

CLINICAL RESEARCH

Reduced expression of Ca²⁺-regulating proteins in the upper gastrointestinal tract of patients with achalasia

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be of lesser importance for regulation of SERCA than in heart. Lower expression of Ca²⁺ storage proteins (CSQ and CRT) might contribute to increased lower esophageal sphincter pressure in achalasia, possibly by increasing free intracellular Ca²⁺.

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Abstract

AIM: To compare expression of Ca²⁺-regulating proteins in upper gastrointestinal (GI) tract of achalasia patients and healthy volunteers and to elucidate their role in achalasia.

METHODS: Sarcoplasmic reticulum Ca²⁺ ATPase (SERCA) isoforms 2a and 2b, phospholamban (PLB), calsequestrin (CSQ), and calreticulin (CRT) were assessed by quantitative Western blotting in esophagus and heart of rats, rabbits, and humans. Furthermore, expression profiles of these proteins in biopsies of lower esophageal sphincter and esophagus from patients with achalasia and healthy volunteers were analyzed.

RESULTS: SERCA 2a protein expression was much higher in human heart (cardiac ventricle) compared to esophagus. However, SERCA 2b was expressed predominantly in the esophagus. The highest CRT expression was noted in the human esophagus, while PLB, although highly expressed in the heart, was below our detection limit in upper GI tissue. Compared to healthy controls, CSQ and CRT expression in lower esophageal sphincter and distal esophageal body were significantly reduced in patients with achalasia ($P < 0.05$).

CONCLUSION: PLB in the human esophagus might

INTRODUCTION

Intracellular Ca²⁺ regulates contractility in striated, smooth, and heart muscle. In the heart, expression of the most important proteins for intracellular Ca²⁺ homeostasis has been studied extensively. β -adrenoceptor agonists elevate Ca²⁺ by activation of cAMP-dependent protein kinase (PKA) and subsequent phosphorylation of regulatory proteins. The predominant protein phosphorylated in cardiac sarcoplasmic reticulum (SR) by PKA is phospholamban (PLB). The best characterized function of phospholamban is the regulation of the activity of the SR or endoplasmic reticulum (ER) Ca²⁺-pump (SERCA). In isolated guinea-pig hearts, β -adrenergic stimulation leads to phosphorylation of PLB and, at the same time, Ca²⁺-uptake into the SR is increased^[1]. PLB in its dephosphorylated state lowers the Ca²⁺ affinity of SERCA, while phosphorylation of PLB reduces the affinity of SERCA for Ca²⁺. Therefore, PLB phosphorylation increases the rate of Ca²⁺-transport into the SR. As a consequence, the rate of cardiac relaxation as well as contraction increase^[2,3]. SERCA itself is coded by three genes in mammals: SERCA 1, 2, and 3^[4]. SERCA 1 usually is expressed in fast-twitch skeletal muscle and SERCA 3 in non-muscle cells. For SERCA 2, two splice variants are known: SERCA 2a and SERCA 2b, the latter containing 49 additional amino acids at the carboxyterminal end^[5]. In the heart SERCA 2a

is the predominant isoform, whereas SERCA 2b is found mainly in smooth muscle, but smaller amounts have been recognized also in other tissues like heart^[6].

Intracellular rapidly exchanging Ca^{2+} stores provide the possibility for release, uptake, and storage of Ca^{2+} transported into SR by SERCA. Ca^{2+} -binding proteins with high capacity and low affinity for Ca^{2+} -binding permit rapid Ca^{2+} release. At least two families of these Ca^{2+} -binding proteins have been described, namely calsequestrin (CSQ) and calreticulin (CRT). CSQ is found in skeletal muscle and heart, while CRT is more widely distributed^[7,8]. CSQ is coded by two genes, the skeletal muscle isoform (CSQ1) coding for a 62-ku protein, and the cardiac isoform (CSQ2) coding for a 51-ku protein^[9,10]. Differences within the amino acid sequences might be functionally relevant, because the deduced amino acid sequence of cardiac calsequestrin is consistent with its ability to bind larger amounts of Ca^{2+} ^[10]. In contrast, CRT is coded by one gene and splice variants have not yet been reported^[11].

