The sarco(endo)plasmic reticulum calcium ATPase SERCA2b is an alternate isoform encoded by the SERCA2 gene. SERCA2b is expressed ubiquitously and has a higher Ca\(^{2+}\) affinity compared with SERCA2a. We made transgenic mice that overexpress the rat SERCA2b cDNA in the heart. SERCA2b mRNA level was approximately ~20-fold higher than endogenous SERCA2b mRNA in transgenic hearts. SERCA2b protein was increased 8–10-fold in the heart, whereas SERCA2a mRNA/protein level remained unchanged. Confocal microscopy showed that SERCA2b is localized preferentially around the T-tubules of the SR, whereas SERCA2a isoform is distributed both transversely and longitudinally in the SR membrane. Calcium-dependent calcium uptake measurements showed that the maximal velocity of Ca\(^{2+}\) uptake was not changed, but the apparent pump affinity for Ca\(^{2+}\) \((K_{d})\) was increased in SERCA2b transgenic mice \((0.199 \pm 0.011 \text{ mM})\) compared with wild-type control mice \((0.269 \pm 0.012 \text{ mM}, p < 0.01)\). Work-performing heart preparations showed that SERCA2b transgenic hearts had a higher rates of contraction and relaxation, shorter time to peak pressure and half-time for relaxation than wild-type hearts. These data show that SERCA2b is associated in a subcompartment within the sarcoplasmic reticulum of cardiac myocytes. Overexpression of SERCA2b leads to an increase in SR calcium transport function and increased cardiac contractility, suggesting that SERCA2b plays a highly specialized role in regulating the beat-to-beat contraction of the heart.

The sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA\(^1\) family of proteins is encoded by three separate genes: SERCA1, SERCA2, and SERCA3. Each of these genes is transcribed in a tissue-specific manner, and alternate splicing results in at least six different isoforms. SERCA1a is expressed in adult fast-twitch skeletal muscle \((1, 2)\). SERCA2a is expressed at high levels in cardiac and slow-twitch skeletal muscle \((3)\). SERCA2b, the so-called "housekeeping" isoform, is expressed virtually in every cell type \((4, 5)\). The SERCA3 isoforms are expressed in specialized non-muscle cells \((6, 7)\).

SERCA2b is ubiquitously expressed; it is responsible for maintaining intracellular Ca\(^{2+}\) stores and plays an important role in regulating Ca\(^{2+}\) signaling in vital tissues including neurons, pancreas, and other secretory cells. Although SERCA2b protein is detected in the heart tissue, it has not been shown to be expressed specifically in cardiac myocytes, and the role of SERCA2b in muscle has never been clearly defined. For example, in pancreatic and salivary gland cells, specific and polarized expression of SERCA isoforms has been observed \((8)\). In pancreatic acinar cells, SERCA2a is found in the luminal pole, whereas SERCA2b is expressed in the basolateral membrane and nuclear envelope. In salivary gland cells, SERCA2b is found in the luminal pole, whereas SERCA3 is expressed in the basolateral membrane. By analogy to its role in other cell types, SERCA2b might be expected to play such a unique role in cardiac myocytes. No such intracellular compartmentalization or specialized role has yet been shown.

SERCA2b has specific structural and functional properties that might facilitate a specialized role in cardiac myocyte calcium handling. The SERCA2b isoform is identical to SERCA2a except for the carboxy terminus. The four COOH-terminal amino acids of SERCA2a are replaced by an extended hydrophobic sequence of 49 amino acids in SERCA2b \((9)\). Epitope tagging and immunocytochemical analysis \((10)\) shows that the extended carboxy terminus of SERCA2b spans the endoplasmic reticulum membrane an additional \((i.e. 11th)\) time compared with SERCA2a. The carboxy-terminal of SERCA2a is in the cytosol and that of SERCA2b is in the endoplasmic reticulum lumen.

Detailed comparison of the enzymatic properties of various SERCA isoforms has been made in a COS cell expression system \((11–13)\). By measuring calcium-dependent calcium uptake at various Ca\(^{2+}\) concentrations, SERCA2b was shown to have a higher apparent Ca\(^{2+}\) affinity \((K_{d})\) than SERCA2a. Verboomen et al. \((13)\) estimated a \(K_{d}\) of 0.17 \pm 0.01 \(\mu\)M for SERCA2b versus 0.34 \pm 0.01 \(\mu\)M for SERCA2a. The enzymatic turnover rate has been assessed for these two SERCA isoforms by measurement of oxalate-facilitated Ca\(^{2+}\) uptake during the linear portion of the uptake curve normalized to total covalent phosphorylated enzyme intermediate \((E\cdot P)\). Enzymatic turnover for SERCA2b in a COS expression system is about 0.5 times that of SERCA2a \((11, 13)\).

