

# Expression of SERCA2a is not regulated by calcineurin or upon mechanical unloading in skeletal muscle regeneration

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Received 16 November 2004; revised 17 December 2004; accepted 22 December 2004

Available online 11 January 2005

Edited by Felix Wieland

**Abstract** This study investigates to what extent the expression of the slow myosin heavy chain (MyHC1) isoform and the slow type sarcoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA2a) isoform are co-regulated in fibers of regenerating skeletal soleus muscle. Both overexpression of cain, a calcineurin inhibitor, or partial tenotomy prevented the expression of MyHC1 but left SERCA2a expression unaffected in fibers of regenerating soleus muscles. These data complement those from different experimental models and clearly show that the expression of MyHC1 and SERCA2a – the major proteins mediating, respectively, the slow type of contraction and relaxation – are not coregulated in regenerating soleus muscle.

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*Keywords:* SERCA2a; MyHC1; Calcineurin; Tenotomy; Regeneration

## 1. Introduction

Skeletal muscle contraction takes place when the head of the myosin heavy chain (MyHC) pulls actin filaments toward the middle of the sarcomere. Conditions for muscle relaxation are established when the sarcoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA) decreases the Ca<sup>2+</sup> concentration in the sarcoplasm by re-accumulating Ca<sup>2+</sup> into the sarcoplasmic reticulum. The slow type of myosin heavy chain isoform (MyHC1) and the slow type of sarcoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA2a) are characteristic markers of slow-twitch skeletal muscle and are normally co-expressed in slow type fibers. Further, MyHC1 and SERCA2a levels mostly change in parallel during fast to slow transition of fiber types [1], upon denervation [2], during regeneration [3–5], and in adaptations to overload [6] and passive stretch [7]. The expression of the slow type MyHC1 is entirely dependent on the corresponding slow type of innervation and is mainly regulated at the gene transcriptional level [8,9]. It has also been suggested that calcineurin coregulates MyHC1 and SERCA2a expression during muscle remodeling, because oral application of cyclosporin A (CSA), a calcineurin inhibitor, decreased the ratio of (slow) fibers coexpressing MyHC1

and SERCA2a in the soleus [10]. Indeed, calcineurin mediates the slow-nerve dependent determination of the slow fiber identity and the expression of MyHC1 [9]. In contrast, the expression of SERCA2a appears to be neuronal-independent and controlled mainly posttranscriptionally [11]. Taken together, these observations make it less likely that calcineurin controls SERCA2a expression. However, during muscle regeneration, the most pronounced increase in the SERCA2a protein level occurs between days 10 and 21, but it is not accompanied by a comparable change in its mRNA level [12,4,13]. The possibility of a post-transcriptional calcineurin-mediated regulation of SERCA2a expression cannot be excluded. We investigated this possibility by overexpressing cain, a calcineurin inhibitor, in fibers of regenerating muscle at days 12 and 21 and showed that the expression of SERCA2a is unaffected, in contrast to that of MyHC1 [9].

Calcineurin controls switching of fiber type in mechanically overloaded muscle [14]. The levels of MyHC1 also change in coordination with those of SERCA2a in fibers of regenerating plantaris muscles exposed to overload [6]. The level of MyHC1 in regenerating muscle is lowered by tenotomy [15] but, to our knowledge, there are no reports on changes of SERCA2a expression in tenotomized regenerating muscle. The reports only concern the effect of overload on normal [16] and regenerating muscle [6]. We now used partial tenotomy as a model for muscle fiber unloading to further compare the regulation of SERCA2a and MyHC1 expression in fibers of regenerating soleus. The experiments reported here provide further evidence for a distinct regulation of expression of the slow type contractile elements (MyHC1) and the corresponding slow type of relaxation apparatus (SERCA2a) in skeletal muscle.

## 2. Materials and methods

Treatment of male Wistar rats (300–360 g) and the induction of muscle regeneration were the same as reported previously [11]. 50 µg of the plasmid expressing the calcineurin inhibitory domain of cain from CMV promoter [17] was injected intramuscularly in 50 µl of 20% sucrose on day 4 of regeneration as in [11]. The efficiency of in vivo transfection was tested in parallel experiments using enhanced GFP controlled by the CMV IE promoter and terminated by the SV40 polyadenylation signal (GFP cassette of the pEGFP-C1 vector, Clontech). In other experiments, the proximal tendon of soleus was partially cut with fine scissors to achieve partial tenotomy immediately before the induction of regeneration. Both cain-injected and tenotomized soleus muscles were dissected at days 12 and 21 of regeneration.

