Expression of SERCA2a is independent of innervation in regenerating soleus muscle

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SKELETAL MUSCLE IS A TISSUE composed of a variety of functionally diverse fiber types (for recent review, see Ref. 22). The rate of contraction of a skeletal muscle largely depends on the myosin heavy chain isoforms (MyHC), whereas the relaxation is initiated and maintained by the sarcoplasmic reticulum Ca²⁺-ATPases (SERCA). The expression of the slow muscle-type myosin heavy chain I (MyHCI) is entirely dependent on innervation, but, as we show here, innervation is not required for the expression of the slow-type sarcoplasmic reticulum Ca²⁺-ATPase (SERCA2a) in regenerating soleus muscles of the rat, although it can play a modulator role. Remarkably, the SERCA2a level is even higher in denervated than in innervated regenerating soleus muscles on day 7 when innervation is expected to resume. Later, the level of SERCA2a protein declines in denervated regenerated muscles but it remains expressed, whereas the corresponding mRNA level is still increased. SERCA1 (i.e., the fast muscle-type isoform) expression shows only minor changes in denervated regenerating soleus muscles compared with innervated regenerating controls. When the soleus nerve was transected instead of the sciatic nerve, SERCA2a and MyHCI expressions were found to be even more uncoupled because the MyHCI nearly completely disappeared, whereas the SERCA2a mRNA and protein levels decreased much less. The transfection of regenerating muscles with constitutively active mutants of the Ras oncogene, known to mimic the effect of innervation on the expression of MyHCI, did not affect SERCA2a expression. These results demonstrate that the regulation of SERCA2a expression is clearly distinct from that of the slow myosin in the regenerating soleus muscle and that SERCA2a expression is modulated by neuronal activity but is not entirely dependent on it.

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muscles that were denervated by transecting either the sciatic nerve or the soleus nerve. Our results demonstrate that the SERCA2a mRNA and protein are highly expressed in sciatic-denervated (whole limb denervated) regenerating soleus fibers, whereas the MyHCl mRNA and the protein are absent. The same absolute dependence on innervation of MyHCl expression, but not of SERCA2a expression, was inferred from regeneration experiments after the soleus nerve was cut (selective denervation). In this latter model, the weight of soleus was not decreased after 10 days, unlike in sciatic-denervated regeneration. Furthermore, we show that in vivo transfection of innervated regenerating muscle with a dominant negative Ras mutant or transfection of denervated regenerating muscles with a Ras mutant that selectively and constitutively activates the MAPK pathway only affects MyHCl expression (19) in the fibers but not SERCA2a expression.

MATERIALS AND METHODS

Many of the methods used here have been described before (17, 35, 36). However, a few important considerations are detailed below.

Animals and treatments. Experiments with animals were approved by the Ethics Committee of Animal Treatment of the Medical Faculty of the University of Szeged.

The soleus muscles of the left hindlimb of male Wistar rats 3 mo of age and weighing 300–360 g were treated with notexin as described previously (34). For transfection experiments, the muscles were reinjected 4 days later with 50 μg of plasmid DNA in 50 μl of 20% sucrose (19). Eight days after plasmid transfaction (12 days after notexin injection), the muscles were dissected and frozen in isopentane cooled in boiling liquid nitrogen. The muscles were kept at −70°C until use. Dissecting a 1-cm-long part of the sciatic nerve high in the thigh was used to denervate the hindlimb. In some experiments, a selective disruption of the innervation of the soleus muscle was obtained by removing a 2- to 3-mm-long piece from the soleus nerve leading to the proximal end of the muscle.

