The rates of calcium loss, measured in SR vesicles passively loaded with $^{45}$Ca, were similar when assayed in SR obtained from ventricles of both hypertensive and normal rats. These results enable us to suggest that in hearts of rats presenting spontaneous hypertension, the function of the SR system could account for a normal handling of cytosolic calcium. They might support the absence of mechanical alterations described in hearts of young rats of the SHR strain.

Key words: spontaneously hypertensive rats; cardiac hypertrophy; sarcoplasmic reticulum; Ca$^{2+}$ uptake; Ca$^{2+}$ ATPase activity

Introduction

Myocardial hypertrophy is present in spontaneously hypertensive rats (SHRs) either before the development of arterial hypertension of very early in its course (4, 5, 19). In the young SHRs, this cardiac hypertrophy is not accompanied by modifications of mechanical cardiac behaviour; a depression of the contractile state (15, 18) and an impairment of relaxation (8) have been reported only after 12 months of established cardiac hypertrophy and arterial hypertension. However, in SHRs of about 5 months of age, the cardiac sarcoplasmic reticulum has been found to be impaired in its capacity to accumulate calcium ions (2, 12, 13). The lack of a mechanical correlation to this finding has yet to be explained.

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The variables analyzed in the present work – calcium uptake in homogenates; amount of SR protein isolated from these homogenates; calcium uptake in SR microsomes, and calcium dependence of the microsomal SR calcium pump – enable us to state that in hearts of young rats presenting spontaneous hypertension, the function of the SR system can be accounted for by a normal handling of cytosolic calcium.

Methods

Hypertensive rats were obtained by mating spontaneously hypertensive males of the Aoki-Okamoto strain with females rats of our Wistar colony. The male rats that developed arterial hypertension were separated into two groups: those showing mild and severe hypertension. Sex-matched normotensive Wistars were used as controls. Age of the rats: 16 to 20 week-old (control groups); 20 to 24 week-old (hypertensive groups).

Systolic pressure was measured by tail-cuff plethysmographic technique, and mean arterial pressure by inserting, under pentobarbital anesthesia, a catheter connected with an Alitech MS10 pressure transducer into the carotid artery.

Determination of hypertrophy

Left ventricular hypertrophy was assessed in two ways: by comparing the ratios of left ventricular weight/body weight (LVW/BW) obtained in the control and hypertensive groups, and comparing the LVW of each rat (normotensive or hypertensive) with a theoretical LVW value, calculated from an equation derived by computed linear regression of 32 LVWs and BWs measured in Wistar rats. In this case, the results were expressed as percentage deviation of the measured LVWs with respect to the calculated LVWs (LVW (mg) = 117 + 1.37 × BW (g)).

Homogenates and SR microsomes

After anesthetizing with sodium pentobarbital, 80 mg/kg i.p., the heart was quickly separated, washed with cold saline solution (0.9 g/l NaCl) and the left ventricle, including the interventricular septum, dissected. The left ventricle was washed with the same cold saline solution, blotted and weighed. The tissue was cut into small pieces and homogenized three times for 15 s, with 30-s rest intervals, in 3 ml of 10 mM NaCO3 H or 20 mM Tris-Maleate buffer, pH 6.8. A motor drive teflon pestle homogenizer was used. The homogenate was diluted to 8 ml with the homogenization medium and processed for the isolation of sarco plasmic reticulum according to the method of Harigaya and Schwartz (9). 0.5 ml of the homogenate of those preparations, initially diluted in 20 mM Tris-Maleate buffer, pH 6.8, was saved, to determine its protein concentration and oxalate supported calcium uptake. Protein concentration in homogenates and in SR preparations was measured according to the method of Lowry et al. (14).

Measurement of calcium uptake

Calcium uptake was assessed by measuring the amount of 45Ca entrapped in the preparations. The reactions were carried out in a medium containing (in mM): Tris-Maleate buffer (pH 6.8) 20, KCl 100, NaNO3 5, MgCl2 5, Na2ATP 5, K oxalate 1, EGTA 0.100 and 45Ca 0.040 (0.612 µM of free Ca²⁺) and started by the addition of homogenate or SR proteins. Variable amounts of 45Ca were used when calcium uptake was measured as a function of the calcium concentration (0.103 to 18 µM of free Ca²⁺). The reactions were stopped after 3 min of incubation at 37 °C by filtering the medium through Millipore membranes (HA 0.45 µm). The same reaction conditions were used to determine the unspecific 45Ca retention by incubating protein in the absence of ATP. At each calcium concentration assayed, the value of the unspecific 45Ca retained was substracted from the 45Ca retained by the protein incubated with ATP.