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Immunostaining on parallel frozen sections was done as in [11]. The cain expression was assessed by the myc-tag using anti-myc antibody (mouse, Roche, 1:25). The slow myosin and SERCA2a were stained with BA-D5 mouse monoclonal (1:50) and the R-15 (goat, 1:500) antibodies, respectively, as in [11]. The HRP coupled secondary antibodies, anti-mouse (rabbit, 1:100) and anti-rabbit (goat, 1:100), were purchased from DAKO (A/S Denmark). The immunoreaction was visualized by DAB-peroxidase staining which, in case of anti-myc, was enhanced by nickel ammonium sulfate. Muscle fibers were counted on whole cross-sections using the Analysis program (Soft Imaging System GmbH, Münster, Germany). Each set of experiments was repeated on three or four muscles.

### 3. Results

To investigate whether calcineurin also controls SERCA2a expression, soleus muscles were transfected on day 4 of regeneration with a plasmid expressing the calcineurin inhibitor domain of cain/cabin-1 protein. In spite of the fact that in our assay overexpression of cain could only be detected in a few fibers (i.e., in 2–3 times less fibers than GFP expression in a control experiment, see footnotes in Table 1), it apparently inhibited the expression of slow myosin in many fibers but showed little or no effect on SERCA2a expression at day 12 (Fig. 1(a)–(c), Table 1). Also at day 21 of regeneration, SERCA2a expression was not different between fibers expressing slow myosin and those that were slow myosin-negative (Fig. 1(e)–(g)). On a total of 202 MyHCI-negative fibers counted in a sample of three cain-injected muscles, only three fibers were found negative for SERCA2a on day 12 of regeneration (Table 1), but even those rare SERCA2a-negative fibers were not cain-positive. This clearly shows that MyHCI (i.e., a slow contraction element) and SERCA2a (a slow relaxation element) are differentially reacting to calcineurin in the regenerating soleus.

Calcineurin controls the expression of slow myosin [9] and also controls the fiber-type switch upon mechanical overload [14]. Therefore, we mechanically unloaded regenerating muscle by partial tenotomy, in order to assess the effect of unloading on SERCA2a expression during regeneration. MyHCI and SERCA2a are present in many fibers on day 10 of regeneration [4,11] and are uniformly coexpressed in most fibers on day 12 of intact regenerating soleus (Fig. 2(a) and (b), Table 1). In partially tenotomized regenerating soleus, the fibers affected by the tenotomy can be readily identified by their lack of

MyHCI expression [15] on day 12 of regeneration (Fig. 2(c), Table 1). However, SERCA2a is still present in most fibers and hence its expression appears to react independently from

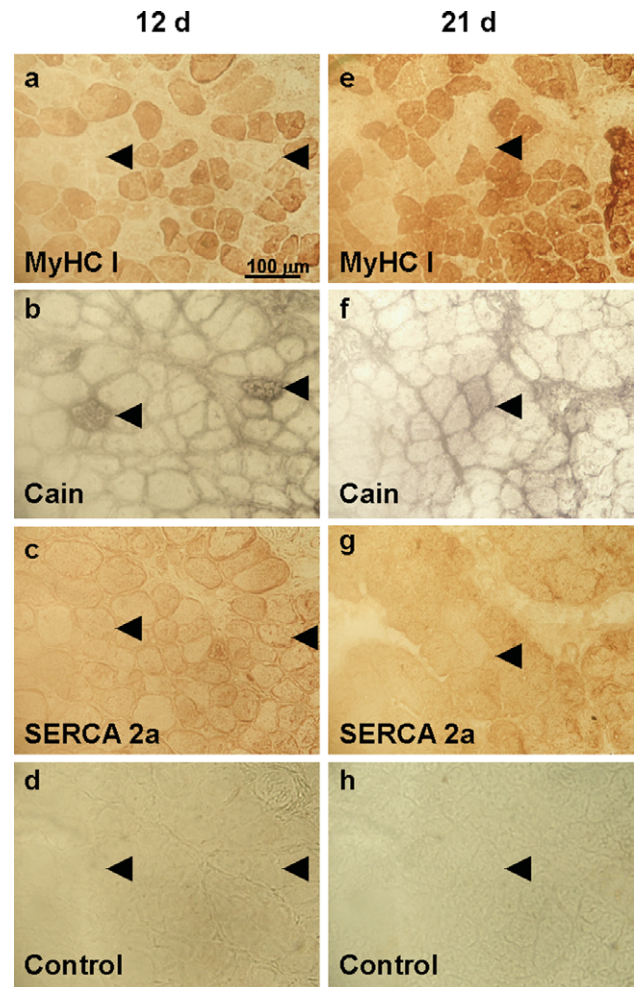


Fig. 1. Cain/cabin-1 abolishes MyHCI levels but leaves SERCA2a expression unaffected in regenerating rat soleus. The expression of MyHCI (a, e), cain (b, f) and SERCA2a (c, g) in fibers of 12 (a–d) and 21-days regenerating (e–h) soleus muscles transfected with plasmid expressing cain/cabin-1. Control sections (d, h) were immunostained as for SERCA2a except for the omission of the primary antibody. Arrows mark identical fibers on parallel sections.