In heart, regulation of SERCA2a activity by expression and phosphorylation of PLB is well established, and disturbances of this system might be involved in pathophysiological events leading to heart failure^[3,12,13]. Indeed, targeted over-expression of CSQ or CRT in the heart of transgenic mice led to cardiac hypertrophy, dilated cardiomyopathy, and heart failure^[14-16]. However, expression, function, and pathophysiological role of Ca^{2+} -processing proteins in the human gastrointestinal (GI) tract and their alterations in diseased states have not been examined before.

There are few diseases of the GI tract where altered Ca^{2+} homeostasis might be involved. One of the possible entities is achalasia. Achalasia is a rare motility disorder characterized by increased pressure of the lower esophageal sphincter (LES) and simultaneous contractions of the esophageal body (EB) leading to impaired swallowing. There is evidence that gastric motility is also impaired in achalasia^[17,18]. Common treatments of achalasia are invasive procedures like pneumatic dilatation and laparoscopic myotomy. For medical therapy, smooth muscle relaxants as nitrates and Ca^{2+} -channel blockers of the dihydropyridine-type are effective in the treatment of achalasia^[19]. Hence, we tested the hypothesis that the expression of SR-proteins involved in Ca^{2+} handling in the GI tract is altered in achalasia in humans.

MATERIALS AND METHODS

Animals

Male Wistar rats (body weight, 150-250 g) and rabbits which had fasted overnight were sacrificed. Organs (esophagus, heart) were prepared and immediately frozen in liquid nitrogen.

Human cardiac tissue

Samples were taken from left ventricles of non-failing hearts which were obtained from prospective organ donors whose hearts could not be used. The study was performed in accordance with the guidelines from the Local Ethics Committee.

Patients

Manometrically proven 9 patients with achalasia (age, 19-52 years), and 6 healthy controls (age, 22-49 years) participated in the study. None of the control group had a history of GI disease or complained of any symptom of a GI disease. All patients underwent gastroduodenoscopy and ¹³C-urea breath test to exclude gastroduodenal disease or infection with *H pylori*. During endoscopy, biopsies of esophagus and LES were taken. Biopsies were immediately frozen in liquid nitrogen. The study had been approved by the Local Ethics Committee, and all patients gave written informed consent.

Esophageal manometry

Patients were fasted overnight before manometric examination was performed using a low-compliance, water-perfused system. The Arndorfer catheter-with four side-holes oriented radially, 0.5 cm apart, located distally for LES (lower esophageal sphincter) examination and four more orifices located every 5 cm above the distal ones thus allowing for the assessment of the motility pattern in the middle and distal part of the EB (esophageal body)-was attached to force transducers. The recorded signals were amplified (Polygraph VIII, Synectics, Stockholm, Sweden) and stored for further analysis with a specially designed software (Gastrosoft, Irving, Texas, USA). The motility patterns of distal esophagus were examined with (wet swallows) and without (dry swallows) swallowing of 5 mL of water bolus and expressed as mean values of the amplitude (mm Hg), duration (s) and propagation (cm/s) as calculated for five wet and five dry swallows. The LES resting tone was assessed by pull-through technique and the mean value, expressed in mm Hg above the mean intragastric pressure, was calculated from three consecutive measurements. The LES relaxation was measured in response to 5 mL of water bolus, while the distal catheter orifices were located in the LES high-pressure zone and results were documented as complete or incomplete relaxation of the LES.

SDS-PAGE and Western blot analysis

Gel electrophoresis was performed according to the method described by Laemmli^[20]. Briefly, samples were homogenized in 10 mmol NaHCO_3 /50 g/L SDS using a microdismembrator (Braun, Melsungen, Germany) and the protein content of homogenates was measured according to Lowry *et al.*^[21]. After SDS polyacrylamide gel electrophoresis, the separated proteins were transferred onto nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany) as described previously^[22]. For immunostaining, the following primary antibodies were used: monoclonal anti-PLB (2D12), monoclonal anti-SERCA 2a (2A7-A1), polyclonal anti-CSQ (all kindly provided by L.R. Jones, Krannert Institute of Cardiology, Indianapolis, USA), polyclonal anti-SERCA 2b (kindly provided by L. Raeymaekers and Wuytack, University of Leiden, Belgium), and polyclonal anti-calreticulin (Alexis, Grünberg, Germany). Thereafter, bound primary antibodies were detected with [¹²⁵I]-labeled protein A (ICN, Meckenheim, Germany). Visualization and

