Targeted Disruption of the ATP2A1 Gene Encoding the Sarco(endo)plasmic Reticulum Ca\(^{2+}\) ATPase Isoform 1 (SERCA1) Impairs Diaphragm Function and Is Lethal in Neonatal Mice*

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Mutations in the ATP2A1 gene, encoding isoform 1 of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA1), are one cause of Brody disease, characterized in humans by exercise-induced contraction of fast twitch (type II) skeletal muscle fibers. In an attempt to create a model for Brody disease, the mouse ATP2A1 gene was targeted to generate a SERCA1-null mutant mouse line. In contrast to humans, term SERCA1-null mice had progressive cyanoysis and gasping respiration and succumbed from respiratory failure shortly after birth. The percentage of affected homozygote SERCA1\(^{-/-}\) mice was consistent with predicted Mendelian inheritance. A survey of multiple organs from 10-, 15-, and 18-day embryos revealed no morphological abnormalities, but analysis of the lungs in term mice revealed diffuse congestion and epithelial hypercellularity and studies of the diaphragm muscle revealed prominent hypercontracted regions in scattered fibers and increased fiber size variability. The \(V_{\text{max}}\) of Ca\(^{2+}\) transport activity in mutant diaphragm and skeletal muscle was reduced by 80% compared with wild-type muscle, and the contractile response to electrical stimulation under physiological conditions was reduced dramatically in mutant diaphragm muscle. No compensatory responses were detected in analysis of mRNAs encoding other Ca\(^{2+}\) handling proteins or of protein levels. Expression of ATP2A1 is largely restricted to type II fibers, which predominate in normal mouse diaphragm. The absence of SERCA1 in type II fibers, and the absence of compensatory increases in other Ca\(^{2+}\) handling proteins, coupled with the marked increase in contractile function required of the diaphragm muscle to support postnatal respiration, can account for respiratory failure in term SERCA1-null mice.

Sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs) are 110-kDa membrane proteins that catalyze the ATP-dependent transport of Ca\(^{2+}\) from the cytosol to the lumen of the sarco(endo)plasmic reticulum (1). Three different ATP2A genes encode six different sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) proteins (2–5). SERCA1a and SERCA1b, the developmentally regulated isoforms of the ATP2A1 gene, arise through alternative splicing at the 3' end of the ATP2A1 transcript (6). SERCA1a accounts for more than 99% of SERCA isoforms expressed in adult rat fast twitch skeletal muscle, whereas SERCA1b is predominant in neonatal muscle (7). SERCA2a is the major isoform in heart and slow twitch skeletal muscle, whereas SERCA2b and SERCA3 are more ubiquitously expressed.

Brody disease is a rare inherited disorder of skeletal muscle, resulting in exercise-induced impairment of skeletal muscle relaxation, stiffness, and cramps (8). Sarcoplasmic reticulum Ca\(^{2+}\) uptake and Ca\(^{2+}\)-ATPase activities in muscle samples obtained from Brody's patients are reduced to levels ranging from 0 to 50% compared with activities measured in normal controls (9–13), suggesting that Brody disease might result from defects in the ATP2A1 gene. Sequencing of ATP2A1 DNA from Brody disease patients has revealed a number of frame-shift mutations that truncate SERCA1 (14, 15) as well as a missense mutation (16). All of these mutations lead to loss of SERCA1a function. However, mutations in ATP2A1 account for only about half of Brody disease cases, and the genetic basis for the other Brody syndrome patients remains to be discovered (17).

Recent advances in transgenic mouse technology have made it possible to address the physiological relevance of increases or decreases in SERCA expression. Overexpression of SERCA2 in the myocardium resulted in enhanced myocardial function (18–20). By contrast, the ablation of ATP2A2 was lethal, and heterozygous SERCA2\(^{-/-}\) mice manifested impaired cardiac contractility and delayed cardiomyocyte relaxation (21). Ablation of the ATP2A3 gene encoding SERCA3 was not lethal, but defects were noted in endothelium-dependent relaxation of vascular smooth muscle and endothelial cell Ca\(^{2+}\) signaling in SERCA3-null mice (22).

