

Molecular Cloning and Characterization of a $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent Adenosine Triphosphatase from Rat Cardiac Sarcoplasmic Reticulum

Regulation of its Expression by Pressure Overload and Developmental Stage

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Abstract

To investigate the regulation of expression of cardiac $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase (Ca^{2+} -ATPase) in sarcoplasmic reticulum (SR), we isolated cDNA (pHA6) encoding a Ca^{2+} -ATPase of rat cardiac SR. The clone consisted of 2,311 mRNA-derived nucleotides, which covered half the coding region and the entire 3'-untranslated regions. The nucleotides and deduced amino acid sequences of pHA6 showed striking homology, 89 and 98%, respectively, to those of rabbit Ca^{2+} -ATPase of the slow-twitch form. Northern blot analyses revealed that the mRNA levels of Ca^{2+} -ATPase were decreased by pressure overload and became 32% of sham in 1 mo. During the developmental stage the mRNA levels were very low in the fetal period and steeply increased around birth. These changes in mRNA levels were correlated with the corresponding protein levels. These results suggest that the expression of cardiac Ca^{2+} -ATPase in SR is regulated by pressure overload and the developmental stage, at least in part, at the pretranslational level.

Introduction

Intracellular calcium (Ca^{2+}) plays a substantial role in myocardial contraction, and its concentration is determined by the Ca^{2+} flux across the sarcolemma, together with Ca^{2+} release and uptake by the intracellular organelles (1). Cardiac sarcoplasmic reticulum (SR) is the major determinant that sequesters intracellular Ca^{2+} and influences the relaxation and the tension development of myocardium. Calcium uptake by the SR is driven by a $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase (Ca^{2+} -ATPase),¹ which is a membrane protein with a molecular mass of 100,000 D and constitutes 35–40% of the protein in cardiac SR (2, 3).

Many changes in mechanical performance and excitation-contraction coupling phenomena have been observed in the

hypertrophied heart. In cardiac hypertrophy induced by pressure overload, there is a decrease in the maximal velocity of shortening and in peak isometric tension development, and a prolonged duration of isometric contraction and time-to-peak tension (4, 5). Abnormalities of the diastolic properties of the left ventricle, especially diastolic relaxation and compliance, also exist under cardiac hypertrophy (6–8). The decreased velocity of shortening could be explained by the isozymic transition of cardiac myosin (9), whereas prolonged duration of isometric contraction and a decrease in peak isometric tension development were thought to be due to impaired Ca^{2+} handling. Since a prolonged duration of isometric contraction in the hypertrophied muscles correlated with a similar prolongation of the calcium transient, Gwathmey and Morgan thought that the rate of sequestration and perhaps release of Ca^{2+} by intracellular stores is decreased in hypertrophy (10). Many studies have shown the depression in Ca^{2+} uptake and Ca^{2+} -ATPase of SR in hypertrophied hearts (11–13); however, there have been quite a few reports concerning the mRNA levels of Ca^{2+} -ATPase. In addition, although it is generally accepted that there are many differences in myocardial function between fetus and adult (14), developmental regulation of the cardiac Ca^{2+} -ATPase of SR remained unclear.

Recently, MacLennan et al. have cloned and sequenced cDNA encoding a Ca^{2+} -ATPase of rabbit skeletal muscle SR (15–17). In the present study, to know the regulation of expression of cardiac Ca^{2+} -ATPase, we have isolated and characterized the cDNA encoding a Ca^{2+} -ATPase of rat cardiac SR, and using the cDNA as a probe we investigated its expression in hearts during hypertrophy and development at the mRNA level. In addition, we prepared SR fractions and examined the changes of Ca^{2+} -ATPase content and Ca^{2+} uptake at the protein level.

Methods

Screening of the rat heart cDNA library. Rat cardiac Ca^{2+} -ATPase cDNA was identified from the Sprague-Dawley rat heart cDNA library, purchased from Clontech Laboratory Inc., Palo Alto, CA., by the phage plaque hybridization method. In brief, the library was screened with Pst I/Pst I restriction fragment of rabbit Ca^{2+} -ATPase cDNA plasmid (15), which was a generous gift from Dr. MacLennan, University of Toronto, Toronto, ON, as a probe. Probes were prepared by using the random-priming procedures. Phages were plated at a density of 1.5×10^4 /150-mm plate, and plaques were transferred to nitrocellulose filters. Approximately 1.5×10^5 plaques were screened with the ^{32}P -labeled cDNA probe. Hybridization with ^{32}P -labeled cDNA (1×10^7 cpm/ml) was performed at 42°C for 24 h in a solution containing 40% (vol/vol) formamide, 2× standard saline citrate (SSC) ($1 \times \text{SSC}$ is 150 mM NaCl, 15 mM sodium citrate), 5× Denhardt's solution ($1 \times \text{Denhardt's}$ solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 0.1% SDS, 1 mM EDTA, and 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA. Filters were washed to a stringency of $0.1 \times \text{SSC}$,

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1. Abbreviations used in this paper: Ca^{2+} -ATPase, $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase; SR, sarcoplasmic reticulum; TES, *N*-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid.

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0.1% SDS at 42°C, and exposed for 24 h at -70°C to XAR-5 film (Eastman Kodak Co., Rochester, NY). Positive recombinant phages were purified by sequential screening at low plaque density. Phage DNA was purified according to Maniatis et al. (18). Eco RI-excised cDNA inserts were subcloned into a plasmid vector pUC13 and characterized by restriction endonuclease mapping.

cDNA sequence analysis. Appropriate restriction fragments were isolated from agarose gels and subcloned either into bacteriophage M13 or plasmid vector pUC19. DNA sequencing was performed by the dideoxynucleotide chain-termination method of Sanger et al. (19).