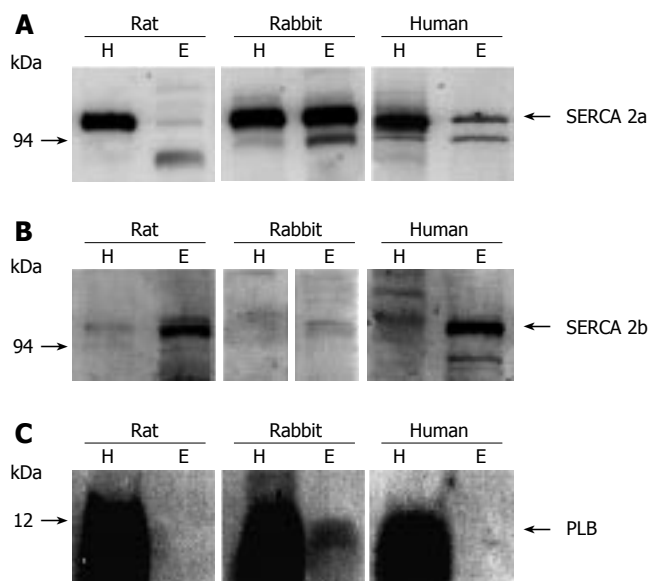


Figure 1 Protein expression of SERCA 2a (A), 2b (B), and PLB (C) in esophageal and ventricular homogenates of rat, rabbit, and human. Protein loading for esophagus (100 μ g) was 5 times higher than for heart (20 μ g). For documentation of PLB, sensitivity of the PhosphorImager was optimized for detection of very weak signals. Therefore, signals in heart samples are overexposed. H: heart; E: esophagus.

quantification of protein bands were performed with a PhosphorImager system (Molecular Dynamics, Krefeld, Germany). Differences in protein loading were corrected by densitometric quantification of Ponceau-stained membranes.

Statistical analysis

Results were presented as mean \pm SE. The significance of differences between means was evaluated using Mann-Whitney test with a confidence value at $P < 0.05$.

RESULTS

First, we studied the expression of Ca^{2+} -regulatory proteins in the upper GI tract. For comparison, we used cardiac tissues where the distribution of Ca^{2+} -regulatory proteins has been established before^[23,24]. Finally, we were interested in their putative pathophysiological role in man. Of note, to our knowledge, expression of these proteins has not been studied before in the human GI tract. However, as more data on expression are available for animal tissues than for human tissues, we studied, where possible, human and animal tissues in parallel. First, we analyzed the expression of SERCA in homogenates from various tissues. Figure 1 depicts protein expression of SERCA 2a and 2b in the esophagus and heart of different species (human, rat, and rabbit). Although, only 20 μ g of ventricular protein was loaded compared to 100 μ g for esophagus, it is obvious that SERCA 2a protein expression was highest in the cardiac ventricle in all three species analyzed (Figure 1A). In rabbit esophagus, the SERCA 2a signal was quite pronounced, whereas in human and rat esophageal expression of SERCA 2a was hardly detectable (Figure 1A). Although, primary sequences of SERCA 2a

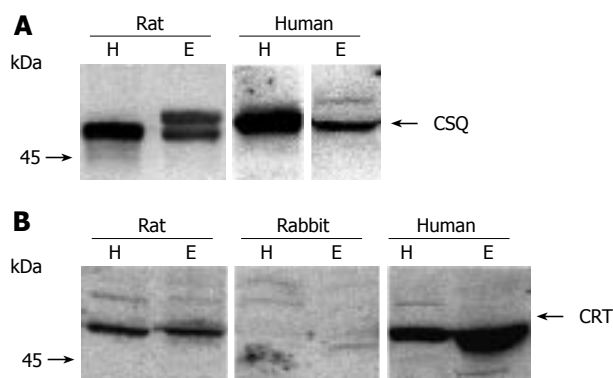


Figure 2 Protein expression of CSQ (A) and CRT (B) in esophageal and ventricular homogenates of rat, rabbit, and human. Protein loading was 50 μ g/lane for CSQ and 100 μ g/lane for CRT. H: heart; E: esophagus.