RT-PCR. RNA extraction and reverse transcription were carried out as described previously (34–35). Primers and PCR conditions are shown in Table 1. All the PCR cycles were carefully adjusted to the log/near phase of amplification. The ratio RT PCR of SERCA1/SERCA2 mRNA was done as described previously (33). Shortly, primers hybridizing to both SERCA1 and SERCA2 simultaneously amplified two fragments of the same size, and a restriction enzyme digest distinguished between the fragments. To radiolabel the PCR fragments for quantification, 5 μl (i.e., one-tenth of the total volume) of the primary PCR mixture was transferred to a new tube containing 50 μl of the same amplification buffer, except that [α-32P]dCTP was added. Two additional PCR cycles were executed with the same cycle parameters used in the primary PCR. The fragment of SERCA1 was cut by Msc I, which left SERCA2 intact, whereas NcoI, which did not cut SERCA1, digested the fragment of SERCA2. The ratio of SERCA2/SERCA1 mRNAs was calculated from the level of uncut fragments. The amplification products were analyzed on a 6% (wt/wt) polyacrylamide gels, which were then either stained with Vistra Green and the bands quantified by means of a model 840 Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or air-dried and the 32P spots quantified by means of the Typhoon 9400 Variable Mode Imager program (Molecular Dynamics). When [α-32P]dCTP was used for labeling, the band intensities were corrected for the CG (cytosine and guanine base) content of the amplified fragment.

Immunoblotting. The SERCA2a and MyHCl were measured on extracts from the same muscles. When denervated regenerating muscles were used, the volume of extracting buffer was decreased in proportion to their lower fresh weight. SERCA2a immunoblots were made from the mitochondrial-microsomal fraction as in Ref. 36. The pellets of the first centrifugation (1,000 g for 10 min) were used to extract myosin according to Ref. 9. Equal parts of the muscles were loaded on each lane of the gel for both SERCA and myosin analysis. The SERCA2a antisera R-15 (rabbit, 1:500; Ref. 32) and MyHCl antibody (BA-D5, mouse, 1:100, Ref. 25) were used as primary antibodies in the immunoblot analysis. The bands were either visualized by Ni-DAB staining (33) and then quantified by densitometry on Gel Doc (Bio-Rad Laboratories, Hercules, CA) or stained with the Vistra ECF system (Amersham Biosciences), following the protocol of the manufacturer, and quantified by model 840 Storm Chemiluminescence Imager (Molecular Dynamics).

Ras plasmids. A number of vectors expressing mutant Ras proteins were kindly provided by Dr. A. Serrano (Padua, Italy). The constitutively active Ras mutants H-RasV12 and H-RasV12S35 in the pDPC expression vector under the control of the CMV promoter were used (23, 24). These Ras mutants had a hemagglutinin (HA) epitope added at their COOH termini. An additional mutant used in this experiment was the dominant negative H-RasN17, which had no HA tag.

Immunostaining. Cryosections of 20 μm thickness obtained from regenerating muscles were stained by peroxidase immunohistochemistry (as in Ref. 36) by using the BA-D5

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Expected Fragment Size, bp</th>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′GAPDH</td>
<td>tctgccacacacaactgttagcc</td>
<td>377</td>
<td>94-60-72°</td>
</tr>
<tr>
<td>3′GAPDH</td>
<td>tagccagattcctccatggg</td>
<td></td>
<td>1-1-1 min/22 cycles</td>
</tr>
<tr>
<td>5′MyHCl</td>
<td>tatctccaggtctcagatttg</td>
<td>277</td>
<td>94-60-72°</td>
</tr>
<tr>
<td>3′MyHCl</td>
<td>taaatagatcactgacgagga</td>
<td></td>
<td>1-1-1 min/17 cycles</td>
</tr>
<tr>
<td>5′SERCA2a/SERCA2b</td>
<td>ctcctcaggtctcagatttg</td>
<td>231/328</td>
<td>94-55-72°</td>
</tr>
<tr>
<td>3′SERCA2a</td>
<td>agcagacagactatccatc</td>
<td></td>
<td>1-1-1 min/22 cycles</td>
</tr>
<tr>
<td>3′SERCA2b</td>
<td>gggtactccagatgg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5′SERCA1/SERCA2</td>
<td>gagcaggtgggtgagcgc</td>
<td>194/194</td>
<td>94-60-72°</td>
</tr>
<tr>
<td>3′SERCA1/SERCA2</td>
<td>gggtggtgagcgc</td>
<td></td>
<td>1-1-1 min/22 cycles</td>
</tr>
</tbody>
</table>
SERCA2a, but not MyHCI, is expressed in soleus muscles that are denervated before regeneration. De-
nervated regenerating rat soleus muscles do not ex-
press the slow isoform of myosin heavy chain (MyHCI) (31). In contrast, our immunohistochemical analysis
clearly indicated that after 12 days of regeneration, the
slow type of sarcoplasmic reticulum Ca²⁺-ATPase (SERCA2a) was expressed to nearly the same extent in
muscles that are denervated before regeneration. De-
sellous muscle.