Cells must regulate their Ca\(^{2+}\) within a very tight physiological range; too little Ca\(^{2+}\) results in impaired function, and
too much results in apoptosis and cell death. With this as motivation we sought to answer the question: what is the role of SERCA2b in cardiac myocytes, cells that express SERCA2a at much higher levels? Why is SERCA2b expression required in these cells, and what, if any, specific function does SERCA2b have? Overexpression of SERCA isoforms in the heart or in isolated cardiac myocytes provides a powerful model system to assess the specific functional role of these molecules in muscle contraction and relaxation (14–19).

Our hypothesis is that SERCA2b, with its different Ca\(^{2+}\) handling properties, contributes in a unique way to intracellular calcium homeostasis in the myocyte and helps to regulate beat-to-beat function of the heart. Although SERCA2b has been termed the housekeeping isoform, its essential role in heart is virtually unknown. In fact, it has not been previously demonstrated that SERCA2b is expressed in cardiac myocytes as opposed to non-cardiac cells. In addition, it is not known whether SERCA2b is physically associated with subcompartments of the SR, or whether its intracellular localization differs from that of SERCA2a. To test this hypothesis in an animal model, we have generated transgenic mice that overexpress SERCA2b and studied their structural and functional differences from wild-type controls.

**EXPERIMENTAL PROCEDURES**

**Generation of Transgenic Mice**—The rat SERCA2b cDNA (clone RB 2–5, gift of G. Shull (Ref. 20)) was cloned into the plasmid a-MHC26 (gift of J. Robbins (Ref. 21)), which contains the upstream promoter and the lower band to SERCA2a mRNA, which is not significantly altered when normalized with GAPDH mRNA.

Restriction sites and a human growth hormone polyadenylation site. The entire SERCA2b coding sequence and 2 kilobase pairs of 3'-untranslated region and polyadenylation site of the SERCA2b cDNA were included in the construct. The sequence immediately surrounding the methionine initiation codon of the SERCA2b cDNA was changed to better adhere to the Kozak translation consensus initiation sequence. The construct was linearized with NotI, gel-purified, and injected into the male pronucleus of fertilized eggs (mouse strain FVB/N). The eggs were implanted into pseudo-pregnant females. Sixty-nine founder pups were obtained, 7 of which were transgenic.

**Genotype Analysis**—Transgenic animals were identified by Southern blotting using the NdeI/SalI fragment of a-MHC21, or with polymerase chain reaction using a sense primer specific for a-MHC (5'–GCCCA-CACCAGAAATGACAGA-3') and an antisense primer (5'–ATCA-CAAGTTCACAGAAG-3') specific to the 5' end of SERCA2.

**RNase Protection Assays**—RNA probes specific for SERCA2a and SERCA2b mRNA were derived from mouse genomic DNA fragments encoding the different 3'-untranslated regions of SERCA2a and SERCA2b (22) cloned into pBluescript. 32P-Labeled riboprobes were synthesized from linearized pBluescript plasmids using T7 RNA polymerase (Maxiscript\textsuperscript{TM}, Ambion, Inc., Austin, TX). Total RNA (20 µg)
purified from total hearts of 12–14-week-old transgenic and non-transgenic littermate mice was hybridized simultaneously with RNA probes for SERCA2a and SERCA2b, and digested with RNase T1 and RNase A (RPA III™, Ambion, Inc.). The protected fragments were separated by electrophoresis on a 5% denaturing polyacrylamide gel and analyzed by autoradiography as described previously (22). Although the SERCA2b probe is derived from mouse, it hybridized well with mRNA encoded by the rat SERCA2b transgene due to high sequence similarity.