Animals and surgical procedures. To make pressure overload-induced hypertrophy, male Wistar rats (40 d old, weighing 150–180 g) were anesthetized with diethyl ether and the upper part of the abdominal aorta was constricted with a hemoclip. Of 204 operated rats, 158 survived the procedures and were killed at predetermined times after the operation (0.5, 2, 4, 8, 12, 24, 48, and 72 h, 1 wk, and 1 mo; $n = 16, 18, 16, 18, 14, 18, 15, 13, 15,$ and $15,$ respectively). Sham-operated animals underwent identical procedures except for placement of the hemoclip and were killed after different time intervals postoperatively. To investigate the developmental change, hearts of 12-, 15-, and 18-d embryos, 5-d-old neonates, and 40- and 200-d-old adults were examined.

RNA preparation. The body weight was measured and the hearts were excised. The atria, great vessels, and right ventricular free walls were removed. The left ventricles were opened, rinsed with cold saline, weighed, and quickly frozen in liquid nitrogen. Total cellular RNA was extracted from three or four left ventricles by the lithium urea method (20). Poly(A⁺)RNA was enriched by oligo(dT)-cellulose chromatography. Total RNA was also extracted from soleus and hindlimb muscles of 40-d-old rats.

Northern blot analysis. 20 µg of total RNA or 3 µg of poly(A⁺)RNA was denatured at 60°C for 7 min, fractionated by electrophoresis through 1.2% agarose gels, and transferred to nylon membrane. Membranes were exposed to ultraviolet rays for 2.5 min, prehybridized, and hybridized at 42°C with ³²P-labeled cDNA probe. Prehybridization was performed in a solution containing 40% formamide, 5× SSPE (0.9 M NaCl, 40 mM NaOH, 50 mM Na₂HPO₃, 5 mM EDTA) buffer, 5× Denhardt's solution, 1% SDS, 10% dextran sulfate, and 100 µg/ml of heat-denatured salmon sperm DNA for 24 h at 42°C. Hybridization was performed in the same solution with the addition of 5 × 10⁷ cpm/ml of ³²P-labeled probe for 24 h at 42°C. Membranes were washed twice at 42°C with 2× SSC and 0.1% SDS and twice at 42°C with 0.2× SSC and 0.1% SDS, air dried, and exposed to x-ray film for 24 h at -70°C with intensifying screen. Relative amounts of Ca²⁺-ATPase expression were determined by a densitometric scanner. Control hybridizations were carried out using a 1.1-kb mouse α -actin cDNA (21).

Isolation of SR, gel electrophoresis, and assay of Ca²⁺ uptake. SR was isolated using a method of Nakanishi and Jarmakani (14). Eighty 15-d-old fetal hearts, four 40-d-old adult hearts that received pressure overload for 2 wk or 1 mo, and four sham-operated hearts were used for SR isolation. The left ventricles were minced with scissors and homogenized three times with a polytron tissue processor at a setting of 2 for 5 s in 4 vol of the isolation solution containing 10 mM Na₂HCO₃ (pH 7.1 with 0.1 N HCl). The homogenate was centrifuged at 1,000 g for 10 min. The supernatant was centrifuged at 14,000 g for 20 min three times to remove mitochondria. The supernatant from the third spin was centrifuged at 45,000 g for 30 min. The pellet was suspended in a solution containing 0.6 M KCl and 10 mM *N*-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid (TES; pH 7.1) using a glass-Teflon homogenizer. The suspension was centrifuged at 45,000 g for 30 min. The resulting pellet was suspended in solution containing 150 mM KCl and 1 mM TES (pH 7.1) and used as SR fractions. The SR fractions were separated by electrophoresis with 7.5% acrylamide according to the procedure of Laemmli (22) and stained with Coomassie blue. The density of the protein band corresponding to the 100,000-D Ca²⁺-ATPase protein was measured by a densitometric scanner. Ca²⁺ uptake by SR was measured by the Millipore technique

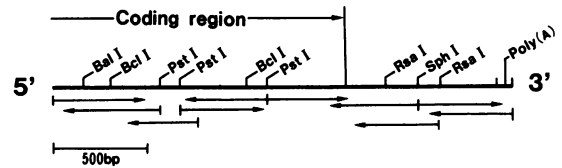


Figure 1. Restriction map and sequencing strategy for the cDNA insert of clone pHA6. The extent and direction of sequencing are indicated by the arrows. Sequence was determined by the dideoxy methods.

as in Ito et al. (13) at 25°C in buffer containing 10 mM Tris-histidine (pH 7.2), 5 mM MgCl₂, 100 mM KCl, 5 mM sodium azide, 5 mM sodium oxalate, 50 µM CaCl₂, 50 µg of SR protein, and 5 mM sodium ATP. Na⁺-K⁺-ATPase activity and cytochrome *c* oxidase activity were measured as marker enzymes by the method of Bers (23) and Wharton and Tzagoloff (24), respectively.

Results

Isolation of rat cardiac Ca²⁺-ATPase clones. Approximately 110 positive clones were identified from among ~ 1.5 × 10⁵ recombinant phages. The Eco RI-digested fragments from nine of these clones were subcloned into the Eco RI restriction site of plasmid pUC13. These clones contained long inserts (> 1,800 bp) and had the same restriction maps in regions where they overlapped. Then two clones, designated pHA6 and pHA12, were subjected to sequencing.

Restriction mapping and sequence analysis of pHA6 clone. Fig. 1 shows a restriction endonuclease cleavage map and sequence strategy for the pHA6 clone. Nucleotide and deduced amino acid sequences are shown in Fig. 2. Clone pHA6 consists of 2,311 mRNA-derived nucleotides, which encode half the amino acid of Ca²⁺-ATPase, including the entire 3'-untranslated region and a portion of the poly(A⁺) tail. Clone pHA12 consists of 2,166 mRNA-derived nucleotides, and they were identical to those of the pHA6 clone. Fig. 3 shows the amino acid comparison between the pHA6 clone and rabbit skeletal Ca²⁺-ATPase clones. The extensive homology was recognized between pHA6 and rabbit slow-twitch skeletal Ca²⁺-ATPase (pCA4; 15). In coding regions, 89% of the nucleotide positions were identical and the corresponding amino acid positions for this region showed 98% homology. Furthermore, even in the untranslated regions, there was high homology between cardiac and slow-twitch skeletal Ca²⁺-ATPase, in spite of the species difference. Alignment of DNA sequences to obtain maximum homology reveals 88% homology. In contrast, degree of the homology was relatively low between cardiac and fast-twitch skeletal Ca²⁺-ATPase clones (pFA4; 16). Direct comparison of the DNA sequences of clones pHA6 and pFA4 demonstrates 80% nucleotide and 81% amino acid homology within the translated regions. More numerous nucleotide mismatches were observed within the 3'-untranslated region and at least 35% nucleotides were divergent.