Table 1

Number of fibers not expressing cain/cabin-1, MyHCI and SERCA2a in cain transfected (three muscles) and partially tenotomized regenerating muscles (four muscles) on days 12 and 21

| Muscle treatment                                       | Fibers          |        |                 |
|--|-----------------|--------|-----------------|
|  | Cain+           | MyHCI– | SERCA2a–        |
| 12 d regenerating + cain transfected <sup>a</sup>      | 11 <sup>b</sup> | 202    | 3 <sup>c</sup>  |
| 21 d regenerating + cain transfected <sup>d</sup>      | 13 <sup>b</sup> | 104    | 14 <sup>c</sup> |
| 12 d regenerating + partially tenotomized <sup>a</sup> | –               | 1021   | 78              |
| 21 d regenerating + partially tenotomized <sup>d</sup> | –               | 15     | 12              |
| 12d Regenerating <sup>a</sup>                          | –               | 7      | 5               |
| 21d Regenerating <sup>d</sup>                          | –               | 22     | 15              |

<sup>a</sup>Total fiber number is around 2400 per transversal section, in parallel transfection experiments GFP is expressed in a total of 38 (11, 12 and 15, respectively) fibers of three separate 11-days regenerating muscles.

<sup>b</sup>All cain-positive fibers are MyHCI negative.

<sup>c</sup>Found only in cain-negative fibers.

<sup>d</sup>Total fiber number is around 2700 per transversal section, in three individual 21-days regenerating soleus GFP is expressed in a total of 25 (6, 8 and 11, respectively) fibers.

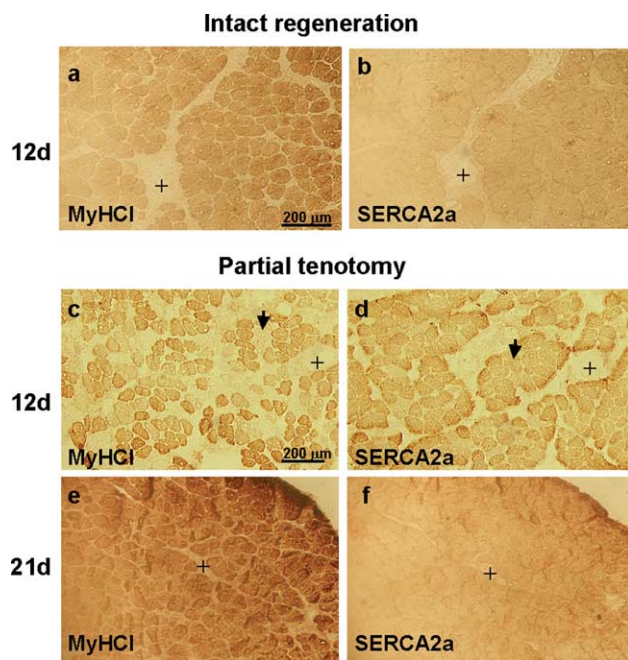


Fig. 2. MyHCI and SERCA2a expressions are not regulated in coordination upon partial tenotomy of regenerating soleus. The expressions of MyHCI (a,c,e) and SERCA2a (b,d,f) in fibers of regenerating (a,b) and partially tenotomized regenerating (c–f) soleus muscles on days 12 (a–d) and 21 (e,f). Arrows indicate identical fibers and + marks similar positions on consecutive transversal sections.

that of the slow myosin (Fig. 2(d), Table 1). When after 21 days of regeneration MyHCI increased again, the co-expression of MyHCI and SERCA2a was re-established (Fig. 2(e) and (f), Table 1). This supports the view that the expressions of SERCA2a and MyHCI are differentially regulated in regenerating soleus muscle.