Statistics. The Newman-Keuls test was used to test for
significant differences. The numbers of SERCA2a- and My-
HCl-expressing fibers were compared by using a t-test.

RESULTS

SERCA2a and SERCA1 mRNAs in denervated re-
genrating soleus muscle. Figure 2 shows that in de-
nervated regenerating soleus, MyHCI mRNA was ab-
sent, but the SERCA2a mRNA was clearly expressed on
days 7 and 10 and elevated on day 21. In the innervated regenerating control muscles, both MyHCI and SERCA2a mRNAs increased gradually from days 7 to 21. Denervation apparently decreased the levels of
SERCA2a mRNA 3- and 0.25-fold, respectively, on
days 10 and 21 compared with the innervated controls
(Fig. 2A). The ratio of SERCA2/SERCA1 mRNAs was
determined by a ratio RT-PCR technique (33, 35) (Fig. 
2B). We found that denervation suppressed SERCA1 
mRNA nearly completely by day 7 and, therefore, 
strongly increased the SERCA2a/SERCA1 mRNA ra-
tio. However, at later stages of regeneration the level of 
SERCA1 mRNA increased again. These observations 
are in line with earlier ones showing that denervation 
of a slow muscle promotes the fast phenotype (7, 31).

It should be remarked that the PCR primers used in 
all of these assays would coamplify SERCA2a and 
SERCA2b cDNAs. However, under our experimental 
conditions, the SERCA2a isoform accounted for practi-
cally all SERCA2. Tests with SERCA2b-specific prim-
ners showed the near absence of this isoform, so 
SERCA2a accounted for nearly all SERCA2.

In denervated regenerating soleus, a pronounced in-
crease of SERCA2a protein is followed by a gradual 
decrease. Next, we compared the time course of 
SERCA2a, SERCA1, and MyHCI proteins during re-
generation. In innervated control muscles, SERCA2a 
proteins gradually increased during regeneration and 
followed an expression pattern similar to that of the 
corresponding mRNA. Remarkably, denervation led to 
a three-fold higher level of SERCA2a compared with 
the innervated control on day 7 of regeneration (Fig. 3). 
Subsequently, SERCA2a expression gradually de-
clined at days 10 and 21. This shows that although 
inervation is clearly not a prerequisite for the expres-
sion of SERCA2a, it is needed for maintaining a long-
term expression of the Ca²⁺ pump.
The expression of the fast-type sarcoplasmic reticulum pump protein SERCA1 was less affected by denervation than that of SERCA2; it showed a transient increase by 20% on day 10 in the denervated regenerating soleus compared with the innervated control, but it declined back to the normal level afterward (Fig. 3). The expressions of SERCA2a and MHCI are also uncoupled in selectively denervated regenerating soleus.

In the experiments described above, denervation was done by removing a piece of the sciatic nerve. A drawback of this method is that it paralyzes the hindlimb and results in a decrease of both the fresh weight and the fiber diameter of regenerating soleus muscles. This side effect can be partially circumvented by selectively disrupting the soleus nerve instead of the main branch sciatic nerve. In such selectively denervated regenerating muscles, the denervation atrophy was somewhat delayed. After 10 days, the fresh weight, RNA, and protein content were similar to those in innervated regeneration (Table 2). However, the fiber mean CSA was smaller (1,010 ± 308 μm² vs. 1,944 ± 534 μm²) and similar to that of hindlimb denervated (1,031 ± 156 μm²) regenerating muscles. In selectively denervated regeneration, the slow myosin was nearly abolished, whereas SERCA2a was still present in most fibers (Fig. 4A, a and b). During innervated regeneration, MyHCI and SERCA2a were coexpressed, but some fibers expressed more MyHCI and SERCA2a than others (Ref. 33; Fig. 4A, c and d). By day 12 of regeneration, these isoforms were expressed in most fibers (see Fig. 1, A and B). In selectively denervated regenerating soleus, the MyHCI mRNA were nearly absent and the extracted MyHCI protein levels were extremely low (Fig. 4B); nonetheless, the SERCA2a mRNA level was less decreased than in sciatic denervation (87 ± 17.7% vs. 33.8 ± 13.7% of normal, P < 0.05). The levels of SERCA2a protein did not change significantly compared with those of the innervated regenerating muscle. This confirms the view that the denervated soleus muscle maintains SERCA2a expression but loses MyHCI expression. Furthermore, it shows that the uncoupled expression of MyHCI and SERCA2a is not linked to the degenerative changes like, e.g., the loss of fresh weight, RNA, and protein content of the muscle.