The purpose of the present study was to investigate both the role of SERCA1 in physiological functions and the mechanisms by which SERCA1 mutations could cause Brody disease. Since the ATP2A1 gene is expressed almost exclusively in fast-twitch fibers, it was of particular interest to investigate the effects of...
its disruption on the function of muscles such as the diaphragm, where fast twitch fibers make up a large fraction of the total fibers. SERCA1−/− mice were born with normal body weight and normal gross morphology. However, affected mice developed cyanosis and gasping respiration and died shortly after birth. Histopathological analysis of mice at term revealed congestion and hypercellularity of the lung, consistent with failure of the respiratory musculature to produce sufficient chest expansion to open the lung alveoli after birth. Analysis of the diaphragm muscle revealed an increased fiber size variability and prominent hypercontracted regions in scattered muscle fibers. The demonstrable loss of SERCA1 protein from diaphragm and skeletal muscles was accompanied by a dramatic reduction in Ca\(^{2+}\) uptake activity in homogenates from these tissues. No compensatory responses were detected in mRNA or protein levels for other Ca\(^{2+}\) regulatory proteins. A significant impairment of the contractile response of isolated diaphragm muscles to electrical stimulation was also measured. These results demonstrate that the loss of SERCA1 in the diaphragm of mice leads to neonatal death from respiratory failure.

**EXPERIMENTAL PROCEDURES**

**Preparation of the Targeting Construct**—A phage clone containing part of the mouse ATP2A1 gene was isolated from a strain 129/SvJ genetic library (a gift of Dr. J. Rossant, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada) and partially characterized by Southern blot analysis, restriction mapping, and DNA sequence analysis. The cloned 15-kb fragment contained the 3′-end of the promoter and the sequence corresponding to exons 1 to 9 of the human ATP2A1 gene, which encode amino acids 1–365. In over 154 codons sequenced, no amino acid substitutions were observed in the mouse sequence relative to that of human SERCA1, thereby confirming the identity of the gene (23). This conclusion was strengthened when a partial sequence of the corresponding region was retrieved from the murine genome via the Ensembl Genome Browser (available on the World Wide Web at www.ensembl.org). Strikingly, the similarity between the human and mouse ATP2A1 gene regions extended beyond the conserved sequences within the exons; the overall size of the introns was very similar in the two species.

The targeting construct was prepared with the plBluescript II SK− vector as a backbone. The PGK-Neo and PGK-TK cassettes in plasmids pGEM7(KJ1)SalI-R and pGEM7(TK)SalI-R, respectively, were used in construction. The targeting vector was constructed by replacing 4.2 kb of genomic sequence with the PGK-Neo expression cassette (see Fig. 1A). The cassette was inserted between a 5.2-kb EcoRI-AvaII gene fragment located 1.8 kb upstream of exon 1 and a 0.9-kb BamHI-HindIII fragment containing exon 5. The orientation of the PGK-Neo cassette was the same as that of the ATP2A1 gene. The PGK-TK expression cassette was inserted at the HindIII site 0.7 kb downstream from exon 5. Gene Targeting and Generation of Mutant Animals—The construct was linearized at the NotI site in the vector and transfected by electroporation into ES cells. Twenty-four hours after electroporation, the cells were exposed to 4818 at 200 μg/ml and 2 μg gancyclovir to select cells containing the neo gene and lacking the HSV-tk gene, respectively. Three hundred clones resistant to both drugs were picked and expanded 10 days after electroporation. DNA from individual ES cell clones was digested with EcoRI and analyzed by nonradioactive Southern blotting (24) using two different probes. Probe 1 was a 1.8-kb HindIII-XbaI fragment located 2.8 kb downstream from exon 5. Probe 2 was a 2.9-kb fragment specific for the neo gene. Fourteen ES cell lines containing the disrupted ATP2A1 gene were used for blastocyst-mediated transgenesis. Blastocyst injection was carried out by Dr. M. Rudnicki (MacMas-ter University, Hamilton, Canada). Five chimeric mice with 50–70% chimerism were produced and mated to C57Bl mice. Germ line transmission of the recombinant gene was achieved with only one chimera, and this mouse was used as a founder of the murine line with targeted ablation of the ATP2A1 gene.

**Western Blot Analysis**—Total homogenates like those used in Ca\(^{2+}\) transport assays were separated by SDS-PAGE and then transferred to a nitrocellulose membrane. Primary monoclonal mouse antibodies used were as follows: A52 (diluted 1:5000) against SERCA1; 2A7-A1 (diluted 1:100) against Ryanodine receptors (Affinity Bioreagents); and R3F1 (diluted 1:500) against SERCA2a (Affinity Bioreagents Inc.). Western blotting was performed as described in the kit (Invitrogen). Trace genomic DNA contamination from total RNA was removed by 2 units of DNase I treatment (Ambion) for 7 min, and stopped by filtration through a 0.3-μm Millipore filter. RNA samples were quantified using the Bio-Rad method using bovine serum albumin as a standard.