Western Blotting—Cardiac microsomes (23) were prepared from whole cardiac homogenates from transgenic and non-transgenic animals (n = 4, each group). Serial dilution of each microsome preparation were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Immobilion, Millipore, Bedford, MA). Antipeptide antibodies specific for SERCA2a and SERCA2b (gift of F. Wuytack, Ref. 24) were used to quantify SERCA expression. Antibodies against sarcomeric α-actin and tubulin (Sigma) were used in control blotting experiments. Peroxidase-labeled secondary anti-mouse or anti-rabbit antibodies were used to detect primary antibody binding with ECL (Kirkegaard and Perry, Gaithersburg, MD).

Quantification of signals was performed by densitometry of scanned autoradiographs using NIH Image (version 6.1) software.

Immunostaining of Isolated Cardiomyocytes—Isolated myocytes were obtained from SERCA2b transgenic and non-transgenic control littermates (12–15 weeks old, n = 3 each group) by perfusion with trypsin and collagenase and processed for immunostaining and confocal microscopy (25) as described previously. Isolated myocytes were permeabilized with 0.5% Triton X-100, fixed in 4% paraformaldehyde, col-lected on small nylon mesh, and fixed to charged glass slides (mesh side out). Cells were exposed to 10% goat serum followed by SERCA2a or SERCA2b rabbit polyclonal antibodies (24). A secondary fluorescein-conjugated goat anti-rabbit antibody was used to detect the primary antibody. A mouse monoclonal antibody against α-actinin (Sigma A7811) was simultaneously used to stain the same SERCA2b transgenic and wild-type myocytes to localize Z-line sarcomeric staining. A goat anti-mouse rhodamine-conjugated antibody was used to detect α-actinin staining. Composite images were made by exposing the same autoradiographs using NIH Image (version 6.1) software. Immunofluorescence assay was performed with an Olympus FV 300 confocal laser scanning microscope with a 40× objective (N.A. 1.30).

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Mice were anesthetized with 30 mg/kg pentobarbital sodium intraperitoneally and anticoagulated with 500 units of heparin. The aorta and pulmonary vein were cannulated, and a catheter was placed through the pulmonary vein and partially pushed through the left ventricular apex in order to measure left ventricular wall pressures. Physiological parameters were recorded on a six-channel P7 Grass polygraph (Grass Instruments, Quincy, MA) and digitized. Custom-designed software calculated and displayed heart rate, mean aortic pressure, intraventricular pressure, peak systolic pressure, mean diastolic and end-diastolic pressure, time to peak pressure (TPP), half-time of relaxation (RT,1/2), the first derivative of systolic and diastolic interventricular pressure (+dP/dt, −dP/dt), aortic and coronary flow, venous return, left ventricular minute work, stroke volume, stroke work, left atrial pressure, and perfusate temperature.

RESULTS

We have generated mice that overexpress a SERCA2b transgene under the control of cardiac α-MHC gene promoter (21). This promoter has been demonstrated to drive high levels of expression of a variety of transgenes in the postnatal mouse heart, and has been successfully used to overexpress two other SERCA isoforms in our laboratory (15, 16). We used the rat SERCA2b cDNA (20) (gift of Dr. Gary Shull), which is nearly identical to that of the mouse. The SERCA2b transgene construct includes the entire coding and 3’-untranslated region of the rat SERCA2b cDNA (5 kilobase pairs) downstream of the (~5.5-kilobase pair) α-MHC promoter and followed by an additional polyadenylation signal sequence from the human growth hormone gene.

Southern blot analysis using tail clips from the founders (F0) indicated that 7 out of 59 initial F0 mice carried the transgene (lines 1, 21, 22, 25, 44, and 54). Southern blotting showed that three of the lines (25, 44, and 54) had high copy numbers (approximately 30 copies) (Fig. 1) of the transgene and were chosen for this study. Transgenic lines that had low copy numbers of the transgene (one or two copies) were not analyzed.

 steady state levels of endogenous mouse SERCA2a and transgenic SERCA2b mRNA were quantified in SERCA2b transgenic and wild-type litter mate hearts by an RNase protection assay. Probes derived from genomic sequences encoding the 3’-untranslated regions of the two isoforms allowed unambiguous detection of each SERCA isoform. Figure 2 shows two representative non-transgenic mice compared with three SERCA2b transgenic littermates. Quantitation of signals after normalization with GAPDH mRNA revealed that the steady-state level of SERCA2b mRNA was increased approximately 20-fold in SERCA2b transgenic mice compared with wild-type littermate mice. There was no significant down-regulation of
endogenous SERCA2a mRNA in SERCA2b transgenic animals.