Ras mutants do not affect SERCA2a expression. Murgia et al. (19) showed that the in vivo expression of a dominant negative Ras mutant, RasN17, is able to mimic the effect of denervation on MyHCI expression in regenerating soleus muscle. Fibers constitutively expressing this mutant are deprived of Ras pathways transmitting nerve influence to the slow myosin transcription. We transfected regenerating soleus with RasN17 to investigate its effect on SERCA2a expression. Our hypothesis was that if SERCA2a and MyHCI were coregulated by the Ras pathway, they would both follow similar kinetics. However, we found that MyHCI-negative fibers (Fig. 5Aa) still expressed...
SERCA2a with the same intensity as the MyHCI-positive fibers (Fig. 5Ab). This showed that whereas Ras affects MyHCI expression, it apparently does not affect that of the SERCA2a isoform.

The effect of denervation on MyHCI in regenerating soleus can also be reversed by the ectopic expression of RasS35, a constitutively active form of Ras that specifically stimulates the MAPK pathway (19). In vivo expression of this RasS35 mutant in regenerating soleus (Fig. 5Bc) did not stimulate SERCA2a expression above the normal level (Fig. 5Bb) in the MyHCI-expressing regenerating fibers (Fig. 5Ba). A similar result was obtained with the overexpression of the Ras12V mutant (data not shown). Ras12V is also a constitutively active Ras mutant known to stimulate MyHCI expression but which, in contrast to RasS35 besides the MAPK pathway, also targets the phosphatidyl inositol-3-OH kinase pathway and ralGDS, the guanine dissociation stimulator for the GTPase Ral (19). The above observations confirm that the expression of SERCA2a is independent from Ras.

DISCUSSION

Our results clearly demonstrate that innervation is not required for the expression of the slow-type sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase SERCA2a in regenerating soleus muscles of the rat, whereas the maintenance of SERCA2a protein level is dependent on innervation. This dependence is probably indirect because SERCA2a has to incorporate into the sarcoplasmic reticulum, which itself shows a nerve-dependent development (3). Our results are consistent with the early expression of the SERCA2a protein on day 5 of regeneration, i.e., before the new innervation of regenerating soleus muscle is established (36), and with the reported SERCA2a and MyHCI mismatch in fibers of muscles adapting after spinal cord injury (29). However, our data are at variance with the report of Germinario et al. (7), who claim that the expression of the SERCA2a and of MyHCI proteins is strictly dependent on innervation. Indeed, Germinario and coworkers did not detect SERCA2a between days 5 and 10 of regeneration, whereas, in the experiments reported here or previously, we clearly did (36). The time course of muscle regeneration after bupivacain induced-necrosis as used by Germinario et al. (7) appears slightly different from that after notexin-induced necrosis because the cellular events accompanying muscle regeneration depend on the nature of injury (15). Moreover, bupivacain probably elicits a less complete necrosis than notexin, and therefore the regeneration starts earlier. Innervation of new fibers is established on day 5 after bupivacain (13, 19, 28) and only on day 7 after notexin injection (8).