**Semiquantitative RT-PCR Analysis**—Total RNA isolated from diaphragm or limb muscles using Triazol reagent, following the method described in the kit (Invitrogen). Trace genomic DNA contamination from total RNA was removed by 2 units of DNase I treatment (Ambion) in a 100-μl reaction for 10 min at 37 °C and then extracted once with phenol/chloroform and twice with chloroform. The integrity of each of the RNA samples was assessed by electrophoresis (25) and the concentration was estimated by spectrophotometry using the A\(_{260}\)/A\(_{280}\) ratio. Micrograms of total RNA from each sample were subjected to hexamer random primed first-strand cDNA synthesis in a volume of 20 μl using Superscript II reverse transcriptase (Invitrogen), according to the guidelines of the manufacturer. The absence of contaminating DNA from each RNA sample was confirmed by PCR by omitting reverse

### Table I

| Isoform | Product size | Diagonal restriction enzyme | Cleavage products |
|--------|--------------|----------------------------|------------------|
| bp     |              |                            |                  |
| SERCA1 | 192          | NcoI                       | 113, 79          |
| SERCA2 | 192          | BamHI                      | 113, 79          |
| SERCA2 | 213          | PstII                      | 117, 96          |
| SERCA2 | 213          | AvaI                       | 126, 87          |
| SERCA2a| 265          | BgIII                      | 181, 84          |
| SERCA1b| 223          | AvaII                      | 147, 76          |
| SERCA2a| 215          | DelI                       | 162, 53          |
| SERCA2b| 211          | MspI                       | 155, 56          |
| PLN    | 204          | DpnI                       | 149, 55          |
| SN     | 230          | AvaII                      | 155, 115         |
| GAPDH  | 254          | BglI                       | 177, 77          |

<http://www.jbc.org/content/13368.109/S11001>
transcriptase from the RT reaction.

Equal amounts (2 μl) of the reverse transcription product were subjected to PCR amplification of SERCA1a, SERCA1b, SERCA2a, SERCA2b, SERCA3, phospholamban, and sarcolipin. Analysis of the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was carried out to normalize the level of expression of the genes of interest. The step-cycle program was adjusted individually. To measure the SERCA1 to SERCA2 and SERCA2 to SERCA3 transcripts are listed in Table I. To measure the relative ratio of SERCA1a to SERCA1b expression, SERCA1a and SERCA1b primers were used to co-amplify the SERCA1a and SERCA1b fragments. Since the two fragments differ in length by 42 bp, with the longer fragment representing the adult form, SERCA1a, they could be distinguished and quantified. To determine the levels of SERCA2a and SERCA2b, sets of specific primers were used to amplify SERCA2a and SERCA2b individually. To measure the SERCA1 to SERCA2 and SERCA2 to SERCA3 mRNA ratios, the co-amplified products were digested using the specific enzymes listed in Table I to produce digestion fragments that could be used for quantification.

Electrical Stimulation and Muscle Contractile Measurements—Experiments were performed on isolated diaphragm muscle strips from newborn homozygous ATP2A1 mice and their wild-type littermates. Isometric contractile properties of diaphragm muscles were measured in vitro from neonatal mice within 0.5–5 h following delivery by caesarian section on day 19 or 20 of embryogenesis. Each diaphragm muscle was exposed, removed with intact ribs and tendons, and placed into oxygenated Krebs solution (95% O2, 5% CO2) containing 118 mM NaCl, 25 mM NaHCO3, 11 mM glucose, 1.2 mM KH2PO4, 1.9 mM CaCl2, and 1.2 mM MgSO4, pH 7.4, and maintained at 4–10 °C. Each

