Changes in mRNA levels of the sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase isoforms in the rat soleus muscle regenerating from notexin-induced necrosis

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The relative mRNA levels corresponding to the different sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase isoforms (SERCA1a, SERCA1b, SERCA2a, SERCA2b and SERCA3) were measured by reverse transcriptase–PCR in rat soleus muscles regenerating after notexin-induced necrosis. The succession of appearance of the different types of SERCA mRNA species in regenerating muscle largely recapitulates those observed during normal ontogenesis. The mRNA levels of the muscle-specific isoforms SERCA1a and SERCA2a became very low on the first and third days after injection of the snake venom. It was only on the fifth day of regeneration that the mRNA of the neonatal variant of the fast-twitch skeletal SERCA1b isoform began to rise, well before the other SERCA transcripts. At 7 and 10 days, i.e. at a time when the new myofibres normally become re-innervated, the mRNA level of SERCA1a and SERCA2a increased markedly, but the fast-twitch skeletal SERCA1a isoform was still the most prominent. On day 21, in the advanced stage of regeneration, a switch in the relative expression levels of SERCA1a and SERCA2a mRNA was observed and the ratio of both isoforms became similar to that found in the normal soleus muscles. This was followed by a decline in the level of all SERCA mRNA species, so that on day 28 the levels of the sarcoplasmic/endoplasmatic-reticulum Ca^{2+}-pump RNAs was again lower but their ratio remained similar to that of the untreated control soleus.

INTRODUCTION

The sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) pumps Ca^{2+} from the sarcoplasm into the SR. This function is essential to the relaxation of skeletal muscle. Three types of SERCA gene are known [1,2]. SERCA1 is expressed mainly in fast-twitch skeletal muscle, SERCA2 is in the slow-twitch skeletal muscle, in cardiac muscle and to a limited extent in smooth muscle, whereas the protein of the SERCA3 gene is found mainly in platelets, lymphoid cells, mast cells and epithelial and endothelial cells in various organs [3–5]. As a result of tissue or developmental stage-dependent alternative transcript processing, the SERCA1 and SERCA2 genes each encode two protein isoforms that differ in their C-terminal ends. Two different mature SERCA1 mRNA species have been described that are each translated to their corresponding protein isoform. The SERCA1a isoform is expressed mainly in the adult fast-twitch skeletal muscle, whereas SERCA1b represents the neonatal isoform [6–8]. The situation is more complex for SERCA2. Here four different transcripts have been found [9–11]. The class 1 transcript is translated into SERCA2a, a protein characteristic of cardiac muscle, slow-twitch skeletal muscle and smooth muscle. The class 2–4 mRNA species differ only in their 3' -untranslated region and are all translated into the SERCA2b protein, the ubiquitous 'housekeeping' isoform of muscle and non-muscle tissues. At present only one splice variant is known for the SERCA3 gene.

One of the most amenable ways of assessing the changes in relative levels of transcripts of the SERCA genes is by means of reverse transcriptase–PCR (RT–PCR) [12–14]. Although making RT–PCR quantitative is not easy [15] it can, when care is taken not to saturate the PCR system, be a very quick and sensitive method to measure changes in transcript levels. In a modified version of this method (ratio RT–PCR) cDNA fragments are co-amplified from similar mRNA species using a set of primers which are common to both isoforms and the fragments amplified from the different isoforms distinguished by restriction enzyme digestion [4,16]. In this way it is possible to measure the ratio of the two similar SERCA transcripts. As the level of SERCA transcripts changes during skeletal muscle differentiation [6,17], one might expect similar alterations to occur during muscle regeneration. Indeed, we report here marked changes in the levels of SERCA transcripts in the regenerating muscle, which recapitulate in many aspects those observed during development. To induce regeneration we injected snake venom (containing notexin) into the soleus muscle of the rat. The muscle thereby undergoes necrosis, followed by a relatively fast regeneration in which the satellite cells are activated, proliferate, fuse into myotubes and develop into mature fibres [18–21].

MATERIALS AND METHODS

Animals and treatment

Wistar rats (300–360 g) were used for the experiments. The rats were narcotized by injection of 1 ml or 0.5% sodium pentobarbital per 100 g of body weight. A small incision was made in

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT–PCR, reverse transcriptase–PCR; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase.

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Table 1 Total RNA yield from rat soleus muscles regenerating from notexin-induced necrosis

Total RNA was isolated as described in the Materials and methods section. The means ± S.E.M. for three different animals are shown.

