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Expression of SERCA2a is independent of innervation in regenerating soleus muscle

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Zádor, Ernő, and Frank Wuytack. Expression of SERCA2a is independent of innervation in regenerating soleus muscle. *Am J Physiol Cell Physiol* 285: C853–C861, 2003. First published May 28, 2003; 10.1152/ajpcell.00592.2002.—The speed 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 Ca^{2+}-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 Ca^{2+}-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 increasing. 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.

slow type sarcoplasmic reticulum Ca^{2+} pump; MyHCI; nerve influence

SKELETAL MUSCLE IS A TISSUE composed of a variety of functionally diverse fiber types (for recent review, see Ref. 22). The rate of contraction and of relaxation of a skeletal muscle fiber is largely determined by the myosin heavy chain (MyHC) and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) isoforms, respectively (4, 26). The slow-twitch fibers of rat soleus predominantly express MyHCI and SERCA2a (17, 30, 36). In a number of experimental models in which muscle undergoes phenotypic transitions, the expression of both proteins apparently changes in coordination, e.g., upon chronic low-frequency stimulation of normal muscles (9) or after denervation of muscles of euthyroid rabbits, (10), but in other models the coordination is less clear or absent, resulting in mismatched MyHCI and SERCA2a expression (29). It is well documented that the expression of the slow MyHCI form in soleus is strictly dependent on neuronal activity and that its regulation occurs mainly at the transcriptional level. However, the relative contribution of various signaling pathways in this process remains to be established, because both a calcineurin-dependent pathway (28) and a Ras/MAPK pathway (19) can control this excitation-transcription coupling. Calcineurin relays changes in neuronal influence by dephosphorylating NFAT, a transcriptional activator, and thereby helps it to translocate to the nucleus, where NFAT may stimulate the transcription of MyHCI and various other genes related to the slow-twitch phenotype (5; reviewed in Ref. 21). Feeding rats with a diet containing an inhibitor of calcineurin like cyclosporin-A decreased the number of slow fibers expressing MyHCI and SERCA2a (2), which suggested that MyHCI and SERCA2a are coregulated by a calcineurin-dependent pathway. However, SERCA2a and MyHCI follow different transient expression patterns in fibers of human muscles phenotypically adapting after spinal cord injury (29, 37). Furthermore, during regeneration of rat soleus muscles from notexin-induced necrosis (12), SERCA2a is already expressed on the fifth day (36), whereas MHCI only appears after innervation has been established on *day 7* (6, 8, 13, 31). The new innervation after notexin-induced necrosis is often made by axon collaterals (11), which might explain why the proportion of slow-type fibers increases in the predominantly slow-type soleus after regeneration (31). One report describes a similar dependence of SERCA2a expression on nerve influence as for MyHCI (7); however, these authors could not detect SERCA2a before *day 15* in the regenerating soleus, and they admit the limited sensitivity of their SERCA immunodetection.

In the present study, we followed the mRNA and protein levels of SERCA2a in regenerating rat soleus

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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 MyHCI mRNA and the protein are absent. The same absolute dependence on innervation of MyHCI 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 MyHCI 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 transfection (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/linear 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-^{32}P]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 *Mse* I, which left SERCA2 intact, whereas *Nco*I, 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 ^{32}P spots quantified by means of the Typhoon 9400 Variable Mode Imager program (Molecular Dynamics). When [α-^{32}P]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 MyHCI 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 antiserum R-15 (rabbit, 1:500; Ref. 32) and MyHCI 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 Chemifluorescence 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 pDCR 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 1. *Primers and PCR conditions*