**Targeting of SERCA1**

**Fig. 1. ATP2A1 gene targeting strategy to disrupt SERCA1, verification of targeting, and characterization of SERCA1−/− mice.** A, targeting strategy. **Top,** organization of the relevant region of the wild-type gene with a partial restriction map of the locus. **Middle,** targeting construct with the PGR-Neo expression cassette replacing ~4.2 kb of genomic sequence including the first four exons of the ATP2A1 gene. The herpes simplex virus thymidine kinase gene (TK) was included for negative selection of ES cell clones. **Bottom,** targeted ATP2A1 gene and location of probe 1 and probe 2 for Southern blot analysis. The EcoRI fragments (14.2 kb for wild-type allele; 6.4 kb for mutant allele) detected by Southern blot analysis are indicated. Restriction enzyme sites are as follows. A, AvrII; B, BamHI; E, EcoRI; H, HindIII; X, XbaI. Black boxes represent exons 1–9. The arrows indicate the transcriptional orientation of the inserted expression cassettes. B, Southern blot analysis of EcoRI-digested genomic DNA from ES cells (genomic DNA from tail clips of the offspring from heterozygous matings was hybridized with probe 1, as indicated). C, PCR of genomic DNA from tail clips from mice of all three genotypes with the primers specific to wild-type (lanes 1, 3, and 5) or mutant (lanes 2, 4, and 6) alleles. The PCR product generated by the mutant allele is 1.4 kb, and that generated by the wild-type allele is 3 kb. D, lethality of homozygous SERCA1−/− newborn mice shortly after birth. A mating between SERCA1−/− parents yielded the littermate progeny with gross phenotypes representative of SERCA1−/−, SERCA1−/+, and SERCA1+−/− newborn mice. Note the similar size and normal appearance of the littersmates, except that the two SERCA1−/− mice are cyanotic and appear light purple in color.
Fig. 2. Histological analysis of multiple organs in SERCA1−/− newborn mice. Histological analysis of heart (A and E), lung (B and F), longitudinal sections of diaphragm (C and G), and cross-sections of diaphragm (D and H) are presented from SERCA1−/− (A–D) and SERCA1−/− (E–H) newborn mice. Note that a lack of SERCA1 expression in SERCA1−/− mice results in a congested and hypercellular appearance in lung (F), scattered degeneration and focal hypercontraction in diaphragm (G), and variation in diaphragm fiber size (H). Heart sections exhibit histology that is similar in newborn mice of both genotypes (A and E).

Supramaximal stimulation voltage was used (110–120 V) with a pulse duration of 0.2 ms. Force data were collected on-line using a 640A signal interface (Aurora Scientific Inc.) connected to a National Instruments 16-bit A/D card and analyzed using the Dynamic Muscle Control and Data Acquisition (DMC) and Dynamic Muscle Analysis (DMA) Software (Aurora Scientific). Muscle length was adjusted to obtain maximal isometric twitch force. Peak isometric force amplitude (g) and the maximal rates of force development (+dF/dt) and relaxation (−dF/dt) were determined during a twitch and across a range of stimulation frequencies from 10 to 100 Hz. Diaphragm fatigability was also assessed using a 3-min stimulation protocol consisting of 350-ms contractions at 100 Hz, once per second. Fatigue data are expressed as a percentage of initial force data. After the contractile and fatigue properties were measured, the diaphragm muscle strips were trimmed of the remnants of the central tendon and rib, blotted on filter paper, and weighed on an analytical balance. Total muscle fiber cross-sectional area of each muscle strip was determined by dividing the muscle mass (mg) by the product of muscle length and 1.06 mg/mm³, the density of mammalian skeletal muscle. Force data were normalized for total muscle fiber cross-sectional area.

Light and Electron Microscopic Level Analysis—After genotyping, samples from animals in some 2 dozen litters were collected and processed by various pathological techniques: 1) paraffin embedding for histopathological evaluation of entire 10-, 15-, and 18-day embryos and term neonatal mice; 2) paraffin embedding of limb and diaphragm muscles from neonatal term mice; and 3) electron microscopy of limb and diaphragm muscles of term mice. For paraffin studies, whole bodies of neonatal or embryonic mice were fixed by immersion in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and analyzed by light microscopy. Diaphragm and limb muscles were dissected immediately after death, flash-frozen in OCT blocks cooled by isopentane in liquid nitrogen, and cut into 5-μm sections for analysis. For electron microscopy, diaphragm and limb muscles were fixed in situ by injection of 2% paraformaldehyde and 2% glutaraldehyde (in 200 mM Sørensen’s phosphate buffer, pH 7.4) into the thoracic and abdominal cavities immediately after death. The diaphragm and limb muscles were then dissected into 1 × 1-mm cubes and immersed again in the same fixative at 4 °C. Muscle was processed for electron microscopy by postfixation with 1% osmium tetroxide in water for 1 h, washed in water, and dehydrated with ethanol and propylene oxide. Dehydrated tissues were embedded in plastic, sectioned at 60 nm, and stained with 5% uranyl acetate and 1% lead. Sections were viewed and photographed on a Hitachi60 electron microscope.