Determination of calcium dependent ATPase activity

ATPase activities were measured by determination of the liberated inorganic phosphate (11). The reaction conditions were the same as for calcium uptake, except that K oxalate and labeled calcium were
Table 1. Characterization of cardiac hypertrophy and protein content of left ventricles in normotensive and hypertensive rats.

<table>
<thead>
<tr>
<th></th>
<th>Normotensive control (n = 9)</th>
<th>Mildly hypertensive (n = 6)</th>
<th>Severely hypertensive (n = 9)</th>
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<tbody>
<tr>
<td>Systolic arterial pressure (mm Hg)</td>
<td>130 ± 3</td>
<td>168 ± 4*</td>
<td>202 ± 6*</td>
</tr>
<tr>
<td>Body weight (BW, g)</td>
<td>276 ± 12</td>
<td>382 ± 13*</td>
<td>349 ± 12*</td>
</tr>
<tr>
<td>Left ventricular weight (LVW, mg)</td>
<td>529 ± 26</td>
<td>958 ± 59*</td>
<td>952 ± 46*</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>2.09 ± 0.07</td>
<td>2.55 ± 0.15*</td>
<td>2.71 ± 0.10*</td>
</tr>
<tr>
<td>Percentage of hypertrophy (%)</td>
<td>3 ± 3</td>
<td>36 ± 8*</td>
<td>41 ± 6*</td>
</tr>
<tr>
<td>Homogenate protein/g wet left ventricle (mg/g)</td>
<td>175.4 ± 8.4</td>
<td>165.4 ± 9.4</td>
<td>210.0 ± 31.0</td>
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</table>

Results represent mean ± SE for the number of experiments indicated in each group (n). Average of the mean arterial pressure values of the mildly and severely hypertensive groups: 136 ± 3 and 182 ± 7 mmHg respectively. The percentages of hypertrophy were calculated as described in Methods. * indicates P < 0.05; N.S.: without statistical significance.

omitted from the incubation medium. The reactions were stopped by the addition of 0.6 ml of 5 % TCA. To obtain the Ca\(^{2+}\)-activated ATPase values, the phosphate liberated in the presence of 1 mM EGTA (no calcium added to the medium), was subtracted from the phosphate liberated by the same amount of protein at each free Ca\(^{2+}\) concentration assayed.

Assays of plasma membrane marker enzyme

The activity of the enzyme 5' nucleotidase was measured in both homogenate and SR proteins. Unspecific hydrolysis of 2' and 3' AMP was substrated from 5' AMP hydrolysis. In order to evaluate the enrichment of SR preparations in plasma membranes, the value of 5' nucleotidase activity measured in the SR isolated from each homogenate was divided by the value of 5' nucleotidase activity measured in each homogenate. These ratios were calculated for control and hypertensive groups in order to establish if the SR microsomes presented the same degree of plasma membrane contamination in both groups.

All data of the above described measurements were obtained from duplicate determinations.

Measurement of the calcium efflux

Passive load of SR microsomes with \(^{45}\)Ca was performed, incubating 0.05 to 1.5 mg of SR protein at 0°C overnight in 1 ml of a medium containing 100 mM KCl, 20 mM Tris-Maleate buffer (pH 6.8) and 3 mM \(^{45}\)CaCl\(_2\) (about 1000 cpm/nmol Ca\(^{2+}\)). After incubating, 0.8 ml of the medium was filtered through a 0.22 μm Millipore filter (Ø 13 mm); the labeled medium remained in the holder system was carefully eliminated by flowing air. Then the filter was washed at a constant volume of 2 ml/min by using a Sage Instrument tubing pump, model 375. The washing medium contained 100 mM KCl, 20 mM Tris-Maleate buffer (pH 6.8), 0.1 mM MgCl\(_2\) and 2 μM of free calcium. The effluent was collected in scintillation vials, each 15 s for 2 min. The solution remaining in the holder system was collected in a separate vial before removing the filter to another vial. All vials were heated at 70°C until complete evaporation of the collected liquid; then filled with 10 ml of a scintillation cocktail, shaken for 30 s and counted. Standards were obtained by measuring the radioactivity of 20 μl of the protein free filtrate. This method makes it possible to determine the amount of \(^{45}\)Ca passively loaded by the SR microsomes and the time course of calcium loss, which was expressed as a percentage of the total loaded calcium.
**Table 2. Comparison of oxalate supported calcium uptake in homogenates and SR microsomes obtained from hearts of normotensive and spontaneously hypertensive rats.**