Northern blot hybridization analysis of rat muscle mRNA. Fig. 4 A shows Northern blots of adult rat skeletal and cardiac muscle RNA hybridized with the Pst I-Pst I restriction fragment (nucleotide residues 607–1,156) from the coding region of the clone pHA6. The hybridizations were observed with a higher mol wt transcript in cardiac muscle and with the lower mol wt transcript in the hindlimb muscle. Two messages of different sizes bound the probe in the soleus muscle, which

* 1 60*
TCA.CGT.GAT.CGA.AAA.TCA.ATG.TCC.GTC.TAC.TGT.ACA.CCA.AAC.AAA.CCG.AGC.CGG.ACG.TCC
Ser-Arg-Asp-Arg-Lys-Ser-Met-Ser-Val-Tyr-Cys-Thr-Pro-Asn-Lys-Pro-Ser-Arg-Thr-Ser

* 61 120*
ATC.AGC.AAG.ATG.TTT.GTG.AAG.GGT.GCT.CCA.GAA.GGT.GTC.ATC.GAC.AGG.TGC.ACC.CAC.ATC
Met-Ser-Lys-Met-Phe-Val-Lys-Gly-Ala-Pro-Glu-Gly-Val-Ile-Asp-Arg-Cys-Thr-His-Ile

* 121 140*
CGA.GTT.GGA.AGT.AAC.GTC.CCC.ATG.ACG.GCT.GGT.GTT.AAA.CAG.AAG.ATT.ATG.TCT.GTC
Arg-Val-Gly-Ser-Thr-Lys-Val-Pro-Met-Thr-Ala-Gly-Val-Lys-Gln-Lys-Ile-Met-Ser-Val

* 181 240*
ATT.CGG.GAG.TGG.GGC.ACT.GGC.AGC.GAC.ACA.CTG.CGG.TGC.CTG.GCT.CTG.GCC.ACT.CAT.GAC
Ile-Arg-Glu-Trp-Gly-Ser-Gly-Ser-Asp-Thr-Leu-Arg-Cys-Leu-Ala-Leu-Ala-Thr-His-Asp

* 241 300*
AAC.CCG.CTG.AGG.AGA.GAG.GAG.ATG.CAC.CTG.GAG.GAT.TCT.GCG.AAC.TTC.ATT.CAA.TAT.GAG
Asn-Pro-Leu-Arg-Arg-Glu-Glu-Met-His-Leu-Glu-Asp-Ser-Ala-Asn-Phe-Ile-Gln-Tyr-Glu

* 301 360*
ACC.AAT.CTG.ACT.TTC.GTT.GGC.TGT.CTG.GGC.ATG.CTG.GAC.CCT.CCC.AGG.ATT.GAA.CTG.GCC
Thr-Asn-Leu-Thr-Phe-Val-Gly-Cys-Val-Gly-Met-Leu-Asp-Pro-Pro-Arg-Ile-Glu-Val-Ala

* 361 420*
TCT.TCT.GTG.AAG.CTG.TGC.CGG.CAA.CGC.GCC.ATC.CGA.GTC.ATC.ATG.ATC.ACT.GGG.GAT.AAC
Ser-Ser-Val-Lys-Leu-Cys-Arg-Gln-Ala-Ala-Ile-Arg-Val-Ile-Met-Ile-Thr-Gly-Asp-Gly-Val-Asn-Asp

* 421 480*
AAA.GGC.ACT.GCT.GTG.GCC.ATC.TGT.CGC.CGC.ATT.GGC.ATC.TTT.GGG.CAG.GAT.GAG.GAT.CTG
Lys-Gly-Thr-Ala-Val-Ala-Ile-Cys-Arg-Arg-Ile-Gly-Ile-Phe-Gly-Gln-Asp-Glu-Asp-Val

* 481 540*
ACA.TCA.AAG.GCT.TTT.ACA.GGG.CGA.GAA.TTT.GAT.GAA.TTA.AGC.CCC.TCA.GCC.CAG.AGA.GAC
Thr-Ser-Lys-Ala-Phe-Thr-Gly-Arg-Glu-Phe-Asp-Glu-Leu-Ser-Pro-Ser-Ala-Gln-Arg-Asp

* 541 600*
GCC.TGC.TTA.AAT.GCC.CGT.TGT.TTT.GCT.CGA.GTT.GAA.CCT.TCC.CAC.AAG.TCT.AAG.ATC.GTT
Ala-Cys-Leu-Asn-Ala-Arg-Cys-Phe-Ala-Arg-Val-Glu-Pro-Ser-His-Lys-Ser-Lys-Ile-Val

* 601 660*
GAG.TTC.CTG.CAG.TCC.TTT.GAT.GAG.ATC.ACA.GCT.ATG.ACT.GGT.GAT.GGT.GTG.AAC.GAC.GCC
Glu-Phe-Leu-Gln-Ser-Phe-Asp-Gly-Ile-Thr-Ala-Met-Thr-Gly-Asp-Gly-Val-Asn-Asp-Ala

* 661 720*
CCC.GCT.CTG.AAG.AAG.TCG.GAA.ATC.GGG.ATT.GCC.ATG.GGC.TCA.GGG.ACT.GCA.GTG.GCT.AAG
Pro-Ala-Leu-Lys-Lys-Ser-Glu-Ile-Gly-Ile-Ala-Met-Gly-Ser-Gly-Thr-Ala-Val-Ala-Lys