Skeletal Muscle Culture—Primary myoblast cultures were established from the limbs of E19 SERCA1−/− and SERCA1−/− embryos, as described in Ref. 27. Muscle tissue dissected from all four limbs was dissociated by fine mincing with microdissecting scissors, followed by treatment with 0.125% trypsin and 0.5% pancreatin and 0.01% of DNase (Life Technologies, Inc.) for 30 min at 37 °C. The enzymatic digestion was stopped by the addition of Ham’s F-10 medium containing 20% fetal bovine serum. Contaminating fibroblasts were removed selectively by preplating the cell suspension for 1 h at 37 °C. Suspensions enriched in myoblasts were then plated on dishes coated with 0.1% gelatin and grown in 20% fetal bovine serum in Ham’s F-10 medium, supplemented with 5 mM basic fibroblast growth factor. Cells were fed the following day and every third day thereafter. To induce differentiation, the growth medium was changed to 2% horse serum in Ham’s F-10.
RESULTS

Generation of Mice with a Targeted Disruption of the ATP2A1 Gene—The strategy illustrated in Fig. 1A was followed to disrupt the ATP2A1 gene. The targeting construct of 7.5 kb (middle panel of Fig. 1A) consisted of mouse genomic sequence in which the neo gene substituted for 4.2 kb of the ATP2A1 gene, corresponding to a 3’ portion of the promoter sequence and a 5’ fragment of the coding sequence, which included exons 1–4. After electroporation of ES cells, 300 colonies survived positive-negative selection in G418 and ganciclovir. Southern blot analysis using probe 1 (Fig. 1B) and probe 2 (data not shown) revealed that 14 clones contained the 6.4-kb EcoRI fragment that was diagnostic of a targeted allele. Blastocyst-mediated transgenesis yielded five male chimeric mice, but the progeny of only one of these mice carried the targeted allele in its germ line after breeding with wild-type females. After five generations of cross breeding with CD1 strain mice, heterozygous mice were mated to produce wild-type, heterozygous, and homozygous mutant offspring, as demonstrated by PCR analysis of tail DNA (Fig. 1C).

Gross Phenotype and Characterization of SERCA1-deficient Mice—Genotype analysis of 299 offspring of heterozygous matings yielded 81 (27%) wild-type, 144 (48%) heterozygous, and 74 (25%) homozygous mutant mice, which is almost identical to a normal 1:2:1 Mendelian ratio. All pups were born alive, and SERCA1−/− mice were indistinguishable from wild-type SERCA1+/+ littermates in their gross appearance at birth. The average birth weight (g) of SERCA1+/+ (1.4 ± 0.08), SERCA1−/− (1.37 ± 0.11), and SERCA1+/+ (1.38 ± 0.13) mice did not differ significantly. Within minutes, SERCA1−/− animals displayed abnormal signs that were characterized by gasping respiration, limited chest wall movements, and progressive cyanosis. Affected mice demonstrated slow limb movements and delayed relaxation of skeletal muscles that was reminiscent of the cramping that characterizes Brody patients. Unfortunately, contractile measurements on skeletal muscles in these mice were not feasible due to the fragile texture and small size of the limb muscles. We assume that the contractile properties of the limb muscles would be similar to those observed with diaphragm muscle and would, therefore, be consistent with our observations on the movements of the live animals. SERCA1−/− mice died within 30 min to 2 h after birth.

Histological Analysis of SERCA1-deficient Mice—A survey of multiple organs from 10-, 15-, and 18-day SERCA1−/− embryos revealed no significant morphological abnormalities (data not shown). Studies of term SERCA1−/− mice that died shortly after birth of respiratory failure revealed prominent hypercontracted regions in scattered fibers (Figs. 2, G and H) and increased fiber size variability (Fig. 2H) in diaphragm muscle. Morphological analysis of hind limb muscles of SERCA1−/− and SERCA1−/− mice revealed uniform fiber size and no pathological alterations in myofiber architecture (data not shown).
Targeting of SERCA1

Analysis of the lung of SERCA1−/− mice revealed diffuse congestion and hypercellularity (Fig. 2F). In contrast, analysis of SERCA1+/− animals revealed uniformly open alveolar spaces in lungs (Fig. 2B), absence of regional hypercontraction and a uniform size of muscle fibers in the diaphragm (Fig. 2D). Analysis of cardiac muscle of SERCA1−/− and SERCA1+/− mice revealed no pathological abnormalities (Fig. 2, A and B).

