Function of the Sarcoplasmic Reticulum and Expression of Its Ca^{2+}-ATPase Gene in Pressure Overload–Induced Cardiac Hypertrophy in the Rat

Diane de la Bastie, Dmitri Levitsky, Lydie Rappaport, Jean-Jacques Mercadier, Françoise Marotte, Claudine Wisniewsky, Victor Brovkovich, Kitty Schwartz, and Anne-Marie Lompré

The reduction in Ca^{2+} concentration during diastole and relaxation occurs differently in normal hearts and in hypertrophied hearts secondary to pressure overload. We have studied some possible molecular mechanisms underlying these differences by examining the function of the sarcoplasmic reticulum and the expression of the gene encoding its Ca^{2+}-ATPase in rat hearts with mild and severe compensatory hypertrophy induced by abdominal aortic constriction. Twelve sham-operated rats and 31 operated rats were studied 1 month after surgery. Eighteen animals exhibited mild hypertrophy (left ventricular wt/body wt < 2.6) and 13 animals severe hypertrophy (left ventricular wt/body wt > 2.6). During hypertrophy we observed a decline in the function of the sarcoplasmic reticulum as assessed by the oxalate-stimulated Ca^{2+} uptake of homogenates of the left ventricle. Values decreased from 12.1±1.2 nmol Ca^{2+}/mg protein/min in sham-operated rats to 9.1±1.5 and 6.7±1.1 in rats with mild and severe hypertrophy, respectively (p < 0.001 and p < 0.001, respectively, vs. shams). This decrease was accompanied by a parallel reduction in the number of functionally active Ca^{2+}-ATPase molecules, as determined by the level of Ca^{2+}-dependent phosphorylated intermediate: 58.8±7.4 and 48.1±13.5 pmol P/mg protein in mild and severe hypertrophy, respectively, compared with 69.7±8.2 in shams (p < 0.05 and p < 0.01, respectively, vs. shams). Using SI nuclease mapping, we observed that the Ca^{2+}-ATPase messenger RNA (mRNA) from sham-operated and hypertrophied hearts was identical. Finally, the relative level of expression of the Ca^{2+}-ATPase gene was studied by dot blot analysis at both the mRNA and protein levels using complementary DNA clones and a monoclonal antibody specific to the sarcoplasmic reticulum Ca^{2+}-ATPase. In mild hypertrophy, the concentrations of Ca^{2+}-ATPase mRNA and protein in the left ventricle were unchanged when compared with shams (mRNA, 93.8±10.6% vs. sham, NS; protein, 105.5±14% vs. sham, NS). In severe hypertrophy, the concentration of Ca^{2+}-ATPase mRNA decreased to 68.7±12.9% and that of protein to 80.1±15.5% (p < 0.001 and p < 0.05, respectively), whereas the total amount of mRNA and enzyme per left ventricle was either unchanged or slightly increased. The slow velocity of relaxation of severely hypertrophied heart can be at least partially explained by the absence of an increase in the expression of the Ca^{2+}-ATPase gene and by the relative diminution in the density of the Ca^{2+} pumps. We cannot, however, exclude that another mechanism could also be involved in the depression of the sarcoplasmic reticulum function of moderately hypertrophied hearts. (Circulation Research 1990; 66:554–564)

A adaptation of the heart to chronic overload depends on quantitative and qualitative changes in the gene expression of myocar-

dial cells that rapidly induce hypertrophy.1–5 During the compensatory period of hypertrophy secondary to pressure overload, the duration of contraction is

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Address for reprints: Anne-Marie Lompré, INSERM U 127, Hôpital Lariboisière, 41 Bd de la Chapelle, 75010 Paris, France.

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increased and the maximum velocities of shortening and lengthening of the muscle are decreased.6–8 In the rat, the alteration in the velocity of shortening is correlated with a modification in myosin heavy chain phenotype9,10 that arises from changes at the pretranslational level11 of two myosin heavy chain genes linked on the genome.12 A slower13,14 and more efficient15 contraction results. This mechanism depends on the initial ventricular phenotype; indeed, in some animal species and in humans the changes in the isoform of myosin appear to be less important,16 and other factors must contribute to the alteration of the contractile activity of the hypertrophied ventricle.