* 721 780*
ACT.GCC.TCT.GAG.ATG.GTG.CTG.GCC.GAC.GAC.AAC.TTC.TCC.ACC.ATC.GTG.GCC.GCT.GTT.GAG
Thr-Ala-Ser-Glu-Met-Val-Leu-Ala-Asp-Asp-Asn-Phe-Ser-Thr-Ile-Val-Ala-Ala-Val-Glu

* 781 840*
GAG.GGG.CGC.GCC.ATC.TAC.AAC.AAC.ATG.AAG.CAG.TTC.ATC.CGC.TAC.CTC.ATC.TCC.TCC.AAC
Glu-Gly-Arg-Ala-Ile-Tyr-Asn-Asn-Met-Lys-Gln-Phe-Ile-Arg-Tyr-Leu-Ile-Ser-Ser-Ser

* 841 900*
GTG.GGG.GAG.GTG.GTC.TGT.ATC.TTC.CTG.ACG.GCA.GCC.CTT.GGG.TTT.CCT.GAG.GCT.TTG.ATT
Val-Gly-Glu-Val-Val-Cys-Ile-Phe-Leu-Thr-Ala-Ala-Leu-Gly-Phe-Pro-Glu-Ala-Leu-Ile

* 901 960*
CCT.GTC.CAG.TTA.CTC.TGG.GTC.AAT.CTG.GTG.ACG.GAT.GGT.CTG.CCT.GCC.ACT.GCG.CTG.GGG
Pro-Val-Gln-Leu-Leu-Trp-Val-Asn-Leu-Val-Thr-Asp-Gly-Leu-Pro-Ala-Thr-Ala-Leu-Gly

* 961 1020*
TTC.AAT.CCT.CCA.GAT.CTG.GAC.ATC.ATG.AAC.AAA.CCC.CCA.CGG.AAC.CCA.AAA.GAA.CCG.TTG
Phe-Asn-Pro-Pro-Asp-Leu-Asp-Ile-Met-Asn-Lys-Pro-Pro-Arg-Asn-Pro-Lys-Glu-Pro-Leu

* 1021 1080*
ATC.AGC.GGG.TGG.CTC.TTT.TTC.CGT.TAC.CTG.GCT.ATT.GGC.TGT.TAT.GTT.GGC.GCT.GCC.ACC
Ile-Ser-Gly-Trp-Leu-Phe-Phe-Arg-Tyr-Leu-Ala-Ile-Gly-Cys-Tyr-Val-Gly-Ala-Ala-Thr

* 1081 1140*
GTG.GGT.GCT.GCT.GCG.TGG.TGG.TTC.ATC.GCT.GCT.GAC.GGT.GGT.CCG.AGA.GTC.TCC.TTC.TAC
Val-Gly-Ala-Ala-Ala-Trp-Trp-Phe-Ile-Ala-Ala-Asp-Gly-Gly-Pro-Arg-Val-Ser-Phe-Tyr

* 1141 1200*
CAG.CTG.AGT.CAT.TTC.CTG.CAG.TGT.AAG.GAG.GAC.AAC.CCA.GAC.TTC.GAA.GGA.GTG.GAT.TGT
Gln-Leu-Ser-His-Phe-Leu-Gln-Cys-Lys-Glu-Asp-Asn-Pro-Asp-Phe-Glu-Gly-Val-Asp-Cys

* 1201 1260*
GCA.ATC.TTT.GAG.TCC.CCG.TAT.CCG.ATG.ACA.ATG.GCA.CTT.TCT.GTT.CTA.GTC.ACC.ATA.GAG
Ala-Ile-Phe-Glu-Ser-Pro-Tyr-Pro-Met-Thr-Met-Ala-Leu-Ser-Val-Leu-Val-Thr-Ile-Glu

* 1261 1320*
ATG.TGC.AAT.GCC.CTC.AAC.AGC.TTG.TCT.GAA.AAC.CAG.TCC.CTG.CTG.AGG.ATG.CCC.CCG.TTG
Met-Cys-Asn-Ala-Leu-Asn-Ser-Leu-Ser-Glu-Asn-Gln-Ser-Leu-Leu-Arg-Met-Pro-Pro-Trp

* 1321 1380*
GAG.AAC.ATC.TGG.CTG.CTG.GGC.TCC.ATC.TGC.TTG.TCC.ATG.TCC.CTT.CAC.TTC.TTG.ATC.CTC
Glu-Asn-Ile-Trp-Leu-Val-Gly-Ser-Ile-Cys-Leu-Ser-Met-Ser-Leu-His-Phe-Leu-Ile-Leu

* 1381 1440*
TAC.GTG.GAA.CCT.TTG.CCA.CTC.ATT.TTC.CAG.ATC.ACA.CCG.CTG.AAT.CTG.ACC.CAG.TGG.CTG
Tyr-Val-Glu-Pro-Leu-Pro-Leu-Ile-Phe-Gln-Ile-Thr-Pro-Leu-Asn-Leu-Thr-Gln-Trp-Leu

* 1441 1500*
ATG.GTG.CTG.AAA.ATC.TCC.CTG.CCT.GTG.ATC.CTC.ATG.GAC.GAG.AGC.CTC.AAG.TTT.GTG.GCC
Met-Val-Leu-Lys-Ile-Ser-Leu-Pro-Val-Ile-Leu-Met-Asp-Glu-Thr-Leu-Lys-Phe-Val-Ala