Electron Microscopy of SERCA1-deficient Mice—Electron microscopy of limb muscles revealed central nuclei, lakes of unbound glycogen, and prominent mitochondria, all of which were present to a similar degree in wild-type term littermates (data not shown). Examination of the diaphragm muscle in SERCA1−/− newborn mice revealed prominent hypercontracted regions in scattered fibers (Fig. 3, A and B). The hypercontractility in some sections was profound, decreasing sarcomere length to as short as 600 nm. At this length, both thick and thin filaments were forced into the Z-band region, leading to increased Z-band density. Thus, abnormal findings were restricted to changes in diaphragm muscle architecture in affected mice.

RT-PCR and Western Blot Analysis of SERCA1-deficient Mice—To demonstrate the loss of SERCA1 protein expression and any consequent compensatory responses, mRNA and total homogenates prepared from diaphragm and hind limb muscles were examined by parallel semiquantitative RT-PCR and Western blot analysis, respectively. Fig. 4A shows through RT-PCR analysis that SERCA1α and -β mRNAs are expressed in diaphragm and hind limb muscles from SERCA1+/+ mice but are absent in SERCA1−/− mice. It also shows that the expression of SERCA2a, -2b, -3, and -3 and their regulators, sarcoplasmic reticulum Ca2+/Na+ exchanger. Thus, abnormal findings were restricted to changes in diaphragm muscle architecture in affected mice.

SERCA1+/− Update Activity in Sarcoplasmic Reticulum Preparations from Skeletal Muscle from SERCA1-deficient Mice—Homogenates were prepared from diaphragm and hind limb muscle from SERCA1+/− and SERCA1−/− newborn mice and assayed for Ca2+ dependence of Ca2+ uptake by the sarcoplasmic reticulum. As expected with the ablation of SERCA1, maximal Ca2+ uptake was reduced (p < 0.05) by 80% in diaphragm and hind limb muscle homogenates of SERCA1−/− mice compared with SERCA1+/+ mice (9.72 ± 0.2 and 1.8 ± 0.1 nmol of Ca2+/mg of protein/min versus 2.26 ± 0.2 and 0.49 ± 0.01 nmol of Ca2+/mg of protein/min, respectively, mean ± S.E.) (Fig. 5A). This decrease in Ca2+ uptake by the sarcoplasmic reticulum is consistent with the decreased overall SERCA1 protein levels in SERCA1−/− mice. In diaphragm, the apparent Ca2+ affinity, expressed as K0.5 in pCa units, was decreased (p < 0.05) in samples from SERCA1−/− mice, compared with samples from SERCA1+/+ mice (5.85 ± 0.12 versus 6.31 ± 0.09, mean ± S.E.) (Fig. 5B).

Contractile Activities in Diaphragm Preparations from SERCA1-deficient Mice—Isometric contractile properties of skeletal muscle of SERCA1 knockout mice were studied on muscle strips dissected from the diaphragm of newborn SERCA1−/− and SERCA1+/+ mice. Fig. 6A shows representative records of force obtained from 30-Hz tetani produced in a muscle strip from a SERCA1−/− and a SERCA1+/+ mouse. Tetanic force was dramatically lower in the SERCA1−/− muscle strip. Time of relaxation was markedly longer in the SERCA1−/− muscle strip. The force-frequency curve was shifted downward significantly in SERCA1−/− muscle strip compared with a SERCA1+/+ muscle strip at all frequencies of stimulation, with the exception of 10 Hz (Fig. 6B). The maximum corrected tetanic force output from SERCA1−/− muscle strips was 8.5 ± 1.5 g/mm2, compared with 2.6 ± 0.4 g/mm2 from SERCA1+/+ muscle strips. The frequency-dependent maximal rates of contraction (+dF/dt) and relaxation (−dF/dt) are shown in Fig. 6C and D, respectively. At all stimulation frequencies, both +dF/dt and −dF/dt were significantly (p < 0.05) depressed in SERCA1−/− mice compared with SERCA1+/+ mice. The response to a stimulation protocol that measures fatigue (not shown) also showed an increased susceptibility to fatigue in diaphragm muscle from SERCA1−/− mice.

Differentiation in Vitro of Myoblasts from SERCA1-deficient Mice—Primary myoblast cultures (Fig. 7, A and B) derived from the limbs of E19 embryos of SERCA1−/− and SERCA1+/+ mice were cultured in vitro under differentiation conditions for 5 and 20 days. The rate of fusion and extent of differentiation of myoblasts isolated from SERCA1−/− and SERCA1+/+ mice were similar (Fig. 7, C–F).