#### 4. Discussion

Here, we clearly showed that in regenerating muscle the expressions of slow type myosin heavy chain and the slow type sarcoplasmic reticulum calcium ATPase are not coregulated by calcineurin. Also upon partial tenotomy, a model of selective fiber unloading affecting only a subset of fibers leaving the others untouched, MyHCI and SERCA2a expressions are dissociated. This suggests that the coordinated change of MyHCI and SERCA2a is only phenomenal and the result of long-term adaptation of skeletal muscle. The muscle phenotype is defined by the expression of pattern of a set of proteins belonging to multigenic families, which are changing in coordination during the transition from the fast glycolytic to the slow oxidative type [18]. Similar transitions also exist in the regenerating soleus muscle where initially the neonatal and fast-type protein isoforms are expressed, which are later gradually replaced by the slow-type isoforms [3,13]. As is shown by the experiments reported here, the expressions of the corresponding SERCA2a and MyHCI isoforms are no longer coordinated properly in fibers of regenerating soleus, which have been transfected with cain or are affected by partial tenotomy. Whereas the expression of the slow myosin isoform is effectively abolished by

the calcineurin inhibitor cain, practically no change of SERCA2a is observed in the cain transfected fibers, meanwhile in the whole muscle the level of SERCA2a increases dramatically between days 12 and 21 of regeneration. MyHCI expression is predominantly regulated at transcriptional level by the calcineurin-NFAT pathway. The specification of the slow fiber type, whose identity is routinely defined by the expression of MyHCI, is prevented by the expression of cain, a calcineurin inhibitor protein [9]. Calcineurin dephosphorylates NFAT and MEF-2 permitting them to translocate to the nucleus and to be involved in transcription of the MyHCI gene (reviewed by [19]). NFAT is a key transcriptional factor in the formation of slow fiber identity and a sensor of slow-type neuronal activity [20]. Previously, we have shown that the mRNA level of SERCA2a is not dependent on innervation [11]. However, calcineurin shows a pleiotropic effect in muscle differentiation where it also acts on channels and receptors regulating the cytosolic  $Ca^{2+}$  level [19] and this type of regulation in principle could affect SERCA2a expression. Therefore, it cannot be excluded that calcineurin acting on the level of translation increases the expression of SERCA2a between days 10–21 of muscle regeneration. Investigating this possibility we detected little, if any, influence of cain expression on SERCA2a in fibers of 12-days regenerating soleus, which were negative for MyHCI. On day 21 of regeneration when the level of SERCA2a reached its maximum [4], the SR  $Ca^{2+}$  pump was uniformly present in fibers irrespective of MyHCI or cain expression. Therefore, these experiments were not compatible with a post-transcriptional effect of calcineurin on SERCA2a expression. However, it must be noted that in our assays overexpression of cain was detected only in about 5–12% of the MyHCI-negative fibers. This could be due to the decline of cain expression after accomplishing a block of slow type differentiation or to the fact that the physiologically effective level of cain fell below the lower limit of immunodetection. Indeed, control experiments run in parallel where GFP, controlled by the same CMV promoter, was transfected under similar experimental conditions in regenerating soleus, showed expression of the reporter in 2–3 times more fibers than cain. Regarding the high number of MyHCI-negative fibers, it can in principle also not be ruled out that the expression of cain elicits an autocrine/paracrine loop of regulation in myotubes similar to that observed in the myoblast recruitment by IL-4 [21]. Such a mechanism has, however, not been reported to affect myosin expression.

The level of slow MyHCI is increased in mechanically overloaded regenerating muscle, together with the relative fraction of slow fibers coexpressing slow myosin and slow SERCA2a [6]. However, in this model the coordinated changes in myosin and  $Ca^{2+}$ -pump isoforms might be phenotypical and do not necessarily result from a common regulatory mechanism. The expression of slow myosin is decreased by total tenotomy of regenerating soleus [15], therefore, in the partially tenotomized muscles the lack of MyHCI expression marks those fibers that are specifically affected by the tenotomy. In our experimental model with partial tenotomy, the majority of the fibers have still an intact tendon and therefore express the slow myosin isoform, while the fibers of which the tendon connection was severed did no longer express MyHCI and were passively moved by the intact fibers. However, both groups of fibers were similar in terms of SERCA2a expression. This shows that the slow myosin and the SERCA2a expression are not directly coregulated by passive stretch either.

Signaling pathways known to control the expression of the slow myosin isoform in regenerating muscle fibers are the ras-MAPK and the calcineurin-NFAT pathways [22,9]. Our previous [11] and present work does not support involvement of any of these pathways in the regulation of slow SERCA expression.

*Acknowledgments:* We thank S. Schiaffino and S.H. Snyder for providing the cain/cabin-1 plasmid. This work was supported by the B-9/02 grants from TÉT, Hungary and the BIL02/18 Ministerie Vlaamse Gemeenschap, Belgium.

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