Days after notexin injection	Yield (μg per soleus muscle)
Normal soleus	95 ± 26
1	93 ± 3.4
3	98 ± 55
5	185 ± 63
7	194 ± 46
10	174 ± 46
21	106 ± 31
28	115 ± 23

Table 3 Amplified fragments and their identifications

Isoform	Product size (bp)	Diagnostic restriction enzyme	Restriction products (bp)
SERCA1	194	*Nco*I	102, 92
SERCA2	194	*Mse*I	127, 67
SERCA2	206	*Bsa*HI	178, 28
SERCA3	209	*Sty*I	176, 33
SERCA1a	248	*Ava*II*	103, 145
SERCA1b	206	*Ava*II*	103, 103
SERCA2a	231	*Hin*fI*	149, 82
SERCA2b	328	*Hin*fI*	246, 82
GAPDH	377	*Bst*XI*	196, 181

* Only used for diagnostic purpose, the quantification is done on the full-length PCR fragments.

the m. gastrocnemius, the soleus muscle slightly lifted from its bed and slowly injected at a point approx. one-third of the total length from the distal end with 20 μg of venom of the mainland tiger snake (*Notechis scutatus scutatus*) in 200 μl of 0.9 % NaCl (Sigma) with a 27G 3/4 injection needle. After injection, the wound of the gastrocnemius and the skin was closed with a suture. At times ranging from 1 day to 28 days after injection the entire soleus muscle was dissected and the animals were killed with an overdose of sodium pentobarbital. Control samples were taken from separate animals where the soleus muscle was injected with 0.9 % NaCl.

Collection and staining of the soleus muscles with haematoxylin–eosin

The dissected soleus muscles were frozen in isopentane cooled with liquid nitrogen and kept at −70 °C. A biopsy was taken from the central part of each frozen soleus before homogenization; it was freeze-sectioned and stained with haematoxylin–eosin to monitor the necrosis and the subsequent regeneration process. The time course of regeneration and the dose of snake venom were established in a set of experiments and found to be reproducible.

RNA isolation and RT–PCR

Total RNA was isolated from normal and regenerating soleus muscles as described in [22]. The yields of extractions are shown in Table 1. A 2 % portion of the total RNA of each soleus was subjected to oligo(dT)-primed first-strand cDNA synthesis in a volume of 20 μl [4]. A 1 μl portion of the first-strand cDNA mixture was subjected to PCR under the conditions specified in Table 2, as described in [4]. The number of PCR cycles was adjusted carefully to avoid saturation of the amplification system. Amplification products were identified by their sizes. Additionally the fragmentation pattern obtained with the selected restriction enzymes listed in Table 3 was checked for conformity with those predicted from the published rat cDNAs. To radiolabel the PCR fragments for quantification, 5 μl (i.e. one-tenth of the 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. Quantification of SERCA1a/SERCA1b, SERCA2a/SERCA2b and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was done on the full-length amplified fragments. The SERCA1/SERCA2 and SERCA2/SERCA3 ratios were obtained from the corresponding digestion fragments obtained with the enzymes listed in Table 3, as described in [4]. The amplification products

Table 2 Oligonucleotide primers and PCR conditions used for amplification of reverse-transcribed RNA

Primer	Name	Sequence	PCR cycles
SERCA1/SERCA2	8	5′-GAC/TGAGTTTGGGGAACAGCT-3′ (nt 760–779*; nt 1264–1283†)	94-60-72 °C/1-1-1 min/21 cycles
	9	5′-GAGGTGGTGATGACAGCAGG-3′ (nt 934–953*; nt 1438–1457†)	
SERCA2/SERCA3	12	From [4]	94-55-72 °C/1-1-1 min/22 cycles
	13		
SERCA1a/SERCA1b	20	5′-TTCCATCTGCCTGTCCATGTC-3′ (nt 2805–2825)*	94-60-72 °C/1-1-1 min/21 cycles
	23	5′-CTGGTTACTTCCTTCTTTCGTCTT-3′ (nt 3029–3052)*	
SERCA2a	U_f	From [13]	94-55-72 °C/1-1-1 min/22 cycles
	C_1		
SERCA2b	U_f	From [13]	94-55-72 °C/1-1-1 min/22 cycles
	C_2		
GAPDH	G+	5′-TCCTGCACCACCAACTGCTTAGCC-3′ (nt 528–551)‡	94-60-72 °C/1-1-1 min/22 cycles
	G−	5′-TAGCCCAGGATGCCCTTTAGTGGG-3′ (nt 880–904)‡	