Relaxation is also modified, and until recently, the molecular mechanisms responsible for these alterations have been poorly studied. The reduced maximum velocity of shortening of the mechanically overloaded heart is accompanied by abnormal Ca2+ handling17,18 and by modifications of the tension-independent heat production, which reflects the energy cost of calcium cycling (for review, see Reference 19). The role of the sarcoplasmic reticulum (SR) in these alterations has been stressed in many studies conducted on isolated microsomal vesicles. A reduction in the calcium transport by the SR has been observed in experimental compensatory cardiac hypertrophy induced by mechanical overload, and the transport capacity further decreased in failing hearts.20–22 An elevated calcium transport by SR was, however, reported in mild hypertrophy induced in rat by pressure overload.27

This work was undertaken to investigate the function of the SR and to define more precisely the molecular defects leading to an alteration of its function in hypertrophy secondary to pressure overload. Abdominal aortic constriction sufficient to induce varying degrees of left ventricular hypertrophy in the rat was used as a model because the mechanical properties of hearts from this species have been well defined.5,7

We first studied the function of the SR Ca2+-ATPase by measuring the rate of oxalate-stimulated Ca2+ uptake concomitantly with the level of acylphosphate intermediate of its SR Ca2+-ATPase. We used crude homogenates for these studies because only a minor fraction of SR could be isolated by standard procedures, and, moreover, marked loss of activity has been reported on isolated vesicles of SR.28–31 Since there are different isoforms of SR Ca2+-ATPase, and their expression is both tissue specific32–38 and developmentally39 regulated, we then looked for changes in the pattern of expression of isoforms at the mRNA level by using S1 nuclease mapping analysis and complementary DNA (cDNA) probes specific for the rat heart SR Ca2+-ATPase messenger RNA (mRNA). Finally, we quantified the level of expression of the SR Ca2+-ATPase gene at both mRNA and protein levels.

Our results indicate that hypertrophy is not accompanied by a switch of the isoforms of SR Ca2+-ATPase. In severe hypertrophy, although the total amount of enzyme present in the left ventricle was unchanged or slightly increased, there is a decrease of both SR function and Ca2+-ATPase concentration (mRNA and protein) that may explain some of the modifications of the relaxation properties of the hypertrophied hearts.

Materials and Methods

Animals

Coarctation of the abdominal aorta was performed in 8-week-old male Wistar rats (weight, 180–200 g), as described previously,10 by placing a partially occluded Weck hemoclip (internal section, 0.6 mm2) around the abdominal aorta between the renal and superior mesenteric arteries. Sham-operated controls were processed as the operated animals but without placement of the clip. All the animals were allowed regular rat chow and tap water ad libitum and were kept under identical housing conditions.

One month after surgery, animals were distributed into two groups. Group A, composed of six sham-operated and 17 coarctated animals, was used to measure the Ca2+ uptake and the phosphorylated intermediate of the enzyme and to determine the total amount of Ca2+-ATPase by immunologic methods. Group B, which included six sham-operated and 14 operated animals, was used for RNA analysis.

Immediately after the rats were killed, the hearts were excised, rinsed with saline, and blotted dry. The right ventricle was isolated from the rest of the ventricular mass by dissecting along its septal insertion, and the left ventricle was weighed. The degree of hypertrophy of the left ventricle of each coarctated rat was estimated by comparing the ratio left ventricular weight (LVW) versus body weight (BW) of operated versus sham-operated animals. The hearts from group A were immediately processed (see below). The tissues from group B were rapidly frozen in liquid nitrogen and stored at −70°C.

Because the relaxation rate varies depending on the increase in cardiac mass and is more pronounced in severe hypertrophy,8 each group was further divided into three subgroups to distinguish mild from severe hypertrophy. Subgroup I consisted of sham-operated rats, subgroup II of animals in which the LVW/BW ratio was less than 2.6, and subgroup III of animals in which the LVW/BW ratio was higher than 2.6.

Preparation of Crude Homogenates for Measurement of Activity and Phosphorylated Intermediate of the Ca2+-ATPase and Ca2+-ATPase Quantification

The hearts of rats in group A were cooled in 0.9% NaCl solution, wiped on filter paper, and weighed. Pieces of left ventricles (600–700 mg) were minced and homogenized two times (5 seconds each) with a Polytron homogenizer (generator PT 10, Kinematica AG Littau, Lucerne, Switzerland) in 10 ml isolation medium containing 30% glycerol, 5 mM sodium azide, and 20 mM Tris-HCl (pH 7.4). The homogenate was centrifuged for 10 minutes at 2,500 rpm, and
the supernatant was collected. Ten milliliters of the isolation medium was added to the pellet, and the whole procedure was repeated twice. The two supernatants and the final homogenate were pooled and kept frozen at −70°C. All operations were performed at 0°C. We checked that the freezing did not affect SR functions.