The three-fold increase of SERCA2a level after 1 wk of regeneration in denervated muscles compared with the innervated controls can clearly not be ascribed to a direct neuronal effect on SERCA2a expression. This is an indirect effect, which might rather be the result of apoptotic changes (14) causing the dramatic loss of fresh weight of denervated regenerating muscles. Remarkably, the time-dependent changes in the ratio of SERCA2/SERCA1 mRNA levels paralleled changes in the level of the SERCA2a protein rather than that of the SERCA2a mRNA level. The ratio of SERCA2/SERCA1 mRNAs is a possible indicator of the progress of soleus muscle regeneration: it is lower than 1 shortly after the onset of regeneration (day 5) but increases to values >3 when regeneration is accomplished (35).

The increase of the SERCA2a mRNA on day 21 in the denervated regenerating muscle further indicates that the expression of this Ca²⁺ pump can occur in the absence of neuronal activity. The lack of correlation between SERCA2a mRNA and its corresponding protein suggests an important posttranscriptional component in the regulation of SERCA2a expression (reviewed in Ref. 18). However, in denervated soleus the...
SERCA1 mRNA level gradually increased during regeneration and became even higher than that of SERCA2a on days 10 and 21. Hence, the ratio of SERCA2/SERCA1 mRNA declined on days 10 and 21. Also, the SERCA2a protein was lower on days 10 and 21. A similar decrease of SERCA2a protein has been reported in denervated developing muscles (27) and in denervated soleus muscle (16, 20). It has been shown that the expression of fast myosin heavy chains MyHCIIx/d (and MyHCIIa) is upregulated in denervated developing/regenerating muscle and that only after the muscle becomes subject to an electrical stimulation pattern typical for slow-twitch muscle does a switch to the expression of the slow MyHC1 form occur (19, 31). Surprisingly, a corresponding upregulation of the SERCA1 mRNA and protein levels was not observed in denervated regenerating muscles on day 7. Instead, the SERCA1 mRNA level was practically abolished on day 7, than increased dramatically on day 10, whereas the SERCA1 protein level did not decrease on day 7 and increased by 20% on day 10 in denervated regenerating muscle.

### Table 2. Fresh weight, RNA, and protein content of innervated and denervated regenerating soleus muscles

<table>
<thead>
<tr>
<th>Days of Regeneration</th>
<th>Innervated Regenerated</th>
<th>Hindlimb-Denervated Regenerated</th>
<th>Selectively Denervated Regenerated</th>
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<tbody>
<tr>
<td>Fresh weight, mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>115 ± 5</td>
<td>52 ± 2.5***</td>
<td>103 ± 3</td>
</tr>
<tr>
<td>10</td>
<td>120 ± 5.7</td>
<td>56.7 ± 8.8**</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>143 ± 3.3**</td>
<td>55 ± 5.4***</td>
<td></td>
</tr>
<tr>
<td>RNA content, µg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>194 ± 46</td>
<td>18.5 ± 3.3*</td>
<td>79.7 ± 16.3</td>
</tr>
<tr>
<td>10</td>
<td>174 ± 43</td>
<td>38.5 ± 3.2***</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>106 ± 31</td>
<td>15.5 ± 5.8*</td>
<td></td>
</tr>
<tr>
<td>Protein content, µg</td>
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<tr>
<td>7</td>
<td>2541 ± 184</td>
<td>857 ± 78***</td>
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<tr>
<td>10</td>
<td>3433 ± 428</td>
<td>902 ± 234***</td>
<td></td>
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<tr>
<td>21</td>
<td>2708 ± 11</td>
<td>600 ± 92***</td>
<td></td>
</tr>
</tbody>
</table>

The RNA was measured from the total RNA extracts and the protein from the homogenate of the microsomal and myosin extractions. Values are means ± SE, n = 6 for the fresh weight and n = 3 for the RNA and protein contents. *Significant (P<0.05), **very significant (P<0.01), and ***highly significant (P<0.001) difference compared with the innervated regenerating muscle; *significant and **very significant difference during regeneration.