Western blotting analysis was performed on membrane preparations isolated from homogenates of hearts from each of the three high copy number SERCA2b transgenic lines. An antibody specific to SERCA2b (24) was used to identify the level of this isoform. This SERCA2b-specific antibody was produced by immunizing rabbits with a synthetic peptide derived from the unique 49-amino acid COOH-terminal tail of SERCA2b and is therefore highly specific for this isoform. Quantification of blots revealed that SERCA2b protein is increased approximately 8–10-fold over control hearts in transgenic lines 25, 44, and 54 (Fig. 3A). On the same Western blot, staining for tubulin was used as a control and did not show any major changes in the hearts of transgenic animals. Duplicate immunoblots were stained with an antibody specific for SERCA2a isoform. These blots showed that there were no large changes in the expression level of endogenous SERCA2a protein (Fig. 3B). Sarcomeric actin was also used as an internal control. It is evident that there were no significant changes in sarcomeric actin expression in the transgenic animals.

These same highly specific polyclonal antibodies against SERCA2a and SERCA2b were used to perform immunostaining and confocal microscopy on fixed, isolated myocytes from both wild-type and mice overexpressing SERCA2b (Fig. 4). Immunostaining showed that SERCA2b distributes horizontally in a highly organized pattern and has the same general distribution as α-actinin. This staining pattern suggests that SERCA2b is localized near the transverse tubules (T-tubules) in the SR that overlie the myofilament Z lines, as delineated by the control staining with α-actinin. In comparison with SERCA2b staining (Fig. 4), SERCA2a staining was not localized only near the T-tubular area, but was also distributed longitudinally. This may suggest that SERCA2a is localized both around the terminal SR (near the T-tubules) and in the so-called free or longitudinal SR that surrounds the myofilament bundles. Although the immunostaining images cannot exclude the presence of SERCA2b in the longitudinal SR, at a similar overall intensity of staining of the transverse elements of the SR, there is little or no staining of longitudinal elements observable with the SERCA2b antibody. Staining of both wild-type myocytes and SERCA2b transgenic myocytes for SERCA2b showed a very similar overall staining pattern.

There was no difference in heart/body weight ratio in SERCA2b transgenic mice versus non-transgenic littersmates, suggesting that significant cardiac hypertrophy is absent in the SERCA2b transgenic mice. Histologic analysis of hearts from lines 25, 44, and 54 also revealed no evidence of myocyte hypertrophy or fibrosis (data not shown).

In order to determine the effect of overexpression of the SERCA2b protein on SR Ca\(^{2+}\) transport, the initial rate of oxalate-facilitated Ca\(^{2+}\) uptake was measured in cardiac homogenates from SERCA2b transgenic and non-transgenic hearts, using \(^{45}\)Ca\(^{2+}\) as a tracer (Fig. 5). There was no change in the maximal velocity of calcium uptake when normalized for total homogenate protein between wild-type and TG hearts (V\(_{\text{max}}\) for TG: 60.16 ± 4.89 nmol of Ca\(^{2+}\)/mg of protein/min; for NTG, 60.91 ± 3.37 nmol of Ca\(^{2+}\)/mg of protein/min). There was, however, a significant leftward shift in the calcium uptake curve. The \(K_{d}\) of SR Ca\(^{2+}\) uptake occurred at a much lower Ca\(^{2+}\) concentration in SERCA2b-overexpressing mice compared with controls (TG, 0.199 ± 0.011 μM; NTG, 0.269 ± 0.012 μM). This suggests that enzymatically active SERCA2b was indeed expressed in the hearts of the SERCA2b transgenic mice and it can alter the apparent pump affinity for calcium.

To understand whether SERCA2b overexpression has modified cardiac contractility, “work-performing heart preparrations” were used to evaluate baseline cardiac contractility in isolated hearts (Fig. 6). Compared with wild-type FVB/N control littersmates, the SERCA2b transgenic hearts had significantly increased \(+dP/dt\) (40%) and lower TPP, consistent with improved contractility. The \(-dP/dt\) was significantly increased (+30%), and \(R_{\text{T}}\) was reduced suggestive of improved diastolic function as well.