**Measurements of Ca^{2+} Uptake and of the Phosphorylated Intermediate of the Enzyme (E-P)**

The rate of Ca^{2+} uptake was measured at 30°C in 0.5 ml of a medium containing (mM) KCl 100, ATP 5, MgCl\(_2\) 6, K-oxalate 15, ethylene glycol-bis(β-aminoethyl ether) \(N,N',N''\)-tetraacetic acid (EGTA; Titrilex VI, Merck, West Point, Pennsylvania) 0.2, Tris-HCl buffer 30 (pH 6.85), and \(^{45}\)CaCl\(_2\) 0.150, giving a free calcium concentration of 1.25 μM as determined using the Fabiato's computer program.\(^{40}\) Sodium azide (5 mM) was added to inhibit mitochondrial Ca^{2+} uptake. The reaction was initiated by adding 20–50 μg total protein. After 6 minutes, 0.4-ml aliquots were filtered through Millipore filters (HAWP 0.45 μm, Bedford, Massachusetts). The filters were washed in 15 ml of a solution containing (mM) KCl 100, EGTA 1, and histidine 10 (pH 6.4) and counted in an Intertechique SL 3000 counter. Ca^{2+} uptake was linear for at least 6 minutes.

Ca^{2+}-dependent phosphorylation was measured at 0°C as previously described\(^{28,41}\) in 1 ml of a medium containing (mM) KCl 100, [γ-32P]ATP 0.010, MgCl\(_2\) 5, sodium azide 5, Tris-HCl 20 (pH 7.4), and either CaCl\(_2\) 0.05 or EGTA 1. The reaction was initiated by the addition of 100–150 μg homogenate and quenched after 15 seconds by the addition of 1.5 ml of 6% trichloroacetic acid, 0.3 mM ATP, and 1 mM KH\(_2\)PO\(_4\). The mixture was centrifuged for 10 minutes at 1,000g, and 1 mg bovine serum albumin was used as a carrier. The pellet was washed three times with the quenching solution and solubilized in 0.5 ml of 2 M KOH. Cerenkov radiation was counted in a scintillation counter. The Ca^{2+}-dependent phosphorylation is the difference between the amount of phosphate incorporated in the presence of Ca^{2+} and that measured in the presence of EGTA.

To characterize the proteins phosphorylated in the homogenates, polyacrylamide gel electrophoresis of phosphorylated homogenates was performed. The samples were prepared as above except that albumin was omitted. The final pellet was washed twice with 10% sucrose and solubilized by a mixture of 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol. Preparation of the gels and conditions of electrophoresis at pH 2.4 have been described previously.\(^{42}\) Gel slabs were sliced into 2.5-mm pieces, and the incorporated radioactivity was counted.

**Quantification of Ca^{2+}-ATPase**

Quantification was performed by immunological means using a monoclonal antibody to dog heart SR. Among the monoclonal antibodies previously charac-

**Characterization of Ca^{2+}-ATPase mRNA**

Total cellular RNA was prepared from the rat hearts of group B according to Chirgwin et al.\(^{46}\) RNA blots were obtained by size fractionation on 1% agarose gels containing 3% formaldehyde and blotting onto nylon membrane (Pall Biodyne TM-A transfer membrane, Pall Ultrafine, Glen Cove, New York).

The amount of Ca^{2+}-ATPase mRNA was determined by dot blot analysis. Total RNA was denatured in 15X SSC (1X SSC=0.15 M sodium chloride, 0.015 M sodium citrate) by heating at 65°C for 15 minutes and cooling on ice. Serial dilutions were then spotted onto nitrocellulose filters, using a Schleicher & Schuell minifold apparatus (Keene, New Hampshire). The filters were prehybridized in 50% formamide, 0.1% bovine serum albumin, 0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.05 M sodium phosphate (pH 6.5), 5X SSC, 0.1% SDS, 250 μg/ml salmon sperm DNA at 42°C and hybridized under the same conditions with \(^{32}P\)dCTP-labeled probes. The RNA and dot blots were hybridized with a 1.5-kilobase (kb) cDNA probe complementary to the rat cardiac Ca^{2+}-ATPase mRNA (nucleotides [nt]=50 to +1,446), random prime labeled (Multiprime labeling system, Amersham, Arlington Heights, Illinois) to a specific activity of 1–3×10\(^6\) dpm/μg. This cDNA probe has been previously described.\(^{47}\) To determine the actual amount of RNA present on the filters, blots were dehybridized and rehybridized with a 24-mer oligonucleotide specific to the rat ribosomal 18S RNA (nt 1,046–1,070).\(^{48}\) The oligonucleotide was 5' end
labeled using $[^{32}P]ATP$ and T4 kinase to a specific activity of $10^6$ dpm/μg and diluted using cold oligonucleotide to a specific activity of about $5 \times 10^5$ dpm/μg. The hybridization was performed as described previously but without formamide. Washes were performed at 48°C in 0.5X SSC, 0.1% SDS for 10 minutes for the Ca$^{2+}$-ATPase probe and in 2X SSC at room temperature for 20 minutes for the 18S probe. The blots were exposed to Cronex 4 film (Du Pont, Wilmington, Delaware) using Quanta III intensifying screens (Du Pont) at $-70^\circ$C. Three different exposure times were chosen to obtain densitometric scans within the linear response range of the radiographic film. The optical density measured using the Ca$^{2+}$-ATPase probe was divided by the optical density obtained using the 18S probe. The relative amount of Ca$^{2+}$-ATPase message over 18S RNA in each sample was calculated as a percentage of the mean value observed for sham-operated animals.