* Positions in the sequence of the SERCA1 cDNA [23].
† Positions in the sequence of the SERCA2 cDNA [24].
‡ Positions in the sequence of the GAPDH cDNA [25].

were analysed by electrophoresis on 6% (w/v) acrylamide gels. The gels were air-dried and ^{32}P spots quantified by means of a PhosphorImager model 425 (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Because [α-^{32}P]dCTP was used for labelling, the band intensities were corrected for the CG content of the amplified sequence.

RESULTS

Strategy for PCR analysis of the samples

Ratios of two similar mRNA species can be obtained by means of similar ratio RT–PCR [4]. In this analysis PCR primers are designed in such a way that they exactly match sequences in the reverse-transcribed cDNA separated by a few hundred base pairs. Fragments of both cDNAs are then co-amplified and discriminated from each other by restriction analysis. We have used this principle for the analysis of the SERCA1/SERCA2/SERCA3 forms of the rat. For each of these the corresponding cDNA sequences have been published and are available from the EMBL Database. Figure 1 shows the position of primers 8/9 used to co-amplify SERCA1/SERCA2 fragments and of primers 12/13 used to co-amplify SERCA2/SERCA3 fragments. Table 3 lists the enzymes and fragment sizes used to discriminate SERCA1 from SERCA2, and SERCA2 from SERCA3. To measure relative SERCA1a/SERCA1b and SERCA2a/SERCA2b ratios we had to follow a different approach. SERCA1a (the adult fast-twitch skeletal muscle form) differs from SERCA1b (the neonatal form) because it has a 42 bp optional exon. By using primer set 20/23 we could amplify fragments differing in length by 42 bp. The longer fragment represents the adult form. To determine the SERCA2a/SERCA2b levels we used a set of three primers, $U_f/C_1/C_2$. The U_f/C_2 couple amplifies the SERCA2b (non-muscle form, classes 2–4 cDNA); the U_f/C_1 couple amplifies the SERCA2a form (muscle form, class 1 cDNA).

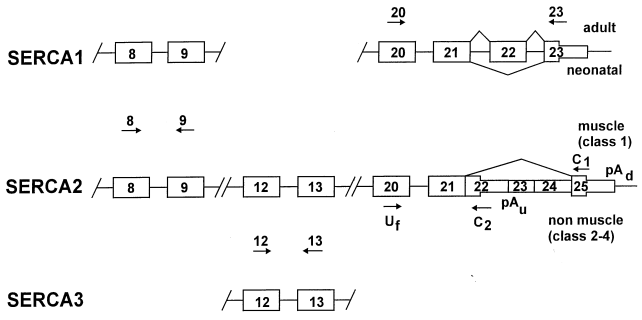

Figure 1 Position of primers used to amplify the SERCA1/SERCA2, SERCA2/SERCA3 ratio and SERCA1a, SERCA1b and SERCA2a (class 1) and SERCA2b (class 2) transcripts

Partial gene structures of the SERCA1, SERCA2 and SERCA3 genes are depicted. Constitutive or optional exons are shown as boxes. The thin lines represent constitutive introns. Large boxes represent translated exon sequences. The numbering of the exons is copied from the numbering in SERCA1 [8], because the exon/intron layout seems to be largely conserved between the SERCA genes. The positions of primers 8 and 9 used to co-amplify SERCA1 and SERCA2 and of primers 12 and 13 used to co-amplify SERCA2 and SERCA3 are indicated as arrows between the amplified sequences. For SERCA1 primers 20 and 23 are used. Exon 22 is retained only in the adult transcript because exon 21 is spliced to exon 23 in the neonatal form. In the muscle-specific transcript of SERCA2 (class 1, amplified by U_f and C_1 primers) exon 21 is spliced to exon 25, whereas the non-muscle transcripts (classes 2–4, amplified by U_f and C_2 primers) might contain exons 22–24 (detailed in [13]). The drawing is not to scale. pA_u and pA_d are upstream and downstream polyadenylation sites respectively.