DISCUSSION

SERCA1 is a member of a family of Ca2+ pumps expressed in the sarco(endoplasmic reticulum of mammalian tissues. ATP2A1, which encodes SERCA1a and SERCA1b, is highly
up-regulated during myoblast differentiation, although its expression is largely limited to fast twitch muscle (2–5). Frameshift or missense mutations in the ATP2A1 gene that lead to loss of SERCA1 function, often with loss of expression of SERCA1 protein, have been shown to be causal of the human muscle disorder Brody disease (14, 28). Brody disease is not life-threatening in humans but is characterized by a lifelong history of exercise-induced muscle contracture and painless muscle cramping without myotonia (9–13).

In the present study, targeted ablation of the ATP2A1 gene produced mice lacking SERCA1 in skeletal muscle so that structural and functional consequences of the elimination of SERCA1 could be assessed in vivo. The absence of detectable mRNA encoding SERCA1α and -β (Fig. 4A) and the absence of SERCA1 protein (Fig. 4B) in homozygous SERCA1−/− mice confirmed that our targeting strategy had produced a null mutation. SERCA1−/− mice were born alive in the predicted Mendelian ratio, demonstrating that SERCA1 ablation is not embryonic lethal. SERCA1−/− neonatal mice were indistinguishable from wild-type littermates in their gross appearance and in their body weight at birth (Fig. 1D). Within minutes, however, SERCA1−/− mice displayed abnormal signs that were characterized by infrequent gasping respiration, limited chest wall excursions, and progressive cyanosis. In addition, SERCA1−/− mice exhibited slow limb movements and apparent contracture that was reminiscent of the contractures observed in the skeletal muscle of Brody patients. Affected mice died within 2 h after birth of respiratory failure. Thus, in contrast to humans, SERCA1 is essential for survival in mice.

Sections of hind limb and cardiac muscle from both SERCA1+/+ and SERCA1−/− newborn mice did not differ in morphology from wild-type littermates (Fig. 2, A and E). However, examination of the diaphragm muscle in newborn SERCA1−/− mice revealed prominent hypercontracted regions in scattered fibers, indicative of impaired muscle fiber relaxation (Fig. 2, G and H). Analysis of the lung revealed pulmonary congestion, hypercellularity of the alveolar epithelium, and atelectasis, indicating failure of expansion of alveoli due to impaired respiration. These findings indicate that absence of SERCA1 in mice leads to respiratory failure due to profound impairment of diaphragmatic function. There are no pathological studies of diaphragm muscle in human Brody disease patients. However, respiratory failure is not a clinical feature of the disease. Thus, there is a marked discrepancy in severity of the SERCA1a deficiency between affected humans and mice, which suggests either that superior compensatory mechanisms for Ca2+ regulation occur in human Brody disease patients or that significant physiological differences exist between mice and humans. Compensatory changes may involve Ca2+-removal by the plasma membrane Ca2+-ATPase pump or by Na+/Ca2+ exchangers in the plasma membrane, mitochondrial Ca2+ uptake, or proliferation of the sarcoplasmic reticulum containing compensatory levels of SERCA2 or SERCA3. Of these possible compensatory processes, only the last would be predicted to result in Ca2+ loading of the sarcoplasmic reticulum, a process necessary for subsequent muscle contraction (29).
The percentage of fast twitch type II fibers in mouse diaphragm at birth is significantly higher than the percentage of slow twitch type II fibers in human diaphragm. Over 90% of muscle fibers in the mouse diaphragm are fast twitch type II fibers, and only 10% are type I (35). By comparison, in humans and in rats, fast twitch type II fibers account for about 60% of fibers in the diaphragm, and 40% are slow twitch type I (36).

In line with these observations, SERCA2 mRNA (expressed in slow twitch fibers) represents ~43% of the total SERCA mRNA measured in diaphragm in rats and presumably also in humans (37). We suggest that the levels of SERCA2 and SERCA3 that were detected in the mouse diaphragm did not contribute enough functional activity to rescue contraction and relaxation in the diaphragmatic muscle of SERCA1−/− mice, but the greater numbers of slow-twitch type I fibers, expressing the SERCA2 isoform, may do so in humans.