S1 nuclease mapping analyses were performed with different probes covering most of the Ca$^{2+}$-ATPase mRNA sequence. Uniformly labeled, single-stranded probes were obtained following the protocol recommended by New England Biolabs (Beverly, Massachusetts) by transcribing single-stranded M13 templates using either the universal M13 primer or oligonucleotides synthesized from a known sequence. The M13 DNA was then linearized using a restriction endonuclease located 3' to the newly synthesized strand. The labeled fragment was dissociated from the M13 template on a 6% acrylamide gel. A double-stranded, 3' end-labeled probe was obtained by filling in the EcoRI sites with $[^{32}P]dATP$ and $[^{32}P]dTTP$ using DNA polymerase (Klenow Enzyme, Boehringer, Mannheim, FRG). Hybridization of the probes with total RNA was performed as previously described.49 S1 nuclease digestion was performed at 25°C C for 1 hour using 10 units S1 nuclease (Biolabs or Amersham) per microgram of RNA. The DNA-RNA hybrids were separated on 6% acrylamide sequencing gels.

**Statistical Analysis**

The statistical significance of differences between the various groups was determined by one-way analysis of variance and group-to-group comparison by Student's $t$ test. Correlations were tested by linear regression analysis using the least-squares method. The test was considered significant for $p<0.05$. The values presented are mean±SD unless otherwise indicated.

**Results**

**Cardiac Hypertrophy**

Thirty days after abdominal aortic constriction, varying degrees of increase in cardiac mass could be demonstrated in these rats (Table 1). Subgroups IA and IIA did not differ significantly from the corresponding B groups, whereas the LVW/BW ratio was slightly higher in group IIA than in group IIB.

**Ca$^{2+}$ Uptake and Acylphosphate Intermediate Determination in Crude Homogenates**

The absolute values of Ca$^{2+}$ transport are presented in Table 2. The rate of Ca$^{2+}$ uptake, expressed in milligrams of protein, was decreased to 74.7±12.7% in moderate hypertrophy, and in severe hypertrophy

<table>
<thead>
<tr>
<th>TABLE 1. Characterization of Cardiac Hypertrophy</th>
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<td><strong>Group A</strong></td>
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<td>Subgroup</td>
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<tr>
<td>GI A</td>
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<td>(n=6)</td>
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<td>GII A</td>
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<td>GIII A</td>
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<th><strong>Group B</strong></th>
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<td>GI B</td>
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<td>$F$</td>
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<td>NS</td>
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Data are expressed as mean±SD. NS, not significant.

The significance of the $F$ test done for the three groups is indicated below $F$ values. When significant, a $t$ test was performed between each group of hypertrophy and the group of sham. *p<0.001.

<table>
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<tr>
<th>TABLE 2. Ca$^{2+}$ Uptake and E-P Levels in Control and Hypertrophied Hearts</th>
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<tr>
<td><strong>Ca$^{2+}$ uptake</strong></td>
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<td>(nmol Ca$^{2+}$/mg protein/min)</td>
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<td>GI A</td>
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<td>&lt;0.001</td>
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For each animal, the uptake and phosphorylated intermediate of the enzyme (E-P) level was the mean value of three determinations; $n$=number of animals in each group. Values are mean±SD.

Significance is indicated below the $F$ values and was determined using Student's $t$ test for each mean of hypertrophied heart group vs. mean of sham-operated group.

* $p<0.001$.
† $p<0.05$.
‡ $p<0.01$. 
the activity decreased further to 55.5±10% of the value observed in the sham-operated animals (p<0.001 vs. sham for both groups).

To determine which protein of the rat heart homogenate is phosphorylated at 0°C in a Ca2+-dependent manner, phosphorylated proteins were separated electrophoretically at acidic pH, which stabilizes acylphosphate bonds. In the presence of calcium ions, 32P was incorporated into two major components of approximate molecular weights 100 and 50 kDa (Figure 1). In the EGTA-containing medium, only the low-molecular weight component was phosphorylated. Since phosphorylation of the 50-kDa component was much less pronounced in microsomal preparations, as already pointed out (Reference 41 and unpublished observations [D.L.]), we concluded that this phosphorylated product did not belong to the sarcoplasmic reticulum membranes. The position of the component phosphorylated in the Ca2+-dependent manner corresponds to that of a Ca2+-ATPase polypeptide as demonstrated by analysis of the slabs stained with Coomassie brilliant blue. Thus, the level of Ca2+-dependent phosphorylation in the homogenate reflected the amount of phospho-enzym e formed in the SR Ca2+-ATPase reaction.