Changes in total RNA and GAPDH mRNA level during muscle regeneration

The total RNA (rRNA, tRNA and mRNA) level of the soleus changed during notexin-induced regeneration (Table 1). There was no increase during the first 3 days, but there was an increase on days 5, 7 and 10 and, later, a decrease on days 21 and 28. The level of housekeeping enzyme, GAPDH, gave an estimate of the general rate of transcription. In the normal muscle the GAPDH mRNA level was slightly higher than in the regeneration stages, but this level was variable from muscle to muscle. Throughout regeneration the level of the transcript of this important glycolytic enzyme remained remarkably constant, except that it declined slightly at the end (Figure 2F). This might be an indication of change from fast-glycolytic to the slow-oxidative metabolic character.

Changes in SERCA1/SERCA2 mRNA ratio during muscle regeneration

The fresh weight of one soleus was about 220 mg. After injection with notexin, this decreased to about one-half of the original value on the third day but thereafter increased again and regained about the original weight on day 28. cDNA made from aliquots representing 2% of total muscle RNA was used for the PCR reactions. A first pair of primers was designed to amplify 194 bp homologous fragments of SERCA1 and SERCA2. The primers were carefully selected such that they had identical binding sites both in SERCA1 and SERCA2, but the amplified sequences in between the primers, although of the same length, showed some differences in composition. The distinction between fragments amplified from SERCA1 and SERCA2 could therefore be made by digestion with restriction enzymes (Figure 3). Note, however, that this analysis does not permit discrimination between the SERCA1a and SERCA1b splice variants, nor between the SERCA2a and the SERCA2b splice variants, because the amplified SERCA1/SERCA2 fragments each belong to parts of the transcripts that are common in the respective splice variants (Figure 1). *Nco*I hydrolysed the SERCA1 fragment (sum of SERCA1a- and SERCA1b-related messengers) into 102 bp and 92 bp fragments, leaving the SERCA2 fragment intact. The SERCA2 amplification product (sum of SERCA2a-and SERCA2b-related messengers) was hydrolysed by *Mse*I into 127 bp and 67 bp fragments, leaving the SERCA1 fragment intact. The total amount of radioactivity incorporated from [α-^{32}P]dCTP in the uncut amplified fragments was measured, corrected for CG content and used for the calculations (Figure 2A). In this way the transcript of the slow-type Ca^{2+}-pump (SERCA2) was found to be more than twice as abundant in the normal soleus as that of the fast-type pump (SERCA1). On the first day of regeneration the level of both transcripts was very low; it started to rise from the fifth day. In the first stages of regeneration, the fast-type SERCA1 mRNA showed a higher level than that of the slow type until day 21, when the fast isoform decreased by 40% while the slow isoform increased by 60% and reached its maximum. This resulted in the slow isoform's reaching a level twice that of the fast isoform. On day 28 the level of both isoforms declined, but the slow isoform still dominated over the fast one.

Changes in the SERCA1a/SERCA1b mRNA ratio during muscle regeneration

The SERCA1 transcripts undergo a developmental-stage-dependent splicing. A 42 bp optional exon is removed from the neonatal SERCA1b isoform but is retained in the adult