**DISCUSSION**

The goal of this study was to understand the role of SERCA2b in the beat-to-beat function of the heart. In this study, we have overexpressed SERCA2b in cardiomyocytes of the mouse heart. The SERCA2b transgenic mice overexpress SERCA2b mRNA approximately 20-fold in the heart as compared with wild-type littersmates. The SERCA2b protein level is increased ~8–10-fold in the heart. At the same time, there is little change in the amount of endogenous SERCA2a protein
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level. This is in contrast to mice that overexpress SERCA1 (fast twitch skeletal muscle-specific isoform), in which there is a 50% down-regulation of SERCA2a protein in cardiac myocytes (16, 28). This may indicate that SERCA2a and SERCA2b do not compete for the same “sites” within the sarcoplasmic reticulum. This is further corroborated by the immunostaining of isolated myocytes using SERCA2a- and SERCA2b-specific antibodies, which shows distinct distribution for these two isoforms. Our results suggest that SERCA2b is localized in a subcompartment of the SR (terminal SR) near the transverse tubules (T-tubules) as delineated by the control staining with α-actinin. On the other hand, SERCA2a staining was observed both transversely and longitudinally. This strongly suggests that there is compartmentalization of the SR with regard to the distribution of SERCA2a and SERCA2b isoforms.

The difference between the two SERCA2 isoforms lies in the carboxyl-terminal tail (50 amino acids in SERCA2b and 4 amino acids in SERCA2a). This suggests that one of the roles of extended carboxyl-terminal of SERCA2b may be to limit its distribution in the SR membrane. The extended SERCA2b carboxyl-terminal tail may result in an additional membrane span for the enzyme compared with SERCA2a. Because the SERCA2b carboxyl-terminal is in the SR lumen (10), there is an opportunity for additional protein–protein interactions to occur, which may regulate the ATPase activity and its location. Thus, the COOH-terminal sequence difference between SERCA2a and SERCA2b may affect both the enzymatic properties and the intracellular localization of the protein.

When expressed in a COS cell system, SERCA2b has a higher apparent Ca\(^{2+}\) affinity and lower enzymatic turnover rate than SERCA2a (11, 12). The SR Ca\(^{2+}\) uptake measurement in SERCA2b transgenic hearts show that there is a higher apparent affinity for Ca\(^{2+}\) as compared with that of wild-type littermates. The magnitude of this shift in Ca\(^{2+}\) affinity suggests that overexpressed SERCA2b is functional in transgenic hearts. We do not see, however, a significant change in the maximal rate of Ca\(^{2+}\) uptake (V\(_{\text{max}}\)) in SERCA2b transgenic hearts. The observation that SERCA2b has 2-fold slower enzymatic turnover rate compared with SERCA2a suggests one explanation for this phenomenon (11–13). If SERCA2b transports Ca\(^{2+}\) at a significantly slower rate than SERCA2a, an increase in SERCA2b over its basal level may not have a significant effect on the observed V\(_{\text{max}}\) of Ca\(^{2+}\) uptake. However, SERCA2b overexpression results in enhanced rate of Ca\(^{2+}\) uptake at lower Ca\(^{2+}\) concentrations, as observed by a leftward shift in the apparent affinity for Ca\(^{2+}\). These data demonstrate that SERCA2b is inserted into cardiac SR membranes and can contribute to SR calcium transport.

If SERCA2b is an important determinant of diastolic calcium levels and the rate of calcium reuptake into the SR, an increase in the relative abundance of SERCA2b should affect intrinsic cardiac performance. We therefore used the isolated work-performing heart preparation to measure intrinsic myocardial contractility in the SERCA2b overexpressing lines. The SERCA2b lines exhibit a significant increase in both base-line systolic and diastolic cardiac function, as compared with wild-type littermate control mice. This increase in intrinsic myocardial performance suggests that SERCA2b may play an important role in the regulation of cardiac contractility.

Our studies on SERCA overexpression and SERCA2 knockout (+/−) mice reveal that a alteration in SERCA pump level results in an increase or decrease in myocardial contractile function (15, 16, 29). These changes in contractility can be correlated with increase or decrease in SERCA protein level in the heart and with corresponding changes in SR calcium uptake. It appears therefore that the level of functional SERCA protein in the SR is one of the fundamental determinants of cardiac contractility. In conclusion, our results showing that increased expression of SERCA2b can increase intrinsic myocardial contractility suggest that SERCA2b may play an important role in determining in vivo cardiac contractility.

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