The amount of this phosphorylated protein, which corresponds to the steady-state level of Ca2+-dependent phospho-enzyme, dropped from 69.7±8.2 pmol P/mg protein in shams to 58.8±7.4 and 48.1±13.5 (p<0.05 and p<0.01) in groups II and III, respectively (Table 2). The Ca2+ uptake and the number of phosphorylated ATPases obtained for all the animals in the three groups are linearly correlated (r=0.75) (Figure 2), thus suggesting that the decrease in SR function was due to alterations of the Ca2+-ATPase rather than to leakiness of the membrane. The diminished concentration of phosphorylated ATPase molecules suggested a lower density of pumps although the possibility of the expression of other isofoms with a lower activity could not be excluded.

**Qualitative Analysis of the Ca2+-ATPase mRNA of Hypertrophied Hearts**

To look for the presence of a new isofom of ATPase mRNA, two different techniques were used: RNA blot analysis and S1 nuclease mapping analysis.

**RNA blot analysis.** The 5' end portion of the rat cardiac Ca2+-ATPase cDNA (nt -50 to +1,445) was used to probe an RNA blot containing 10 μg of total RNA from control, sham-operated, and operated animals (Figure 3). Whatever the physiological state of the heart, only one size of Ca2+-ATPase mRNA was detected. This mRNA was approximately 4 kb long (it migrates just below the 28S RNA), which corresponds to the main Ca2+-ATPase mRNA species reported for rabbit35 and rat36,50 heart. The 3.7-kb Ca2+-ATPase mRNA described exclusively in fast skeletal muscle35,39 was never detected in our experiments. The lower signals correspond to hybridization with the 18S RNA probe and represent the amount of RNA loaded for each sample. It is apparent from Figure 3 that the same amount of total RNA was loaded on each lane; however, the intensity of the hybridization signal with the Ca2+-ATPase probe was lower in hypertrophied than in control hearts.
thus suggesting a relative decrease in the level of the Ca\(^{2+}\)-ATPase mRNA (see below).

11 nuclease mapping analysis. RNA samples from ventricles of sham-operated and pressure-overloaded animals were hybridized to a uniformly labeled, single-stranded 5' end probe (Figure 4A). This 1,100-bp probe extends from nt +858 to nt -207 of the Ca\(^{2+}\)-ATPase mRNA and contains 35 bp of polylinker. The protected fragments obtained with RNA from hypertrophied and sham-operated animals were of the same size as that obtained with RNA from controls which was, as expected, a little smaller (1,065 bp) than the probe itself. Identical results were obtained with a longer 5' end probe (nucleotides +1,093 to -207, not shown). Thus, the Ca\(^{2+}\)-ATPase mRNAs expressed in both normal and hypertrophied hearts share the same 5' end sequence.

The 3' portion of the mRNA was characterized using a double-stranded, 2.4-kb fragment covering nt +1,446 to the end of the mRNA, 3' end labeled at the EcoRI site (Figure 4B). We verified that in the hybridization conditions adopted, there was no reannealing of the two strands of the probe, since no radioactive fragment was detected when Escherichia coli transfer RNA was used. A fully protected fragment was observed whether the RNA was isolated from normal or hypertrophied hearts, and identical results were obtained with a single-stranded, uniformly labeled fragment covering nucleotides +2,622 to 3,307. Taken together, these data indicate that the same Ca\(^{2+}\)-ATPase mRNA is expressed in both normal and hypertrophied cardiac ventricles.

Relative Level of Accumulation of Ca\(^{2+}\)-ATPase mRNA and Protein

The expression of the Ca\(^{2+}\)-ATPase gene was quantitated by measuring the accumulation of both the mRNA and the corresponding protein. Dot blots of total cardiac RNA from sham-operated and pressure-overloaded animals of group B were probed with the same Ca\(^{2+}\)-ATPase cDNA fragment that was used in Figure 3 and then with an oligonucleotide specific to the ribosomal 18S RNA in order to calculate the relative amount of Ca\(^{2+}\)-ATPase message compared with 18S RNA. No significant change in the relative level of Ca\(^{2+}\)-ATPase mRNA was observed in moderate hypertrophy. By contrast, in severe hypertrophy the proportion of mRNA decreased to 68.7±12.9% of the value observed in shams (p<0.001) (Table 3).