Primer	Sequence (5′→3′)	Expected Fragment Size, bp	PCR Conditions
5′GAPDH	tcctgcaccaccaactgcttagcc	377	94-60-72°
3′GAPDH	tagcccaggatgccctttagtggg		1-1-1 min/22 cycles
5′MyHCI	tatcctcaggcttcaagatttg	277	94-60-72°
3′MyHCI	taaatagaatcacatggggaca		1-1-1 min/17 cycles
5′SERCA2a/SERCA2b	ctccatctgcttgtccat	231/328	94-55-72°
3′SERCA2a	agaccagaacatatcact		1-1-1 min/22 cycles
3′SERCA2b	gcggttactccagtattg		
5′SERCA1/SERCA2	gacgagtttggggaacagct	194/194	94-60-72°
3′SERCA1/SERCA2	gaggtggtgatgacagcagg		1-1-1 min/22 cycles

antibody (mouse 1:50) for MyHCI and the R-15 antiserum (rabbit, 1:400) for SERCA2a. The expression from the HA-tagged Ras plasmids was identified by HA antibody (mouse 1:200; Calbiochem) and expression of the dominant negative Ras by the pan Ras (Ab-1) antibody (mouse 1:20; Santa Cruz).

Fiber cross-sectional area. Cross-sectional area (CSA) was measured on hematoxilin/eosin-stained sections by the Olympos DP-soft, version 3.2 program (Olympus, Hamburg, Germany).

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

RESULTS

SERCA2a, but not MyHCI, is expressed in soleus muscles that are denervated before regeneration. Denervated regenerating rat soleus muscles do not express 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 Ca^{2+}-ATPase (SERCA2a) was expressed to nearly the same extent in the fibers of denervated and innervated muscles (Fig. 1, *B* and *D*). When the nerve is left intact, new fiber innervation is established by *day 7* in soleus muscle regenerating from a notexin-induced necrosis (8). Our results, therefore, indicate that unlike for MyHCI (Fig. 1, *A* and *C*), the expression of SERCA2a is not strictly dependent on innervation. To better assess the extent of neuronal independence of SERCA2a expression, we followed its mRNA and protein levels during regeneration of denervated and innervated muscles. Because, in particular, in the early phase of regeneration soleus also expresses SERCA1, and because the SERCA2a/SERCA1 protein ratio is typically high in slow-twitch muscle and low in fast-twitch muscle, we also monitored the mRNA and protein levels of the SERCA1 pump (35, 36).

SERCA2a and SERCA1 mRNAs in denervated regenerating soleus muscle. Figure 2 shows that in denervated regenerating soleus, MyHCI mRNA was absent, 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. 2*A*). The ratio of SERCA2/SERCA1 mRNAs was determined by a ratio RT-PCR technique (33, 35) (Fig. 2*B*). We found that denervation suppressed SERCA1 mRNA nearly completely by *day 7* and, therefore, strongly increased the SERCA2a/SERCA1 mRNA ratio. 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 practically all SERCA2. Tests with SERCA2b-specific primers showed the near absence of this isoform, so SERCA2a accounted for nearly all SERCA2.

In denervated regenerating soleus, a pronounced increase of SERCA2a protein is followed by a gradual decrease. Next, we compared the time course of SERCA2a, SERCA1, and MyHCI proteins during regeneration. 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 declined at *days 10* and *21*. This shows that although innervation is clearly not a prerequisite for the expression of SERCA2a, it is needed for maintaining a long-term expression of the Ca^{2+} pump.