are somewhat different between rat, rabbit, and human, these expressional differences are valid, as comparisons only were done within one species^[4]. However, the SERCA 2a protein sequence expressed in esophagus and heart of a given species is identical. In rat and human, where SERCA 2a expression was barely detectable in 100 μ g of esophagus protein, a strong signal was visible in 20 μ g of cardiac proteins (Figure 1A). This strongly argues for tissue differences in expression between esophagus and heart at least in rat and, more importantly, in human. In contrast, SERCA 2b expression could be found predominantly in rat and human esophagus, while rabbit esophagus did contain only very low levels of SERCA 2b (Figure 1B). Only a very weak signal for SERCA 2b was detectable in heart, as expected (Figure 1B). For subsequent quantification, it was important to establish the linearity of protein detection by Western blotting. Detection of SERCA 2b in human left ventricle and esophagus as well as in rat samples was linear over a range of 50 to 200 μ g of protein loaded per lane (data not shown).

Next, we compared PLB expression in rat, rabbit, and human samples (Figure 1C). Strong signals could be detected in all homogenates of cardiac ventricles, while in rat and human esophagus, expression of PLB was below our detection limit (defined as 5% over background). Only in rabbit esophagus, a weak PLB signal was detectable (Figure 1C).

Both CSQ isoforms were expressed in esophagus of human and rat (Figure 2A). In the heart, only the low molecular weight isoform was present (Figure 2A). The high molecular weight band was identified as the skeletal muscle isoform and the lower band as the cardiac isoform of CSQ, based on the literature^[7].

We studied protein expression of CRT in all tissues. Expression of CRT in rat was nearly the same in esophagus and cardiac ventricle (Figure 2B). In humans, CRT expression was lower in heart than in esophagus (Figure 2B). In rabbit, CRT signal was much weaker in both tissues compared to human and rat. Conceivably this was a problem of the specificity of the antibody we used for detection of CRT.

After determination of protein expression of SERCA, PLB, CSQ, and CRT in different species, we posed the

Table 1 Ratios of CRT/CSQ and CRT/SERCA 2b in esophageal body (EB) and lower esophageal sphincter (LES) and LES pressure of achalasia patients and healthy controls (mean \pm SE)

	EB		LES	
	Control (n = 6)	Achalasia (n = 9)	Control (n = 6)	Achalasia (n = 9)
Pressure (mm Hg)	ND	ND	31.2 \pm 2.9	49.2 \pm 3.8 ^a
CRT/CSQ	0.63 \pm 0.08	0.61 \pm 0.04	0.88 \pm 0.10	0.78 \pm 0.08
CRT/SERCA 2b	1.07 \pm 0.20	0.77 \pm 0.10	1.47 \pm 0.09	1.07 \pm 0.08 ^a

CRT: Calreticulin; CSQ: Calsequestrin; SERCA: Sarcoplasmic reticulum Ca²⁺ ATPase; ND: Not done. ^aP < 0.05 vs controls.

question, whether expression of these proteins might be altered during GI disease. Ca²⁺ plays an important role in GI motility. Hence, we compared SERCA 2b, CSQ, and CRT expression in healthy controls and patients with achalasia (LES pressure was elevated by 58%, Table 1). In biopsies from LES and EB, SERCA 2b expression was not obviously different between controls and patients (Figure 3). However, CSQ expression in LES and EB was found to be significantly reduced in patients with achalasia (LES reduced by 19%, and EB by 22%) (Figure 3). CRT expression was reduced by 26% in LES as well as in EB of patients with achalasia (Figure 3). CRT/CSQ ratio was not markedly different between both groups, while CRT/SERCA 2b ratio was significantly reduced by 27% in LES of achalasia patients (Table 1).