The amount of Ca\(^{2+}\)-ATPase molecules present in the homogenates of rat hearts of group A was determined by immunooquantitation. The specificity of the immunologic reaction is presented in Figure 5, which shows that the radioactivity detected on dot blots represents the specific binding of the Ca\(^{2+}\)-ATPase antibody. Figure 5 (left panel) is an example of the quantitation of the ATPase in homogenates from one control and one hypertrophied heart, using purified rabbit skeletal ATPase as a standard. The results of such quantitation for all the animals are summarized in Table 3. No significant change was observed in subgroup II, whereas the amount of ATPase dropped to 80.1±15.5% (p<0.05) of the control value in subgroup III (Table 3).

Comparison of Function of Ca\(^{2+}\)-ATPase and Levels of Accumulation of the Enzyme and Its mRNA

Figure 6 summarizes the data concerning the velocity of Ca\(^{2+}\) uptake (in nanomoles of Ca\(^{2+}\) per milligram of protein per minute), the concentrations of active Ca\(^{2+}\)-ATPase molecules (E-P, in picomoles...
P per milligrams of protein), of total Ca²⁺-ATPase molecules (as a proportion of total protein), and of its mRNA (as a proportion of the 18S RNA) in the hypertrophied compared with control hearts. It emerges from these data that the relative decrease in Ca²⁺ uptake observed in severe hypertrophy is accompanied by a relative decrease in phosphorylated enzyme and in Ca²⁺-ATPase gene expression measured at both the mRNA and protein levels. By contrast, in moderate hypertrophy there is a decrease in Ca²⁺ transport, with no changes in the expression of the Ca²⁺-ATPase gene.

Because the Ca²⁺ transport measurements and the quantification of the pumps were carried out on total homogenates, we could calculate the absolute Ca²⁺ transport and the level of expression of the gene per ventricle. In the severely hypertrophied hearts, the rate of Ca²⁺ transport per left ventricle was the same as in controls (1.17±0.1 and 1.21±0.2 μmol Ca²⁺/LV/min in subgroups I and III, respectively). The number of phosphorylated ATPase molecules per ventricle is slightly increased in group III compared with sham (8.75±1.13 vs. 6.4±0.68 nmol P/LV, respectively, p<0.05). The total number of ATPase molecules also increased (148.85±26.6% vs. 98.5±9.43%, respectively, p<0.01), but the absolute level of Ca²⁺-ATPase mRNA calculated by multiplying the percentage of remaining Ca²⁺-ATPase mRNA (68.7%) by the increase in cardiac mass (152.8%) was unchanged (104.4%).

Discussion

The issues we have addressed in this study are determining whether changes in SR Ca²⁺-ATPase can account for the alteration of Ca²⁺ movements observed in pressure overload–induced cardiac hypertrophy and identifying the possible mechanisms leading to such changes. We show that the Ca²⁺ transport capacity of SR per unit mass (or per milligram of protein) is reduced, while the total capacity per heart is unchanged. In severe hypertrophy this can be accounted for by a relative modulation of the level of accumulation of a single Ca²⁺-ATPase isoform.

Previous data have demonstrated either decreased20–26 or enhanced27 Ca²⁺ transport and Ca²⁺-ATPase activities of microsomal preparations purified from hypertrophied hearts. The yield of proteins varied, however, in the microsomal preparations from normal and hypertrophied hearts.24,25,51 Moreover, when standard isolation procedures are used, a...
major fraction of the Ca\(^{2+}\) pump system remains in the pellet after low-speed centrifugation. Thus, micromosal preparations may not represent the total SR fraction in normal and pathological hearts. Furthermore, a significant loss of activity occurs during SR preparation, but the extent of loss in control hearts is different from that in pathological hearts. To avoid such problems in comparing SR Ca\(^{2+}\) transport capacity in normal hearts with that in pathological hearts, a technique has been developed that allows measurement of the SR function on whole homogenates. Oxalate-supported Ca\(^{2+}\) uptake is considered to reflect exclusively the Ca\(^{2+}\) pump activity of sarcoplasmic (endoplasmic) reticulum of the cell. Velocity of the Ca\(^{2+}\) transport in the presence of oxalate is linear for several minutes, and the uptake surpasses Ca\(^{2+}\) accumulation by the SR in the absence of precipitating anions. We thus measured the velocity of ATP-dependent Ca\(^{2+}\) accumulation in the presence of oxalate on whole homogenates from normal and hypertrophied hearts and observed a decrease in the rate of Ca\(^{2+}\) transport (expressed per milligram of protein) in both mild and severely hypertrophied hearts.