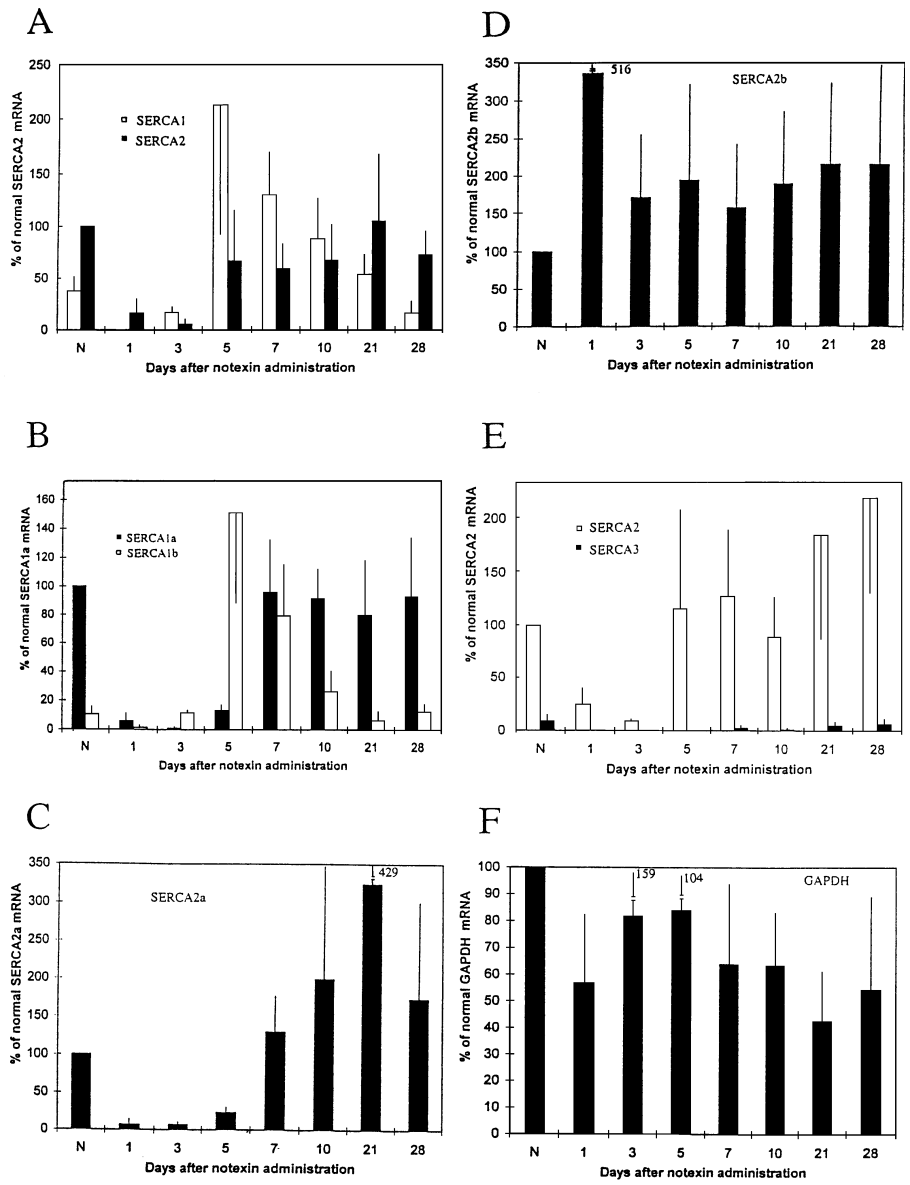

Figure 2 Reverse transcriptase-PCR-detected changes in SERCA and GAPDH mRNA levels during notexin-induced regeneration of the rat soleus muscle

The column labels represent: N, normal soleus; 1–28, days after administration of the toxin. The values given in the ordinate are relative values that cannot be compared between panels. Note, from (**A**), that during regeneration an initial expression of SERCA1 is gradually replaced by SERCA2. (**B**) Early stages of regeneration express predominantly the neonatal SERCA1b isoform. SERCA2a expression (**C**) shows greater changes during regeneration than SERCA2b (**D**). (**E**) The ratio of SERCA2 and SERCA3; note that only in the normal soleus, and on days 7, 21 and 28 after the administration of the toxin, SERCA3 is slightly above the detection level. (**F**) Expression of the GAPDH in different stages of regeneration. Three different animals were used for each time point; vertical bars indicate the S.E.M.

SERCA1a form. The ratio of both forms of transcripts can be measured by using primers that encompass the optional exon. A set of such primers used in our experiments amplified a 248 bp adult fragment and a 206 bp neonatal fragment. In the control soleus the adult transcript predominated (Figure 4). On the third and fifth days of regeneration, at the time when SERCA1 expression again became apparent, first the neonatal SERCA1b form showed up and dominated over the adult form, and later the adult form became again dominant (Figure 2B). Hence in its initial stage of regeneration the soleus muscle recapitulates the ontogeny of the fast-twich skeletal muscle. Splicing of SERCA1 transcripts is controlled similarly during regeneration and normal development.

Changes in SERCA2a and SERCA2b levels during muscle regeneration

As with SERCA1, the SERCA2 transcripts are also subject to an alternative splicing process. However, in contrast with SERCA1, the SERCA2 splicing is dependent on the tissue not the developmental stage. In addition, in SERCA2 this splicing involves