<table>
<thead>
<tr>
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<th>Severely hypertensive (n = 9)</th>
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<tr>
<td>Calcium uptake in homogenate protein (nmol Ca(^{2+})/mg)</td>
<td>1.15 ± 0.26</td>
<td>2.12 ± 0.30</td>
<td>2.21 ± 0.46</td>
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<tr>
<td>Calcium uptake in SR protein (nmol Ca(^{2+})/mg)</td>
<td>7.83 ± 1.98</td>
<td>11.96 ± 2.51</td>
<td>12.53 ± 2.09</td>
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<tr>
<td>SR/homogenate calcium uptake ratio</td>
<td>9.17 ± 2.09</td>
<td>6.77 ± 1.99</td>
<td>7.08 ± 1.12</td>
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<td>Calcium uptake in homogenate obtained per gram of LV (nmol Ca(^{2+})/g LV)</td>
<td>200 ± 41</td>
<td>338 ± 30</td>
<td>502 ± 180</td>
</tr>
<tr>
<td>Calcium uptake in SR fraction obtained per gram of LV (nmol Ca(^{2+})/g LV)</td>
<td>17.37 ± 5.79</td>
<td>51.62 ± 10.06</td>
<td>64.99 ± 12.94</td>
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Results represent mean ± S.E. for the number of experiments indicated in each group (n). Calcium uptake values are for 3 min incubation. Systolic arterial pressures for each group are given in Table 1. Calculation of calcium uptake in homogenates obtained per gram of left ventricle (nmol Ca\(^{2+}\)/g LV): Calcium uptake in homogenate protein (nmol Ca\(^{2+}\)/mg) × amount of protein in homogenates (mg)/left ventricular weight (g).

Calculation of calcium uptake in SR fractions obtained per gram of left ventricle (nmol Ca\(^{2+}\)/g LV): Calcium uptake in SR protein (nmol Ca\(^{2+}\)/mg) × amount of SR protein obtained per gram of left ventricle (mg/g).

* indicates P < 0.05; N.S.: without statistical significance.

Free calcium concentrations were calculated by computation as described by Fabiato and Fabiato (7). Na\(_2\)ATP, vanadium free and EGTA were obtained from Sigma chemical Co. \(^{45}\)Ca as the chloride salt was obtained from New England Nuclear. All other reagents were of analytical grade.

**Results**

Table 1 summarizes the data corresponding to systolic arterial pressure, body weight, left ventricular weight, indexes of hypertrophy and protein content of the homogenates. Both groups, mildly and severely hypertensive, evidenced left ventricular hypertrophy in relation to the control group. The extent of hypertrophy assessed either by the LVW/BW ratio, or by an increase in the LVW%, was similar for the two hypertensive groups, in spite of the differences in arterial pressure values. None of the three groups differed in the protein content of the homogenates normalized per gram of mass ventricle.

Data on calcium uptake measured in homogenates and SR microsomes are shown in Table 2. In both preparations, calcium uptake values increased when obtained from hypertrophic ventricles, while statistical significance was only attained in homogenates of the mildly hypertensive group. After the isolation procedure, SR microsome enrichment was similar in hypertensive and control groups, since SR/homogenate calcium uptake ratios showed no statistical differences between the groups. Data on SR protein obtained per gram of left ventricle are presented in Fig. 1. The capacity of calcium uptake resulting from the SR protein obtained per gram of left ventricle, was calculated using the values on Fig. 1A, and
Fig. 1. (A) SR protein (mg) obtained per gram of wet homogenized ventricle. (B) Percentages of the homogenate protein corresponding to the SR protein. Data from same control (□), mildly hypertensive (■) and severely hypertensive (■) groups presented in Tables 1 and 2. Bars are mean ± S.E. * indicates P<0.05.

the calcium uptake measured in SR microsomes. A higher capacity for calcium accumulation was demonstrated in ventricles of the hypertensive groups (last line on Table 2). These results were not due to differences in the purity of preparations: as shown in Table 3, control and hypertrophic ventricles did not differ in the ratio values of the plasma membrane marker enzyme activity assayed in homogenates and SR preparations.