DISCUSSION

For phasic smooth muscles, it was shown that SR Ca²⁺ cycling can play a major role in modulating mechanical activity^[25]. As achalasia, a human GI motility disorder, can be treated with drugs modulating intracellular Ca²⁺, one can speculate that impaired Ca²⁺-homeostasis might be involved in development or deterioration of this disease. Therefore, aim of this study was to compare protein expression of Ca²⁺-regulating proteins in upper GI tract from different species and moreover, to ascertain if they could play a role in human diseases of the upper GI tract. In rats, as well as in humans, SERCA 2a was predominantly expressed in cardiac tissue, while expression was hardly detectable in esophageal tissue. However, rabbit esophagus contained high amounts of SERCA 2a, while SERCA 2b was not detectable in both, cardiac and esophageal, homogenates of this species. Our data concerning the relative expression of SERCA 2a and SERCA 2b in rat cardiac and esophageal homogenates are in accordance with observations by Wu *et al.*^[26] who reported a similar ratio of SERCA 2a/2b expression in rat heart (30:1) and esophagus (1:4) at the mRNA level. Similar distribution of SERCA 2a and 2b RNAs in rat stomach and heart have been described^[27]. However, esophagus was not analyzed in this study. Another study, using the sensitive nuclease protection assay, determined a SERCA 2a/2b mRNA ratio of 20:1 in rabbit cardiac tissue, whereas in most smooth muscle and non-muscle tissues SERCA 2b was expressed predominantly^[28]. Surprisingly, in rabbit esophagus, SERCA 2a accounted for 80% of SERCA 2 content^[28].

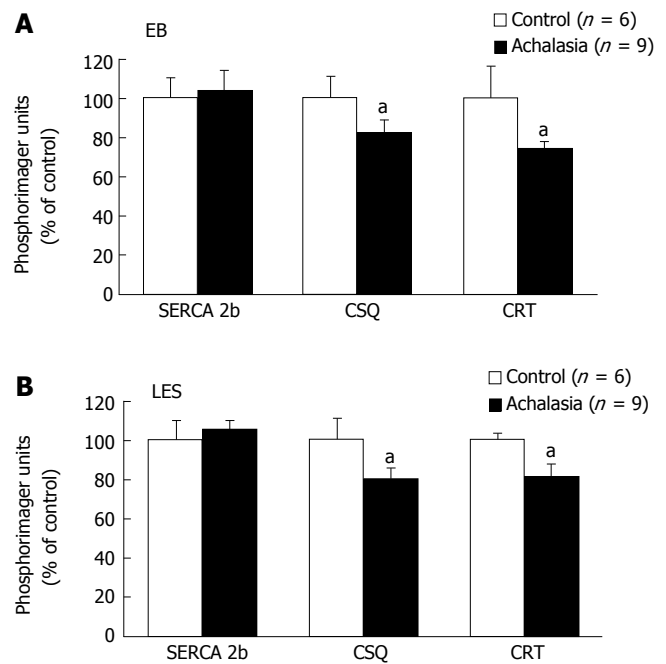


Figure 3 Protein expression of SERCA 2b, CSQ, and CRT in biopsies of esophageal body (EB, **A**) and lower esophageal sphincter (LES, **B**) from patients with achalasia or healthy controls. Protein expression was quantified using [¹²⁵I]-labeled protein A for detection of primary antibodies. Radioactive bands were visualized and quantified using a PhosphorImager. Data are presented as mean \pm SE of n patients. ^aP < 0.05 vs control.

These data are in accordance with our observation that in rabbit esophagus SERCA 2a expression was much more pronounced than in esophagus of rats and humans (compared to heart of the same species). Correspondingly, the SERCA 2b signal was significantly weaker in rabbit than in rat and human esophagus. Although, species specificity of the antibody (the alternatively spliced 50 amino acid carboxy terminal tail exhibits 10 differences between rabbit and man, 3 between rat and human and 10 between rabbit and human, most of these changes being conservative replacements^[28]) might contribute to this phenomenon, it is conceivable that the weaker expression of SERCA 2b might be due to a lesser importance of this isoform in rabbit esophagus. In summary, there are differences in the relative expression of SERCA 2a and 2b. Hence, splicing of SERCA 2 exhibits distinct tissue and species differences. Similar results at the protein level had been published elsewhere^[29]. But esophageal tissue was not analyzed so far.

In our study, expression of PLB was abundant in cardiac tissue of all species analyzed. A weak PLB signal could be obtained in rabbit esophagus. In the esophagus of rats and humans, PLB was below the detection limit. Only sparse data on PLB expression in the GI tract are available. Varying amounts of PLB mRNA and protein with a strong signal in gastric smooth muscle of pigs, but no signal in aorta were noted^[30]. In canine smooth muscle cells of ileum, PLB was detected by immunogold microscopy, but only ileum, not esophagus, was analyzed^[31]. Another group detected PLB in pig stomach, rat aorta and dog aorta, but not in pig aorta. They concluded from their experiments that PLB might exist in many, but not all muscle tissues^[32].