Decrease in the Ca\(^{2+}\) transport capacity could be a consequence of modifications at the level of the pump itself, such as a lower concentration of active pumps or changes in the nature of the enzyme. It could also be due indirectly to the pump by a modification of factors that influence the enzyme activity, such as energy supply, phosphorylation of phospholamban, and leakiness of the membrane. Ca\(^{2+}\) pump activity was thus compared with the steady-state level of phosphorylated Ca\(^{2+}\)-ATPase. The latter value reflects the amount of active Ca\(^{2+}\)-ATPase molecules in crude cardiac membrane preparations. A good correlation is observed between the amount of active ATPase and the Ca\(^{2+}\) transport capacity of SR, which is in favor of a modification of the Ca\(^{2+}\) transport due to the Ca\(^{2+}\)-ATPase itself rather than to leakiness of the membrane. Moreover, previous studies have shown that the dependence of the enzyme on the substrate (Ca\(^{2+}\), ATP) does not vary with the physiological or pathological state of the heart.

The decrease in Ca\(^{2+}\) transport thus seems to be due to changes at the level of the ATPase. The lower concentration of phosphorylated pumps in hypertrophied hearts could result from the presence of an ATPase with a different kinetic cycle, possibly due to a different structure. This could also be the consequence of a failure of the hypertrophied cell to accumulate new pump molecules to the same extent as the other proteins.

The existence of several isoforms of Ca\(^{2+}\)-ATPase that differ in their primary structure is now well established and during the development of skeletal muscle three different isoforms are sequentially expressed. The possibility of the existence in the hypertrophied heart of an ATPase of a different primary structure was approached at the mRNA level by S1 nuclease mapping analysis using specific rat heart cDNA probes. This technique, which was sensitive enough to detect a new isoform of Ca\(^{2+}\)-ATPase in smooth muscle, enabled us to conclude here that the Ca\(^{2+}\)-ATPase mRNA was identical in normal and hypertrophied hearts. Similar conclusions were drawn by Nagai et al., who used the same technique to explore the complexity of the Ca\(^{2+}\)-ATPase mRNA in pressure overload induced in rabbits. It should be pointed out that since our probe does not extend to the transcription start site, the possibility of differences in the 5' noncoding region still exists, but this would not change the structure of the enzyme. Thus, in the hypertrophying heart, an isoform switch in Ca\(^{2+}\)-ATPase analogous to those demonstrated for myosin heavy chains, light chains, actin, tropomyosin, and creatine kinase does not occur. During ontogenic development of the heart, and in contrast to what has been shown for skeletal muscle, only one Ca\(^{2+}\)-ATPase isoform is detected (unpublished observation [D.B., A.-M.L.], Reference 56). Moreover, the fast skeletal muscle isoform was not detected in heart during thyrotoxicosis. All these data indicate that, whatever the physiopathologic situation, a single Ca\(^{2+}\)-ATPase mRNA is expressed in heart; posttranslational modifications of the enzyme cannot, however, be ruled out.

We have looked for a quantitative modification in the expression of the Ca\(^{2+}\)-ATPase gene at both the mRNA and the protein levels. A relative decrease in the accumulation of the pump and of its mRNA is observed in severe hypertrophy; however, the absolute level of accumulation is unchanged or only slightly increased. Hypertrophy of the adult heart occurs without any cell division; therefore, the
number of ATPase molecules per cell in hypertrophied myocytes remains similar to its value in controls. This has to be related to morphological studies which have shown that, among the numerous alterations in cellular morphology that occur during myocyte hypertrophy, the smooth endoplasmic reticulum and T-system components retain their normal conformations and remain normally distributed around the contractile network, but both their volume and membrane surface area are significantly increased (×2).62,63 Thus, since the surface of the SR membrane doubled while the total number of ATPase molecules increased at most by 48.8±26.6%, we concluded that the density of the Ca\(^{2+}\) pumps was decreased. The level of accumulation of the mRNA is parallel to that of the protein, which suggests a pretranslational level of regulation. The expression of the Ca\(^{2+}\)-ATPase gene does not, however, follow the overall increase in gene expression, which results in a decrease in the relative proportion of ATPase molecules. Other factors such as thyroid hormone can, however, upregulate the expression of the SR Ca\(^{2+}\)-ATPase gene and increase the level of accumulation of the Ca\(^{2+}\)-ATPase mRNA.50,56