* 1501 1560*
CGA.AAC.TAC.CTG.GAG.CCT.GCA.ATA.CTG.GAG.TAACCGCTTCTCAACATCGCAAAATGTAAGGGTGTTCG
Arg-Asn-Tyr-Leu-Glu-Pro-Ala-Ile-Leu-Glu
CTTGCCTGATGTCCTTGTGTAGCAACACATCTCCAGCCCTCTGATGACTGACGTGGGGAAGAGAAATAGA
1645 1719
ACAGCCCCCAGCTCACTGTGTGTGTGTGGAGAAATGTGATTACAAGTGGGGTTTATGCTGTGAGTCAAAATAAT
1720 1794
AACAGGTGTACAATTTAGCATAAGAAATCGGAGAGCTCCAGAGAAAGTGGGTTCTTTGCTCGAAGAAAGT
1795 1869
AGGTTCTGAACCTTATCCCAAGAACAGAAGCCATCAGCCCAAGTCTCCACATTTCTCTGCAAAATGTTGAGCCCT
1870 1944
CTATAACTGTATGATAGTGTAAATGCATGCCTCAGTTGTAAGTGGCCAGATCGCGCTACAGTGACATTGAAACC
1945 2019
TGCTCTTAATGGCCCTGTACAGTTGGCTTATTTAAATTCATTTAAACACACACAGCTGTGAATGGTACA
2020 2094
ACCTAGGCCTCCGGTCTCAACTCAAGTGTCTCCCTGGTGGCAGCCAGCTGTCCCAACATGATTTATTGTAA
2095 2169
CTATTTAGTGAAGTCAGAACAGTAGGACAGATGTTGGTGGCAATACAAGTATGTTGTCATTTATCGTAATAA
2170 2244
AGTCGTCGGCTGGGTCAGTTCCTCAGCTTCTCAGTGCATCTGCTGACTGTAGCTGTGTAATAGAGGTCAG
2245 2311
TGTCCTGTCTGAACAGGTATCGATCGCACAGACATGATTCAGTAAATAAATCGATCTCAGAT-poly(A)

Figure 2. Nucleotide sequence and deduced amino acid sequence of clone pHA6. The nucleic acids are numbered beginning at the first triplet of the cloned Ca²⁺-ATPase sequence.

consists of both slow-twitch fibers and fast-twitch fibers. The mRNA level of the cardiac Ca²⁺-ATPase was slightly less than those of the skeletal Ca²⁺-ATPase. In contrast, Northern blot analysis revealed that a cDNA probe from the 3'-untranslated region, Sph I-Eco RI fragment, of pHA6 hybridized only to the higher mol wt transcript in both soleus and cardiac muscle but

did not hybridize to mRNA in hindlimb muscle or to the lower mol wt transcript in soleus muscle (Fig. 4 B).

Northern blot hybridization analysis of Ca²⁺-ATPase in pressure overload-induced hypertrophy. The mean aortic pressure in the aortic-constricted animals showed an increase of 46±6 mmHg after 4 h (n = 4) and 62±6 mmHg after 1 mo (n

pHA6: SRDRKSMVSYCTPNKPSR TSMSKMFVKGAPEGVIDRCTHIRVVGSTKVPMTAGVKQKIMSVIREWGSQSD 70
pCA4: SRDRKSMVSYCTPNKPSR TSMSKMFVKGAPEGVIDRCTHIRVVGSTKVPMTAGVKQKIMSVIREWGSQSD
pFA4: SRDRKSMVSYCTPNKPSR AAVGNKMFVKGAPEGVIDRCTHIRVVGSTKVPMTAGVKQKIMSVIREWGSQSD

TLRCLALATHDNLPLRREEMHLKDSANF I QYETNLTFVCGVGMDDPPRIEVAASSVKLCRQAAIRVIMITGD 140
TLRCLALATHDNLPLRREEMHLKDSANF I KYETNLTFVCGVGMDDPPRIEVAASSVKLCRQAGIRVIMITGD
TLRCLALATHDNLPLRREEMHLKDSANF I QYETDLTFVCGVGMDDPPRIEVAASSVKLCRQAGIRVIMITGD

NKGTAVAI CRRIGIFGQEDVTS KAFTGREFDELSPSAORDACLNARCFARVEPSHKSKI VEF LQSFDEI 210
NKGTAVAI CRRIGIFGQEDVTS KAFTGREFDELSPSAORDACLNARCFARVEPSHKSKI VEF LQSFDEI
NKGTAVAI CRRIGIFGQEDVTS KAFTGREFDULPABORACRRAACCFARVEPSHKSKI VEF LQSFDEI

TAMTGDGVNDAPALKKSEIGIAMSGTAVAKTASEMVLADDNFSTIVA AVEEGRAIYNNMKQFIRYLIS 280
TAMTGDGVNDAPALKKSEIGIAMSGTAVAKTASEMVLADDNFSTIVA AVEEGRAIYNNMKQFIRYLIS
TAMTGDGVNDAPALKKSEIGIAMSGTAVAKTASEMVLADDNFSTIVA AVEEGRAIYNNMKQFIRYLIS

NVGEVVCIFLTAALGFPEALIPVQLLWNLVTDGLPATALGFNPPDLDIMNKPPRNKPEPLISGWLF 350
NVGEVVCIFLTAALGFPEALIPVQLLWNLVTDGLPATALGFNPPDLDIMNKPPRNKPEPLISGWLF
NVGEVVCIFLTAALGFPEALIPVQLLWNLVTDGLPATALGFNPPDLDIMDRPPRNKPEPLISGWLF

LAIGCYVGAATVGA AAWWFIAADGGPRVSYFQLSHFLQCKEDNPDFEGVDCAIFESPYPMTMALSVLVTI 420
LAIGCYVGAATVGA AAWWFIAADGGPRVSYFQLSHFLQCKEDNPDFEGVDCAIFESPYPMTMALSVLVTI
LAIGCYVGAATVGA AAWWFIAADGGPRVSYFQLSHFLQCKEDNPDFEGVDCAIFESPYPMTMALSVLVTI

EMCNALNSLSENQSLRMPWNIWLVGSI CLSMLHLFLILYVEPLPLIFQITPLNLTQWMLVKISLPV 490
EMCNALNSLSENQSLRMPWNIWLVGSI CLSMLHLFLILYVEPLPLIFQITPLNLTQWMLVKISLPV
EMCNALNSLSENQSLRMPWNIWLVGSI CLSMLHLFLILYVEPLPLIFQITPLNLTQWMLVKISLPV

ILMDET LK F VARNYLEPAILE *
ILMDET LK F VARNYLEPAILE *
IGLDET LK F VARNYLEDPEDERK *

Figure 3. Amino acid sequence homology among the rat cardiac and the rabbit slow- and fast-twitch skeletal muscle forms of the Ca²⁺-ATPase. The amino acid sequence of the rat cardiac Ca²⁺-ATPase of SR (pHA6) was compared with those of the rabbit slow- (pCA4) and fast-twitch (pFA4) skeletal Ca²⁺-ATPase (15, 16). The sequence of the cardiac and slow Ca²⁺-ATPase is shifted to the right by one residue at residue 19 to allow realignment after the difference in sequence length. Identical amino acids are boxed.