The calcium-dependent enzyme activity was higher in SR preparations obtained from hypertrophic ventricles than in SR preparations from control ventricles (Fig. 2A). In the two SR preparations the activation by calcium was maximal at the lowest free Ca$^{2+}$ concentration assayed. The ATPase activity independent of Ca$^{2+}$ (inset of bars) was similar in both SR preparations. Calcium uptake did not attain significant statistical differences between the

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<th>Table 3. Comparison of 5' nucleotidase activity in homogenates and microsomal fractions obtained from hearts of normotensive and spontaneously hypertensive rats.</th>
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<tr>
<td>Homogenate 5' nucleotidase activity</td>
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<td>Microsomal 5' nucleotidase activity</td>
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<td>Microsomal/homogenate 5' nucleotidase activity ratio</td>
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Values are mean ± S.E. of 5' nucleotidase activity measured in the number of preparations indicated in each group (n). The enzyme activity is expressed in μmol Pi/mg protein/30 min. Hydrolysis of 5' AMP and 2' and 3' AMP was measured incubating homogenate or SR proteins at 37°C in a medium containing 50 mM Tris-Maleate buffer (pH 7.5), 5 mM MgCl$_2$ and 5 mM 5' AMP, or 5 mM 2' and 3' AMP respectively. The ratio values of the plasma membrane marker enzyme activity did not differ statistically between the hypertensive and normotensive groups. Mean systolic arterial pressure values of the control and spontaneously hypertensive groups were 126 ± 4 and 204 ± 14 mm Hg respectively. N.S.: without statistical significance.
two groups on calcium saturation curves (Fig. 2B). When in each individual SR preparation, absolute calcium uptake values were expressed as relative to the maximal calcium uptake measured, SR from the hypertensive group exhibited higher affinity for calcium than SR from the normotensive group (Fig. 3A). Figure 3B shows the results from the normalization of the same absolute calcium uptake values to the calcium uptake measured at the lowest free Ca$^{2+}$ concentration assayed (0.103 μM). The change in the affinity for calcium can be demonstrated with data expressed as relative to both maximal and minimal calcium uptake values.

Fig. 3. Absolute values of oxalate supported calcium uptake of Figure 2B expressed as relative to: (A) maximal, and (B) minimal calcium uptake values. (○) Control; (■) spontaneously hypertensive.
Fig. 4. Results of SR microsomes passively loaded with $^{45}$Ca. SR preparations from control (○) and spontaneously hypertensive (■) rats were filtered after equilibrating with 3 mM $^{45}$CaCl$_2$ (see Methods). (A) $^{45}$Ca present in the filter with entrapped SR protein (nmol/mg); the values at each time was obtained, adding the radioactivity measured in the effluent after that time and the radioactivity remaining in the filter after 2 min washing. (B) Radioactivity measured in the effluent each 15 s, expressed as percentage of the $^{45}$Ca present in the filter with entrapped SR protein before starting the wash. Values are mean ± S.E. of five different preparations for each group. Systolic arterial pressure of the spontaneously hypertensive group: 216 ± 11 mm Hg. * indicates P < 0.05.

The results obtained with the SR microsomes passively loaded with $^{45}$Ca are shown in Fig. 4. The hypertensive group showed lower values of $^{45}$Ca passively loaded per mg of SR protein than the normotensive group (Fig. 4A). The rate of calcium loss during washing of vesicles was not different between hypertensive and control groups (Fig. 4B).

Discussion

Our spontaneously hypertensive rats developed similar ventricular hypertrophy under both mild and severe arterial pressure elevations. The hypertrophic ventricles did not differ from control ventricles in their protein content per unit of mass tissue, but yielded more sarcoplasmic reticulum protein. This was not due to differences in the degree of purification of the preparations: The enrichment in plasma membranes or sarcoplasmic reticulum membranes was similar in hypertensive and normotensive groups. The results suggest that in hypertrophic hearts of rats presenting spontaneous hypertension, the sarcotubular membranes grow more than the whole cell; a similar difference has been already reported for other types of cardiac hypertrophy (17).

In addition to the greater amount of SR membranes in homogenates obtained from hypertrophic ventricles, the higher capacity to accumulate calcium exhibited by these homogenates could also be due to the presence of a more active calcium pump in their SR membranes. In agreement with previous reports (2, 12, 13), we found that in SR preparations of spontaneously hypertensive rats the enzyme activity related to calcium transport was enhanced. However, we did not observe an improvement of calcium uptake in these microsomes; they showed an increased affinity for Ca$^{2+}$ and similar levels of maximal calcium uptake compared to the control SR microsomes.