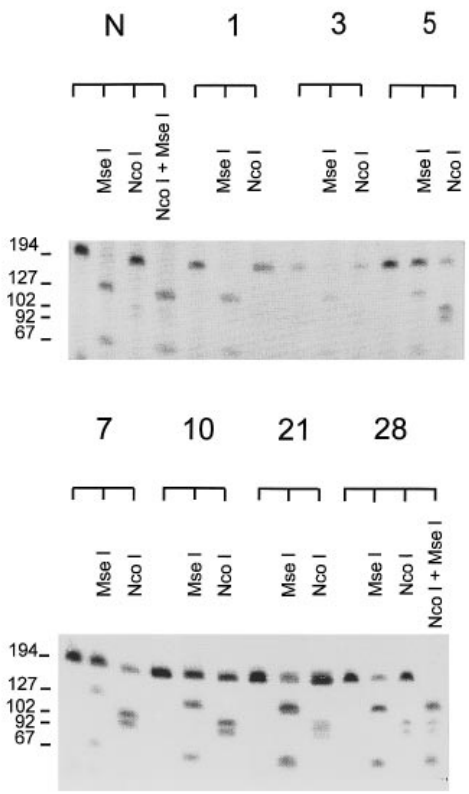

Figure 3 RT–PCR amplification of SERCA1 and SERCA2 mRNA species during the notexin-induced regeneration of the soleus

Lanes of a single amplification are grouped as follows: N, normal soleus; 1–28, days after administration of the toxin. In each group the left lane shows the combined amplification product of SERCA1 and SERCA2 mRNA species. In the second lane the SERCA2 amplification product was selectively cut by *Mse*I into 127 bp and 67 bp fragments (numbers shown at the left), leaving the SERCA1 product intact. In the third lane SERCA1 was cut by *Nco*I into 102 bp and 92 bp fragments, the SERCA2 remaining undigested. In the fourth lane (only given for the normal and the 28-day regenerating soleus) both enzymes were added. Note that no amplification product remained undigested. A 5 μl portion of the amplification product was labelled with [α-^{32}P]dCTP in two cycles; 5 μl of this reaction mixture was digested with the above-indicated restriction enzymes. The total volume of the restriction digest mix was analysed on polyacrylamide gel as indicated in the Materials and methods section. A typical experiment of three is illustrated.

Figure 4 RT–PCR amplification of the adult SERCA1a and the neonatal SERCA1b mRNA species

Lanes are labelled as follows: N, normal soleus; 1–28, days after toxin administration. Numbers at the left indicate lengths in base pairs. The 248 bp bands represent the adult (SERCA1a) isoform. The 206 bp bands correspond to the neonatal (SERCA1b) isoform, which is shorter because it lacks a 42 bp exon. The identities of the expected amplification products were ascertained by restriction digest as described in the Materials and methods section. A typical experiment of three is illustrated.

the removal of an optional exon in muscle (SERCA2a mRNA; class 1 transcript) and its retention in non-muscle cells (SERCA2b; class 2–4). The transcript processing is further

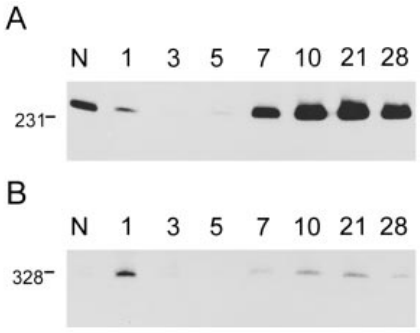

Figure 5 RT–PCR amplification of SERCA2a and SERCA2b mRNA species

(**A**) SERCA2a amplification products; (**B**) SERCA2b amplification products. The identity of the amplification products was ascertained by restriction enzyme digest as indicated in the Materials and methods section. A typical experiment of three is illustrated. Lanes are labelled as follows: N, normal soleus; 1–28, days after notexin administration.

complicated by the use of a polyadenylation site located between the splice donor and acceptor sites in the class 2 mRNA but not in the class 3 mRNA (see Figure 1). Because of the complicated nature of this transcript processing we had to rely on a system of three PCR primers (U_f, C_1 and C_2). The class 1 mRNA was detected as a 231 bp fragment amplified with the U_f and C_1 primers hybridizing with parts of exon 20 and 25 respectively. The level of SERCA2a was low on days 1–5 of regeneration, after which it increased on day 7, reached a maximum on day 21 and declined again on day 28 (Figures 2C and 5A). Interestingly the level of SERCA2b, the non-muscle isoform (detected as a 328 bp fragment by the U_f and C_2 primers), was very low in control soleus, and the largest increase occurred shortly after injection of the toxin (days 1 and 3). However, these changes observed on days 1 and 3 seemed to be variable from muscle to muscle (Figures 2D and 5B).