Fig. 4. The distinct regulation of SERCA2a and MyHC1 in fibers of selectively denervated (transsection of soleus nerve) regenerating soleus muscle after 10 days of intramuscular injection of notexin (A). Note abolished MyHC1 expression of selective denervated (sdr, a) compared with the innervated regenerating soleus (r, c), whereas SERCA2a is expressed at similar intensity in the fibers of selectively denervated (b) and innervated (d) regenerating muscles. B: note decreased mRNA and protein levels of MyHC1, whereas for SERCA2a only the mRNA level is decreased with about 25% of that of the innervated regenerating muscle. Symbols and abbreviations are as in Fig. 2. Sel denerv reg, selective denervated regenerating soleus.

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However, in the innervated soleus, both the SERCA2a and the MyHCI levels gradually increased with the progress of slow muscle regeneration, as could be expected because both are characteristic components of a slow muscle. Calcineurin was suggested as a candidate common regulator of MyHCI and SERCA2a expression because the number of slow fibers coexpressing MyHCI and SERCA2a declines in soleus muscles of rats fed with a cyclosporin A-supplemented diet (2) and because the expression of MyHCI and SERCA2a changed in parallel in fibers of regenerating muscle adapting phenotypically to overload (1). Calcineurin, via transcription factors like NFAT, can mediate excitation-transcription coupling and can therefore regulate slow muscle-type transcription (21). The role of calcineurin in the upregulation of MyHCI expression is well documented as the overexpression of cain, a protein inhibitor of calcineurin, prevents MyHCI expression in the fibers of regenerating soleus (28). However, because denervation does not abolish expression of SERCA2a, it is difficult to perceive a calcineurin-mediated regulatory pathway for this sarcoplasmic reticulum Ca$^{2+}$ pump as well.

Talmadge et al. (29) demonstrated that the expression of SERCA2a and slow myosin is not always coupled in fibers of muscles phenotypically adapting to spinal cord injury. Such fibers have probably lost nerve influence but remain stretched by the surrounding intact fibers. A similar situation might exist in selective denervation of soleus when the functional innervation of the other hindlimb muscles remains intact. Here, we show that in the regenerating soleus when only the soleus nerve is ablated (selective denervation), the SERCA2a mRNA and protein levels are higher than in the case of ablation of the sciatic nerve (hindlimb denervation), whereas in both types of denervation the MyHCI is abolished. This further points to an uncoupling of expression of these two characteristically slow protein isoforms. Another characteristic of the selective denervation is that the muscle keeps its weight, RNA, and protein content, although the size of its individual fibers is decreased compared with the innervated regenerating control, probably because of the retarded regeneration. Therefore, the expression of SERCA2a is less likely to be stimulated by apoptosis-related changes (14) than in the sciatic-denervated regenerating muscles in which these parameters are lower.

We tested the effect of the Ras oncogene, another reported mediator of neuronal influence, on slow myosin expression. This further supported the view that these contraction and relaxation elements are separately reg-
The overexpression of RasS35, which is able to mimic nerve influence on MyHCI expression in the denervated regenerating muscle (19), left SERCA2a expression unaffected. Similarly, the dominant negative Ras mutant, known to mimic denervation affects on MyHCI expression (19), did not influence the expression of the slow SR Ca\(^{2+}\) pump. The fibers expressing dominant negative Ras are probably stretched by the intact fibers; this situation is somewhat similar to that of the selectively denervated regenerating soleus and that of fibers adapting after spinal cord injury. Apparently, the passive stretch did not compensate for the inhibition of MyHCI expression in denervated regenerating muscle (19), left SERCA2a expression unaffected. Similarly, the dominant negative Ras are probably stretched by the intact fibers; this situation is somewhat similar to that of the selective denervation of slow twitch muscle of rat. Apparently, the passive stretch did not compensate for the inhibition of MyHCI expression in denervated regenerating muscle. Cell Struct Funct 22: 147–153, 1997.

It remains at present an open question, what regulates SERCA2a expression in the regenerating soleus muscle. Our results show that this regulation must be more complex than that of MyHCI. SERCA2a expression is regulated at both transcriptional and posttranscriptional levels, unlike that of MyHCI, where the regulation is primarily at the transcriptional level.

We thank Dr. A. Serrano (Padua, Italy) for kindly providing the Ras expression vectors.

DISCLOSURES

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