However, in our experiments, we failed to detect PLB in human and rat esophagus. This has not been studied and reported before. Our observation might be due to the fact that PLB is only of minor functional importance for SERCA regulation in these tissues.

May these discrepancies have functional consequences? Some studies have addressed the question of how SERCA 2a and 2b interact with PLB in different muscle tissues. The effect of cAMP- and cGMP-dependent protein kinases on Ca^{2+} uptake of ER vesicles is smaller in smooth muscle than in cardiac tissue^[33]. Using transfection experiments with COS-1 cells, a slower turnover rate for SERCA 2b was found and a higher apparent affinity for Ca^{2+} was reported for SERCA 2b compared to SERCA 2a. Ca^{2+} -affinity was decreased in both cases by co-expression with PLB^[5,34]. John *et al*^[35] reported similar functional differences of SERCA 2a and 2b isoforms. Additionally, they described the ability of CRT to modulate SERCA 2b-, but not SERCA 2a-activity, most likely by a binding to the additional eleventh transmembrane segment and luminal carboxy terminus of SERCA 2b. Since PLB expression in human and rat esophagus was below the detection limit, in contrast to cardiac PLB expression, esophageal PLB (at least in rats and humans) seems to be of lesser importance for regulation of SERCA than in heart. It is tempting to speculate that regulators other than PLB, possibly CRT, might control SERCA 2b and Ca^{2+} -handling in these tissues. In agreement with this speculation, we detected PLB expression only in rabbit esophagus, where a high amount of SERCA 2a, but no CRT was present, while CRT was detectable in rat and human esophagus, where SERCA 2b, but not PLB, was expressed.

In accordance with the literature, we found two isoforms of CSQ in rat, a low and high molecular weight isoform. In the heart exclusively the low molecular form was expressed, while smooth muscle tissue contained both isoforms^[7]. Here we have demonstrated that human esophagus also contains both isoforms of CSQ. To the best of our knowledge, CSQ protein expression in human upper GI tract has not been described before.

CRT protein expression was found in esophagus of rat and man and, to lesser extent, in cardiac tissue of these species, while it was missing in rabbit tissue. CRT expression, although being detectable in high amounts in various tissues, has not been analyzed in human esophagus. The expression patterns, presented here, encourage the speculation that CRT might be involved in the regulation of esophageal SERCA 2b.

Finally, we found decreased expression of CSQ and CRT in LES and EB of patients with achalasia. Although, antagonists of L-type Ca^{2+} -channels like nifedipine have proven effective in treatment of achalasia, studies on free intracellular Ca^{2+} levels in achalasia have not been published. It might be speculated that decreased CRT and CSQ levels might lead to increased intracellular free Ca^{2+} and therefore to elevated LES pressure. Interestingly, over-expression of CSQ decreases the free Ca^{2+} in the cytosol of cardiac myocytes^[13]. This finding may explain why contractility of CSQ-over-expressing cardiac cells was diminished compared to wild-type cells. In contrast, one can speculate that reduced expression of CSQ might

be accompanied by increased free cytosolic Ca^{2+} followed by enhanced contractility. If this mechanism comes true for smooth muscle cells, reduced expression of CSQ might lead to elevated free Ca^{2+} levels and hence increased tension and therefore to achalasia in patients. Reduced CRT expression might contribute to achalasia by similar mechanisms like PLB. For instance, in PLB-knockout mice, cardiac contractility was enhanced, because loss of PLB relieved any inhibition of SERCA function by PLB: Ca^{2+} is more effectively removed from the cytosol and more Ca^{2+} can be released during the systole leading to enhanced force generation in these hearts^[36]. A similar mechanism might hold true for CRT in esophagus: lower levels of CRT should also loosen its inhibitory modulation of SERCA function in the esophageal cells and more tension might be generated.

In conclusion, our results provide new insights to understand the mechanisms leading to achalasia. Further studies will be necessary to elucidate the role of impaired Ca^{2+} regulation for development of diseases like achalasia. Some questions which have to be answered are why the Ca^{2+} -regulatory proteins CSQ and CRT are down-regulated, and does down-regulation occur before or after manifestation of the disease.

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