Changes in SERCA3/SERCA2 mRNA ratio during muscle regeneration

To estimate the ratio of SERCA2 and SERCA3 mRNA species [4] another ratio RT–PCR experiment was performed. Except for the low amount of expression in the normal soleus and on days 7, 21 and 28 after the injection of the toxin, no SERCA3 transcript was detected during regeneration. The changes in relative levels of SERCA2 throughout the regeneration process deduced from these experiments showed a similar time course to that found in the study of the SERCA1/SERCA2 ratio (Figures 2E and 6). The total SERCA2 levels declined after the injection, remained low on the day 1, 3, increased from day 5 until day 28.

DISCUSSION

The injection of notexin completely degrades the myofibres of the soleus muscle and as a result the transcripts of the slow and fast types of Ca^{2+} pump quickly disappeared from the muscle. This enabled us to study the regeneration-stage-dependent expression of the different SERCA mRNA species. Because the sarcoplasmic/endoplasmic-reticulum Ca^{2+} pumps are essential for muscle function, one might expect that considerable transitions in the levels of SERCA transcripts accompany muscle necrosis/regeneration. In support of this assumption the levels of SERCA mRNA species were found to rapidly recover during the regeneration of the rat soleus. In the freshly necrotized muscle

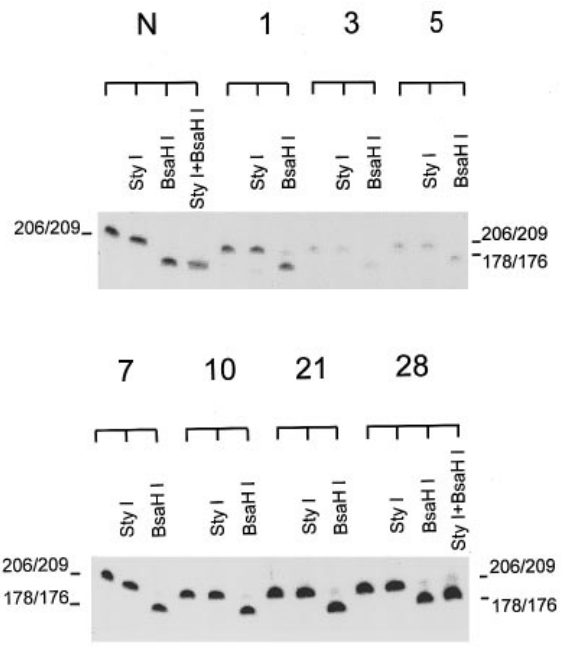

Figure 6 Reverse transcriptase-PCR amplification of SERCA2 and SERCA3 mRNA species

Lanes of a single amplification are grouped as follows: N, normal soleus; 1–28, days after administration of the toxin. In each group the left lane represents the combined amplification product of SERCA2 and SERCA3 mRNA species. The second lane shows the SERCA3-specific digestion by *Sty*I, leaving the SERCA2 mRNA intact. The third lane shows the SERCA2-specific digestion with *Bsa*HI, leaving the SERCA3 fragment intact. In the fourth lane (shown only for the normal and the 28-day regenerating soleus) both *Sty*I and *Bsa*HI were used [4]. A typical experiment of three is illustrated.

the SERCA transcripts normally found in the adult muscle subsided and at the beginning of regeneration were first replaced by a relatively small peak of the ubiquitous non-muscle SERCA2b transcript and later on by an early rise in the neonatal SERCA1b mRNA. In the normal soleus muscle controls we found slightly higher levels of SERCA2 transcript than of SERCA1 (ratio 2.8:1). There exists some discrepancy in the literature on the relative expression levels of SERCA1 and SERCA2 in soleus. Some authors [26] found a similar ratio between the two isoforms to that described here, whereas others [5,23] detected more SERCA1 than SERCA2 transcript. This might be ascribed to the difference in methodology. However, we consider that the ratio RT–PCR method used in our experiments is one of the most reliable methods now available for assessing the ratios of expression of similar transcripts (i.e. in our case SERCA1/SERCA2 or SERCA3/SERCA2) and has proved to be successful [4,13,14].