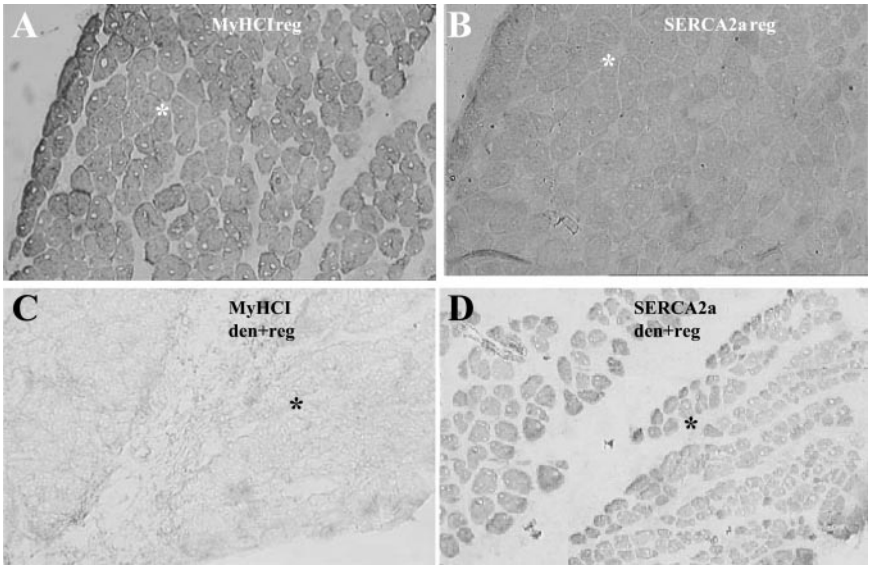

Fig. 1. The slow-type sarcoplasmic reticulum Ca^{2+}-ATPase SERCA2a was not changed by denervation in the soleus fibers after 12 days of regeneration (*B* and *D*), whereas myosin heavy chain I (MyHCI) was abolished (*A* and *C*). Reg, innervated regenerating muscle; den + reg, denervated regenerating muscle. *Identical positions on consecutive sections.

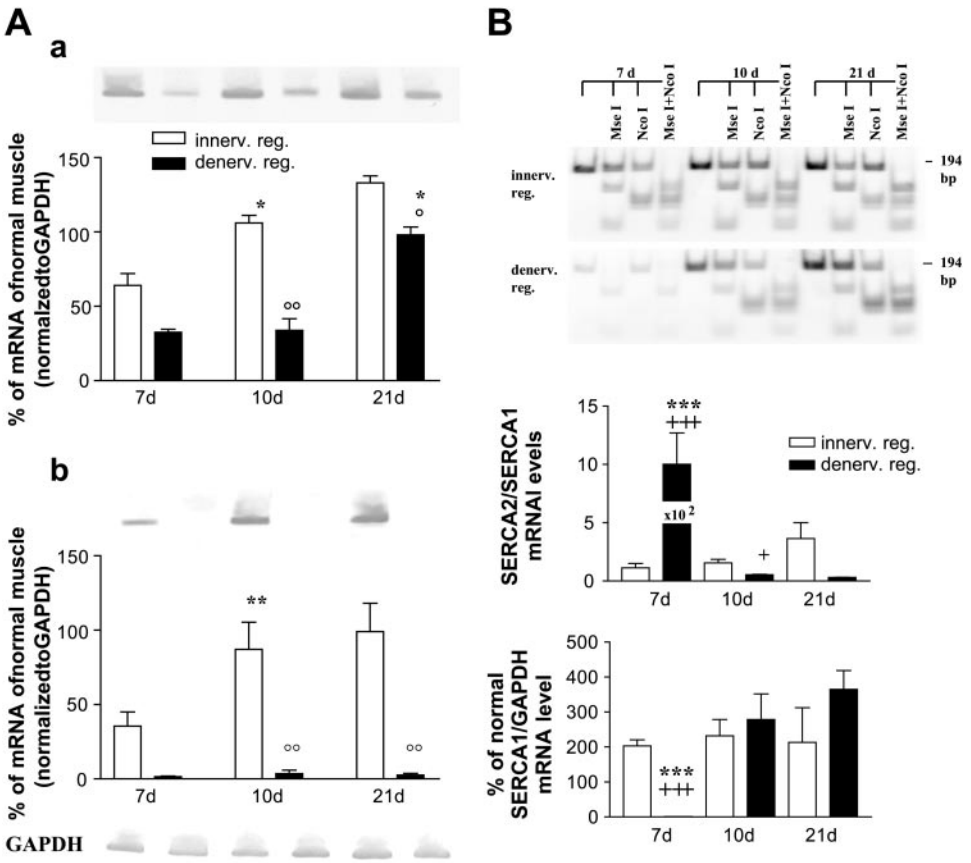

Fig. 2. *A*: mRNA levels of SERCA (*a*) and MyHCI (*b*) isoforms. *B*: ratio of SERCA2 and SERCA1 mRNAs and levels of SERCA1 mRNA in denervated and innervated regenerating soleus. Values are means ± SE, $n = 3$. *Significant ($P < 0.05$), **very significant ($P < 0.01$), and ***highly significant ($P < 0.001$) changes during regeneration. °Similar differences between innervated and denervated regenerated samples. Innerv. reg., innervated regenerating; denerv. reg., denervated regenerating muscle. Note that *Mse*I hydrolizes SERCA1 and *Nco*I hydrolizes SERCA2 fragment amplified by ratio RT PCR. The *inset* shows 1 representative gel of the 3 repeated experiments. In *B*, the column representing SERCA2/SERCA1 ratio on *day 7* denervated regenerated muscle is 10^2 times higher than the scale of the ordinate, which is indicated in the column.