The absence of any change in the relative level of accumulation of both mRNA and protein in moderate hypertrophy (<40%), in which the SR Ca\(^{2+}\) transport and the number of ATPases phosphorylated were depressed, could be due to a difference in the sensitivity of the technique used to measure the function of the ATPase and the expression of the gene. In fact, a 15–25% decrease in the rate of Ca\(^{2+}\) uptake and in the E-P level, as described in moderate hypertrophy, is approximately the limit of sensitivity of the dot blot technique. Another regulatory mechanism, such as a lower activation of the ATPase by phospholamban, could also occur. Phospholamban when phosphorylated increases the SR Ca\(^{2+}\)-ATPase activity in intact myocardium by increasing the affinity and decreasing the \(K_m\) for Ca\(^{2+}\) (for review, see Reference 64). Phosphorylation occurs via several kinases (for review, see Reference 65) and especially the cyclic AMP (cAMP)-dependent kinase. The decrease in density of the \(\beta\)-receptors described in the heart after aortic constriction66 could influence the activity of the Ca\(^{2+}\)-ATPase through a lesser activation of the cAMP-dependent protein kinase. Limas and Cohn23 proposed that the decreased Ca\(^{2+}\) accumulation in cardiac SR from spontaneously hypertensive rats might be explained by the reduced cAMP-dependent protein kinase activity since a strong correlation between both parameters was observed. However, phosphorylation by exogenous cAMP and protein kinase of microsomal preparations from aortic stenosis–induced hypertrophied and control rabbit hearts increased Ca\(^{2+}\) uptake in both types of preparations but did not abolish the difference in Ca\(^{2+}\) transport found between them.25

Our results can explain some of the modifications of Ca\(^{2+}\) movements and relaxation characteristics that have been described in hypertrophied cardiac cells. The duration of the intracellular Ca\(^{2+}\) movements, measured using aequorin, are indeed modified in hypertrophied hearts.17,18 Some of these perturbations have been attributed to a dysfunction of both the sarcolemma and the SR. The authors hypothesized that the sarcolemma increased Ca\(^{2+}\) entry, possibly via Ca\(^{2+}\) channels, whereas the SR caused a decreased rate of sequestration of Ca\(^{2+}\).18 The increased number of dihydropyridine receptors observed by Wagner et al67 in hypertrophied hearts from cardiomyopathic hamsters is consistent with an increased Ca\(^{2+}\) entry. In the same model of hypertrophy that we used, Mayoux et al68 observed an increase in the total number of dihydropyridine receptors, while their density is unchanged. The reduced density of Ca\(^{2+}\)-ATPase and the slower Ca\(^{2+}\) uptake that we found are also consistent with SR dysfunction. In addition, in hypertrophied rat heart, the Na\(^{+}\)-dependent Ca\(^{2+}\) influx and efflux are decreased, and the sensitivity of the exchange to Ca\(^{2+}\) is lower than in normal hearts.69 Moreover, in renal hypertension in rat, a decrease in Na\(^{+}\)-Ca\(^{2+}\) exchange and in sarcolemmal Ca\(^{2+}\)-ATPase is also described.70 This could also contribute to a modification of the Ca\(^{2+}\) movements. On the other hand, myothermal measurements have shown that the tension-independent heat (the heat liberated per gram of tissue by Ca\(^{2+}\) cycling) is decreased in hypertrophied cardiac muscle (for review, see Reference 19). A reduction in the density of the Ca\(^{2+}\) pump molecules by decreasing the energy used per unit mass would contribute to the decrease in heat production. Moreover, the reduction in SR Ca\(^{2+}\) pump density could be one of the mechanisms that contribute to the reduction in relaxation rate of hypertrophied heart fibers. In hypertrophied rat heart, the relaxation rate is reduced but still load sensitive, as in the normal heart. By contrast, pressure overload–induced hypertrophy in guinea pigs results in the disappearance of the sensitivity of relaxation to the loading conditions.8 It has been hypothesized that in rat heart the SR is still functional, whereas in guinea pig heart there is a major impairment of Ca\(^{2+}\) reaccumulation by the SR. The modifications we have observed might thus be species-specific and even more important in other species, such as guinea pigs or humans.

In conclusion, our results indicate that at least in severe hypertrophy, the expression of the Ca\(^{2+}\)-ATPase gene does not follow the overall increase in gene expression that results in cardiac growth. This leads to a decrease in the density of the Ca\(^{2+}\) pumps, which results in an alteration in the function of the SR and in an impairment of Ca\(^{2+}\) movements in the hypertrophied myocardium. Other mechanisms are also probably involved in the modification of the function of SR during moderate hypertrophy.

Our data are in general agreement with two very recently published papers. Komuro et al71 showed that 1 month after induction of pressure overload in rat, the rate of SR Ca\(^{2+}\) transport was decreased by
24%. This was accompanied by a parallel decrease (35%) in the density of Ca\(^{2+}\) pumps and by a very marked diminution (70%) of the Ca\(^{2+}\)-ATPase mRNA. In rabbit, Nagai et al\(^{56}\) demonstrated that the same Ca\(^{2+}\)-ATPase isofrom is expressed in control and pressure-overloaded hearts and that the level of its mRNA is decreased in hearts from operated as compared with sham-operated animals.

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Function of the sarcoplasmic reticulum and expression of its Ca2(+-)-ATPase gene in pressure overload-induced cardiac hypertrophy in the rat.

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