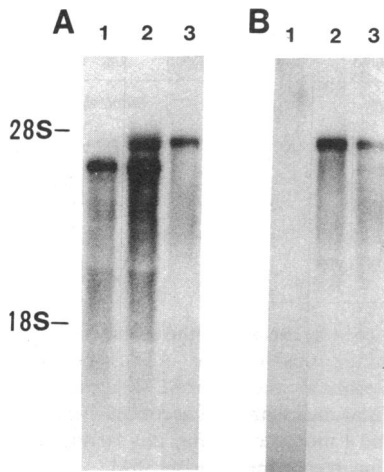


Figure 4. Northern blot analysis of rat muscle mRNA. The coding region, Pst I-Pst I restriction fragment (A), and the untranslated region, Sph I-Eco RI restriction fragment (B), of the pHA6 clone were hybridized with the rat muscle RNA. The same filter was probed sequentially for A and B. Each lane contained 20 μ g of total RNA from hindlimb (1), soleus (2), and cardiac muscle (3), respectively. The size of rRNA is indicated as a marker.

= 4), compared with the sham controls ($n = 4$). The cardiac hypertrophy was already evident at 72 h after the operation. The percent hypertrophy score (mean experimental left ventricular weight - mean sham-operated left ventricular weight)/mean sham-operated left ventricular weight, was 29% at 72 h after the operation, and 59% hypertrophy was observed 1 mo after aortic constriction. The mRNA levels of cardiac Ca^{2+} -ATPase were markedly decreased by pressure overload compared with sham controls (Fig. 5 A). Sham operation did not change the mRNA levels of Ca^{2+} -ATPase (data not shown). The decrease was first detected at 4 h after the operation, gradually progressed to 72 h, and after that the lower level of the expression was continued. By densitometer scanning the mRNA level at 4 h was $82 \pm 5\%$ (mean \pm SEM, $P < 0.05$), and at 1 mo it was $32 \pm 7\%$ ($P < 0.001$) compared with sham (Fig. 5 B). The mRNA levels of Ca^{2+} -ATPase in the hindlimb muscle did not change in the same pressure-overload conditions (Fig. 5 C).

Northern blot hybridization analysis of Ca^{2+} -ATPase during development. Fig. 6 A shows Northern blots of rat cardiac muscle mRNA of different developmental stages hybridized with the Eco RI-Eco RI 2.3-kb fragment. The expression level of Ca^{2+} -ATPase was significantly low during the early embryonic period, and from 2 d before birth it was steeply increased. The mRNA levels in 12-d fetuses and 5-d neonates were $8 \pm 3\%$ and $82 \pm 9\%$, compared with 200-d-old adults by densitometer scanning (Fig. 6 B).

Gel electrophoretic analysis of SR and assay of Ca^{2+} uptake. The yield of the SR protein in the fetus was significantly less than in the adult (0.12 ± 0.08 mg/g in fetus vs. 0.84 ± 0.10 mg/g in 40-d-old adult, $P < 0.01$) and that in the hypertrophied hearts was similar to the sham-operated hearts (2 wk after the operation, 0.89 ± 0.10 ; 1 mo, 0.94 ± 0.16 ; sham, 0.90 ± 0.12 mg/g). Na^+ - K^+ -ATPase activity and cytochrome *c* oxidase activity in the SR fraction were $< 1\%$ of the total activities and were similar in all groups. PAGE of the SR fraction isolated from the hypertrophied hearts showed a marked decrease in the concentration of 100,000-D protein compared with the sham-operated hearts, while the density of the Ca^{2+} -ATPase protein in the fetal SR was similar to that in the adult SR (Fig. 7). Ca^{2+} uptake by SR was determined in the presence of ATP and oxalate. Ca^{2+} uptake was linear up to 5

min, and the value at 5 min was used to calculate the rate of Ca^{2+} uptake. The rate of Ca^{2+} uptake by SR in the hypertrophied hearts (76 ± 5 nmol/mg protein per min) was significantly less than that in the sham-operated hearts (105 ± 6 nmol/mg protein per min), while that in the fetal hearts (96 ± 5 nmol/mg protein per min) was not different from that in the adult hearts (102 ± 7 nmol/mg protein per min; Table I).

Discussion

We isolated and sequenced a 2.3-kb cDNA clone encoding a Ca^{2+} -ATPase of rat cardiac SR. Significant homology was recognized between rat cardiac and rabbit slow-twitch skeletal