The expression of the fast-type sarcoplasmic reticulum pump protein SERCA1 was less affected by denervation than that of SERCA2a; 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 μm^2 vs. 1,944 ± 534 μm^2) and similar to that of hindlimb denervated (1,031 ± 156 μm^2) regenerating muscles. In selectively denervated regeneration, the slow myosin was nearly abolished, whereas SERCA2a was still present in most fibers (Fig. 4*A*, *a* and *b*). During innervated regeneration, MyHCI and SERCA2a were coexpressed, but some fibers expressed more MyHCI and SERCA2a than others (Ref. 33; Fig. 4*A*, *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. 4*B*); 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. 5*Aa*) still expressed

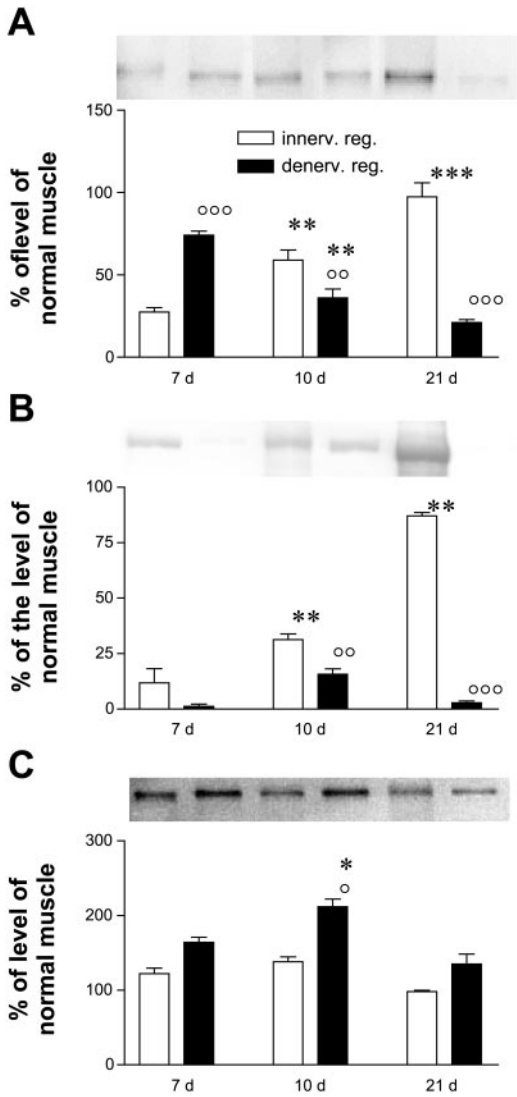

Fig. 3. The level of SERCA2a (*A*), MyHCI (*B*), and SERCA1 (*C*) in the soleus muscles regenerating under denervated (transection of sciatic nerve) and innervated conditions. Symbols are as in Fig. 2.

SERCA2a with the same intensity as the MyHCI-positive fibers (Fig. 5*Ab*). 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. 5*Bc*) did not stimulate SERCA2a expression above the normal level (Fig. 5*Bb*) in the MyHCI-expressing regenerating fibers (Fig. 5*Ba*). 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 Ca^{2+}-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. This difference might be explained by the higher sensitivity of the SERCA2a immunoassay that we used. 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 Ca^{2+} 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

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

	Days of Regeneration	Innervated Regenerated	Hindlimb-Denervated Regenerated	Selectively Denervated Regenerated
Fresh weight, mg				
	7	115 ± 5	52 ± 2.5***	
	10	120 ± 5.7	56.7 ± 8.8**	103 ± 3.3
	21	143 ± 3.3°°	55 ± 5***	
RNA content, μg				
	7	194 ± 46	18.5 ± 3.3*	
	10	174 ± 43	38.5 ± 3.2*°°	79.7 ± 16.3
	21	106 ± 31	15.5 ± 5.8*	
Protein content, μg				
	7	2541 ± 184	857 ± 78**	
	10	3433 ± 428	902 ± 234**	2223 ± 113
	21	2708 ± 11	600 ± 92***°	

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.