Aoki et al. reported that the SR from SHR hearts had low maximum calcium binding with slightly increased Ca$^{2+}$ affinity and high Ca$^{2+}$-dependent ATPase activity (2). To explain the uncoupling between calcium uptake and energy supply, they suggested leakier SR mem-
branes in SHR hearts. Other studies also described a defective calcium transport and an elevated Ca$^{2+}$-dependent ATPase activity for SR microsomes obtained from hearts of SHRs (12, 13). The impaired calcium uptake was related to a lowered cyclic AMP-dependent protein kinase activity detected in these microsomes (12). A diminished cAMP protein kinase activity has also been reported in cardiac muscle of SHRs (3). However, the modulation of calcium transport in SR through phosphorylation of the SR protein phospholamban by cAMP-dependent protein kinases is not accompanied by the uncoupling of the ATP enzyme splitting and the calcium uptake (10, 20). Higher permeability to calcium in SR microsomes obtained from SHR hearts was also proposed in order to explain elevated ATP hydrolysis and lowered capacity to accumulate calcium ions in these preparations (12, 13). Nevertheless, the authors presented very similar results measuring calcium accumulated both in the absence and in the presence of 5 mM Na oxalate (12).

Despite the fact that we measured calcium uptake in the presence of 1 mM of K oxalate, we cannot discard the participation of calcium efflux in determining the amount of calcium retained by the SR microsomes after 3 min of uptake. Actually, the calcium release is an early process in SR microsomes obtained from rat hearts, even when calcium uptake is measured in the presence of oxalate (16). On the other hand, calcium release should be enhanced by increasing the external calcium concentration (6). If, as has been already suggested, the SR microsomes from SHR hearts have an elevated permeability to calcium (2, 12, 13), we could have an underestimation of the calcium uptake values measured at saturating calcium concentrations in our hypertensive SR preparations. If this were true, the calcium uptake determined at submaximal calcium concentrations might be overestimated when expressed as relative to the maximal uptake value (Fig. 4A), and the higher affinity for calcium observed in the hypertensive preparations would be only apparent and meaningless for the active process of calcium transport. We therefore normalized calcium uptake measured at the different free calcium concentrations assayed, to the value of calcium accumulated at the lowest free calcium concentration used (0.103 μM; Fig. 3B). The assumption was that at this low free calcium concentration in the medium, the calcium efflux would be neglected (6). The increase in the affinity for calcium was still observed for the SR microsomes obtained from hypertensive rat hearts. The normalization of calcium uptake values to maximal or minimal levels did not change the results, thus supporting the notion that SR vesicles obtained from hypertrophied ventricles are not leakier than the vesicles obtained from control ventricles. Our present results on calcium efflux from passively $^{45}$Ca loaded SR microsomes also suggest that calcium permeability is not altered in SR preparations from hypertrophied ventricles. Probably, a higher amount of unsealed vesicles unable to retain Ca$^{2+}$ ions are present in these preparations, since after equilibration with $^{45}$CaCl$_2$ they show lower radioactivity per mg of microsomal protein. The presence of unsealed vesicles, able to hydrolyze ATP but not to accumulate calcium, might be the reason for the enhanced Ca$^{2+}$-dependent ATPase activity, without improvement of calcium uptake, found in the SR preparations from SHR hearts. If the enrichment in open membranes is the same as in sealed membranes – which our results showed without differences between control and hypertensive SR preparations – it is reasonable to think that the initial homogenization produces more open SR membranes in the homogenates from hypertrophic ventricles. It is possible that this effect could be more relevant to the homogenization method used in previous studies, thus supporting the striking disassociation between ATP hydrolysis and calcium uptake found in SHR cardiac SR preparations.

In addition, we have differences from those studies concerning the experimental conditions in which calcium uptake was measured (temperature and concentrations of EGTA plus Ca$^{2+}$). We did not incubate the SR microsomes prior to the reaction but started both calcium uptake and ATPase assays by the addition of protein to the incubation medium. This
procedure pointed to the fact that preincubation of SR microsomes in different Tris-containing buffers impairs the capacity of SR vesicles to retain calcium and enhance their Ca^{2+}-dependent ATP splitting activity (1).

Our results support the notion that cardiac sarcoplasmic reticulum from young spontaneously hypertensive rats (5–6 months of age) presents a normal (or improved) capacity to transport calcium ions. An increase in the amount of sarcotubular membranes, together with a normal (or improved) function of the calcium pump, suggests that the SR of rats presenting spontaneous hypertension might be able to supply and to remove calcium normally during the contraction-relaxation cycle.

References

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