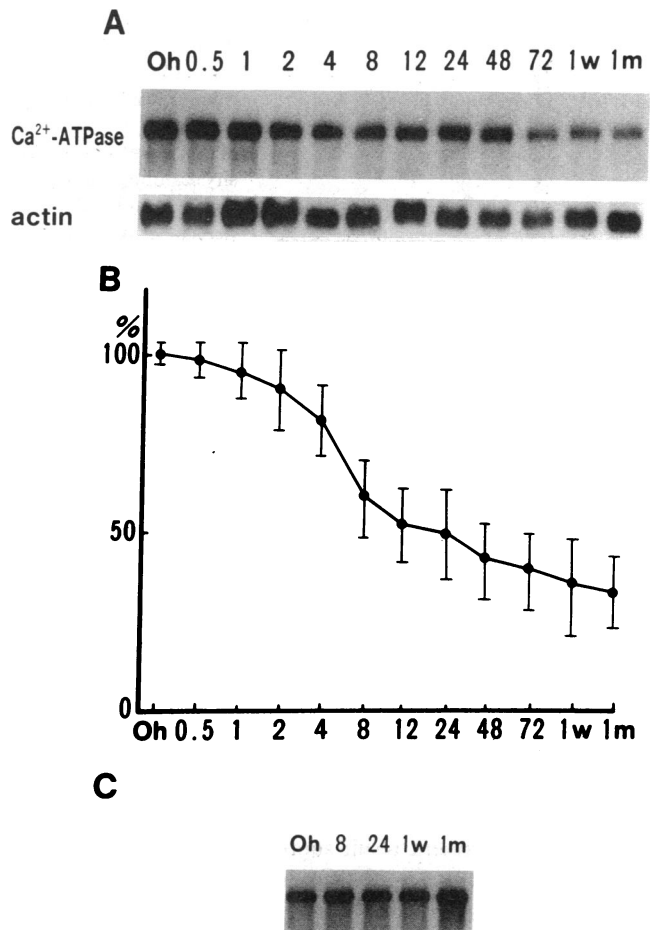


Figure 5. Northern blot analysis of Ca^{2+} -ATPase in pressure overload cardiac hypertrophy. (A) The aorta of male Wistar rats was constricted with a hemoclip. The rats were killed at the indicated times (0, 0.5, 2, 4, 8, 12, 24, 48, and 72 h, 1 wk, and 1 mo) after the operation and RNA was extracted from the hearts. 3 μ g of poly(A)⁺ RNA was separated on 1.2% agarose gel, blotted on nylon membrane, and hybridized with the Eco RI-Eco RI 2.3-kb fragment (pHA6). The membranes were exposed to x-ray film for 24 h at -70°C with intensifying screen (top). The same RNA blot was sequentially hybridized with mouse α -actin cDNA probe to show the integrity of RNA samples (bottom). (B) Relative amounts of Ca^{2+} -ATPase expression were determined by soft laser density scanning. Values are expressed as mean \pm SEM percent of sham control from four separate experiments. (C) The pHA6 cDNA clone was hybridized with the hindlimb muscle RNAs, which were extracted from control rats and pressure-overloaded for 8 and 24 h, 1 wk, and 1 mo.

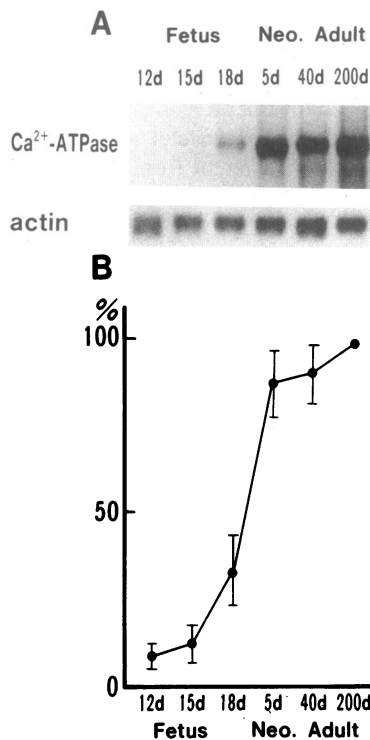


Figure 6. Northern blot analysis of Ca^{2+} -ATPase during development. (A) Hearts of 12-, 15-, and 18-d embryos (Fetus 12 d, 15 d, and 18 d), 5-d-old neonates (Neo. 5 d), and 40- and 200-d-old adults (Adult 40 d and 200 d) were examined. 3 μg each of poly(A⁺) RNA were blotted on each lane and hybridized as described in Fig. 5 A. (B) Relative amounts of Ca^{2+} -ATPase expression were determined compared with 200-d-old adults by soft-laser density scanning. Results are the mean \pm SEM percent of 200-d-old adults for four experiments.

muscle Ca^{2+} -ATPase, whereas the homology between cardiac and fast-twitch skeletal Ca^{2+} -ATPase was relatively low. The mRNA and protein levels of cardiac Ca^{2+} -ATPase decreased by pressure overload. In the developmental stage, the mRNA levels of Ca^{2+} -ATPase and the yields of SR fractions were quite low in the fetal period and steeply increased around birth.

Sequence analyses showed the striking homology, including the 3' untranslated region, between cardiac and slow-twitch skeletal muscle Ca^{2+} -ATPase despite the species difference. Many reports showed the similarities between cardiac and slow-twitch skeletal muscle Ca^{2+} -ATPase, in their concentration in SR (25), its Ca^{2+} transport activity, lower ATPase activity (26), and the existence of the regulatory protein, phospholamban (27). MacLennan et al. suggested the identity between cardiac and slow-twitch muscle Ca^{2+} -ATPase from the same restriction endonuclease map and partial sequence analysis (16, 17). The present result supports these observations and furthermore shows that the Ca^{2+} -ATPase gene is highly conserved throughout evolution.

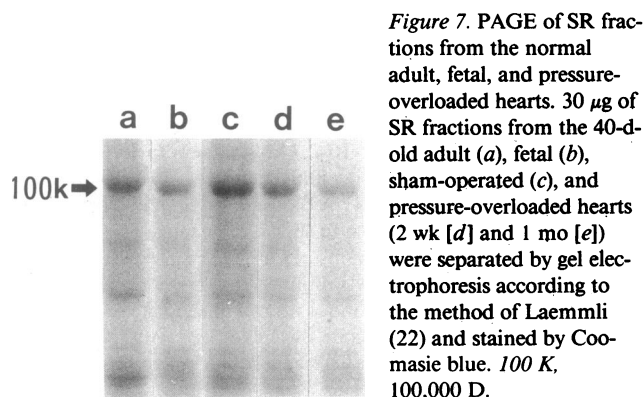


Figure 7. PAGE of SR fractions from the normal adult, fetal, and pressure-overloaded hearts. 30 μg of SR fractions from the 40-d-old adult (a), fetal (b), sham-operated (c), and pressure-overloaded hearts (2 wk [d] and 1 mo [e]) were separated by gel electrophoresis according to the method of Laemmli (22) and stained by Coomassie blue. 100 K, 100,000 D.