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 MyHCI 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.

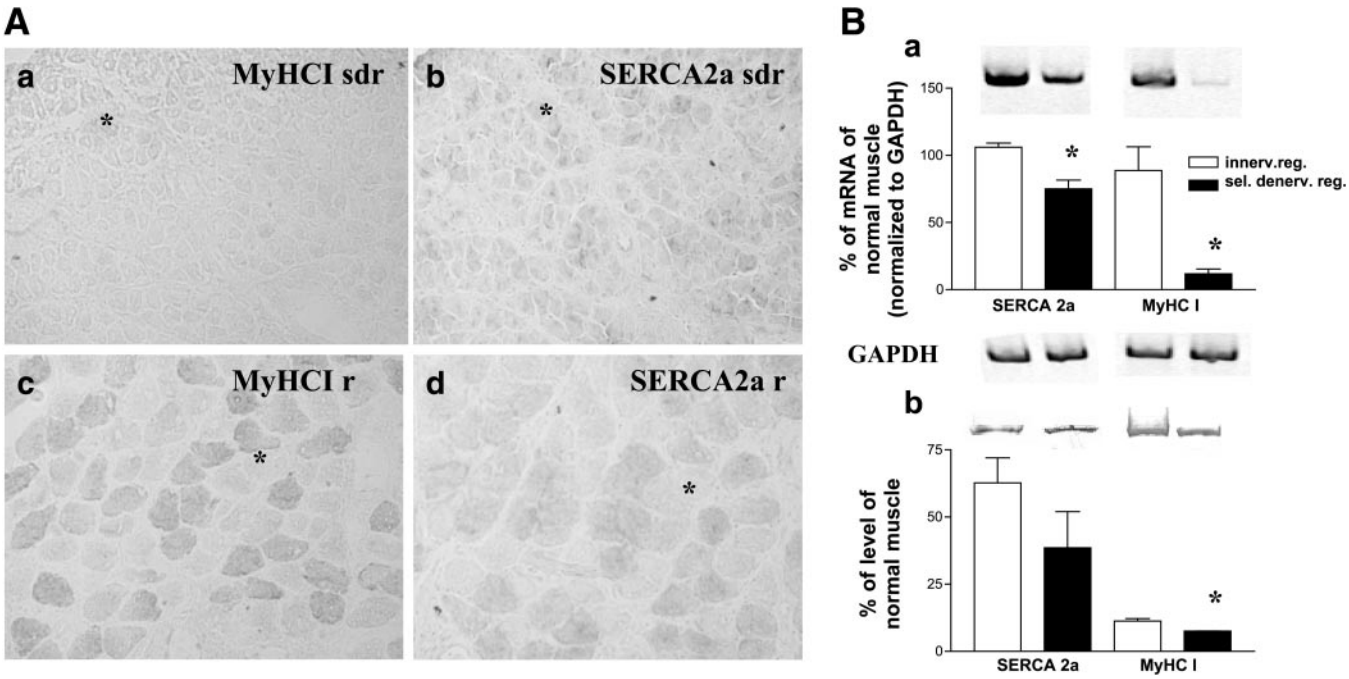

Fig. 4. The distinct regulation of SERCA2a and MyHCI in fibers of selectively denervated (transsection of soleus nerve) regenerating soleus muscle after 10 days of intramuscular injection of notexin (*A*). Note abolished MyHCI expression of selective denervated (sdr, *a*) compared with the innervated 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 MyHCI, 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.