The fact that only the diaphragm muscle showed the defect, whereas hind limb muscle was histologically normal in SERCA1−/− mice, may be due to the diaphragm being the most differentiated muscle at birth (38). Consistent with this view (6, 39, 40), we also demonstrated that the total protein content of SERCA1 in wild-type newborn mice was 5-fold higher in diaphragm than in hind limb muscle by Western blot analysis (Fig. 4B). Our semiquantitative RT-PCR experiments showed that, in the diaphragm, about 50% of the total SERCA1 was the adult SERCA1a isoform, but in the hind limb muscle, SERCA1a accounted for only 20% of total SERCA1 (Fig. 4A). Furthermore, the $V_{\text{max}}$ of Ca$^2+$ transport activity was about 5 times higher in total diaphragm (9.72 ± 0.2 nmol of Ca$^2+$/mg of protein/min) than in total hind limb homogenates (2.26 ± 0.2 nmol of Ca$^2+$/mg of protein/min). This suggests that a high expression of the adult form of SERCA1 (SERCA1a) in the diaphragm at birth is crucial for the diaphragm to generate a force sufficient for respiration after birth.

To test the role of SERCA1 in an early stage of muscle development, we studied myotube formation in primary cultures from hind limb muscles from SERCA1−/− newborn mice. We did not detect any differences in the extent or rate of growth or differentiation of primary myoblasts into myotubes in cultures prepared from muscle obtained from either SERCA1−/− or SERCA1−/− (Fig. 7). When histological analysis of skeletal muscles was carried out in SERCA1−/− mice during embryo development at days 10, 15, and 18, no obvious changes were found. This suggests that SERCA1 is not required for the early stages of muscle development. This is consistent with observations (6, 39, 40) in which SERCA1 was only detected at a late stage of muscle development.

We cannot rule out the possibility, however, that SERCA1 plays a role in lung development in the very late prenatal stage. The preterm mouse lung is normally very cellular and "cuboidal," and this morphology evolves rapidly to adult respiratory epithelium over several days. SERCA1 could be involved either directly or indirectly with the final "terminal sac phase," which occurs between day 18 and term.

The $V_{\text{max}}$ of Ca$^2+$ transport activity in both total diaphragm and limb muscle homogenates of SERCA1−/− mice was reduced to roughly the same extent as the SERCA1 protein levels, confirming a direct relationship between SERCA protein level and the maximal rate of Ca$^2+$ sequestration. We also observed a lower apparent Ca$^{2+}$ affinity for SERCA in homogenates prepared from SERCA1−/− diaphragm. This may be due to an increased ratio of PLN and SLN to SERCA in SERCA1−/− mice due to the unchanged expression of PLN and SLN, as determined by semiquantitative RT-PCR in SERCA1−/− mice (Fig. 4A). Thus, in the SERCA1-deficient mice, not only is there a reduction in the total content of SERCA pumps, but those
pumps that remain are more highly inhibited.

The depression of the maximal rates of force relaxation (Fig. 6, A and D) of the response to electrical stimulation observed in SERCA1−/− diaphragm strips is fully consistent with the slow rate of Ca2+ pumping into the sarcoplasmic reticulum in SERCA1-deficient mice. The depression of the maximal rates of force development (Fig. 6C) and force output (Fig. 6, A and B) is also fully consistent with a decrease in Ca2+ release from the sarcoplasmic reticulum due to a diminished Ca2+ store in the sarcoplasmic reticulum. The impairment of excitation-contraction coupling may lead to elevated resting [Ca2+], which might initiate transient structure alterations and depressed Ca2+ release during contraction (41–43).

In addition to alterations in diaphragm contractile responses that are directly related to disruption of the Ca2+ signal, disruption of metabolic processes may occur as a consequence of loss of Ca2+ homeostasis, thereby contributing to a decline in muscle function. Increases in resting free Ca2+ levels would stimulate glucose transport into skeletal muscle (44), inhibit glycogenolysis, stimulate pyruvate dehydrogenase, and activate mitochondrial function (41). Structural and morphological degradation after exercise has also been proposed to result from increases in the cytosolic Ca2+ concentration. Several Ca2+-dependent proteases have been found to be activated after exercise (45), and myofibrillar proteins have been degraded by lysosomal proteases (46). All of these processes need to be evaluated further in our SERCA1−/− mouse model, which will be valuable for elucidating the mechanism of pathological changes due to the disruption of Ca2+ homeostasis.

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Targeted Disruption of the ATP2A1 Gene Encoding the Sarco(endo)plasmic Reticulum Ca\textsuperscript{2+} ATPase Isoform 1 (SERCA1) Impairs Diaphragm Function and Is Lethal in Neonatal Mice

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