Table I. Yield of SR Fractions, Ca^{2+} -ATPase Content, and Ca^{2+} Uptake

	SR yield	Ca^{2+} -ATPase content	Ca^{2+} uptake
	mg/g	%	nmol/mg per min
Control	0.84 \pm 0.10	100	102 \pm 7
Sham	0.90 \pm 0.12	112 \pm 10	105 \pm 6
2 wk after banding	0.89 \pm 0.10	92 \pm 7	96 \pm 8
1 mo after banding	0.94 \pm 0.16	73 \pm 8	76 \pm 5
15-d fetus	0.12 \pm 0.08	90 \pm 6	96 \pm 5

Ca^{2+} -ATPase content was assessed by measuring the density of the protein band corresponding to the 100,000-D Ca^{2+} -ATPase protein by a densitometric scanner. Results are the mean \pm SEM percent of control. Control, 40-d-old Wistar rats; sham, rats sacrificed 1 mo after sham operation; 2 wk and 1 mo after banding, rats sacrificed 2 wk or 1 mo after aortic banding, respectively, mean \pm SEM.

* $P < 0.01$, † $P < 0.05$.

In pressure overload-induced hypertrophy, there is now much evidence that not only contraction force but also myocardial relaxation are impaired (4–8). Gwathmey and Morgan reported that the hypertrophied myocardium demonstrated a prolonged duration of isometric contraction that correlated with a similar prolongation of the calcium transient. They interpreted that the rate of sequestration and perhaps release of calcium by intracellular stores was decreased in the hypertrophied hearts (10). There have been many studies on the SR function in states of cardiac hypertrophy. Although SR function was dependent on the species and ages of the experimental animals, or the duration and the degree of pressure overload, most studies showed the depression in calcium uptake and calcium ATPase of SR in the hypertrophied hearts (11–13, 28, 29). Suko et al. showed the reduced calcium uptake and Ca^{2+} -ATPase in microsomes prepared from calf right ventricles with 6- to 8-wk pulmonary hypertension (11). Lamers and Stinis reported a significant reduction of microsomal Ca^{2+} uptake in rabbit hearts by aortic constriction for 1 mo (29). In this study we also showed that Ca^{2+} uptake by SR was decreased by pressure overload for 1 mo in parallel to a decrease in the content of Ca^{2+} -ATPase protein. Lamers and Stinis also reported that phosphorylation of the microsomes by excess exogenous cAMP did not correct the decrease of Ca^{2+} uptake in the hypertrophied hearts. These results and observations suggest that the abnormality of Ca^{2+} metabolism might be due to a reduced number of functional Ca^{2+} -ATPase molecules per unit of membrane. The mRNA levels of Ca^{2+} -ATPase decreased from 4 h after aortic banding and gradually progressed to become 32% at 1 mo compared with those of sham-operated animals. The protein content of Ca^{2+} -ATPase decreased to 65% of sham operation by pressure overload for 1 mo. The discrepancy between mRNA and protein-decreased levels of Ca^{2+} -ATPase might imply translational or posttranslational regulation of Ca^{2+} -ATPase in cardiac hypertrophy. Again, if the Ca^{2+} -ATPase protein had a long half-life, the discrepancy might be due to the differences between the half-lives of the mRNA and protein. However, since the mRNA levels decreased markedly, the reduction of the Ca^{2+} -ATPase molecule at the pretranslational level might be one of the major reasons for the decrease of the Ca^{2+} -ATPase protein and its decrease

might be the molecular mechanism for the impaired sequestration of intracellular Ca^{2+} in hypertrophied hearts.

The expression of Ca^{2+} -ATPase was also regulated during the developmental stage at the pretranslational level. The mRNA levels of Ca^{2+} -ATPase were significantly low in the fetus and steeply increased from 2 d before birth. Although the protein contents of Ca^{2+} -ATPase and Ca^{2+} uptake per SR were not changed between the adult and the fetus, the yield of SR fraction was quite reduced in the fetus. Fabiato and Fabiato reported the changes in Ca^{2+} -stimulated Ca^{2+} release between embryonic and neonatal rat ventricle, and they attributed this change to the sudden maturation of the Ca^{2+} transport by the SR around birth (30). In morphometrical studies the SR fractions in the fetal hearts were reported to be less than in the adult hearts (31). Nakanishi and Jarmakani reported that the yield of the SR protein in the fetus was significantly less than in the newborn and the adult, and that the relative value of Ca^{2+} uptake by SR per gram of muscle in the fetus was 17% of that in the adult (14). They also observed that the resting tension, half time to relaxation, and cytosolic Ca^{2+} were increased in the fetus, and they speculated that an age-related change in myocardial contractility is due to the differences in the relative capability of the Ca^{2+} -releasing system and the Ca^{2+} -sequestering system as well as myofibrillar content and ATPase activity. Taken together with the present results and these observations, the decreased Ca^{2+} sequestration and subsequent impaired relaxation of the fetus heart were determined, at least in part, by the decrease in the expression of Ca^{2+} -ATPase of SR at the pretranslational level.

Quite recently it was demonstrated that the mRNA coding for the Ca^{2+} -ATPase of rat cardiac SR is highly influenced by thyroid hormone (32). Although it remains to be proven whether changes in the Ca^{2+} -ATPase mRNA levels are due to changes in transcriptional rate or in mRNA stability, it was demonstrated in the present study that the cardiac Ca^{2+} -ATPase was also regulated by pressure overload and developmental stage at the mRNA level. Since the mRNA levels of Ca^{2+} -ATPase in the skeletal muscle did not change by aortic constriction, the decrease of Ca^{2+} -ATPase mRNA levels in hypertrophied hearts might be due to hemodynamic overload but not humoral factors. Further work is needed, however, to elucidate the precise molecular mechanisms of Ca^{2+} -ATPase gene regulation.

Acknowledgments

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