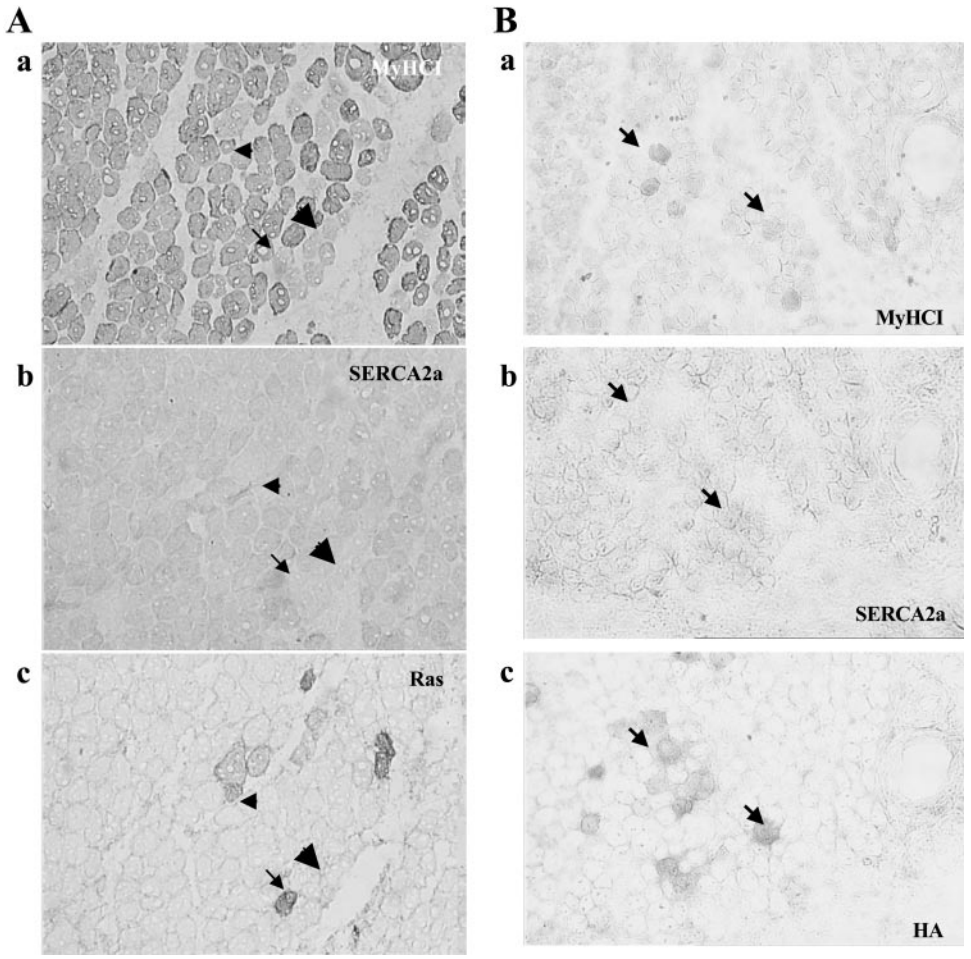

Fig. 5. Expression of MyHCI and SERCA2a in fibers (arrows) of regenerating muscles transfected with Ras. Note that the dominant negative RasN17 mutant abolished (*Aa*), whereas RasS35 induced (*Ba*), MyHCI expression with no obvious effect on SERCA2a (*Ab* and *Bb*). pRSV RasN17 was injected into innervated regenerating and pDCR RasV12 S35 into denervated regenerating muscle. Ras N17 was identified by anti-Ras (*Ac*) and H-Ras V12S35 by anti-hemagglutinin (HA) antibody (*Bc*) on the parallel sections.

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-

ulated. 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 fibers expressing dominant negative Ras; however, it might contribute to the maintenance of the level of SERCA2a expression like it did in the selectively denervated regenerating soleus.

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.