The main prerequisite for quantitative application of this method is that the efficiency of PCR amplification must be the same for the fragments of both isoforms for which the ratio is determined. To fulfil this condition, the binding sites of the primers are selected to be identical in both isoforms, such that the efficiency of primer annealing is rigorously the same. Moreover the length of the fragments amplified for both isoforms is also the same. Although there are some differences in the nucleotide composition of the sequences encompassed by the primers, these minor differences are unlikely to influence the relative efficiency of PCR amplification for both isoforms. However, the efficiency of amplification is not guaranteed to be the same when comparison is made between splice variants of a single SERCA isoform (e.g. when SERCA1a/SERCA1b or SERCA2a/SERCA2b ratios are compared). This is certainly true when a different set of primers is used, as with the SERCA2 isoforms. However, even in those cases a lateral comparison of the intensity of the amplified bands between the different time points of the regeneration process is still possible. During the first week of regeneration the activated satellite cells proliferate, form myotubes and become innervated [18–20]. In myotubes, which are at the initial stage of innervation at day 5, the SERCA1b mRNA was found to be the dominant SERCA isoform. This isoform was earlier reported to be characteristic of the neonatal tissue, although it is present in a small amount in the adult muscle as well [7,8]. Our results therefore suggest that also during regeneration the expression of SERCA1b precedes that of SERCA1a, similar to what has been reported to occur during normal development of fast but not of slow muscles.

Necrosis of the soleus depressed both the levels of SERCA1 and SERCA2 mRNA species and their levels remained low until day 5, whereafter they increased again. This increase coincides with the onset of innervation [18–20] as could be expected because Ca^{2+} transport in muscle is indirectly regulated by neuronal activity [27]. Denervation of slow-twitch muscle of rats and rabbits was reported to lower the levels of SERCA2 mRNA and protein without affecting the expression of SERCA1. Inversely, the SERCA1 mRNA and protein levels decrease in fast-twitch muscle of rats and rabbits after denervation, leaving the expression of SERCA2 unchanged [28,29]. This suggests that although the regeneration process as such elicits some expression of Ca^{2+}-pumps, a functional innervation is a prerequisite for the expression of the proper ratio of isoforms. In the regenerating soleus the largest increases in the transcript levels of SERCA1a and SERCA2a were observed at day 7, when the re-innervation of the new fibres had almost recovered to normal. However, at this stage the SERCA1a isoform was still most prominent. Only after 3 weeks of regeneration did the slow SERCA2 form become dominant over the fast SERCA1 and at that time reached a situation (ratio 3.9:1) found in the normal adult soleus, which contains 90% slow-twitch myofibres [23]. This suggests that during the later stages of redifferentiation SERCA1 gene expression is suppressed whereas SERCA2 expression is stimulated.

A similar switch from SERCA1 to SERCA2 isoform was observed after chronic low-frequency stimulation of fast-twitch muscle [30,31]. In addition, chronic mechanical overload of rat fast-twitch muscle increased the expression of SERCA2 (mRNA and protein) 2-fold and decreased the SERCA1 level [32]. Remarkably similar changes in expression patterns to those that we observed for the SERCA pumps were also reported for the myosin isoforms. Here, too, from the multiple myosin isoforms, i.e. the embryonic, neonatal, fast and slow isoforms, only the slow one became exclusively expressed in the final stage of the notexin-induced regeneration of the innervated soleus [19]. Regeneration of the denervated soleus, however, culminates only in the expression of the fast type of myosin. The interpretation given in [19] was that the regeneration of the soleus started from a homogeneous population of satellite cells that were determined for expression of the fast myosin. Under the influence of innervation these cells changed phenotype and expressed only the slow-myosin isoform. In accordance with this interpretation, the fast SERCA1 type of mRNA appeared first (neonatal SERCA1b followed by adult SERCA1a respectively) also in our experiments, but after reinnervation the slow SERCA2a isoform became dominating. A change from one SERCA transcript isoform to another during normal development (although in the

opposite direction to that which we observed, i.e. from the fast to the slow isoform) has to our knowledge been documented only for the fast-twitch quadriceps muscle but could not be seen in the slow-twitch soleus muscle. In the initial stages of quadriceps development the transcript level of SERCA1 is lower than that of SERCA2a. Later, first SERCA1b is temporally up-regulated and gradually replaced by SERCA1a, whereas the SERCA2a is down-regulated [17].

In conclusion, the expression of Ca^{2+}-pump isoforms in the regenerating soleus recapitulates some aspects of the developing muscle and, in the later stages, of the low-frequency stimulated fast-twitch muscle. This is in agreement with other studies suggesting that the determination of the type of myoblasts that take part in the process of muscle differentiation/regeneration varies according to the muscle type and depends both on intrinsic factors and on external conditions [33].

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