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DISCLOSURES

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corrigendum

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Pages C853–C861: Zador E and Wuytack F. "Expression of SERCA2a is independent of innervation in regenerating soleus muscle." On page C854, right column, line 8, the sentence describing restriction enzymes used in the PCR cycles was in error and should read "The fragment of SERCA1 was cut by *Nco*I, which left SERCA2 intact, whereas *Mse*I, which did not cut SERCA1, digested the fragment of SERCA2." Accordingly, on page C856, Fig. 2 and its legend should appear as shown below. (See http://ajpcell.physiology.org/cgi/content/full/285/3/C652)

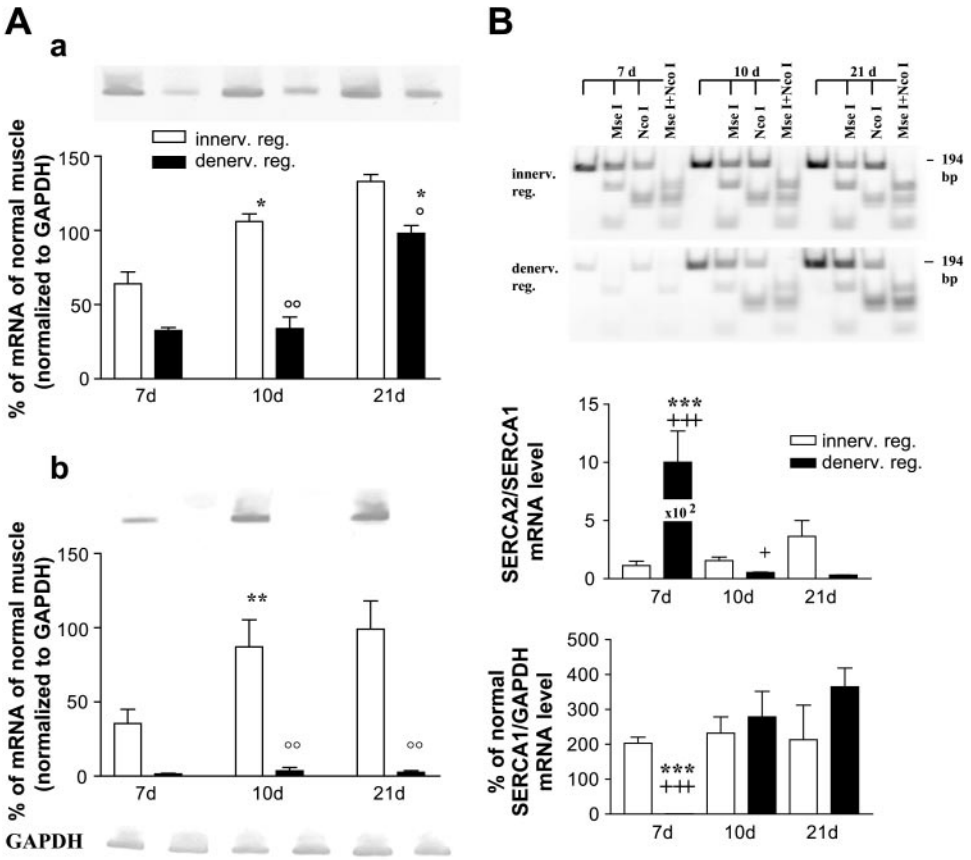

Fig. 2. *A*: mRNA levels of SERCA (*a*) and MyHCI (*b*) isoforms. *B*: ratio of SERCA2 and SERCA1 mRNAs and levels of SERCA1 mRNA in denervated and innervated regenerating soleus. Values are means ± SE, $n = 3$. Significant ($^{*}P < 0.05$), very significant ($^{**}P < 0.01$), and highly significant changes ($^{***}P < 0.001$) during regeneration are indicated. In *A*, significant ($^{\circ}P < 0.05$) and very significant differences ($^{\circ\circ}P < 0.01$), and in *B*, significant ($^{+}P < 0.05$) and highly significant differences ($^{+++}P < 0.001$), between innervated and denervated regenerated samples are indicated. Innerv. reg., innervated regenerating muscle; denerv. reg., denervated regenerating muscle. Note that *Mse*I hydrolizes SERCA2 and *Nco*I hydrolizes SERCA1 fragment amplified by ratio RT PCR. *Inset*: 1 representative gel of 3 repeated experiments. In *B*, the column representing SERCA2/SERCA1 ratio in *day 7* denervated regenerated muscle is 10^2 times higher than the scale of the ordinate; this is indicated in the column.